CALCIUM TRANSPORT IN RAT LIVER MITOCHONDRIA

by

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SUMMARY

In the present study, it was found that various components of the rat cytosol affect Ca^{2+} transport into and out of mitochondria when examined in vitro.

The ^{3}H-palmitate-binding protein when saturated with palmitate caused Ca^{2+} release. The Ca-binding protein and albumin helped prolong Ca^{2+} retention in the mitochondria.

Palmitoyl CoA and palmitate caused release of Ca^{2+} from preloaded mitochondria. On the other hand , palmitoylcarnitine caused Ca^{2+} retention.

The mitochondria were able to accumulate 4 - 9 % of the exter nally added Mg^{2+} . The presence of varying concentration of external Mg^{2+} did not inhibit or increase Ca^{2+} uptake by the mitochondria, however, the extra Mg^{2+} accumulated, prolonged Ca^{2+} retention in the mitochondria. During Ca^{2+} release from the mitochondria, there was a corresponding release of mitochondrial Mg^{2+}. During Ca^{2+} retention, the level of mitochondrial Mg^{2+} remained steady.

ATP or respiratory substrate was required for Ca^{2+} uptake. The presence of both respiratory substrate and ATP favoured Ca^{2+} retention.

During Ca^{2+} release induced by palmitoyl CoA, PEP or glucose plus hexokinase, the total ATP concentration (i.e. that in the medium plus mitochondria) decreased, while the total AMP increased. During prolonged Ca^{2+} retention in the mitochondria, a high concentration of total ATP and a low concentration of total AMP was observed.

The findings of Lehninger et al (1978) (that a more oxidised steady state of the mitochondrial pyridine nucleotide

favours Ca^{2+} release and a relatively reduced state favours Ca^{2+} retention) was confirmed and further extended in this study. The adenine nucleotides were not significantly different from the control during Ca^{2+} release in the presence of the oxidants of mitochondrial NADH such as acetoacetate or oxaloacetate. Bovine serum albumin or ethane-1-hydroxy-diphosphonic acid (EHDP), substances known to help Ca^{2+} retention in the mitochondria, caused the Ca^{2+} released from the mitochondria by oxaloacetate to be taken up again.

Externally added NAD^+ and possibly NADH prolonged Ca^{2+} retention in the mitochondria. On the other hand, NADPH caused an earlier Ca^{2+} release. $NADP^+$ did not affect Ca^{2+} uptake or release.

The effect of cAMP on Ca^{2+} release was reinvestigated in the present study based on the findings of Lehninger et al (1978) and Christiansen (1977). Cyclic AMP caused Ca^{2+} release provided that palmitoyl CoA or palmitoylcarnitine were substrates and that the mitochondria prepared from fed rats were used within 1 hr after isolation. It was also noted in this study that the mitochondria isolated from starved rats had a lower $NADH/NAD^+$ ratio and released their Ca^{2+} earlier than the mitochondria from fed rats. It was suggested that cAMP stimulates Ca^{2+} release from the mitochondria presumably by altering the redox state of the mitochondrial pyridine nucleotides.

Obligatory Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge and belief the thesis contains no copy or paraphrase of material previously published or written by another person, except where due reference is made in the text of this thesis.

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Abbreviations used

ADP - adenosine diphosphate

AMP - adenosine monophosphate

ATP - adenosine triphosphate

BSA - bovine serum albumin

Ca-NTA - NTA = nitrilotriacetic acid or triglycolamic acid

$$N\left[CH_2COO^-\right]_3$$

cAMP - Adenosine 3'5'- cyclic monophosphate

cGMP -cyclic Guanosine 3'5'monophosphate

EDTA - Ethylenediaminetetraacetic acid

EGTA - Ethyleneglycolbis(aminoethylether)-N,N'-tetraacetic acid

EHDP - Ethane-1-hydroxy-diphosphonic acid

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

Murexide - ammonium purpurate;5,5'-nitrilodibarbituric acid, ammonium derivative

MW - molecular weight

NAD(H) - nicotinamide-adenine dinucleotide (reduced)

NADP(H) - nicotinamide-adenine dinucleotide phosphate (reduced)

NEM - N-ethylmaleimide

PEP - phosphoenolpyruvate

POP - 2,5-diphenyloxazole

POPOP - 1,4,Bis(2-(4-Methyl 5 Phenyloxazolyl)Benzene)

SDBS - sodium dodecylbenzene sulphonate

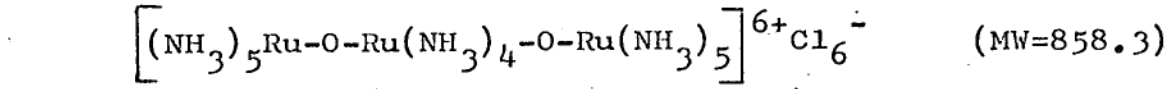
TCA - trichloroacetic acid

TRIS - Tris(hydroxymethyl)aminomethane

Appendix A

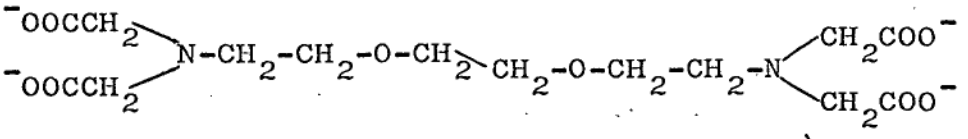
Chemical formulas

Ruthenium red

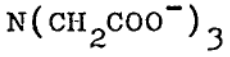


$$\left[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5\right]^{6+}Cl_6^- \qquad (MW=858.3)$$

EGTA (ethyleneglycolbis(aminoethylether)-N-N'-tetraacetic acid)

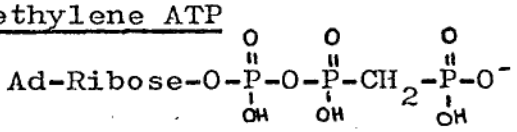


NTA (nitrilotriacetic acid or triglycolamic acid)

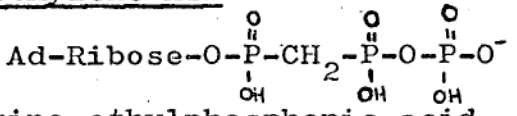


$$N(CH_2COO^-)_3$$

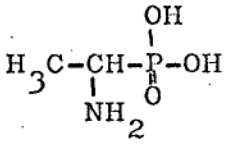
βγMethylene ATP



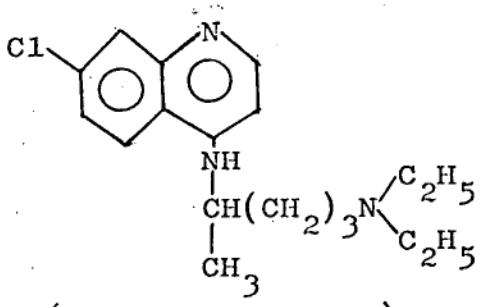
αβMethylene ATP



2-amino-ethylphosphonic acid

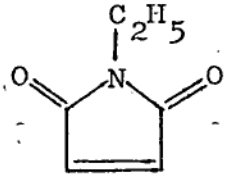


Chloroquine

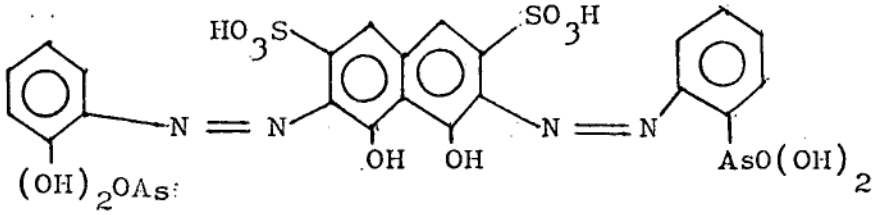


MW 320

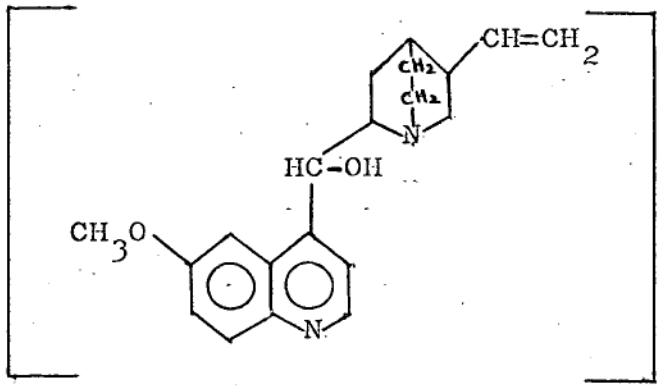
NEM (N-ethylmaleimide)



Arsenazo III



Quinidine sulphate

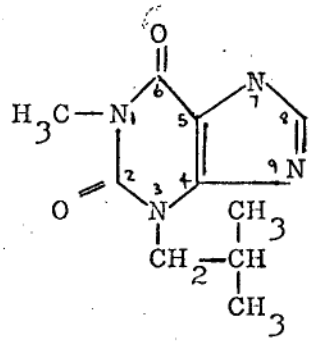


$H_2SO_4 \cdot 2H_2O$

$C_{40}H_{50}N_4O_8S \cdot 2H_2O$

(MW=783)

3-isobutyl-1-methylxanthin



INTRODUCTION

It is now well established that mitochondria from almost all vertebrate tissues such as liver, heart, kidney, and brain can accumulate Ca^{2+} by an energy dependent process (Carafoli and Lehninger, 1971). Apparently the process of Ca^{2+} accumulation in most higher plants and yeasts is not well developed (Bygrave, 1977; Chen and Lehninger, 1973; Moore and Bonner, 1977).

A range of divalent metal ions can be accumulated by the mitochondria. Such ions include (i) Ba^{2+} and Sr^{2+} (Carafoli, 1965; Vainio et al, 1970), (ii) Mn^{2+} (Bartley and Amoore, 1958; Chappell et al, 1962, 1963; Vainio et al, 1970; Gunter et al, 1975; Case, 1975), and (iii) Fe^{2+} (Flatmark and Romslo, 1975). Reports by Vainio et al (1970) and Carafoli (1965) have established that the specificity for bivalent ion transport in rat liver mitochondria is as follows:- $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+}$. Whether these ions are transported via a common carrier system is, however, unknown. According to Gunter et al (1978) the influx mechanism of Mn^{2+} and Ca^{2+} is similar but the efflux mechanism differs. While Mg^{2+} does not appear to be accumulated by liver mitochondria (Chappell et al, 1963; Klingenberg, 1963; Pressman and Park, 1963; Sallis et al, 1963; Carafoli, 1965; Selwyn et al, 1970; Reed and Bygrave, 1974(a)), it can accumulate in heart mitochondria (Brierley et al, 1963a, 1964, 1970).

Most of the studies on mitochondrial Ca^{2+} uptake involve the isolation of mitochondria and their subsequent incubation in a suitable medium. Techniques normally employed for *in vitro* studies of mitochondrial Ca^{2+} transport are:-

(a) Radioassay technique: This technique used radioactive calcium, $^{45}Ca^{2+}$, to monitor mitochondrial calcium transport. The mitochondria are then separated by rapid filtration or centrifugation and $^{45}Ca^{2+}$ is estimated in the mitochondria-free medium or the mitochondria themselves (Lehninger and Carafoli, 1967). The EGTA-ruthenium red quench technique developed by Reed and Bygrave (1974(a), 1975a) ensures discrimination of Ca^{2+} transported into mitochondria from that bound externally to the inner mitochondrial membrane.

(b) Spectrophotometry: The metallochromic indicator, murexide (ammonium purpurate), is used to monitor changes in absorbance at specific wavelengths when Ca^{2+} associates and dissociates from it (Mela and Chance, 1968; Chance, 1972 a; Sordahl, 1975). The indicator mixes very rapidly with Ca^{2+} and its high extinction coefficient makes the system very sensitive to changes in calcium concentration.

(c) Fluorimetry: This technique depends on the change in fluorescence intensity at excitation 400 nm and emission 520 nm wavelength when Ca^{2+} interacts with chlorotetracycline. The fluorescence is enhanced when the antibiotic chlorotetracycline chelates diamagnetic cations such as Mg^{2+} and Ca^{2+} (Schatz, 1969; Caswell and Hutchison, 1971; Schuster and Olson, 1974). Since the fluorescence of the chelator chlorotetracycline is polarity dependent, being higher in the non-aqueous solvents, one can also determine whether the cation is in the membrane or in the matrix of mitochondria (Caswell and Hutchison, 1971). However, recent findings by Luthra and Olson (1978) suggest that caution is needed in interpreting experimental results when chlorotetracycline is used, since they observed that the

chlorotetracycline induced Ca^{2+} release from previously loaded mitochondria.

(d) Calcium selective electrode: This technique was made possible through the development of ion exchangers that selectively extract Ca^{2+} from aqueous solution into an organic membrane phase with useful response to calcium ion activities (Ross, 1967). The specific calcium-electrode is used to monitor changes in Ca^{2+} concentration in the medium, resulting in a continuous recording of Ca^{2+} transport into and out of mitochondria (Johnson and Pressman, 1968; Truesdell and Pommer, 1963). This technique can be used to study the kinetics of Ca^{2+} transport (Madeira, 1975; Yamazaki, 1979) or Ca^{2+} cycling in mitochondria (Lehninger et al, 1978).

Techniques (a) to (d) are perhaps the more common techniques used to study mitochondrial Ca^{2+} transport <u>in vitro</u>. However, to investigate the nature of chemical bonding between Ca^{2+} and mitochondria or the state of hydration of the ion, magnetic resonance techniques are employed. Mn^{2+} is used as the paramagnetic analogue of Ca^{2+} and analysed by electron paramagnetic resonance (EPR) or nuclear magnetic resonance (NMR) (Chappell et al, 1963; Gunter et al, 1975; Case, 1975). This technique can also provide information on the environment inside the mitochondria where the ion is located.

With this background, the mechanism of mitochondrial Ca^{2+} uptake and release and its physiological implication is reviewed below.

I CALCIUM UPTAKE

The earliest report on Ca^{2+} accumulation by mitochondria was that of Slater and Cleland (1953). Since then, there have been numerous reports on Ca^{2+} transport into mitochondria, particularly on aspects of Ca^{2+} uptake. Excellent reviews on the Ca^{2+} transport process in mitochondria are available (Lehninger et al, 1967; Lehninger, 1970; Carafoli, 1974; Chance and Montal, 1971; Bygrave, 1977).

(a) Energy independent Ca^{2+} binding.

The first step in mitochondrial Ca^{2+} transport is energy independent binding of Ca^{2+} to the inner mitochondrial membrane. Results obtained by Reynafarje and Lehninger (1969) from Scatchard plots (i.e. Ca^{2+} bound (nmoles/mg protein)/ Ca^{2+} free, μM versus Ca^{2+} bound (nmoles/mg protein)) of Ca^{2+} binding to respiration-inhibited rat liver mitochondria indicated two classes of Ca^{2+} binding sites, i.e. a low affinity and a high affinity binding site.

The low affinity binding site is non-specific, also binding ions such as K^+, Mn^{2+}, Na^+, and at pH 7.4 binds approximately 40 nmoles Ca^{2+}/mg mitochondrial protein. The binding is rapid and independent of temperature in the range 0 - 30°C. The low affinity binding sites are possibly those associated with the functional groups of lipid and/or proteins of the inner mitochondrial membrane. Scarpa and Azzi (1968) using phospholipid depleted mitochondria showed that Ca^{2+} binding was reversibly lowered. They also found that the use of local anesthetics (e.g. Nupercaine or Pantocaine, which have been observed to bind and inhibit cation binding to

isolated phospholipid (Feinstein, 1964)) inhibited Rb^{+} and Ca^{2+} binding to submitochondrial particles, suggesting the membrane phospholipids as Ca^{2+}-binding sites. Hauser and Dawson (1967) showed that anionic forms of various phospholipids and gangliosides bind Ca^{2+} readily above pH 5.5, particularly phosphatidic acid and phosphatidyl inositol both of which occur in rat liver mitochondria.

According to Reynafarje and Lehninger (1969), the high affinity binding site for Ca^{2+} binds approximately 1 nmole Ca^{2+}/mg mitochondrial protein, and that binding is inhibited by 2,4-dinitrophenol and other uncouplers. Na^{+}, K^{+}, Mg^{2+} neither bind nor interfere with binding but Sr^{2+} and Mn^{2+} inhibit the binding competitively but with a lower affinity than Ca^{2+} for the high affinity binding sites. Reynafarje and Lehninger (1969) further suggest that high affinity Ca^{2+} binding reflects the binding of Ca^{2+} to the active site of calcium-carrier in the inner mitochondrial membrane, and is not simply due to the respiration coupled net transport of small amounts of Ca^{2+} into the mitochondrial matrix as suggested by Akerman et al, 1974; Southard, 1974; Reed and Bygrave, 1974.

A new approach developed by Tew (1977) has indicated two classes of Ca^{2+} binding sites on the mitochondria. The latter worker examined inhibition of respiration-independent Ca^{2+} binding and respiration-dependent Ca^{2+} uptake by lanthanide cations such as Lu^{3+}, Dy^{3+}, Eu^{3+}, Sm^{3+}, Nd^{3+} and Pr^{3+}. The results showed that lanthanide inhibition of respiration-independent Ca^{2+} binding is different from respiration-dependent Ca^{2+} uptake. In the absence of respiration, the

inhibition of Ca^{2+} binding by the lathanide cations increased in a linear manner with increasing ionic radius, possibly indicating a non-specific inhibition. However, in the presence of succinate as energy source, the best inhibitors of Ca^{2+} uptake by the mitochondria are those lanthanide cations with ionic radii similar to Ca^{2+}, suggesting that the binding site is specific for Ca^{2+}. The inhibition decreases as the ionic radius of the lanthanide ions becomes larger or smaller than Ca^{2+}.

Summarising, there are two energy independent Ca^{2+} binding sites, a low affinity binding site and a high affinity binding site. The low affinity binding sites are possibly associated with functional groups of lipid and/or protein constituents of the inner mitochondrial membrane, while the high affinity binding sites are presumably the active site of the Ca^{2+} specific carrier.

(b) Calcium carrier.

It is now a well accepted fact that the energy dependent transport of Ca^{2+} into mitochondria occurs via a specific carrier. The characteristics displayed by the calcium-carrier include:-

(i) high affinity for Ca^{2+}

(ii) sensitive to inhibitors of mitochondrial Ca^{2+} transport, such as ruthenium red (Moore, 1971; Vasington et al, 1972) and lathanium (Mela, 1969)

(iii) those of a low molecular weight, mobile, hydrophobic glycoprotein;

(iv) location in the inner mitochondrial membrane.

Several laboratories have attempted to isolate the calcium-

binding carrier protein from mitochondrial membrane.

For example, Gomez-Puyou et al (1972) extracted a calcium -binding protein from mitochondrial membranes by osmotic shock and further purified it by ammonium sulphate fractionation. The protein isolated was soluble, contained lipids and had a high affinity for Ca^{2+}.

Sottocasa et al (1971, 1972) also extracted a calcium-binding glycoprotein, presumably from the intermembrane space, by osmotic shock or exposure of mitochondria to chaotropic agents. The protein was then purified by preparative polyacrylamide gel electrophoresis.

Blondin (1974) managed to isolate a calcium-binding protein from mitochondria by tryptic digestion of mercurial-treated, lipid-depleted heart mitochondria and then extracted with a mixture of butanol and acetic acid. The properties of the protein isolated were similar to those of calcium ionophores X-537A and A23187. It exhibited a cation-proton exchange and induced the transfer of Mg^{2+} and Ca^{2+} across the inner mitochondrial membrane.

Recently, Jeng et al (1978) isolated a low molecular weight calcium-carrier from calf heart mitochondria. They extracted the protein from the inner mitochondrial membrane with potassium deoxycholate. The carrier named calciphorin has a molecular weight 3000. Using a Pressman cell, they showed that the protein can mediate Ca^{2+} transport through an organic phase. The pressman cell consists of two aqueous phases separated by a stirred organic phase. $^{45}Ca^{2+}$ was added to one of the aqueous phases and the protein was added to the organic phase. The non-radioactive aqueous side was then

assayed for the appearance of radioactivity. Jeng and colleagues observed that the Ca^{2+} translocation in the Pressman cell by calciphorin was selectively driven by a hydrogen ion gradient. The pH gradient from donor to receptor sides was 7.8 to 5.0 . In the absence of a pH gradient, i.e. when both sides were pH 7.8 or pH 5.0, no Ca^{2+} translocation was observed. When the pH gradient was reversed from pH 5.0 to pH 7.8, i.e. at the donor and receptor sides respectively, no Ca^{2+} translocation occurred over a period of 16 hours. Thus, the results suggest that Ca^{2+} movement via the protein carrier, calciphorin, requires a pH gradient, being acidic on the receptor side. The latter workers also observed that the inhibitors of mitochondrial Ca^{2+} transport namely ruthenium red and lanthanum inhibited the calciphorin mediated extraction of Ca^{2+} into the organic phase of the Pressman cell. A selectivity sequence of calciphorin determined from organic solvent extraction experiments for divalent cations was Ca^{2+}, $Sr^{2+} > Mn^{2+}$. The Scatchard plot of calcium binding data was biphasic indicating two classes of binding sites for Ca^{2+}. Since Tyson et al (1976) have shown that phospholipids may function as ionophores in Pressman cell assay, Jeng et al demonstrated that the Ca^{2+} transport properties observed with calciphorin are not due to contaminating phospholipids. Thus, the calcium carrier isolated by Jeng and colleagues seems to exhibit most of the characteristics of a calcium carrier which includes, high affinity for Ca^{2+}, sensitive to inhibitors of mitochondrial Ca^{2+} transport, low molecular weight, situated in the inner mitochondrial membrane, and that Ca^{2+} transport via the carrier is driven by a hydrogen ion gradient.

In order to demonstrate the necessity of the Ca^{2+}-binding protein carrier for Ca^{2+} transport, Panfili et al (1976) used an antibody which is specific to the calcium-binding glycoprotein. According to their data, a low concentration of the antibody appeared to block 45 % of the Ca^{2+} transport in rat liver mitoplast (i.e. mitochondria devoid of the outer membrane) without affecting the electron transport and respiratory control ratio.

An interesting experiment using the isolated calcium-binding glycoprotein was performed by Prestipino et al in 1974. They reconstituted the transport of Ca^{2+} in an artificial lipid bilayer system consisting of a solution of purified egg lecithin in n-decane. Addition of the glycoprotein isolated from mitochondria to the lipid bilayer, decreased the electrical resistance only in the presence of Ca^{2+}, and was sensitive to ruthenium red. The result was, however, not observed in the presence of a variety of commercially available glycoproteins indicating that the response observed is specific to the isolated mitochondrial glycoprotein.

At this point it is worth mentioning the ~~possible~~ existence of a specific Na^{+}/Ca^{2+} carrier in heart mitochondria (Carafoli and Crompton, 1978). The Na^{+} induced Ca^{2+} efflux from heart mitochondria via this carrier is inhibited by La^{3+}. The carrier presumably also catalysed the exchange of extra- and intra-mitochondrial Ca^{2+} in the absence of Na^{+} and in the presence of ruthenium red. Externally added Ca^{2+} inhibits the Na^{+} induced Ca^{2+} release and Na^{+} inhibits the Ca^{2+}/Ca^{2+} exchange (Crompton et al, 1977). Apparently, the Ca^{2+}/Ca^{2+}

exchange is insensitive to ruthenium red but sensitive to La^{3+} (Carafoli and Crompton, 1978).

Thus, several laboratories have reported the existence of a calcium-specific carrier in mitochondrial membrane and its necessity for mitochondrial Ca^{2+} transport. However it is not known for certain whether the calcium-binding protein is a mobile transmembrane carrier or whether it may act as a superficial specific Ca^{2+} receptor. Studies on intra-mitochondrial location of calcium-binding protein have revealed a rather homogenous distribution of the compound throughout the mitochondrial compartments with the exception of the matrix space (Sandri et al, 1974). In a later paper, Sandri et al (1976) presented data showing that the amount of glycoprotein bound to the inner mitochondrial membrane is dependent upon the existence of Ca^{2+} in the medium and is increased by Ca^{2+} transport, suggesting a mobile carrier. However, evidence presented by Carafoli et al (1977) and Carafoli (1975(a)) seemed to suggest that the calcium-binding protein may act as a superficial Ca^{2+} receptor and not as a transmembrane calcium-carrier. The kinetic parameter obtained by Carafoli (1975(a)) could well be accounted for by a specific superficial receptor.

While it is generally accepted that transport of Ca^{2+} into mitochondria is via a carrier, the exact molecular mechanism of Ca^{2+} transport into mitochondria is still uncertain. Nevertheless there is considerable evidence in favour of the uniport mechanism i.e. Ca^{2+} transported into mitochondria via a simple facilitated diffusion with a net charge transfer of 2 for each Ca^{2+} transported (Lehninger, 1974; Lehninger and Brand, 1976; Selwyn et al, 1970; Rottenberg and Scarpa, 1974; Reynafarje and Lehninger, 1977; Vercesi et al, 1978, Akerman, 1978(a)). Reynafarje and Lehninger, 1977, measured simultaneously Ca^{2+} uptake using a Ca^{2+}-sensitive electrode and H^+ movement using a pH glass electrode to determine the no. of H^+ ejected in electro-

neutral exchange for Ca^{2+}. They reported that H^+/Ca^{2+} ratio was very close to 2 when phosphate movements were prevented. Their result also indicated close to $4H^+$/energy conserving site of the respiratory chain in contrast to $2H^+$/site as postulated by the chemiosmotic hypothesis. Akerman (1978a) determined K^+ efflux per calcium influx stoichiometry in the presence of valinomycin and observed $2K^+$ extruded per calcium uptake by the mitochondria i.e. indicating 2 net positive charges per calcium uptake. It is also worth mentioning that the latter worker observed a net transfer of 1 charge per calcium translocated measured during nitriloacetate induced calcium efflux from de-energised mitochondria. This would suggest that Ca^{2+} efflux might be mediated by a different mechanism which would possibly involve H^+/Ca^{2+} exchange. It is also of interest to mention that Carafoli et al (1967) had indicated that during Ca^{2+} transport into mitochondria in the absence of a permeant anion or during transport of small amounts of Ca^{2+}, the Ca^{2+}/site stoichiometry during respiration was higher than 2.0. The effect is termed super-stoichiometry. Since the rapid superstoichiometric Ca^{2+} uptake was reduced in the presence of oligomycin (inhibitor of mitochondrial ATPase), Reynafarje and Lehninger (1974) suggested endogenous ATP as the primary energy source for superstoichiometric Ca^{2+} uptake.

Alternative mechanisms for Ca^{2+} uptake have been proposed which include :-

(i) Symport mechanism : Moyle and Mitchell (1977a) estimated H^+/Ca^{2+} ratio and the results obtained suggested that Ca is transported into mitochondria with only 1 net positive charge. They proposed that this could result from $(Ca_2)^{4+}$-HPO_4^{2-} or Ca-β-hydroxybutyrate symporter or both (Moyle and Mitchell, 1977 a,b). However, Reynafarje and Lehninger, 1974 determined H^+/Ca^{2+} ratio under conditions of Moyle and Mitchell and reported H^+/Ca^{2+} ratio close to 2. The former

workers measured (rather than assumed) the actual amounts of Ca^{2+} released and taken up by the mitochondria.

(ii) Ca^{2+}/H^{+} antiport mechanism whereby the Ca^{2+} carrier catalyses a partially charge-compensated movement of Ca^{2+} by exchange between Ca^{2+} and H^{+} (Reed and Bygrave, 1975). The mechanism was proposed based on the observation that the initial rates of Ca^{2+} uptake were stimulated by Pi (i.e. Pi act indirectly by lowering the intramitochondrial pH during Ca^{2+} transport).

(C) Energy source for calcium transport

Energy for Ca^{2+} transport into mitochondria may be derived from ATP hydrolysis (Fanburg and Gergely, 1965; Bielawski and Lehninger, 1966, Spencer and Bygrave, 1973) or from oxidation of respiratory substrates (Chance, 1965a; Rossi and Lehninger, 1964). Ca^{2+} accumulation into mitochondria with ATP as the energy source is inhibited by oligomycin but not by respiratory inhibitors such as antimycin A. With respiratory substrate as the energy source, Ca^{2+} uptake by the mitochondria is inhibited by respiratory inhibitors but not oligomycin.

It is interesting to note that mitochondrial Ca^{2+} transport utilises endogenous ATP preferentially, depleting it before using the energy from added respiratory substrates such as succinate (Brand and Lehninger, 1975). Apparently the rate of endogenous ATP-supported Ca^{2+} uptake is 2-3 times greater than the maximum rate of Ca^{2+} uptake supported by electron transport (Reynafarje and Lehninger, 1974). Also, in the presence of externally added Ca^{2+} and ADP, mitochondria transports the Ca^{2+} into mitochondria prior to oxidatively phosphorylating the ADP (Rossi and Lehninger, 1964; Lehninger, 1970). This suggests the importance of Ca^{2+} transport in

mitochondria.

Generally ADP competes with ATP for translocation into mitochondria via the adenine nucleotide translocase. In the presence of Ca^{2+}, Spencer and Bygrave (1972) observed that more ATP is transported into the mitochondria. A possible explanation for this is that ATP is transported into mitochondria via an alternative pathway called ATP-ADP-Phosphate pathway. This pathway, proposed recently by Reynafarje and Lehninger (1978) allows inward movement of added ATP into mitochondria in exchange for mitochondrial ADP and inorganic phosphate described by :-

ATP(out) in exchange for ADP(in) + 0.5 phosphate (in). This exchange system is insensitive to N-ethylmaleimide or mersalyl (inhibitors of Pi/OH^- exchange), is inhibited by atractyloside (an inhibitor of adenine nucleotide translocase) and does not appear to promote the exchange of ADP and phosphate in the medium for mitochondrial ATP. The ATP-ADP-phosphate exchange system differs from the Pi/OH exchange system and Pi/dicarboxylate carrier system which are inhibited by mersalyl, and also from the classical ATP/ADP translocase system which is not known to transport phosphate and generally provides entry of ADP and exit of ATP during oxidative phosphorylation. Thus, the ATP-ADP-phosphate pathway presumably allows ATP consuming processes in mitochondria, such as Ca^{2+} accumulation into mitochondria, to take place using the energy obtained from ATP hydrolysis.

The nature of the driving force for Ca^{2+} transport is associated with the mechanism of energy transduction in mitochondria. Most likely the membrane potential is the driving force for mitochondrial Ca^{2+} transport. Scarpa and Azzone (1970) showed that in the presence of metabolic inhibitors, the outward movement of endogenous K^+ from

mitochondria induced by valinomycin can be used to accumulate Ca^{2+} into mitochondria. This has been taken as an indication that Ca^{2+} uptake occurs in response to a membrane potential. Further, swelling experiments performed by Selwyn et al (1970) showed that under de-energized conditions (i.e. in the absence of energy source for Ca^{2+} uptake), the uptake of Ca^{2+} was either by a simple facilitated diffusion mechanism (uniport) or through an electrogenic Ca^{2+} for K^{+} exchange system (antiport), either of which could be driven by a membrane potential. Recent findings by Roos et al (1978) have suggested that the phosphoenolypyruvate induced Ca^{2+} release from mitochondria caused a decrease in mitochondrial membrane potential from 120 mV to 35 mV. Other experimental evidence to show that Ca^{2+} transport in mitochondria is an electrophoretic process driven by membrane potential includes studies by Rottenberg and Scarpa (1974), Heaton and Nicholls (1976), and Lehninger (1974).

Assuming a membrane potential, negative inside of between 160-200 mV in energised mitochondria (Mitchell and Moyle, 1969; Nicholls, 1974) and that the electrophoretic Uniporter transfers 2 charges, one would expect the gradient of Ca concentration between the mitochondrial matrix and the medium to range from 10^5-10^6 (i.e. according to Nernst equation). Indirect estimates from assays of Ca-sensitive enzymes in mitochondrial matrix and cytosol indicated that the gradient may have a value $\leq 10^3$ (Brostrom et al, 1971; Denton et al, 1978). Although it should be noted that the exact value of the gradient of Ca concentration across the mitochondrial membrane is difficult to measure because the concentration of free Ca^{2+} in the matrix is uncertain. Since intramitochondrial Ca^{2+} is probably 10^{-4}M - 10^{-5}M (Rasmussen et al, 1975) and if Nernstian

equilibrium would be reached against -180 mV, the cytosolic free Ca^{2+} would be lowered to an improbable concentration of 10^{-10}-10^{-11}M (c.f. estimated value of 10^{-6}M). The deviation from the Nernstian equilibrium can now be explained by the existence of an independent efflux pathway in addition to the uniporter. The aspect on efflux pathway will be discussed in part II of the Introduction.

Summarising, Ca^{2+} transport in mitochondria is an electrophoretic process in response to membrane potential, negative inside that is generated across the inner membrane as a result of respiratory chain or ATPase activity (Bygrave, 1977).

(d) Permeant anions

The presence of a permeant anion seems to be prerequisite for mitochondrial Ca^{2+} transport. However not all penetrant anions can support Ca^{2+} uptake by mitochondria. Only those which have the ability to donate protons to mitochondrial matrix can stimulate Ca^{2+} transport (Lehninger, 1974). Little or no Ca^{2+} is accumulated in the mitochondrial matrix in the presence of permeant anions such as NO_3^-, SCN^-, ClO_3^- and ClO_4^-. Anions such as acetate, propionate, butyrate and β-hydroxybutyrate pass through the inner mitochondrial membrane in protonated form and these anions can support Ca^{2+} uptake. Elder and Lehninger (1973) reported that bicarbonate can be used as the permeant anion for Ca^{2+} transport. The penetrating species is either HCO_3^- (Selwyn and Walker, 1977) or dissolved CO_2 (Elder and Lehninger, 1973) which will yield CO_3^- in the matrix through the action of mitochondrial carbonic anhydrase. As a result $CaCO_3$ accumulates in the matrix (Elder and

Lehninger, 1973).

The permeant anion which is commonly used for in vitro Ca^{2+} uptake by the mitochondria is phosphate. Mitochondria from mammalian tissues possess 2 phosphate transport systems namely Pi/OH^- transport system (via a phosphate carrier) and Pi/dicarboxylate transport system (via dicarboxylate carrier) (Chappell and Crofts, 1966; Chappell and Haarhoff, 1967). Kinetic studies by Coty and Pedersen (1974) showed that the Pi-carrier is the major system involved in Pi transport. Substrates for the Pi-carrier also include arsenate and monofluoro derivatives of phosphate (Frietag and Kadenbach, 1978; Kadenbach et al, 1978). The carrier is inhibited by various reagents that bind to -SH groups (mercurial, maleimides) suggesting that a sulfhydryl group is probably involved in the catalysis of Pi transport. Phosphate exists in a nearly 1:1 mixture of HPO_4^{2-} and $H_2PO_4^-$ at pH 7.0 . Frietag and Kadenbach (1978) showed that monofluoro phosphate but not the difluoro derivative is transported on the phosphate carrier suggesting that the divalent phosphate (i.e. HPO_4^{2-}) is the ionic species that binds to the carrier and that there are 2 separate proton binding sites on the carrier which must be filled before electroneutral transport can occur (LaNoue and Schoolwerth, 1979). Lehninger (1974) suggested that Pi moved into the mitochondrial matrix in response to an electrochemical gradient of protons generated across the mitochondrial membrane by electron transport. This results in a membrane potential with a negative inside anion gradient and Lehninger postulated that this gradient is possibly the pulling force for Ca^{2+} influx into the mitochondria. He further suggested

that the inward transport of anions presumably precedes and is required for the inward transport of Ca^{2+}.

The mitochondrial preparation normally contain contaminating phosphate. Therefore in order to show the necessity of permeant anions for Ca^{2+} uptake, it is essential to remove or block entry of phosphate and also to prevent internal generation of Pi (Harris and Zaba, 1977). In the presence of 80 μM mersalyl (which inhibits movement of residual endogenous phosphate) and oligomycin (5 μg/mg mitochondrial protein), i.e. to prevent generation of Pi via ATPase, the initial rate and capacity of Ca^{2+} accumulation into the mitochondria is reduced significantly. The inhibition is relieved on adding 2 mM Clelands reagent which regenerates SH-groups and allows endogenous Pi to penetrate into mitochondria and Ca^{2+} uptake is accelerated on providing more Pi (Harris and Zaba, 1977).

Schuster and Olson (1974) reported the necessity of a permeant anion for Ca^{2+} movement into the mitochondrial matrix. A fluorescent chelate probe, chlorotetracycline was used to monitor the association of Ca^{2+} with the mitochondrial membrane (Note: earlier Caswell and Hutchison (1971) observed that the fluorescence of the chlorotetracycline-metal complex is polarity dependent being higher in a non-aqueous environment). In the presence of succinate as the energy source for Ca^{2+} uptake by the mitochondria but in the absence of permeant anion, an increase in chlorotetracycline-associated fluorescence was observed, i.e. an indication of uptake of exogenous Ca^{2+} by mitochondrial membrane. However addition of permeant anion such as Pi in the incubation medium

causes a diminished fluorescence, even though maximal uptake of Ca^{2+} was observed in the presence of Pi when determined using radioassay technique. Thus movement of Ca^{2+} into the matrix appeared to be dependent on a permeant anion.

(e) Kinetics of calcium uptake.

In order to understand the mechanism and role of mitochondrial calcium transport under physiological condition, it is also necessary to study the kinetics of calcium movement in and out of mitochondria *in vitro*. The measurement of the initial rate of calcium uptake requires the estimation of calcium in mitochondria within a short span of time, thus an effective and rapid technique of calcium estimation is a necessity. The earlier techniques employed include the indirect measurements of the rate of oxygen uptake or H^+ release during mitochondrial accumulation (Chance, 1965(b)) or measurement of the shift in the redox state of cytochrome b or flavoprotein accompanying calcium accumulation in mitochondria (Chance and Schoener, 1966; Chance, 1972(b); Carafoli and Azzi, 1971). However these techniques are not reliable. Direct and possibly more reliable techniques for the kinetic study of mitochondrial calcium transport include:-

(a) using a calcium indicator such as murexide or arsenazo III and estimating the change in absorbance on a dual wavelength spectrophotometer in order to minimise non-specific absorbance changes. A stopped flow apparatus which enables rapid mixing of reactants has also been used (Vinogradov and Scarpa, 1973).

(b) using a calcium ion specific electrode (Yamazaki et al, 1979).

(c) employing the radioassay technique in which a ruthenium red/EGTA quenching medium is used, thus distinguishing the Ca^{2+} transported into mitochondria from that bound externally to the inner mitochondrial membrane. Accurately known concentrations of free $^{45}Ca^{2+}$ are generated with Ca^{2+}/nitrilotriacetic acid buffers for the determination of substrate/velocity relationships (Reed and Bygrave, 1975(b)).

A sigmoidal relationship between initial rate of calcium uptake and external Ca^{2+} concentration has been reported for rat liver mitochondria (concentration range 0 - 150 μM) (Vinogradov and Scarpa, 1973; Reed and Bygrave, 1975b) and also heart mitochondria (Ca^{2+} concentration 23 - 218 μM) (Noack and Heinen, 1977). The sigmoidicity observed indicate co-operativity in mitochondrial Ca^{2+} transport (i.e. the existence of more than one binding site for the Ca^{2+} on the carrier and that binding of Ca^{2+} to one binding site facilitates the binding of Ca^{2+} to the subsequent binding site). It is worth noting however that Akerman et al (1977) observed that the plot of initial rate of mitochondrial Ca^{2+} transport versus concentration in a sucrose medium is hyperbolic (in concentration range 5 - 100 μM). In the presence of Mg^{2+} the plot becomes sigmoidal and the effect is enhanced when both Mg^{2+} and K^{+} are present. They further suggested that surface potential which depends on ions such as K^{+} and Mg^{2+} bound to the low affinity sites determines whether the kinetics of Ca^{2+} uptake in mitochondria is sigmoidal or hyperbolic. Also, Williams and Barrie (1978) observed that temperature has an effect on the kinetics of calcium transport by rabbit cardiac mitochondria. At 10°C, the initial rate of calcium transport is a sigmoidal function of free calcium concentration, whereas at 25°C , the sigmoidal relationship is reduced and the Hill coefficient lowered from 1.9 (at 10°C) to 1.3 (25°C).

The K_m values for mitochondrial Ca^{2+} transport (i.e. the concentration of calcium for which half the maximal rate of calcium transport occurred) observed by Reed and Bygrave (1975b) was 4 μM Ca^{2+} at 0°C and pH 7.4 with a Hill coefficient of 1.7. Recently Yamazaki (1979) using a calcium ion sensitive electrode reported a K_m value of 6.5 μM at 25°C and a Hill coefficient = 2.3. Heaton and Nicholls (1976) obtained a K_m value of 4.7 μM at 23°C and pH 7.2 using the divalent cation ionophore A23187 to study the kinetics of energy-dependent calcium uptake by rat liver mitochondria under steady state conditions. Thus, the K_m values obtained by the mentioned researchers are low, infact near physiological cytosolic free calcium concentration. In contrast, Vinogradov and Scarpa (1973) obtained a high K_m value, i.e. between 50 to 60 μM but this may have been due to the presence of inhibitory cations such as K^+ and Mg^{2+} (Hutson et al, 1976) which compete with Ca^{2+} for low affinity binding site.

Useful information may be obtained from kinetic studies of the mitochondrial calcium transport such as the existence of more than one binding site for Ca^{2+} on the carrier (possibly 2 binding sites as indicated by the Hill coefficient value (Koshland, 1970). The rapid rate of calcium uptake is possibly an indication that the mitochondria may play an important role in regulating cell Ca^{2+} by rapidly removing Ca^{2+} from the cytosol whenever there is a sudden increase in the concentration.

(f) Limited and Massive Calcium Loading

There is a need to differentiate between 2 types of in vitro calcium loading in mitochondria, i.e. limited loading and massive loading. Limited loading refers to calcium accumulation in mitochondria

(<100 nmoles Ca^{2+} per mg mitochondrial protein) whereby there is no functional damage to mitochondria and normal respiratory control and phosphorylation efficiency is maintained (Rossi and Lehninger, 1964). In massive calcium loading, mitochondria are exposed to greater than 100 nmoles Ca^{2+} per mg mitochondrial protein, resulting in swelling, membrane damage and organelle lysis (Lehninger et al, 1967; Chappell and Crofts, 1965; Hackenbrook and Caplan, 1969). However it is worth mentioning that the capacity of calcium accumulation in mitochondria of soft tissues is different from that of chondrocytes. Calcium content in chondrocyte mitochondria is over 200 times greater than values reported for mitochondria of cells of non-mineralising tissues (Carafoli and Lehninger, 1971; Shapiro and Lee, 1975). According to Lee and Shapiro (1978) up to 350 nmoles Ca per mg chondrocyte mitochondria performed coupled oxidative phosphorylation. Matthews et al (1970) also observed little evidence of pathlogical damage in chondrocyte mitochondria containing very high concentrations of calcium. It is of interest to note that tumour mitochondria are able to tolerate high concentration of Ca (Mc Intyre and Bygrave, 1974) and that the massive Ca loading did not result in uncoupling of oxidative phosphorylation in these mitochondria (Bygrave 1976b).

When mitochondria accumulate large amounts of calcium with phosphate, the solubility product of calcium phosphate is exceeded and electron dense granules of calcium phosphate form in the mitochondrial matrix adjacent to or on the cristae (Greenawalt et al, 1964). These granules have been observed in the matrix of mitochondria of intact animal tissues particularly in bone forming or bone lysing tissues.

There is also evidence from electron microscopy and electron microprobe analysis that calcium phosphate granules may normally be present in mitochondria of many animal tissues in vivo. According to Posner (Lehninger's personal communication), the X-ray diffraction analysis of rat liver mitochondria which is massively loaded with calcium showed that the granules are non-diffracting and therefore amorphous. Apparently calcium phosphate in mitochondria are always amorphous and appear to be stabilized in this form. Presumably mitochondria of bone are the site of the respiration coupled formation of amorphous $Ca_3(PO_4)_2$ which can pass from mitochondria through cytosol to the extracellular space where it is proposed it serves as precursor of hydroxyapatite in the collagen matrix of bone (Lehninger, 1970). Lehninger et al (1978(a)) suggested that phosphocitrate may act as an inhibitor of hydroxyapatite formation and that it also stabilises amorphous $Ca_3(PO_4)_2$ in mitochondria. Recently, Williams and Sallis of the Biochemistry department, University of Tasmania (1979) have successfully synthesised phosphocitrate. ^{31}P-NMR, ^{14}C-NMR and proton NMR spectral interpretations confirm the identity as phosphocitrate and they have shown that indeed, phosphocitrate is a very powerful inhibitor of the ACP (amorphous Ca-Pi) $\longrightarrow$ Hydroxyapatite transformations. They also managed to isolate phosphocitrate from rat kidney and liver and most likely it is confined to the mitochondria at concentration 1 ng/mg mitochondrial protein. The properties of the isolated phosphocitrate seemed to agree with the synthesized phosphocitrate.

II CALCIUM RELEASE

Little is known about the mechanism of Ca^{2+} release from mitochondria, for example, what regulates Ca^{2+} release, whether it is via a carrier or whether the process is energy dependent. The available information is discussed below under the following sub-headings :

a) Efflux pathway and Ca^{2+} cycling.

b) carrier for calcium release.

c) energy for calcium release.

d) kinetics of calcium release.

e) substances inducing mitochondrial calcium release.

(a) Efflux pathway and Ca^{2+} cycling

There is considerable evidence to indicate that the efflux of Ca^{2+} from mitochondria differs from the influx mechanism. For example :-

(1) Peng (1977) observed that ruthenium red blocked Ca^{2+} uptake and H^{+} production in heart mitochondria but did not prevent dinitrophenol induced efflux of Ca^{2+}. On the other hand, bongkrekic acid, a potent inhibitor of mitochondrial adenine nucleotide translocase (Henderson and Lardy, 1970), inhibited dinitrophenol-induced Ca^{2+} efflux but did not inhibit Ca^{2+} uptake.

(2) Crompton et al (1976) found that the Ca^{2+} influx system in heart mitochondria was inhibited by ruthenium red, however the efflux system required Na^{+} and that ruthenium red promoted the Na^{+}-induced Ca^{2+} efflux from heart mitochondria.

(3) Puskin et al (1976) used Mn^{2+} as a paramagnetic Ca^{2+} analogue. EPR measurements of free Mn^{2+} concentration gradient were made on valinomycin-treated mitochondria and compared with K^{+} and Rb^{+} gradients measured on the same samples. Evidence from these studies suggests that the energy linked uptake of Mn^{2+} deviates from the equilibrium predicted by the Nernst equation. Thus, this evidence favours an inward mitochondrial Ca^{2+} movement in response to an electrochemical activity gradient, and also of another system that can transport ions outward against an activity gradient.

(4) Caroni et al (1978) observed that when rat liver mitochondria were incubated with the uncoupler, FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone) and ruthenium red (inhibitor of Ca^{2+} uptake), Ca^{2+} uptake remained blocked but Ca^{2+} release occurred normally suggesting separate pathways for Ca^{2+} uptake and release in the rat liver mitochondria.

Drahota et al (1965) first suggested that the accumulation of Ca^{2+} by mitochondria might be in a dynamic steady state in which efflux of Ca^{2+} from mitochondria is counterbalanced by Ca^{2+} influx. Reports by Stucki and Ineichen (1974) on energy dissipation in mitochondria by Ca^{2+} recycling and that by Grist and Baum (1974) on halothane-dependent cycle flux of Ca^{2+}, provide further evidence for Ca^{2+} cycling. If Ca^{2+} uptake and release from the mitochondria is via the electrophoretic uniporter only then there would not be any cycling. Therefore an essential proof for Ca^{2+} cycling is the existence of a separate pathway for Ca^{2+} uptake and release (Carafoli, 1979 ; also see earlier discussion on separate pathway).

The efflux pathway simultaneously transport Ca^{2+} in the opposite direction to the Ca influx pathway, resulting in Ca cycling across the inner mitochondrial membrane.

Although Ca^{2+} cycling imposes a slight energy dissipation in the mitochondria (Stucki and Ineichen, 1974), it has its advantages. By analogy with the substrate-cycle concept of Newsholme and Crabtree (1976), the significance of Ca^{2+} cycling includes the following (Bygrave 1978).

(i) the ability to enhance or retard net flux is greatly facilitated e.g. a net inward flux of Ca^{2+} into the mitochondria would result not only from stimulation of the influx component but also from simultaneous inhibition of the efflux component.

(ii) increase sensitivity of Ca-sensitive reactions.

(b) Carrier for calcium release

Apparently, the release of Ca^{2+} from mitochondria is also via a glycoprotein carrier (Sandri et al, 1976). The latter workers observed that the mitochondria were unable to release Ca^{2+} in the presence of the uncoupler pentachlorophenol when this particular calcium-binding glycoprotein was detached from the mitochondria. It is however not known whether the release of Ca^{2+} from mitochondria is via the influx glycoprotein carrier but a different active site, or via a different carrier altogether.

(c) Energy for calcium release

Evidence presented by Gunter et al (1978) suggests that the release of mitochondrial Ca^{2+} is an energy dependent process. They observed that the release of Ca^{2+} from mitochondria is

retarded in the presence of low concentrations of the uncoupler CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) and EGTA (used to block Ca^{2+} influx). In addition, studies on Na^{+}-induced efflux of Ca^{2+} from heart mitochondria (Carafoli et al, 1974; Crompton et al, 1976; Carafoli and Crompton, 1978) have also revealed that the release of Ca^{2+} requires energy. They observed that, on adding respiratory inhibitors such as antimycin A to Ca^{2+} preloaded mitochondria, the release of Ca^{2+} is slow. However Na^{+} accelerates the release and is linked to the efflux rate by the same sigmoidal dependence on concentration as in the presence of energy. They also noted that the maximal rate of Ca^{2+} release by de-energized mitochondria is only between 0.08 and 0.1 nmole/mg mitochondrial protein per sec, i.e. 2.5 to 3 times slower than in energized mitochondria, further implying that energy is required for Ca^{2+} release from mitochondria.

(d) Kinetics of calcium release.

Although there have been extensive studies on the kinetics of mitochondrial Ca^{2+} uptake, little is known about the rate of Ca^{2+} release from mitochondria. Carafoli (1975(b)) has obtained data suggesting that the rate of release is proportional to the amount of Ca^{2+} present in the mitochondria (first-order kinetics). The fact that the exact concentration of internal free Ca^{2+} concentration in the mitochondria is not measureable is one of the main problems faced in the kinetic study of mitochondrial Ca^{2+} release.

(e) Substances inducing mitochondrial calcium release.

Substances that would dissipate the energized state in

mitochondria seemed to induce Ca^{2+} release from mitochondria, e.g. respiratory inhibitors when Ca^{2+} accumulation is supported by oxidation of respiratory substrates (Drahota et al, 1965), uncouplers of oxidative phosphorylation such as dinitrophenol (Drahota et al, 1965), carbonylcyanide m-chlorophenylhydrozone CCCP (Gunter et al, 1978).

Several physiological and non-physiological substances are known to induce Ca^{2+} release from mitochondria when examined in vitro. Chudapongse and Haugaard (1973) showed that phosphoenolpyruvate (PEP) at a concentration as low as 0.1 mM caused Ca^{2+} release from liver and heart mitochondria respiring in the presence of glutamate or pyruvate plus malate. Apparently PEP had little or no effect on the initial rate of Ca^{2+} uptake (Chudapongse and Haugaard, 1973). The effect of PEP on Ca^{2+} release from mitochondria is dependent on the presence of phosphate since Peng (1974) observed that, in the absence of phosphate, even 6 mM PEP did not induce Ca^{2+} release. Chudapongse and Haugaard (1973) also showed that the PEP effect was inhibited in the presence of ATP or atractylate. Bongkrekic acid, a potent inhibitor of mitochondrial adenine nucleotide translocase (Henderson and Lardy, 1970; Erdelt et al, 1972; Klingenberg and Buchholz, 1973) also seemed to inhibit PEP-induced Ca^{2+} efflux. The fact that ATP, atractylate and bongkrekic acid all inhibited the PEP affect suggests that adenine nucleotide translocase is involved. Apparently the action of PEP is dependent on its transport via adenine nucleotide translocase specifically, and not dependent on its penetration into the mitochondrial matrix per se, since benzene-1,2,3-tricarboxylate, an

inhibitor of the tricarboxylate carrier did not inhibit PEP-induced Ca^{2+} release (Roos et al, 1978). Presumably the essential factor of PEP-induced Ca^{2+} release is the removal of endogenous ATP by exchange with PEP. (Note: PEP is transported by both adenine nucleotide carrier and tricarboxylate carrier of rat liver mitochondria (Shug and Shrago, 1973; Robinson, 1971)). Recently Roos et al (1978) showed that the PEP-induced Ca^{2+} release from rat liver mitochondria is associated with a lowering of the electrical potential difference across the inner mitochondrial membrane as determined from the distribution of ^{86}Rb across mitochondrial membrane in the presence of valinomycin.

Sodium has been found also to cause Ca^{2+} release from mitochondria isolated from heart, bovine adrenal cortex and rat brain (Carafoli et al, 1974; Crompton et al, 1976) but not mitochondria from rat liver, rat kidney, rat lung, bovine uterus muscle or rabbit ileum muscle (Crompton et al, 1978). The rate of Ca^{2+} efflux was found to be linked to the concentration of added Na^+ by a sigmoidal relationship at 25°C. Li^+ also seemed to induce Ca^{2+} efflux, but the effect was not as significant as with Na^+ (Crompton et al, 1976). Ca^{2+} release induced by Na^+ is not complete unless ruthenium red is present. However, after maximal Ca^{2+} uptake, ruthenium red on its own did not cause Ca^{2+} efflux, implying that the Ca^{2+} efflux system requires Na^+ and that ruthenium red promotes the Na^+-induced Ca^{2+} efflux from heart mitochondria. Presumably Ca^{2+} efflux in heart mitochondria is via a Na^+/Ca^{2+} exchange system.

In the chick small intestine where there is massive

movement of Ca^{2+}, Hamilton and Holdsworth (1975) showed that calcium-binding protein isolated from chick duodena caused Ca^{2+} release from intestinal mitochondria.

Asimakis and Sordahl (1977) observed that palmitoyl CoA at concentration as low as 3.3 μM caused Ca^{2+} release from rabbit heart mitochondria. Exogenous ATP and carnitine can prevent this releasing effect. They suggested that atractylate and palmitoyl CoA acted similarly in promoting Ca^{2+} efflux from mitochondria, and that their mechanism of action is different from that of classical uncouplers of oxidative phosphorylation and respiratory chain inhibitors. The latter workers also suggested that palmitoyl CoA may act at a site on or near adenine nucleotide translocase. Besides inducing an earlier Ca^{2+} release, palmitoyl CoA also inhibits Ca^{2+} uptake into mitochondria (Asimakis and Sordahl, 1977).

According to Lehninger et al (1978), the oxidation-reduction status of mitochondrial pyridine nucleotides can regulate Ca^{2+} release and retention in mitochondria of rat heart muscle, liver and also Erlich tumour cells. The mitochondrial $NAD^+/NADH$ ratio was lowered by such substrates as β-hydroxybutyrate or raised by such substrates as oxaloacetate or acetoacetate. They observed that, when the mitochondrial pyridine nucleotides are kept in a relatively reduced steady state, this resulted in Ca^{2+} retention while a more oxidised steady state of the pyridine nucleotides caused Ca^{2+} release from mitochondria. Similar results were obtained when using succinate or ascorbate-TMPD as an energy source for mitochondrial Ca^{2+} uptake, or when energy was derived from ATP hydrolysis. Apparently, the oxidation-reduction state of

mitochondrial pyridine nucleotides may be specific for controlling release and retention of Ca^{2+} because Sr^{2+} and Mn^{2+} are not released from previously loaded mitochondria under oxidised steady state. Lehninger et al (1978) managed to obtain cycles of Ca^{2+} release and reuptake in mitochondria on adding alternately 5 mM β-hydroxybutyrate and 0.5 mM oxaloacetate. However it is not known whether NAD^+ or $NADP^+$ is more important in regulation of Ca^{2+} retention and release in mitochondria. Lehninger et al (1978) suggested that Ca-binding to mitochondrial pyridine nucleotides may act as modulator of Ca^{2+} efflux and influx carrier protein. Earlier, Vinogradov et al (1972) had proposed the formation of a Ca-NADH complex in the non-polar region of mitochondrial membrane based on fluorometric observations.

Another physiological substance which appears to induce Ca^{2+} release from isolated rat liver mitochondria is prostaglandin E_1 (Carafoli and Crovetti, 1973; Malstrom and Carafoli, 1975). However a high concentration of prostaglandin E_1 (10 μM) is required to induce release; at this concentration, prostaglandin E_1 uncouples mitochondrial respiration, but only when prostaglandin E_1 is added to mitochondria after Ca^{2+} accumulation. The investigation of the prostaglandin effect was initially based on a suggestion by Kirkland and Baum (1972) that prostaglandin E_1 could act as natural mitochondrial Ca^{2+} ionophore.

Borle (1974) reported that between 0.1 - 3 μM cyclic AMP caused a rapid and massive release of Ca^{2+} from mitochondria of rat liver, heart and kidney. He used 10 mM ATP and 10 mM succinate as energy sources for Ca^{2+} uptake, and estimated

Ca^{2+} in the medium by the fluorometric titration method of Borle and Briggs (1968). The cAMP effect was also obtained by Matlib and O'Brien (1974). However several laboratories were unable to reproduce the effect of cAMP on Ca^{2+} release under conditions described by Borle (1974) or Matlib and O'Brien (1974). Borle in 1976 has since reinvestigated the cAMP effect and out of the 442 experiments performed only 6 % showed a significant release of Ca^{2+} from liver or kidney mitochondria and so this finding of the cyclic AMP effect has now been retracted by Borle (1976).

Studies by Blackmore et al (1979) and Babcock et al (1979) showed that perfusion of rat livers with phenylephrine (an α - adrenergic agonist), vasopressin and angiotensin caused marked loss of Ca^{2+} from mitochondria. On the other hand, glucagon administration to fed rat or to perfused rat liver prolonged Ca^{2+} retention in the mitochondria (Hughes and Barritt, 1978 ; Prpic et al, 1978).

According to Engstrom and De Luca (1962, 1964) vitamin D administered to rats or added <u>in vitro</u> stimulates Ca^{2+} release from kidney, liver and intestine mitochondria but not from heart or brain mitochondria. However the use of unphysiological amounts of the vitamin make any interpretation difficult.

Non-physiological agents that seemed to cause Ca^{2+} release from mitochondria are quinidine and calcium ionophores. Batra (1976) observed that 2 mM quinidine sulphate caused marked Ca^{2+} release from preloaded mitochondria of frog skeletal muscle. Carafoli et al (1974) showed that two Ca^{2+}-specific acid ionophores X-537A and A23187 caused rapid loss of accumulated or endogenous Ca^{2+} from rat liver mitochondria. The concentration of ionophores used was 2 μg/mg mitochondrial protein and succinate was the energy source for Ca^{2+} transport.

III PHYSIOLOGICAL IMPLICATION OF MITOCHONDRIAL CALCIUM TRANSPORT

Up to date the exact concentration of intracellular calcium *in vivo* is not known. Generally the calcium concentration in cells is estimated indirectly by examining the activity of calcium-sensitive enzymes as affected by different concentrations of calcium (Rasmussen and Nagata, 1970; Baker, 1972; Zammit and Newsholme, 1976), and the use of calcium-sensitive indicators such as murexide and aequorin (Ashley and Caldwell, 1974; Baker, 1972, 1976). Measurement of calcium concentration is further complicated by the fact that calcium can exist in several forms in the cell, i.e. exchangeable, non-exchangeable, free (ionised), diffusable but complexed, bound and even precipitated (Borle, 1975). Claret-Berthon et al (1977) studied the distribution of ^{45}Ca in rat liver cells and reported distinct pools of intracellular calcium as shown below.

Pool location	Pool size (μmole/kg liver)
Mitochondria (rapidly exchanging pool)	115
Mitochondria (slowly exchanging pool)	540
Nuclear pool (slowly exchanging pool)	100
Endoplasmic reticulum plus metabolites	270

According to the latter workers, the slowly exchanging calcium pool in the mitochondria is probably in the form of Ca-phosphate precipitated in the mitochondrial matrix. The concentration of ionised Ca^{2+} in the cytoplasm of liver cells ranges from 10^{-5}-10^{-7}M (Rasmussen et al 1975), muscle cells between 10^{-6}-10^{-7}M (Winegrad 1969) and heart cells between 10^{-5}-10^{-8}M (Crompton et al, 1976). The intramitochondrial Ca^{2+} concentration is between 10^{-4}-10^{-5}M and approximately 10^{-4}M Ca^{2+} in the interstitial space

(Rasmussen et al, 1975).

Since numerous reactions in the cells are sensitive to Ca^{2+} in the μM concentration range, it is therefore essential that intracellular free Ca^{2+} concentration is controlled precisely. Intracellular calcium may be regulated in cells by plasma membrane and subcellular organelles like mitochondria, endoplasmic reticulum and sacroplasmic reticulum. (Carafoli and Crompton, 1977; Bygrave, 1978). The cell cytosol also contain numerous calcium chelators such as adenine nucleotides, Pi, citrate and proteins which may help to lower the concentration of intracellular ionic calcium.

Properties of mitochondrial Ca^{2+} transport examined in vitro: the rapid rates of Ca^{2+} uptake, high affinity and capacity to sequester extramitochondrial Ca^{2+} and reversible Ca^{2+} transport; seem to suggest that mitochondria may act as an effective buffer for cell calcium, transporting Ca^{2+} in or out of mitochondria efficiently whenever there is a sudden change in cytoplasmic free Ca^{2+}. Apparently the rate constant of Ca^{2+} efflux out of the cell via plasma membrane (rate constant = 8/min, Borle, 1973) is similar to the rate constant of Ca^{2+} uptake by mitochondria (rate constant = 5/min, Mela and Chance, 1968) suggesting that the Ca-pump transporting Ca^{2+} out of the cell is as efficient as the Ca-uptake carrier in transporting Ca^{2+} into the mitochondria. However, the mitochondria have a larger Ca-transporting area compared to the plasma membrane (Carafoli and Crompton, 1978) suggesting the importance of mitochondria in the regulation of cell calcium.

Although most of the studies on mitochondrial Ca^{2+} uptake have been done in vitro, some data have been derived from in vivo

studies. Carafoli (1967) and Patriarca and Carafoli (1968) studied the distribution of *in vivo* injected ^{45}Ca in the subcellular fractions of rat heart and rat liver, and observed that most of the radioactivity was associated with subcellular organelles, particularly the mitochondria; only a small fraction (approx. 2.5%) was recovered in the soluble phase. However, these investigations where the tissues are cooled and homogenised in ice-cold media may give misleading results, since Ca^{2+} that may be present in the soluble phase is taken up by the mitochondria. Hamilton and Holdsworth (1975) observed that when mucosal cells from chick duodenum were homogenised in the presence of inhibitors of mitochondrial Ca^{2+} uptake such as ruthenium red (i.e. to prevent redistribution of Ca during isolation process), or when homogenised at 22°-28°C, 15-30% of the Ca was found in the soluble cytosol fraction, the remainder being mainly located in the mitochondria.

Kleneike and Stratman (1974) showed that isolated rat cells are able to accumulate Ca^{2+} within 3-4 min in the presence of respiratory substrates and that inhibition of mitochondrial oxidation by antimycin A or addition of ruthenium red suppressed Ca^{2+} accumulation by liver cells (note : ruthenium red inhibits Ca^{2+} uptake by mitochondria but it has no significant effect on that by microsome or plasma membrane (Ash and Bygrave, 1977). The similarities of Ca^{2+} uptake by isolated hepatocytes and isolated mitochondria suggest the important role of mitochondria in accumulation of Ca^{2+} by intact cells.

Direct evidence that mitochondria can accumulate Ca^{2+} *in vivo* has come from the investigations of Rose and Loewenstein (1975) who worked with cells of the salivary gland of *Chironomus* (insect). The distribution of Ca^{2+} in the cytoplasm was visualised

by means of aequorin luminescence and a television system with an image intensifier (Note : aequorin is a Ca^{2+}-sensitive luminescence protein (Shimomura et al, 1962)). Rose and Loewenstein demonstrated that when cytosolic concentration of calcium increased suddenly above the normal level, the distribution of Ca^{2+} in cytosol, as indicated by the glowing of the aequorin, was confined to a specific area in the cell. On the other hand, in the presence of ruthenium red (the specific inhibitor of mitochondrial Ca^{2+} uptake) or when treated with cyanide (an inhibitor of respiratory chain), free diffusion of the Ca^{2+} in the cytosol was observed.

Physiological reactions which may involve mitochondrial calcium transport include :-

(i) Enzymatic reactions :-

Enzymes such as pyruvate dehydrogenase phosphatase and pyruvate dehydrogenase kinase (Severson et al, 1976), isocitrate dehydrogenase (Vaughan and Newsholme, 1969 ; Zammit and Newsholme, 1976) presumably respond to low concentration (0.01-10 μM) of intramitochondrial Ca^{2+}. Cytoplasmic enzymes like pyruvate kinase (Meli and Bygrave, 1972), phosphofructokinase (Wimhurst and Manchester, 1972) fructose 1-6-diphosphatase (Van Tol et al, 1972) and mitochondrial glycerol phosphate dehydrogenase (Hansford and Chappell, 1967) which is located on the outer surface of the inner membrane (Klingenberg and Buchholz, 1970) respond to cytoplasmic Ca^{2+}.

However the low pKd (Ca^{2+}) values for the cytosolic enzymes mentioned suggest that they may not be sensitive enough to physiological concentration of cytosolic Ca^{2+}, with the possible exception of glycerol phosphate dehydrogenase. (pyruvate kinase, pKd = 3.7; phosphofructokinase, pKd = 2.7; fructose 1-6-diphosphatase,

pKd = 4.0, Vaughan et al, 1973; Glycerol phosphate dehydrogenase pKd = 6.7-7.2, Donellan and Beechey, 1969).

The possibility that Ca^{2+} might not act in its free ionic form but rather requires the presence of a binding protein was first suggested by Meyer et al, 1964. Recent studies have revealed the existence of a cytosolic protein that bind with cytosolic Ca^{2+}, the complex it forms with Ca^{2+} modifies the activity of certain enzymes such as cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi et al, 1970), brain adenylate cyclase (Brostrom et al, 1975; Cheung et al, 1975), erythrocyte membrane Ca^{2+}-dependent ATPase (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977), and several protein kinases which include phosphorylase kinase (Cohen et al, 1978), myosin light-chain kinase (Pires et al, 1974) and NAD kinase (Anderson and Cormier, 1978). The Ca-binding protein, now known as calmodulin was formerly termed as the modulator protein, activator protein or calcium-dependent regulator protein. Apparently the activation of the above Ca^{2+}-dependent enzyme requires the formation of Ca-calmodulin complex (Kakiuchi et al, 1973; Teo and Wang, 1973; Lin et al, 1974). Since calmodulin regulates a variety of intracellular enzyme systems and other cellular activities such as microtubule assembly/disessembly (Welsh et al, 1979), neurotransmitter release (Grab et al, 1979) and intestinal ion secretion (Ilundian and Naftalin, 1979), calmodulin may be considered an intracellular receptor for Ca^{2+}. Furthermore it's affinity for Ca^{2+} (Kd = 4-18μM, Lin et al, 1974) is approximately equal to the estimated intracellular free Ca^{2+} suggesting that calmodulin possibly binds with <u>in vivo</u> intracellular free Ca^{2+}. Mitochondrial Ca^{2+} transport may play an important role here, in that it regulates the concen-

tration of free cytosolic Ca^{2+}, which in turn might regulate the formation of the Ca-calmodulin complex.

(ii) Calcification

According to Lehninger (1970) mitochondria may play an important role in calcification of cartilage and kidney. He proposed that Ca^{2+} transported into mitochondria is precipitated in the form of amorphous $Ca_3(PO_4)_2$ which can pass from the mitochondria through the cytosol and to the extracellular space where it serves as a precursor of hydroxyapatite in the collagen matrix of the bone.

(iii) Muscle contraction and relaxation

Carafoli and Crompton (1976) compared initial rates of Ca^{2+} uptake by heart mitochondria and the rates of Ca^{2+} removed from troponin (i.e. the Ca^{2+} receptor in myofibril) during relaxation of heart myofibril. The latter workers reported that during relaxation in rat heart cells, 25 nmoles Ca^{2+}/g tissue is removed from troponin (the relaxation time = 200 ms). The initial rate of Ca^{2+} uptake by the mitochondria during the first 200ms at 10μM external Ca^{2+} concentration and at 38°C is 32-36 nmoles Ca^{2+}/g tissue. Thus it appears that the Ca^{2+} removed from troponin during relaxation is transported into the mitochondria.

Mitochondrial Ca^{2+} transport is therefore an important process in cells, maintaining Ca^{2+} concentration in cytoplasm in μM concentration range and regulating Ca^{2+}-sensitive reactions.

CHAPTER 1

METHODS AND MATERIALS

1.1 Preparation of mitochondria

Mitochondria were prepared by the method of Hamilton and Holdsworth (1975a). Male hooded Wistar rats of approximately 180 - 220 g weight were decapitated using a guillotine, blood drained and livers removed into ice-cold medium containing 0.25 M sucrose, 2.5 mM HEPES, 1 mM $MgCl_2$, adjusted to pH 7.4 with Tris base. The livers were minced by passing through a Harvard Tissue Press (Harvard Apparatus Co. Inc. Millis, Mass, U.S.A.) then homogenised using a Potter-Elvehjem homogeniser fitted with a Teflon pestle at a ratio 1 g to 7 ml of the above medium with the addition of 1 mM EGTA pH 7.2 - 7.3 with Tris base and 0.1 % w/v bovine serum albumin (BSA). (1 mM EGTA was included in the homogenising medium in order to chelate free Ca^{2+} in the homogenate and BSA was used to preserve the mitochondria). The livers were homogenised for 30 s (4 strokes up and down) using the maximum speed (1,200 rpm) on the electric drill. Approximately 25 ml homogenate was layered over 10 ml of 0.5 mM sucrose and centrifuged at 800 x g (1,000 rpm) for 10 min (MSE MISTRAL 6L). A marked separation between the 2 layers was observed, the lower 0.5 M sucrose layer containing cellular fractions such as cellular debris, nuclei, membranes and red blood cells, while the upper 0.25 M sucrose layer contained mitochondria, lysosomes, microsomes and other low molecular weight components. The upper layer was removed and layered over 10 ml of 0.34 M sucrose and centrifuged at 800 x g for 10 min. The separation between the 2 layers was not as marked. The upper layer was then removed and centrifuged at 12,000 x g (10,000 rpm) for 20 min (SORVALL SUPERSPEED RC2-B, Rotor SS-34). The supernatant was discarded together with the

floating lipid layer, and the sedimented mitochondria were suspended in the above medium (without EGTA and BSA) and centrifuged at 12,000 x g for 10 min and the washing repeated. The washed mitochondria were suspended in a suitable volume of the sucrose medium (without EGTA and BSA), an aliquot was removed for protein estimation, and then BSA was added to 0.01 % w/v. The mitochondrial suspension (20-40 mg/ml) was kept in ice until used. (note : the sucrose layering technique was used to obtain "purer" mitochondria. Inclusion of EGTA prevents Ca^{2+} uptake and the presence of Mg^{2+} although also partly chelated by the EGTA, helps protect membrane structure (Hamilton and Holdsworth, 1975a).

1.2 Mitochondrial ADP:O ratio

The ratio of the ADP uptake to oxygen consumption was measured by the procedure of Chance and Williams (1955). The reaction medium contained 5 mM $MgCl_2$, 10 mM KCl, 1.6 mM disodium EDTA, 10 mM Tris HCl pH 7.2, 5 mM KH_2PO_4 pH 7.2 and 200 mM sucrose. The following was added to 2.5 ml of the reaction medium :- 0.2 ml of 25 mM sodium succinate (1.6 mM final concentration), 0.1 - 0.2 ml of mitochondrial suspension (2 - 4 mg mitochondrial protein) and 0.1 ml H_2O. The solution was allowed to equilibriate and a baseline respiration rate was established before adding 50 - 60 µl of 10 mM ADP. The reaction was maintained at 30°C by means of circulating water from constant temperature bath. The concentration of oxygen in the air-saturated iso-osmotic medium was taken to be 240 µM (Hodgman, 1953). Based on the above value, the concentration of oxygen in the reaction medium is 480 ng atoms/ml at 30°C. The amount of oxygen used up over any given period is therefore :-

$$\left(\frac{h}{H} \times 480 \times V\right) \text{ ng atoms}$$

where h = recorder pen deflection over the desired period

H = recorder pen deflection from saturated to anerobic conditions.

V = total volume of reaction mixture (excluding volume of mitochondria added since the mitochondrial suspension is anerobic).

The ADP:O ratio for the substrate was calculated from the amount of oxygen (ng atoms) consumed and amount of ADP (nmoles) added. The ADP:O ratio was determined for each batch of the mitochondrial preparation. (Note : the ADP:O ratio is equivalent to P:O ratio since negligible ADP was used other than oxidative phosphorylation). From the typical result shown in fig 1 the P:O ratio calculated when 1.6 mM sodium succinate was the substrate ranged between P:O 1.6 - 1.8. (Estabrook (1967) obtained a P:O ratio 1.7 during succinate oxidation).

1.3 Calcium uptake and release by mitochondria using the radioisotope technique.

^{45}Ca was used to monitor the movement of calcium into and out of the mitochondra. The standard incubation medium consisted of 0.25 M sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM KH_2PO_4 pH 7.2, 1 mM $MgCl_2$ and 72 mM KCl. Substrates were added to a final volume of 5 ml. The pH of the incubation mixture was checked each time (and found to be pH 7.3 - 7.4). Known amounts of mitochondria were added and preincubated for 1 min at 25°C (unless otherwise stated) before the addition of ^{45}Ca, in different experiments amounts of 100 to 1,000 nmoles $^{45}CaCl_2$ (1 µCi) were used. Using a metallic test tube holder attached to a submersible magnetic stirrer, a maximum number of 8 experiments can be performed at the same time, under the same conditions. All incubation tubes for these particular experiments were washed with 50 % HNO_3 prior to use. A flow of water-saturated O_2 was maintained over the surface of the incubation fluid to ensure that the system did not become anerobic. Incubation was at 25°C and 0.5 ml samples were

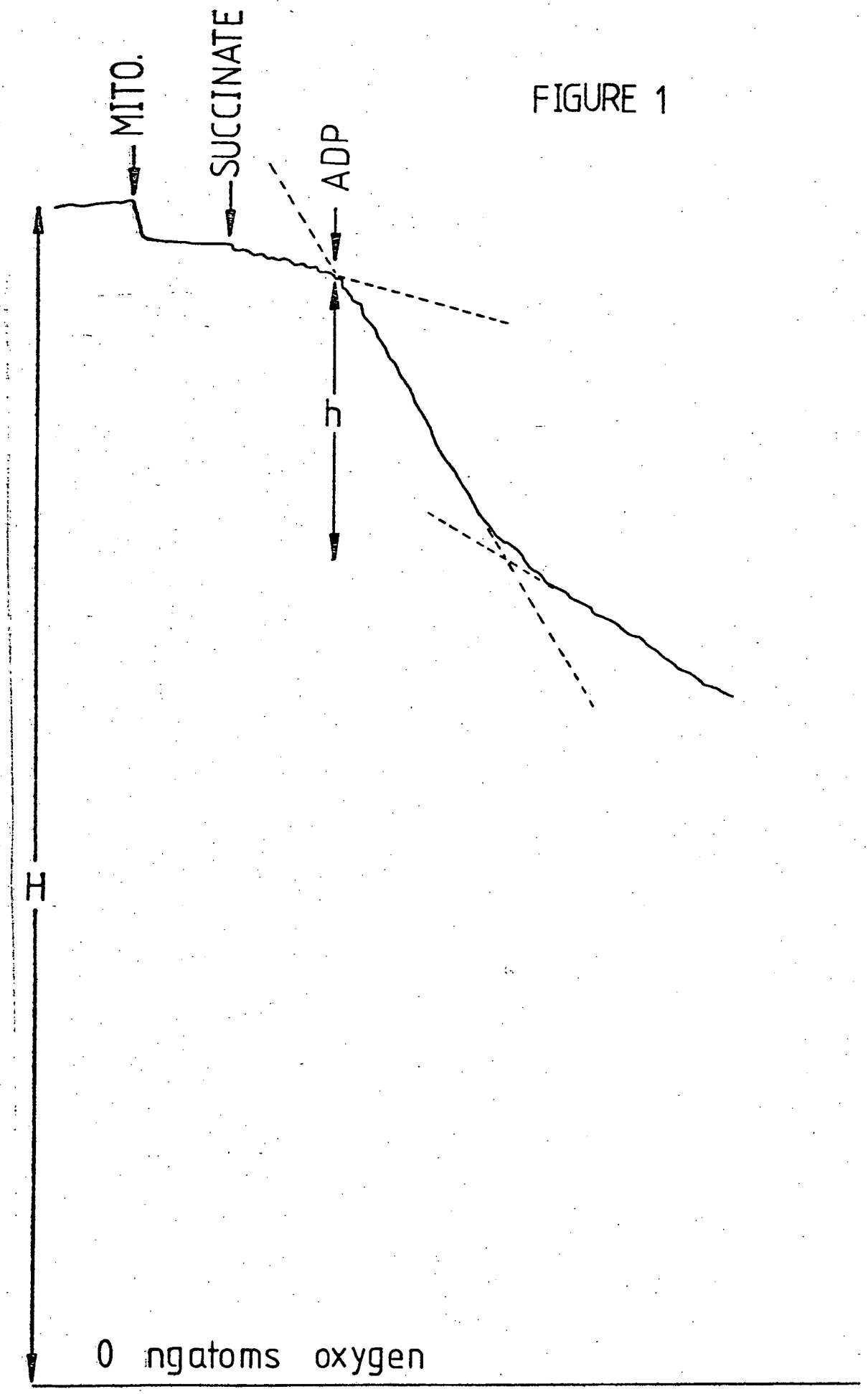
(PTO)

FIGURE 1

Mitochondrial ADP:O ratio

The mitochondrial ADP:O ratio was measured by the procedure of Chance and Williams (1955) as described in section 1.2. The reaction medium (2.5 ml) contained 5 mM $MgCl_2$, 10 mM KCl, 1.6 mM disodium EDTA, 10 mM Tris-HCl pH 7.2, 5 mM KH_2PO_4 pH 7.2 and 200 mM sucrose. At the points indicated, 2 - 4 mg mitochondrial protein (mito.), Na succinate (final concentration = 1.6 mM) and 50 - 60 ul of 10 mM ADP were added.

FIGURE 1



removed at various time intervals into 1 ml of quench medium containing 0.25 M sucrose, 2.5 mM HEPES pH 7.4 with Tris base, 1 mM EGTA and 2 μM purified ruthenium red. Disposable plastic centrifuge tubes (13 mm x 85 mm) were used to contain the quench medium. The quench medium ensured discrimination between Ca^{2+} in the mitochondrial matrix and that externally bound to the outer membrane or the outer side of the inner mitochondrial membrane (Reed and Bygrave, 1975(a)). The mitochondria were separated by centrifugation (Phillips Drucker L-708 Combination Centrifuge, Astoria, Oregon, U.S.A.) at maximum speed (9,750 x g) for 5 min, and ^{45}Ca in the supernatant measured by liquid scintillation counting (Beckman Liquid Scintillation System, Selbys Scientific Ltd., Tasmania). The scintillation fluid consisted of 10 g P.O.P., 100 mg P.O.P.O.P., 1,400 ml toluene and 600 ml Triton X-100. The difference between the total counts and counts in the supernatant gave the counts in the mitochondria. The ^{45}Ca counts in the mitochondrial pellets were also checked as follows:- After centrifugation (Phillips Drucker Centrifuge) at 9,750 x g for 5 min, the supernatant was decanted. The mitochondrial pellets were washed three times gently with 0.25 M sucrose, then 0.5 ml of 1 % SDBS (sodium dodecyl-benzene sulfonate) was added to the mitochondrial pellets and the mixture left for 2 - 3 hr. The solubilised pellets (0.2 ml) were then added to 15 ml of the scintillation fluid and ^{45}Ca measured by scintillation counting. Note: Generally, experiments presented in this thesis were repeated 2 or 3 times to obtain consistent results.

1.4 Calcium uptake and release by mitochondria studied by of a Ca-electrode.

A Ca-electrode was used for some of the mitochondrial calcium transport experiments. The Ca-electrode was particularly useful for continuous monitoring of Ca^{2+} movement in mitochondria, especially when observing initial Ca^{2+} uptake, spontaneous release or when only small changes in free calcium concentration were observed in the medium. On the other hand, the disadvantage of using the Ca-electrode compared to the radio-isotope technique is that there is no distinction between transported Ca^{2+} and Ca^{2+} chelated to other Ca-chelators such as ATP, since the electrode is only sensitive to free Ca^{2+} in the medium. The Radiometer calcium-sensitive electrode type F2112 was used with a 'non-flow' reference electrode (Ionode, type RNFM Selby Scientific Ltd., Hobart, Tasmania). A special high performance amplifier was built around a LH 0022 National semi-conductor (National Semi-conductor Corp., Santa Clara, Calif., U.S.A.). This amplifier was connected to a second amplifier containing an antilog stage and the output of both amplifiers was recorded on two separate 10 mV recorders. The Ca-electrode, its reference electrode and an O_2 electrode (type 506 Triton Pty. Ltd., Braeside, Victoria, Australia) were placed into a 6 ml vessel which was maintained at 25°C. Water-saturated O_2 at 25°C was directed across the surface of the fluid in the vessel. The O_2 supply was adjusted to maintain O_2 saturation of the fluid as monitored by the oxygen electrode. The Ca-electrode was calibrated to give a response of concentration range between 10^{-4}M to 0.5 x 10^{-6}M Ca^{2+} on the log recorder chart and

10^{-5}M Ca^{2+} to 0.5 x 10^{-6}M Ca^{2+} on the antilog recorder chart. The amplified recorder system could be adjusted to give full scale (10") at 5 ± 0.01 mV. In order to obtain accurate values of free Ca^{2+} for calibration, Ca-NTA buffer was used (NTA = nitrilotriacetic acid) (Reed and Bygrave, 1975(a)). The total calcium content required for a given free Ca^{2+} concentration is determined from the equation (see reference by Perrin and Dempsey, 1974):-

$$[Ca^{2+}] = [Ca\text{-}NTA]/\beta' . ([NTA]_{tot} - [Ca\text{-}NTA]),$$

where $[Ca^{2+}]$ = concentration of free Ca^{2+}.

$[Ca\text{-}NTA]$ = concentration of complexed calcium ($>> [Ca^{2+}]$)

$[NTA]_{tot}$ = total ligand concentration, free and complexed.

Stability constant $\beta' = \beta/\alpha_m \alpha_L$

where β = formation constant for the formation of 1:1 complex by unprotonated NTA

$\alpha_m = 1 + 10^{(pH-pKm)}$

where pKm is the pK for the loss of a proton from the hydrated calcium ion

$\alpha_L = 1 + 10^{(pK_1-pH)} + 10^{(pK_1+pK_2-2pH)} + 10^{(pK_1+pK_2+pK_3-3pH)}$

where pK values refer to protonation of the nitrilotriacetic acid. The pKa values given for NTA at 25.3°C are 9.63, 2.49 and 1.89.

With pKm = 12.6 and pH 7.4, α_m can be taken as unity.

The given value for log β = 6.33 at 25.3°C.

Thus the calculated value for $\beta' = 10^4$.

<u>Total calcium content required for a given free calcium concentration at pH 7.4 and 25°C</u> :-

free Ca^{2+}	Total Ca^{2+} in the presence of 10 mM NTA and 10 mM Tris HCl pH 7.4
10^{-4}M	5 x 10^{-3}M $CaCl_2$
10^{-5}M	10^{-3}M $CaCl_2$
10^{-6}M	10^{-4}M $CaCl_2$

The Ca-NTA buffers were used to give a response in the range 10^{-4}M to 10^{-6}M free Ca^{2+} on the log chart recorder. Since the incubation medium used contained high concentrations of ions other than Ca^{2+} such as K^+, Mg^{2+}, ATP, anions, the calibration of the incubation medium was by additions of small amounts (50 nmoles Ca^{2+}) of $CaCl_2$. Unless otherwise stated, the standard incubation medium consisted of 0.25 M sucrose, 2.5 mM HEPES pH 7.4 with Tris base, 1 mM $MgCl_2$, 72 mM KCl and 2 mM KH_2PO_4 at pH 7.2 . The pH of the incubation medium was between pH 7.3 - 7.4 . $CaCl_2$ and various substrates were added to a final volume of 5 ml. The reaction was started by addition of mitochondria. The water and the sucrose solution were freed from contaminating Ca^{2+} by filtration through columns of Chelex-100, 100 - 200 mesh (BIO-RAD, Richmond, Calif.).

Fig 2 shows that in the presence of 400 nmoles Ca^{2+}, 1 mM $MgCl_2$ did not seem to interfere with the sensitivity of the Ca-electrode, however 72 mM KCl and 2 mM KH_2PO_4 pH 7.2 showed a slight but insignificant interference.

1.5 Analytical Methods.

1.5.1 Protein estimation.

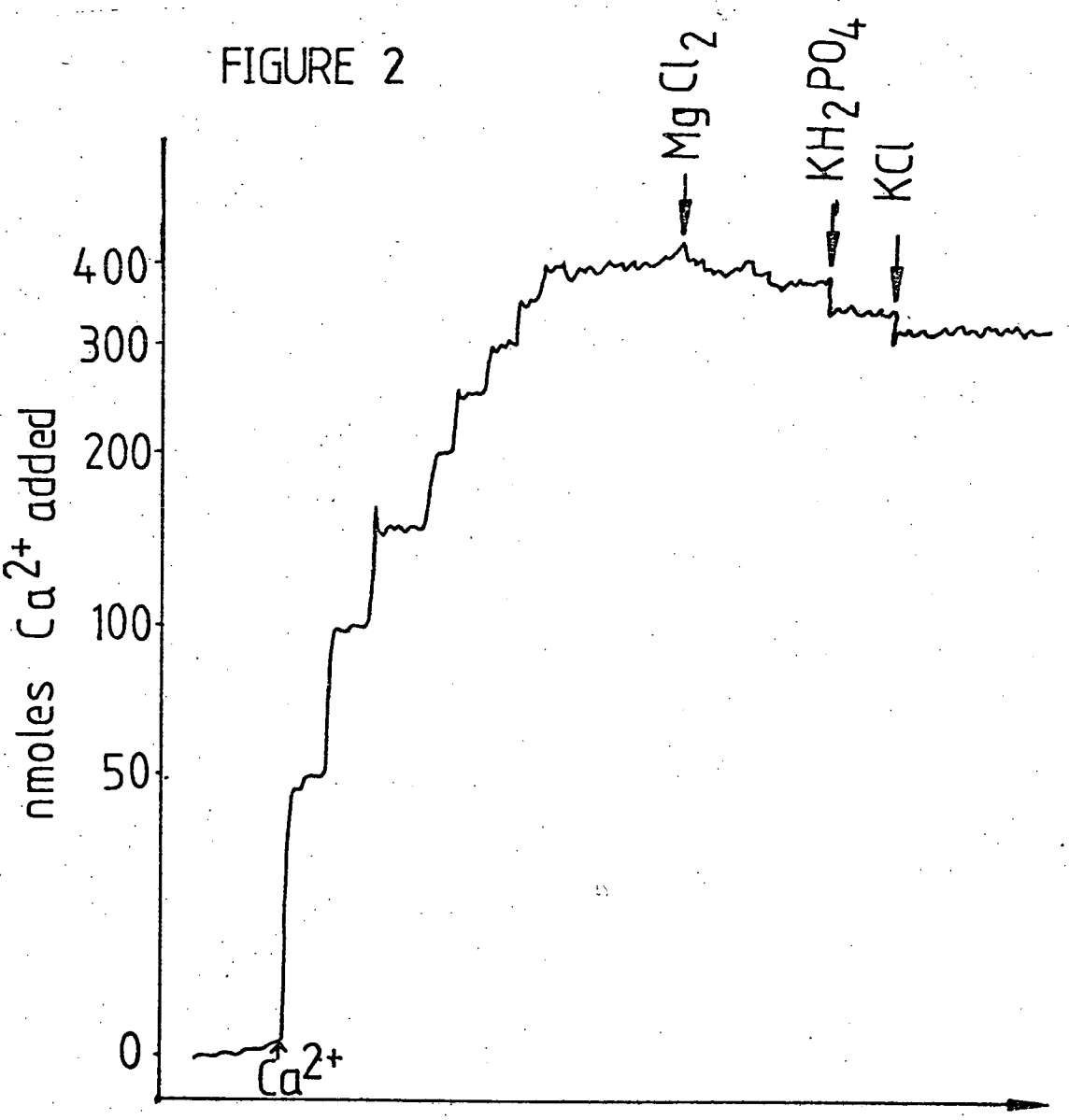
Protein was determined by the Biuret method (Gornall et al, 1949). 0.1 ml and 0.2 ml of the mitochondrial suspension or

FIGURE 2

Effect of various ions present in the standard incubation medium on the sensitivity of the Ca-electrode.

The incubation system (final vol. = 5 ml) contained 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, and 400 nmoles Ca^{2+}. Incubation temp. = 25°C. The Ca^{2+} added is as shown on a log scale. Additions of $MgCl_2$ (final concentration = 1 mM), KH_2PO_4 (final concentration = 2 mM) and KCl (final concentration = 72 mM) were made at the points indicated.

FIGURE 2



25 µl and 50 µl of the rat liver cytosol preparation was made up to 1 ml with 1N NaOH and 2 ml of biuret reagent was then added. Crystalline bovine serum albumin (Calbiochem, La Jolla CA.) was used as the standard, dissolved in the sucrose medium consisting of 0.25 M sucrose, 2.5 mM HEPES, 1 mM $MgCl_2$ adjusted to pH 7.4 with Tris base. The absorbance was read at 560 nm on a Unicam SP 1800 ultraviolet spectrophotometer. Correction was made for non-biuret turbidity. The O.D of protein solution in alkali was measured against alkali blank. The value was then subtracted from that in the presence of Cu.

1.5.2 Inorganic phosphate estimation

Inorganic phosphate was estimated by the method of Taussky and Shorr (1953). Fresh solutions of 2.5 % ammonium molybdate in 1.5N H_2SO_4 and 6 % $FeSO_4$ in 1N H_2SO_4 were prepared. 1.0 ml sample was deproteinised with 0.3 ml of 30 % Trichloroacetic acid, centrifuged (9,750 x g for 2 min), then 25 µl, 50 µl, 0.1 ml of the deproteinised supernatant was made up to 2 ml volume with distilled water and 1.0 ml of 2.5 % ammonium molybdate in 1.5N H_2SO_4 was added and solution mixed, then 1.0 ml of 6 % $FeSO_4$ in 1N H_2SO_4 added. The mixed solution was allowed to stand at room temperature for 10 min and absorbance read at 700 nm (Unicam SP 1800 Ultraviolet spectrophotometer).

1.5.3 Potassium estimation

Potassium was estimated by means of a Beckman Model 105 flame photometer. The lithium working solution (10,000 ppm Li) was prepared by diluting 5 ml of the concentrated lithium solution to 1 litre and the potassium working standard consisted of 1 ml of 10 mM KCl made up to 200 ml with Li-working solution. The solution was set to zero with the Li-working solution.

1.5.4 Magnesium Estimation.

1.5.4.1 Magnesium estimation in the rat cytosol preparation.
Mg^{2+} was estimated by atomic absorption spectrophotometry (Varian Techtron Model 1000) after digestion of the samples in "metal free" (BHD Aristar) concentrated nitric acid (1 ml) and perchloric acid (0.5 ml, 60 % w/v). The digested samples were taken to dryness and dissolved in distilled water. A 1 M ammonium chloride solution containing 15,000 ppm Sr^{2+} was used as the working solution. 20, 40 and 80 nmoles Mg^{2+} standard dissolved in the Sr^{2+} working solution was prepared. The atomic emission of Mg^{2+} was determined at 254.2 nm wavelength.

1.5.4.2 Magnesium estimation in the mitochondrial pellets.
At different time intervals, 0.5 ml sample of the incubation mixtures was layered on 0.4 ml silicone oil (Silicone Fluid M.S.704, Midland Silicone Ltd., Barry, Glamorgan) and centrifuged at 12,000 x g for 4 min (Eppendorf Centrifuge 5412) through the oil into a layer of 0.25 ml 2N $HClO_4$/12.5 % sucrose w/v. The supernatant (i.e. the layer above the silicone oil) and the silicone layer were removed and the side of the centrifugation tube cleaned with cotton wool to remove any Mg^{2+} contamination. 0.2 ml of the lower perchloric/sucrose solution was pipetted to 0.8 ml of the 1 M NH_4Cl solution containing 15,000 ppm Sr^{2+} and Mg^{2+} estimated by atomic absorption spectrophotometry. Magnesium standards were prepared by adding 0.2 ml of the 2N $HClO_4$/ 12.5 % sucrose w/v containing 0 nmole, 20 nmoles, 40 nmoles and 80 nmoles Mg^{2+} respectively were added to 0.8 ml of the NH_4Cl/Sr^{2+} solution. To determine that all of the mitochondrial Mg^{2+} was in the

$HClO_4$/sucrose solution, Mg^{2+} was estimated in the ashed mitochondrial pellets. The atomic emission of Mg^{2+} was determined at 254.2 nm wavelength.

1.5.5 Calcium Estimation.

1.5.5.1 Calcium estimation by atomic absorption spectrophotometry.

Calcium was determined by atomic absorption spectrophotometry (Varian Techtron Model 1000) after digestion of the samples in "metal free" (BHD Aristar) concentrated HNO_3 (1 ml volume) and $HClO_4$ (0.5 ml, 60 % w/v). The digested sample was taken to dryness and dissolved in distilled H_2O. 0.5 ml of La^{3+} acetate pH 4.7 was added to give a final concentration of 10,000 ppm La^{3+}. The atomic emission of Ca^{2+} was determined at 422.7 nm wavelength.

1.5.5.2 Calcium estimation by reaction with Arsenazo III.

After digestion of the samples as described above, the ashed sample was dried over NaOH and dissolved in 3 ml of 0.1 M Tris-HCl pH 7.6 . Calcium was determined by the reaction with Arsenazo III (Gratzer and Beaven, 1977) using the dye which had been purified by the method of Kendrik (1976). (Note: The chemical formula of Arsenazo III is shown in appendix A). Prior to the Ca^{2+} estimation, the cuvettes were washed in 50 % HNO_3 to remove any contaminating Ca^{2+} ions. The concentration of Ca^{2+} in the ashed sample was determined by the addition of 25 µl purified Arsenazo III (10 mg/ml) and titration by the addition of successive portions of 25 µl of 1 mM Chelex treated EGTA until the absorbance at wavelength 650 nm (Unicam SP 800 Ultraviolet Spectrophotometer) coincided with that of the

blank (i.e. minus the sample). The amount of free Ca^{2+} present in the cuvette can be calculated from the volume of 1 mM EGTA added (1 ml of 1 mM EGTA added was equivalent to 1 μmole Ca^{2+}).

1.6 Enzymatic estimation of adenosine 5' nucleotides.

1.6.1 Extraction of total adenosine 5' nucleotides for estimation.

1 ml of the incubation mixtures sampled at various time intervals was added to 0.2 ml ice-cold 3N perchloric acid. The solution was then centrifuged at 9,750 x g for 3 min (Phillips Drucker L-708 Combination Centrifuge) and the clear supernatant neutralised with a few drops of 5N KOH. A drop of bromothymol blue (0.01 %) was added as the pH indicator. The solution was then centrifuged to remove potassium perchlorate. The clear supernatant was stored at -15°C and examined for adenosine 5' nucleotides.

1.6.2 Extraction of adenosine 5' nucleotides from mitochondrial pellets for estimation.

At different time intervals, 1.0 ml sample of the incubation mixtures was layered on 0.4 ml silicone oil (Silicone Fluid M.S. 704) and centrifuged at 12,000 x g for 5 min (Eppendorf Centrifuge 5412) through the oil into a layer of 0.25 ml 1N $HClO_4$/12.5 % w/v sucrose. The layer above the silicone oil was pipetted carefully, then added to 0.2 ml 3N $HClO_4$ and neutralised with 0.12 ml 5N KOH. The solution was centrifuged and the clear supernatant stored at -15°C and examined for extra-mitochondrial adenosine 5' nucleotides. The lower perchloric layer containing the mitochondrial adenosine 5' nucleotides was removed and neutralised with approximately

50 µl of 5N KOH, to pH 7.0 - 7.4 stored at -15°C and examined for adenosine 5' nucleotides.

1.6.3 Enzymatic assay for estimation of adenosine 5' nucleotides.

ATP was determined enzymatically by the method of Lamprecht and Tautschold (1974), and AMP and ADP by the method of Jaworek et al (1974).

1.6.3.1 ADP and AMP Estimation.

The determination of ADP and AMP is based on the following reactions:-

$$AMP + ATP \xrightarrow{\text{myokinase, } Mg^{2+}} 2\ ADP$$

$$2ADP + 2PEP \xrightarrow{\text{pyruvate kinase, } Mg^{2+}, K^{+}} 2ATP + 2\ \text{pyruvate}$$

$$2\ \text{pyruvate} + 2NADH + 2H^{+} \xrightarrow{\text{lactate deH}_2\text{ase}} 2\ \text{lactate} + 2NAD^{+}$$

Oxidation of NADH is measured by a decrease in absorbance at 340 nm. A mixture of 0.18 ml of 1 M $MgCl_2$ (90 mM final concentration), 0.76 ml of 2 M KCl (760 mM final concentration), 0.1 ml of 0.1 M disodium EDTA (5 mM final concentration), 0.4 ml dipotassium phosphoenolpyruvate (20 mg/ml), 0.16 ml dipotassium ATP solution containing 2 mg/ml and 0.3 ml $NADH_2$ solution containing 10 mg $NADH_2$/ml was prepared. The mixture was preweighed in a 5 ml plastic vial, the solution adjusted to pH 7.0 with a few drops of 1N NaOH and distilled water added to a final volume of 2 ml. Triethanolamine HCl buffer for the assay was prepared by dissolving 3.72 g triethanolamine HCl in approximately 8.0 ml of 1N NaOH and distilled water added to a final volume of 100 ml. The pH of the buffer = 7.55 . The following was pipetted into a quartz cuvette:-

1 ml triethanolamine HCl buffer, 0.2 ml of the prepared mixture, 1.8 ml sample plus distilled water and 10 µl lactate dehydrogenase (E.C. 1.1.1.27) (from Calbiochem., San Diego, California). The cuvette contents were mixed thoroughly with a plastic plumper and the absorbance determined at 340 nm wavelength (Unicam SP 800 Ultraviolet Spectrophotometer). The assay reaction was started by adding 5 µl pyruvate kinase (E.C. 2.7.1.40) (from Calbiochem, San Diego, California). Separate plumpers were used on adding different enzymes since the assay was sensitive to trace amounts of enzymes. The absorption at 340 nm was followed until the reaction was completed. The change in absorbance denoted the amount of ADP in the cuvette. (Δ 1.0 abs. unit = 0.489 µmole ADP). Then 2 µl myokinase (EC 2.7.4.3) (from Boehringer Mannheim Australia Pty Ltd., Hardner Rd. Mt. Waverley, Vic.) was added and the absorption followed until the reaction was completed. The amount of AMP present in the cuvette was calculated from the Δ abs. unit (Δ 1.0 abs. unit = 0.244 µmole AMP). Finally 10 µl of 10 mM AMP was added to ensure that the assay system was satisfactory.

1.6.3.2 ATP Estimation.

The determination of ATP is based on the following reactions:-

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase, } Mg^{2+}} \text{glucose-6-P}$$

$$\text{glucose-6-P} + NADP^{+} \xrightarrow{\text{glucose-6-P-deH}_2\text{ase}} \text{6-phosphogluconolactone} + NADPH + H^{+}$$

Production of NADPH is measured by an increase in absorbance at 340 nm.

The following was pipetted into a quartz cuvette:-

1.0 ml triethanolamine buffer (0.1 M, pH 7.55)

0.5 ml $NADP^+$ at 5 mg/ml

25 µl of 1 M $MgCl_2$

0.25 ml D-glucose solution containing 90 mg/ml

1.25 ml sample plus distilled water

10 µl glucose-6-phosphate dehydrogenase suspension (EC 1.1.1.49) (Boehringer Mannheim, Australia Pty Ltd., Hardner Rd. Mt. Waverley, Victoria).

The cuvette contents were mixed thoroughly with a plastic plumper and the absorbance determined at 340 nm wavelength (Unicam SP 800 Ultraviolet Spectrophotometer). The assay reaction was started by adding 10 µl hexokinase suspension (EC 2.7.1.1) (Boehringer) and the absorption followed until the reaction was completed. Δ 1.0 abs. unit = 0.491 µmole ATP.

1.7 Estimation of adenosine 5' nucleotides by means of a High Performance Liquid Chromatography (HPLC)

At specified times, 0.5 ml incubation mixture was added to 1.0 ml of 8 % TCA and centrifuged (9,750 x g for 3 min). The supernatant was then neutralised with NaOH. Nucleotides present in the neutralised TCA extract and standards were estimated on a liquid chromatograph (Waters Associates, Model 204) equipped with a solvent programmer (Model M660), two pumps (Model 600A), a U6K closed loop injector and a dual channel detector (Model 440) at wavelength 340 nm. The column was 30 cm by 0.6 cm containing micro-Bondapak C18 (Waters Associates) reversephase column with 0.05 M $NH_4H_2PO_4$ buffer pH 6.0 as solvent. AUFS = 0.05 (i.e. 0-100 chart divisions = 0.05 abs. units). The estimated concentration of nucleotides in 25 µl of the 1.5 ml TCA extract was expressed as absorbance units (α height of the peak).

1.8 Estimation of pyridine nucleotides

The concentrations of NAD^+ , NADH, $NADP^+$ and NADPH were determined by an enzymatic and fluorimetric assay (Williamson and Corkey, 1969). The NADH and NADPH were estimated within 45 min after extraction due to the lability of the reduced pyridine nucleotides. This was immediately followed by estimation of NAD and NADP.

1.8.1 Acid extraction to determine NAD^+ and $NADP^+$

1 ml of the incubation mixture was added to 0.2 ml of the ice-cold 3N $HClO_4$. The solution was whirlmixed and centrifuged (Phillips Drucker L-708) at top speed (9,750 x g) for 2 min. Then 0.2 ml of 1 M KH_2PO_4 was added to the clear solution and neutralised with a few drops of 5N KOH to pH 7.0 - 7.4. The solution was neutralised immediately to avoid degradation of the pyridine nucleotide. The solution was centrifuged to remove potassium perchlorate. The clear supernatant was then assayed for NAD^+ or $NADP^+$.

1.8.2 Alkaline extraction to determine NADH and NADPH

1 ml of the the incubation mixture was added to 0.5 ml of 0.5N KOH in 50 % ethanol/water v/v. The mixed solution was allowed to remain at room temperature for approximately 30 min, then cooled in ice and approximately 1.0 ml of triethanolamine phosphate buffer (9.3 g triethanolamine HCl, 5.5 g KH_2PO_4, 1.7 g K_2HPO_4 in 100 ml distilled water) added slowly to pH 7.8 - 8.0 (Note : a drop of bromothymol blue (0.01 %) was added to the solution as a pH indicator ; buffer added until bromothymol blue indicator turned green). The solution was

centrifuged for 5 min at 20,000 x g at 0°C (Beckman Model L5-65 Ultracentrifuge, Spinco Rotor 65). The clear solution was then assayed for NADH and NADPH.

1.8.3 NAD^+ Estimation.

The buffer solution contained 4.5 g sodium pyrophosphate, 0.5 g semicarbazide HCl dissolved in 100 ml distilled water, pH of the buffer solution being pH 8.8 . The following were mixed in a cuvette, 1.5 ml buffer, 0.5 ml sample and 10 µl ethanol (100 %). The fluorescence baseline was established (Aminco Bowman Spectrophoto fluorometer, American Instrument Co., Division of Travelnol Lab. Inc., Silver Springs, Maryland) with the following settings:- excitation wavelength = 345 nm, emission wavelength = 470 nm, entrance 0.5 mm slit, exit 1 mm, PM 5, amplifier 0.3 . 10 µl alcohol dehydrogenase suspension (EC 1.1.1.1) was then added and the increase in fluorescence indicated the amount of NAD^+ present in the cuvette. The assay was calibrated with an internal standard of 2 nmoles NAD^+ (i.e. 10 µl of 4 mg NAD^+ ⟶ 25 ml). The enzyme assay was based on the reaction below:-

$$\underset{\text{(alcohol)}}{CH_3CH_2OH} + NAD^+ \xrightleftharpoons{\text{alcohol deH}_2\text{ase}} \underset{\text{(acetaldehyde)}}{CH_3CHO} + NADH + H^+$$

The equilibrium of the reaction favoured the formation of NADH because of the alkaline assay medium and the formation of the semi-carbazone of acetaldehyde.

1.8.4 NADH and NADPH estimation.

For this particular assay, 0.1 M triethanolamine HCl buffer pH 7.4 was used. The fluorometer was first adjusted so that a suitable deflection was produced by 2 nmoles of NADH in 2 ml

buffer. The assay solution consisted of 1.0 ml buffer, 1.0 ml sample and 0.1 ml substrate mixture containing equal volumes of 0.1 M sodium pyruvate, 0.1 M ∝ ketoglutarate neutralised to pH 6.0 - 6.5 and 1 M ammonium sulphate. A steady fluorescence baseline was first established before adding 5 µl lactate dehydrogenase (EC 1.1.1.27) which catalyses the reaction below:-

$$\text{pyruvate} + \text{NADH} + H^+ \longrightarrow \text{L-(+) lactate} + NAD^+$$

The decrease in fluorescence was measured indicating the amount of NADH present in the cuvette. Then 10 µl of glutamate dehydrogenase (EC 1.4.1.4) was added and the change in fluorescence due to NADPH was measured. The fluorometer setting is similar to that described in section 1.8.3 . Glutamate dehydrogenase catalyses the reaction below:-

$$\text{∝-ketoglutarate} + NH_4^+ + \text{NADPH} \longrightarrow \text{L-glutamate} + NADP^+ + H_2O$$

1.8.5 $NADP^+$ estimation.

$NADP^+$ was determined with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) which catalyses the reduction of $NADP^+$ by D-glucose-6-phosphate.

$$\text{D-glucose-6-phosphate} + NADP^+ \longrightarrow \text{6-phosphogluconate} + \text{NADPH} + H^+$$

The following were pipetted into a cuvette:- 1.5 ml of 50 mM triethanolamine HCl buffer containing 10 mM $MgCl_2$, 5 mM EDTA pH 7.4, 0.5 ml sample and 10 µl of glucose-6-phosphate (0.1 M). The reaction was started by the addition of 10 µl glucose-6-phosphate dehydrogenase and the increase in fluorescence followed until the reaction was completed. 2 nmoles of $NADP^+$ (10 µl of 4 mg $NADP^+$ ⟶ 25 ml) was then added as the internal standard.

CHAPTER 2

CALCIUM UPTAKE BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OF RAT LIVER CYTOSOL

2.1 AIM

Most of the experiments on Ca^{2+} uptake by isolated mitochondria have used isolated mitochondria in iso-osmotic sucrose medium or KCl medium. An attempt was therefore made to study Ca^{2+} movement in isolated mitochondria in the presence of rat liver cytosol, and also to investigate factors present in the cytosol that might regulate the calcium content in mitochondria. Since there has not been any reports on preparation of undiluted rat liver cytosol, this study attempts to do so.

2.2 METHODS AND MATERIALS.

2.2.1 Preparation of rat liver cytosol

Rat livers were perfused *in vivo* with 25-30 ml of oxygenated 0.25 M sucrose at 35° (no heparin was used), then washed and cooled in ice-cold 0.25 M sucrose. The livers were crushed by passing through a Harvard Tissue Press (Harvard Apparatus Co., Inc. Millis, Mass. U.S.A.) then homogenised using a Potter Elvehjem homogenizer at a ratio 1 g to 7 ml of 0.25 M sucrose. The homogenate was centrifuged first at 20,000 x g for 20 min (SORVALL RC-2 centrifuge, SS 34 Rotor), then at 40,000 x g for 20 min and finally at 250,000 x g for 60 min (Beckman Model L5-65 Ultracentrifuge, SPINCO, Rotor Ti 70). The supernatant was concentrated over a PM 10 DIAFLO Ultrafilter, MW cut off 10,000 (Amincon. Corp. Lexington, Mass. U.S.A.) to a weight corresponding to the original weight of the liver. This was referred to as the concentrated rat cytosol. The

concentrated rat cytosol and the ultrafiltrate were stored at -15°C. Analyses were made of the 250,000 x g supernatant before and after being concentrated.

2.2.2 Analyses of the rat liver cytosol.

Protein was estimated by the Biuret method, inorganic phosphate by the method of Taussky and Shorr (1953). Potassium was estimated by means of flame photometry, magnesium by atomic absorption spectrophotometry and calcium by reaction with arsenazo III as described in chapter 1, section 1.5 . Adenosine 5' nucleotides in cytosol were determined enzymatically as described in chapter 1, section 1.6 . The osmotic pressure of the rat cytosol was determined by means of an osmometer (Advanced Digimatic Osmometer, Model 3D. Advanced Instruments Inc. Needham Heights, Massachusetts).

2.2.3 Removal of free fatty acid from protein.

2.2.3.1 Using activated charcoal.

Free fatty acids were removed from rat albumin using activated charcoal by the method of Chen (1967). 200 mg rat albumin (Pentex fraction V, Miles Laboratories Inc., Ekhart Indiana, U.S.A.) was dissolved in 2 ml distilled water at room temperature and 100 mg of washed charcoal was then added and the pH of the solution lowered to pH 3.0 with 1N HCl. The solution was then placed in an ice bath and mixed magnetically for 1 hr. The charcoal was then removed by centrifugation for 25 min at 2°C (20,000 x g) (Sorvall RC2 centrifuge, SS 34 Rotor). The clear supernatant was then brought to pH 7.0 with 1N NaOH.

2.2.3.2 Using florisil.

Free fatty acids were removed from rat liver cytosol using florisil. A column 0.4 cm x 5 cm containing 600 mg Florisil (activated magnesium silicate) (100 - 200 mesh) was prepared and 6 ml of rat liver cytosol filtered through the column. One passage served to remove 50 % of the free fatty acids. Free fatty acids from ^{3}H-palmitate-binding protein isolated from the cytosol was also removed using florisil. (Note: Reported composition of florisil:- magnesium oxide 15.5 ± 0.5 %, silicon dioxide 84.0 ± 0.5 % and sodium sulphate 0.5 % - information contained in a bulletin obtained from Floridin Co. Tallahasee, Flo. on the properties and uses of Florisil as a chromatographic adsorbent.) Florisil obtained from Selbys and Co. Pty. Ltd., Notting Hill, Victoria, Australia.

2.2.4 Estimation of free fatty acids by colorimetric method.

Free fatty acids in rat cytosol before and after florisil treatment, were estimated by colorimetric method (Soloni and Sardina, 1973). The method is based on the estimation of copper in a chloroform extract of their cupric salts with oxalic acid bis - (cyclohexylidene-hydrazide). 0.1 ml of the sample was added to a 16 x 100 mm disposable test tube. 50 µl, 20 µl and 10 µl of 50 mg palmitic acid solution/100 ml was used as the standard (corresponding to 25, 10 and 5 mg per 100 ml respectively), 0.3 ml of copper reagent (40 g/litre $Cu(NO_3)_2 \cdot 3H_2O$, 120 ml/litre of 99 % triethanolamine, (pH 8.1)) and 2 ml of chloroform was then added. The test tubes were stoppered with No. 0 neoprene stoppers for minimal evaporation. The tubes were then placed vertical in the vortex multiple shakerhead and shaken for 10 min. Instead of using filter pads

as in the original method, the blue aqueous phase was removed using a Pasteur pipette and the chloroform phase washed with 2 ml of distilled water. 1.0 ml of the chloroform extract was pipetted into a clean disposable test tube. 0.9 ml of cuprizone reagent was then added (cuprizone reagent consisted of oxalic acid bis-(cyclohexylidene-hydrazide), 0.4 g/litre of isopropanol). Then 0.1 ml of 6 % w/v solution of NH_3 was added, stoppered and mixed gently for 10 min. The solution was poured into a spectrophotometric tube and absorbance read at 620 nm wavelength against a reagent set at zero absorbance (Unicam SP 1800 Ultraviolet spectrophotometer).

2.2.5 Determination of calcium-binding activity of cytosolic fractions using Chelex-100 resin.

The method is based on the fact that the resin Chelex-100 resin and the Ca-binding samples are in competition for $^{45}Ca^{2+}$ (Wasserman and Taylor, 1966). The resin, Chelex-100, (BIORAD, Richmond, Calif.) was washed several times with Tris buffer pH 7.2 and diluted in the buffer to a concentration of 0.1 ml resin per 0.2 ml suspension. 0.1 ml and 0.2 ml cytosolic protein fraction was made up to a total volume of 0.7 ml with 0.5 M sucrose HEPES Tris buffer pH 7.4 . Then 0.1 ml of a final concentration of 10^{-3}M, 10^{-4}M and 10^{-5}M $^{45}CaCl_2$ was added and the solution mixed thoroughly. 0.2 ml of the resin (maintained in suspension by vigorous mixing with magnetic stirrer) was then added and the solution mixed thoroughly for 10 min at room temperature after which the mixture was centrifuged (Phillips Drucker) for 5 min (9,750 x g) to remove the Chelex-100. 0.1 ml of the supernatant containing ^{45}Ca bound to the protein was added to 15 ml scintillation fluid

plus 1.0 ml of 0.1N $HClO_4$ and ^{45}Ca measured by scintillation counting. With counting standards and appropriate volume correction, the Ca-binding activity of the protein fraction is calculated and expressed as nmoles Ca-bound per ml protein fraction.

2.2.6 Chromatography of rat liver cytosol.

2.2.6.1 Fractionation of ^{3}H-palmitate and ^{45}Ca labelled rat liver cytosol on AcA 44 column.

5 ml of the concentrated rat liver cytosol was labelled with 25 µl palmitic acid (9:10^{3}H) equivalent to 10 µCi, 30 µmole per g and 5 µl stock ^{45}Ca (2 µCi, 0.4 µmoles per g), then placed on AcA Ultrogel column (LKB Producter, A.B. Stockholm). Dimension of the column: 50 cm x 2 cm. Fractions were eluted with 0.1M Tris HCl pH 7.6 and collected in tubes at 150 drops per fraction. The column was calibrated with 0.4 ml Serva mixture (100 mg/ml) containing ferritin (MW 364,000), conalbumin (MW 86,180), albumin (MW 63,000-67,000), β-Lactoglobulin (MW 35,000), myoglobin (MW 18,000), ribonuclease (MW 14,000), cytochrome c (MW 13,000). Protein in the eluate was determined by measuring the absorbance at 280 nm (Unicam SP 1800 Ultraviolet Spectrophotometer). 1 ml of each fraction was counted for ^{45}Ca and ^{3}H by two channels liquid scintillation counting (Beckman Liquid Scintillation System).

2.2.6.2 Fractionation of ^{3}H-palmitate labelled rat liver on Biogel P 30 column.

45 ml of the concentrated rat liver cytosol (equivalent to 45 g wet weight liver) was labelled with 50 µl ^{3}H-palmitic

acid (100 µCi) and placed onto Biogel P30 column (4 x 60 cm, BIORAD Laboratories, Richmond, Calif. U.S.A.). Fractions were eluted with 50 mM Tris-HCl, pH 7.6 and collected in tubes at 250 drops/fraction (equivalent to 18 ml volume). 1 ml of each fraction was counted for ^{3}H. The column was calibrated with carbonic anhydrase (MW 30,000), ribonuclease (12,600 MW) and vitamin B 12 (MW 1,200).

2.2.6.3 Fractionation of ^{3}H-palmitate labelled rat liver cytosol on Sephacryl column.

10 ml of the concentrated rat liver cytosol (equivalent to 10 g wet weight liver) was labelled with 25 µl ^{3}H-palmitate (50 µCi) and placed on Sephacryl 300 Superfine column (Pharmacia, North Ryde, N.S.W. Australia. 2 cm x 50 cm dimension). Fractions were eluted with 50 mM Tris-HCl pH 7.6 and collected in tubes at 200 drops/tube. The column was calibrated with thyroglobulin (MW 669,000), ferritin (MW 364,000), albumin (MW 63,000 - 67,000), conalbumin (MW 86,180), myoglobin (MW 18,000) and vitamin B12 (MW 1,200).

2.2.6.4 Fractionation of ^{45}Ca labelled freeze dried rat liver rat liver cytosol on Biogel A-0.5 M column.

Instead of concentrating the original 250,000 x g rat liver supernatant over the PM 10 DIAFLO Ultrafilter, 50 ml of the supernatant was dialysed against a litre of distilled water, (MW cut-off of the dialysed tubing 6,000 - 8,000) to remove ions and substances of MW less than 8,000. The dialysed fraction was then freeze dried. 5 g of the freeze dried rat cytosol was dissolved in 20 ml distilled water, labelled with 100 µCi ^{45}Ca and placed on Biogel A-0.5M column (200 - 400

mesh). Fractions were eluted with 100 mM Tris HCl pH 7.2 and collected in tubes at 150 drops/tube. The column was calibrated with ferritin (MW 364,000), lactate dehydrogenase (MW 150,000), myoglobin (MW 18,000) and vitamin B12 (MW 1,200). The freeze dried rat liver cytosol was also fractionated on the same column in the absence of ^{45}Ca.

2.2.7 Immuno-electrophoresis of ^{3}H-palmitate binding fractions. Approximately 5 - 10 µl of the ^{3}H-binding fractions obtained from the Sephacryl column and the AcA 34 column were applied to agarose electrophoresis film (type 470-10-000 Corning Universal, Palo Alto, Calif. U.S.A.) and the electrophoresis film run for 90 min at 80 V, 10 mA in 0.1 ml Na barbital buffer pH 8.6 on a L.K.B. Model 2117 Multiphor apparatus in accordance with the LKB handbook (L.K.B. Produkter, Bromme, Sweden). Charcoal-treated rat albumin (Pentex fraction V, Miles Lab. Inc. Eckhart, Indiana, U.S.A.) was used as the reference.

2.3 RESULTS

2.3.1 Properties of the rat liver cytosol preparation.

The concentration of some of the components of the rat liver cytosol preparations before concentration which might influence mitochondrial Ca^{2+} transport is shown in Table 1. The value for cytosolic protein obtained was 40 g/kg wet weight liver, i.e. approximately half those obtained by organic separation of mitochondria and cytosol (Soboll et al, 1976) suggesting that only 50 % of the cells were broken by the method of preparation . The pH of the rat cytosol preparation was between pH 6.5 - 6.8 and the osmotic pressure as determined by means of the osmometer was 355 milliosmols/kg.

2.3.2 Mitochondrial Ca^{2+} uptake using the Ca^{2+} electrode - Control experiment.

Mitochondrial Ca^{2+} transport was followed by means of the Ca^{2+} electrode described in chapter 1, section 1.4 . Fig 3 shows that when using the standard incubation medium and sodium succinate (2 mM) as the energy source for Ca^{2+} uptake, the mitochondria rapidly reduced the concentration of the Ca^{2+} in the medium to a value corresponding to approximately 1.0×10^{-6}M (calibrated with Ca-nitrilotriacetic acid buffers - Reed and Bygrave, 1975(a)). It is worth noting that the free Ca^{2+} in the medium was monitored on a log scale therefore even though the result seemed to suggest that the mitochondria had accumulated more than the added Ca^{2+}, possibly the excess Ca^{2+} which would only be in the μM concentration range was due to contamination of the incubation medium with Ca^{2+}, despite attempts to remove the Ca^{2+} contamination of the sucrose

TABLE 1

The concentration of some components of the 250,000 x g rat liver supernatant which may influence mitochondrial Ca^{2+} transport.

Rat livers were perfused with 0.25M sucrose and the cytosol prepared and analysed as described in sections 2.2.1. and 2.2.2. The results are given as mean values for 3 samples. The range is as indicated in brackets.

cytosolic components	per kg wet wt. liver
protein	
cytosolic protein	40 g (38 - 42)
cations	
Ca^{2+}	0.15 mmoles (0.14 - 0.16)
Mg^{2+}	0.4 mmoles (0.38 - 0.42)
K^{+}	75 mmoles (73 - 77)
anions	
inorganic phosphate	2.5 mmoles (2.3 - 2.7)
adenine nucleotides	
ATP	0.108 mmoles (0.103 - 0.113)
ADP	0.156 mmoles (0.154 - 0.158)
AMP	0.108 mmoles (0.104 - 0.112)
fatty acids	0.4 mmoles (0.39 - 0.41)

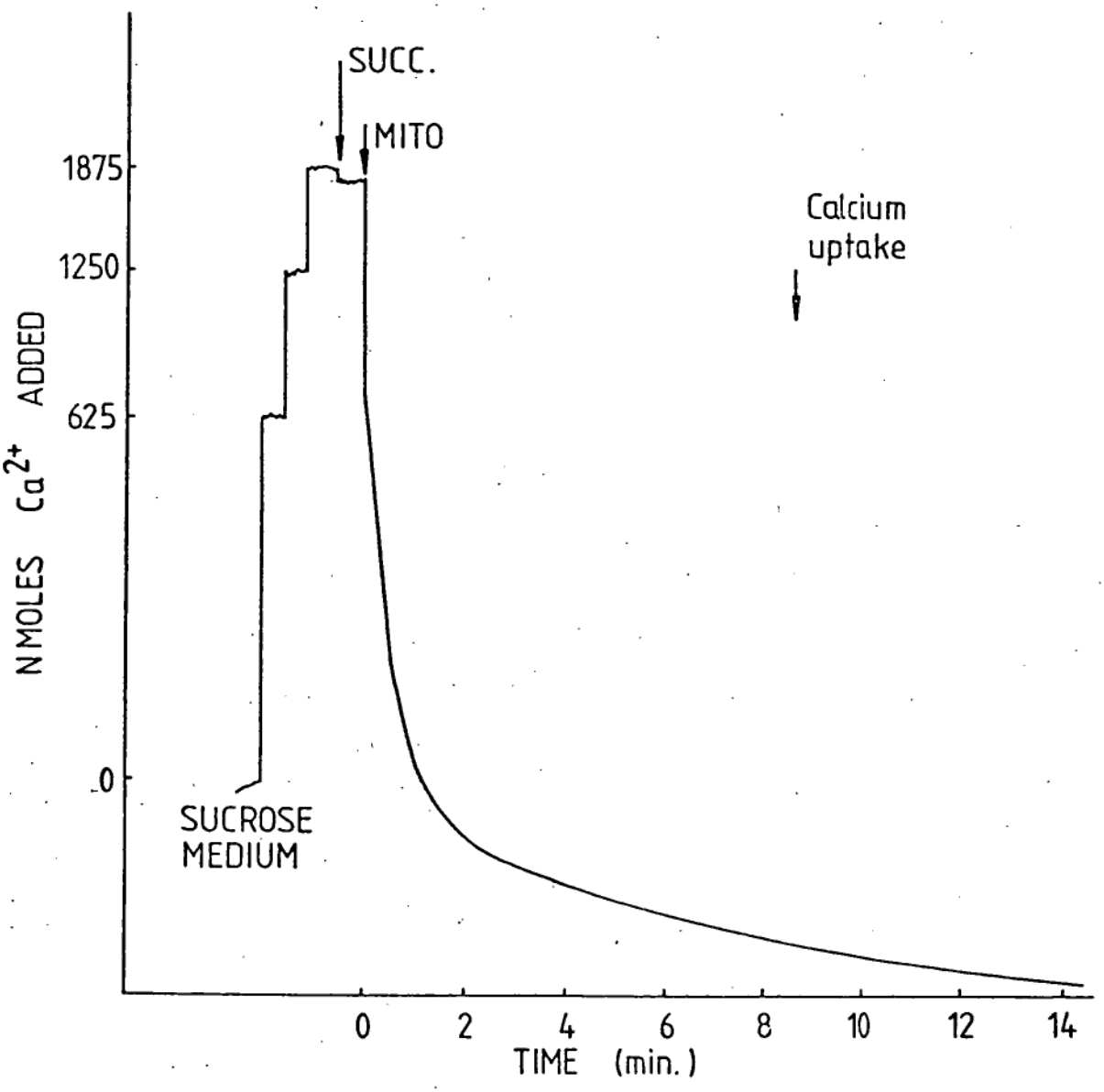
FIGURE 3

Uptake of Ca^{2+} by mitochondria using the Ca-electrode.

Mitochondria (20 mg protein), were added to 5 ml incubation medium containing the standard incubation mixture (i.e. 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM KH_2PO_4 pH 7.4, 1 mM $MgCl_2$, 72 mM KCl), 1875 nmoles Ca^{2+} and 2 mM Na succinate at 25°C. The Ca^{2+} added is shown on a log scale. The electrode was also calibrated with Ca-NTA buffers.

(note: similar result was obtained when 25 mM DL-carnitine HCl was included in the incubation medium)

FIGURE 3


SUCC.
MITO
Calcium uptake
1875
1250
625
0
NMOLES Ca^{2+} ADDED
SUCROSE MEDIUM
0
2
4
6
8
10
12
14
TIME (min.)

solution by filtration through columns of Chelex-100. Approximately 100 nmoles Ca^{2+} was accumulated per mg mitochondrial protein and this was retained by the mitochondria for the 14 min duration of the experiment (fig 3).

2.3.3 The effect of the rat liver cytosol ultrafiltrate (MW < 10,000) on mitochondrial Ca^{2+} uptake and release studied by means of the Ca^{2+}-electrode.

In the presence of 2 ml of the cytosolic ultrafiltrate, a similar result to the control was observed. On adding 10 mg mitochondrial protein, the 1,000 nmoles Ca^{2+} added were accumulated and the mitochondria retained the Ca^{2+} for the 10 min duration of the experiment (Fig 4). The ultrafiltrate contained 1/7 of the concentration present in the original rat cytosol homogenate (i.e. 2 ml ultrafiltrate was equivalent to 0.3 g wet weight liver).

2.3.4 The effect of concentrated rat liver cytosol (MW > 10,000) on mitochondrial Ca^{2+} uptake and release studied by means of the Ca^{2+}-electrode.

1.5 ml concentrated rat liver cytosol (equivalent to 1.5 g wet weight liver) was added to the standard incubation medium (final volume 5 ml). Using 2 mM sodium succinate as the energy source, approximately 66 nmoles Ca^{2+} per mg mitochondrial protein was accumulated (maximal possible mitochondrial Ca^{2+} uptake 100 nmoles Ca^{2+}/mg mitochondrial protein). However, the mitochondria released the accumulated Ca^{2+} almost immediately as shown in fig 5.

FIGURE 4

Effect of the rat liver cytosol ultrafiltrate on mitochondrial Ca^{2+} uptake and release studied by means of the Ca-electrode.

Mitochondria (10 mg protein), were added to 5 ml incubation medium containing the standard incubation mixture, cytosol ultrafiltrate (equivalent to 0.3 g wet wt. liver), 1000 nmoles Ca^{2+} and 2 mM Na succinate at 25°C. The Ca^{2+} added is shown on a log scale.

(note: similar result was obtained when 25 mM DL-carnitine HCl was included in the incubation medium)

succ. = Na succinate

mito. = mitochondria

uf. = ultrafiltrate

FIGURE 4

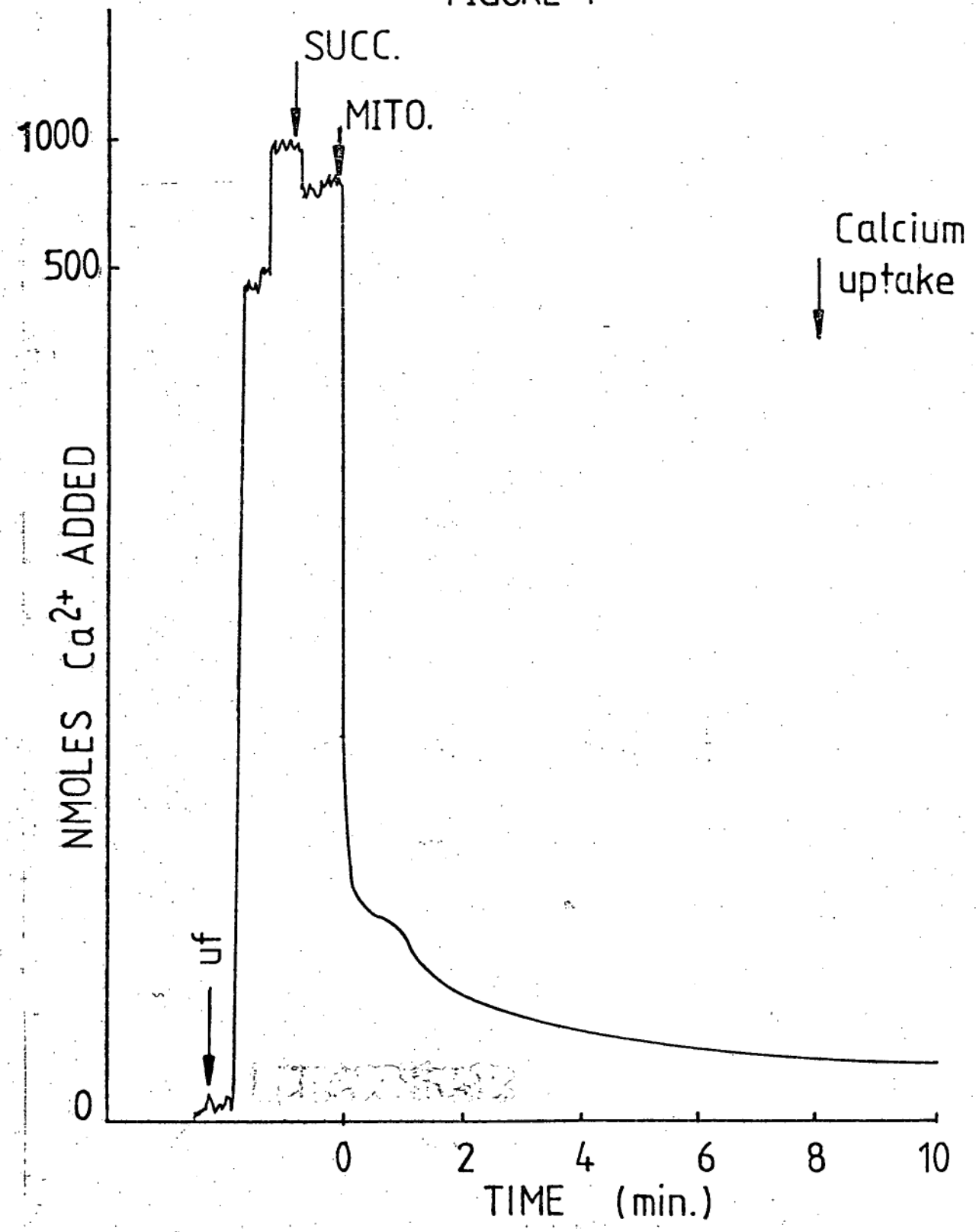

SUCC.
MITO.
1000
500
0
NMOLES Ca^{2+} ADDED
uf
Calcium uptake
0
2
4
6
8
10
TIME (min.)

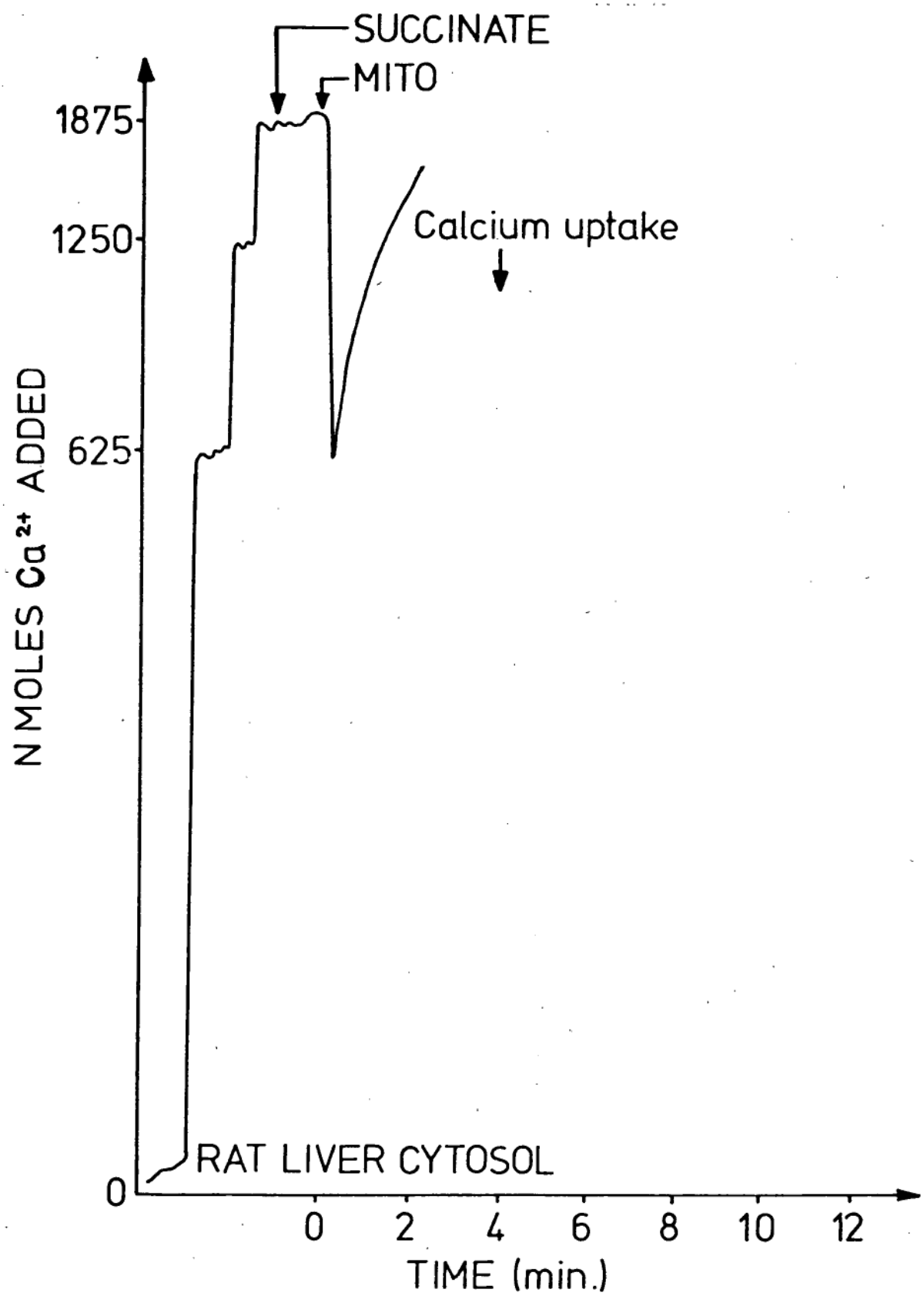
FIGURE 5

The effect of concentrated rat liver cytosol on mitochondrial Ca^{2+} transport studied by the Ca-electrode.

Mitochondria (20 mg protein), were added to 5 ml incubation medium containing the standard incubation mixture, concentrated cytosol (equivalent to 1.5 g wet wt. liver), 1,875 nmoles Ca^{2+} and 2 mM Na succinate at 25°C. The Ca^{2+} added is shown on a log scale.

mito. = mitochondria

FIGURE 5


SUCCINATE
MITO
1875
1250
625
0
Calcium uptake
N MOLES Ca2+ ADDED
RAT LIVER CYTOSOL
0
2
4
6
8
10
12
TIME (min.)

2.3.4.1 Mitochondrial Ca^{2+} uptake in the presence of the concentrated rat liver cytosol and the effect of adding 25 mM carnitine HCl

The inhibitory factors present in the concentrated rat cytosol could be bound fatty acids and/or their derivatives. Carnitine is known to inhibit Ca release from heart mitochondria caused by palmitoyl CoA (Asimakis and Sordahl, 1977) and BSA known to protect mitochondria from the effects of fatty acids (Helinski and Cooper, 1960). Therefore the effect of adding carnitine and BSA to the above experiment (section 2.3.4) was examined.

As shown in fig 6, inclusion of 25 mM carnitine HCl caused almost maximal Ca^{2+} uptake, however the mitochondria began to release the accumulated Ca^{2+} after 4 min. Further additions of carnitine did not cause Ca^{2+} reuptake but the addition of 10 mg BSA (40 µM) did cause reuptake (fig 6). In a separate experiment, the addition of 0.2 ml 25 mM sodium succinate after mitochondrial release induced by the rat liver cytosol, did not cause reuptake as shown in fig 7. Carnitine alone at the concentration used (i.e. 25 mM), had no effect on Ca^{2+} uptake in the control experiment (fig 3) (i.e. standard incubation medium) or in the presence of ultra-filtrate (fig 4). Thus with either the standard incubation medium or in the presence of ultrafiltrate, 25 mM carnitine did not affect results, i.e. maximum mitochondrial Ca^{2+} uptake and Ca^{2+} retention for at least the 10 min duration of the experiment.

2.3.5 The effect of concentrated rat liver cytosol on mitochondrial $^{45}Ca^{2+}$ uptake and release studied by means of the radio-isotope technique

The method and details when using the radio-isotope technique is described in chapter 1, section 1.3. The inhibitory effect of the concentrated rat cytosol on Ca^{2+} uptake was confirmed

FIGURE 6

Mitochondrial Ca^{2+} transport in the presence of the concentrated cytosol and the effect of adding carnitine studied by using the Ca-electrode.

Mitochondria (20 mg protein), were added to 5 ml incubation medium containing the standard incubation mixture, concentrated cytosol (equivalent to 1.5 g wet wt. liver), 2 mM Na succinate, 25 mM DL-carnitine HCl and 1875 nmoles Ca^{2+}. The Ca^{2+} added is shown on a log scale. During Ca^{2+} release from the mitochondria, an additional 25 mM carnitine and 40 µM BSA were added. The opposing arrows indicate the direction of Ca^{2+} uptake and release by the mitochondria. Incubation temp. = 25°C.

succ. = Na succinate

mito. = mitochondria

FIGURE 6

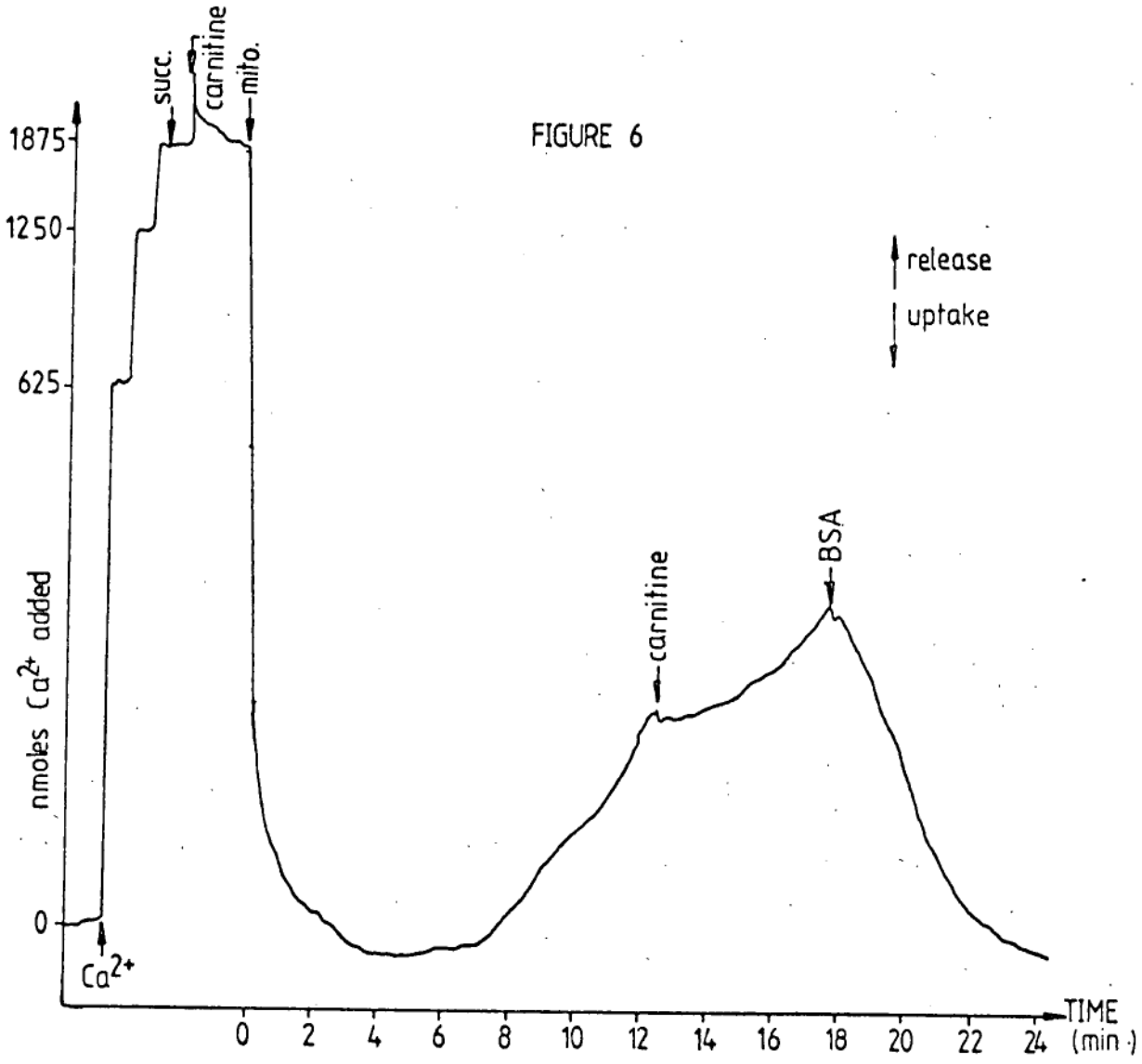

succ.
carnitine
mito.
1875
1250
625
0
nmoles Ca^{2+} added
Ca^{2+}
carnitine
BSA
release
uptake
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME (min)

FIGURE 7

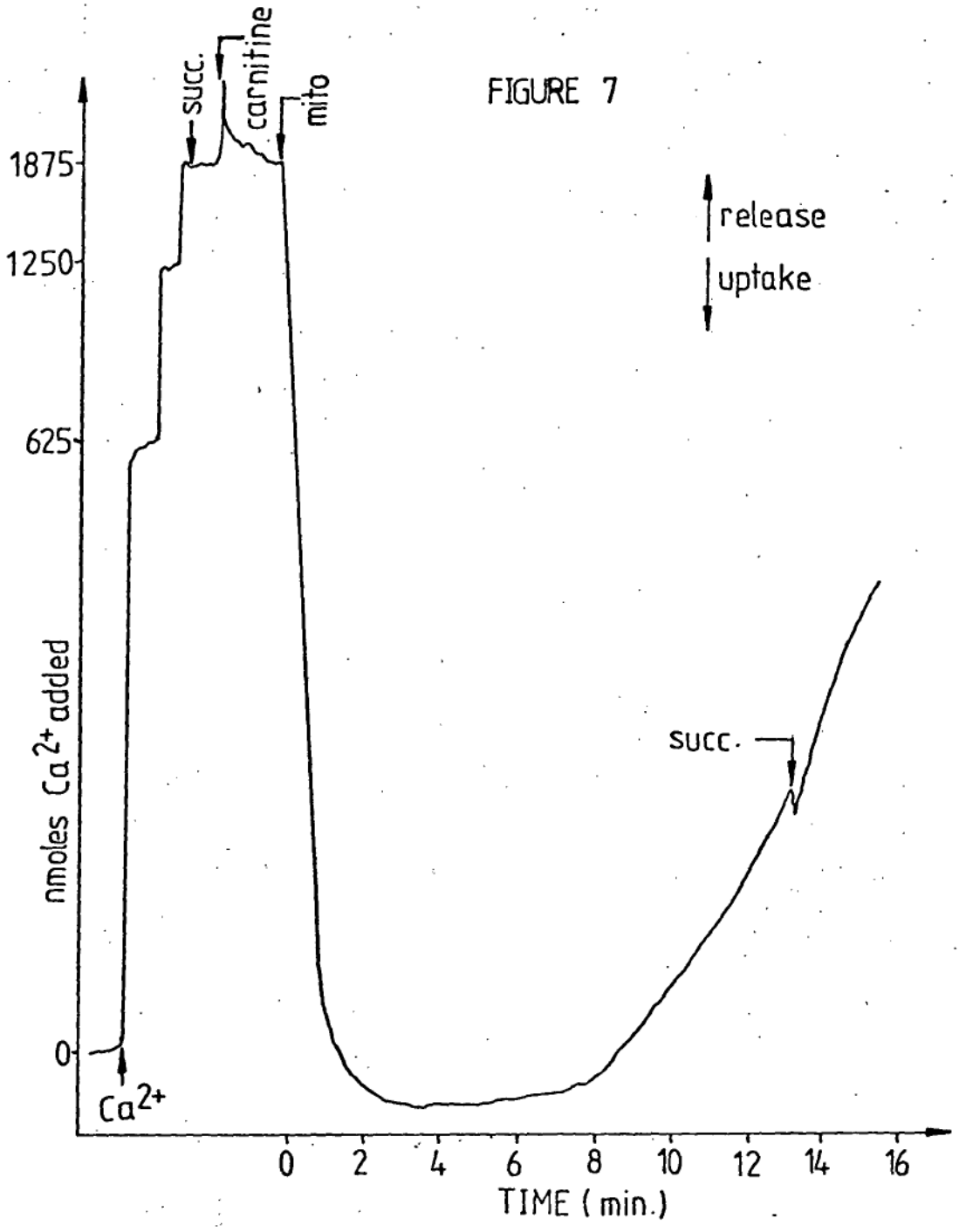
Mitochondrial Ca^{2+} transport in the presence of concentrated cytosol and the effect of adding succinate.

Mitochondria (20 mg protein) were added to 5 ml incubation medium containing the standard incubation mixture, concentrated cytosol (equivalent to 1.5 g wet wt. liver), 1875 nmoles Ca^{2+}, 2 mM succinate and 25 mM DL-carnitine HCl at 25°C. The Ca^{2+} added is shown on a log scale. During Ca^{2+} release from the mitochondria, an additional 2 mM succinate was added. The opposing arrows indicate the direction of Ca^{2+} uptake or release by the mitochondria.

succ. = Na succinate

mito. = mitochondria

FIGURE 7


succ.
carnitine
mito
1875
1250
625
0
release
uptake
nmoles Ca^{2+} added
Ca^{2+}
succ.
0
2
4
6
8
10
12
14
16
TIME (min.)

by the radio-isotope technique. As shown in table 2, no $^{45}Ca^{2+}$ was taken up by the mitochondria in the presence of succinate (2 mM) or when both succinate and ATP were the energy sources.

2.3.5.1 The effect of florisil treatment of concentrated cytosol on mitochondrial Ca^{2+} transport in the presence of carnitine and ATP.

When 50 mM carnitine, 1 mM ATP and 2 mM Na succinate were present together with the concentrated rat liver cytosol, $^{45}Ca^{2+}$ was maximally accumulated (85 % of the total $^{45}Ca^{2+}$ added) and this $^{45}Ca^{2+}$ was retained for as long as 40 min, even longer than the control experiment where the $^{45}Ca^{2+}$ started to be released at 20 min as shown in fig 8.

On partially reducing the fatty acid content of the concentrated rat cytosol in the incubation medium from 200 μM to 100 μM by filtration through florisil, $^{45}Ca^{2+}$ was retained for up to 55 min in the presence of 1 mM ATP, 2 mM succinate and 50 mM carnitine HCl as shown in fig 8.(Note: florisil treatment of concentrated rat cytosol reduced the cytosolic protein from 40 mg/g wet weight liver to 30 mg/g wet weight liver. However on charcoal treatment (Chen, 1967), the cytosolic protein had decreased from 40 mg/g wet weight liver to 14 mg/g wet weight liver and therefore was considered unsuitable for purifying cytosol).

2.3.6 Mitochondrial Ca^{2+} transport and the effect of rat albumin and bovine serum albumin.

It was observed in section 2.3.4.1 that BSA caused reuptake of the Ca^{2+} released by the concentrated rat cytosol. The effect of albumin on Ca^{2+} transport was therefore examined below.

TABLE 2

Effect of concentrated rat cytosol on mitochondrial Ca^{2+} uptake and release studied by means of the radioassay technique.

Mitochondria (20 mg protein) were added to 5 ml incubation medium containing the standard incubation mixture, 1.5 ml concentrated rat cytosol, 2 mM Na succinate or succinate + ATP (1mM) as the energy source/s for Ca^{2+} uptake by the mitochondria, and 1500 nmoles $^{45}Ca^{2+}$. Note: mito = mitochondrial.

Energy source = succinate (2mM)	
Sampling time (min)	nmoles $^{45}Ca^{2+}$/mg mito. protein
1	5
5	5
10	2
15	3
20	2
25	4
Energy source = succinate (2mM) + 1mM ATP	
1	6
5	8
10	7
15	5
20	4
25	3

FIGURE 8

Mitochondrial Ca^{2+} transport in the presence of concentrated cytosol or florisil-treated concentrated cytosol by the radioassay technique.

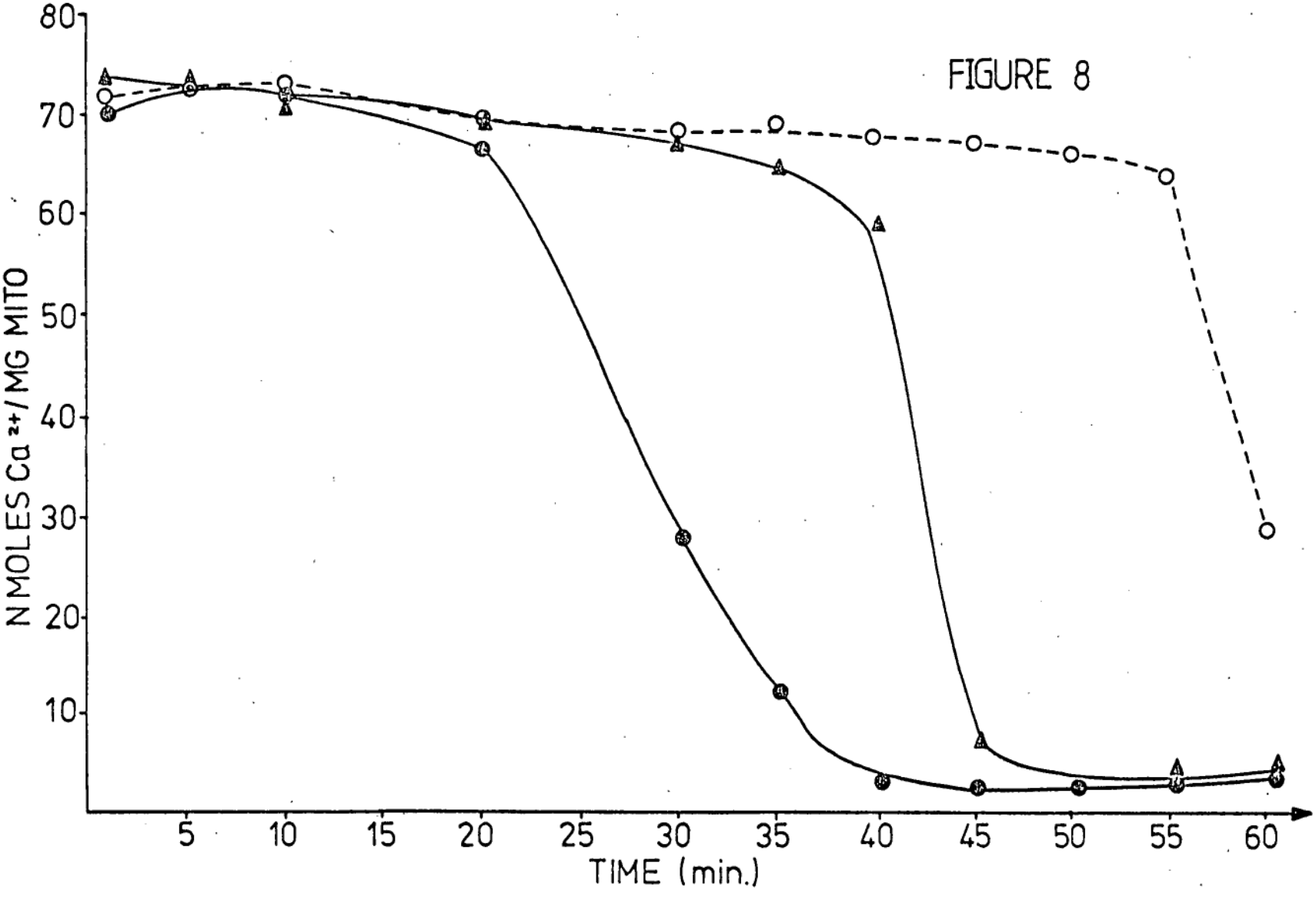
The incubation system (5 ml) contained the standard incubation mixture, 1 mM ATP, 2 mM Na succinate, 50 mM DL-carnitine HCl and mitochondria (29 mg protein). The reaction was started by the addition of 2,500 nmoles $^{45}Ca^{2+}$ (6 µCi). Incubation temp. = 25°C. Additions were made before adding the $^{45}Ca^{2+}$.

—●— control

—▲— + concentrated cytosol (equivalent to 1.5 g wet wt. liver)

---o--- + florisil-treated concentrated cytosol (equivalent to 1.5 g wet wt. liver)

FIGURE 8


80
70
60
50
40
30
20
10
N MOLES Ca 2+/MG MITO
5
10
15
20
25
30
35
40
45
50
55
60
TIME (min.)

2.3.6.1 The effect of charcoal-treated and untreated rat albumin on mitochondrial Ca^{2+} transport studied by means of the Ca^{2+}-electrode.

10 mg rat albumin, as purchased, when added 4 min after uptake of Ca^{2+} by the mitochondria, caused rapid release of Ca^{2+}, the release time observed at 6 min. (2mM Na succinate was used as the energy source for mitochondrial Ca^{2+}-uptake). After treatment of the rat albumin with activated charcoal (chapter 2, section 2.2.3) Ca^{2+} was retained for a longer time and even longer than the control addition of H_2O (fig 9). The control started to release Ca^{2+} at 9 min, while in the presence of charcoal-treated rat albumin, the Ca^{2+} releasing time was 15 min (fig 9).

2.3.6.2 The effect of bovine serum albumin, polyvinyl pyrrolidone and polyethylene glycol on mitochondrial $^{45}Ca^{2+}$ transport studied by means of the radio-isotope technique.

An attempt was made to find out whether the ability of BSA (MW 69,000) to help mitochondrial Ca^{2+} retention was also shared by other high molecular weight polymers such as polyethylene glycol (MW 4,000) or polyvinyl pyrollidone (MW 24,500). The standard incubation medium was used, and for this particular experiment, 2 mM Na succinate and 1 mM ATP were the energy sources for mitochondrial Ca^{2+} uptake. As shown in fig 10, 10 mg BSA (approximately 0.2 µmole) helped $^{45}Ca^{2+}$ retention up to 45 min. However in the presence of 0.2 µmole polyethylene glycol or 0.1 µmole polyvinyl pyrrolidone, $^{45}Ca^{2+}$ release was observed at 15 min similar to the control, suggesting that polyethylene glycol or

FIGURE 9

Effect of charcoal-treated and untreated rat albumin on mitochondrial Ca^{2+} transport studied by means of the Ca-electrode.

Mitochondria (9 mg protein), were added to 5 ml incubation medium containing the standard incubation mixture, 400 nmoles Ca^{2+} and 2 mM Na succinate. Addition of 10 mg untreated rat albumin (solid line(a)) or H_2O (solid line (b)) or 10 mg charcoal-treated rat albumin (solid line (c)) was made at 4 min after adding the mitochondria. Incubation temp. = 25°C.

succ. = Na succinate

mito. = mitochondria

FIGURE 9

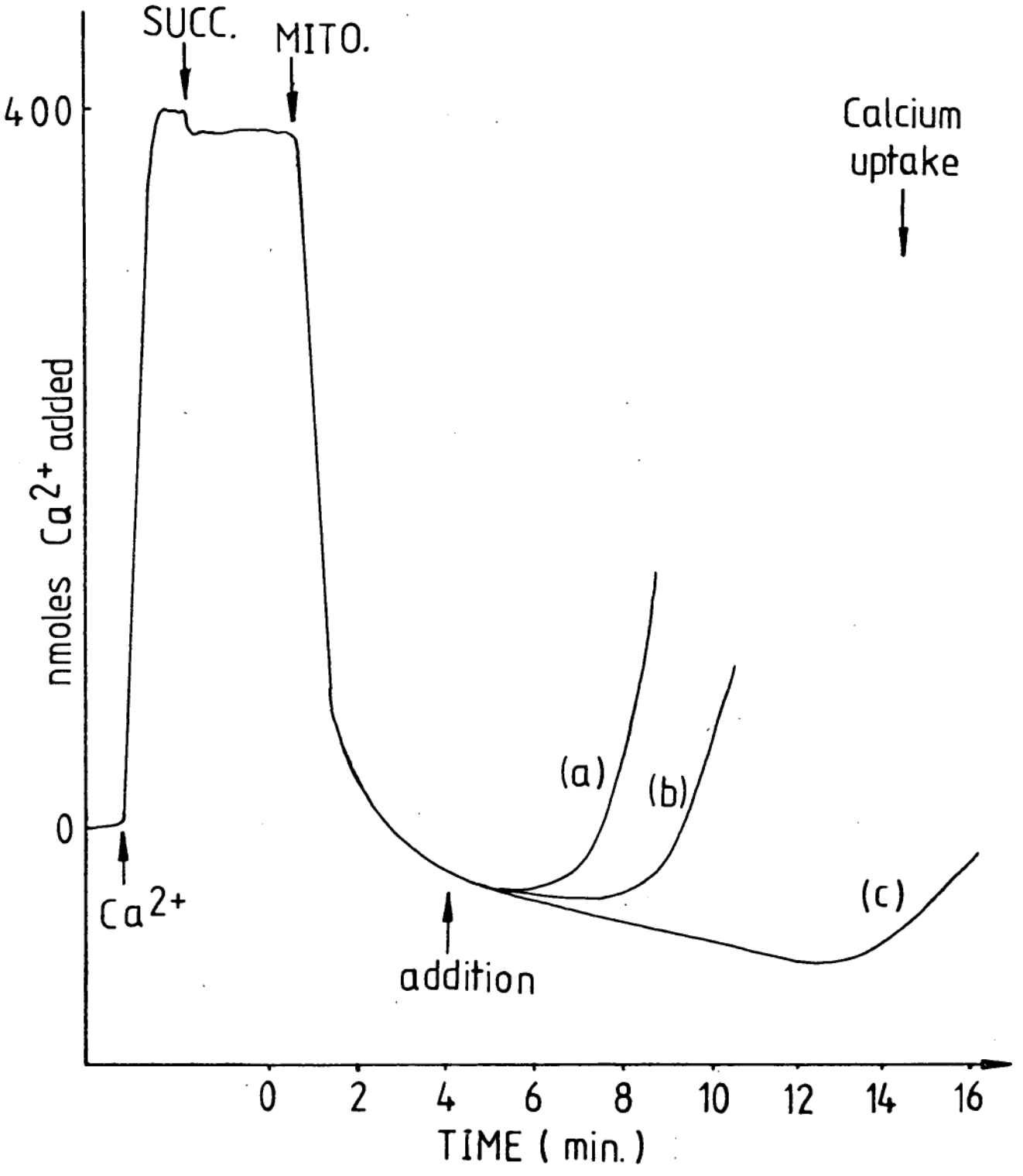

SUCC.
MITO.
400
0
nmoles Ca^{2+} added
Calcium uptake
(a)
(b)
(c)
Ca^{2+}
addition
0
2
4
6
8
10
12
14
16
TIME (min.)

FIGURE 10

Effect of bovine serum albumin (BSA), polyvinyl pyrrolidone and polyethylene glycol on mitochondrial Ca^{2+} transport studied by means of the radioassay technique.

The incubation system (5 ml) contained the standard incubation mixture, 2 mM Na succinate, 1 mM ATP and mitochondria (4.2 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 µCi). Incubation temp. = 25°C. The following additions were made before adding the $^{45}Ca^{2+}$ unless stated otherwise.

—●— control

—▲— + BSA (0.2 µmole)

—■— + polyvinyl pyrrolidone (0.1 µmole)

—○— + polyethylene glycol (0.2 µmole)

---●-- BSA (0.2 µmole) added at 21 min

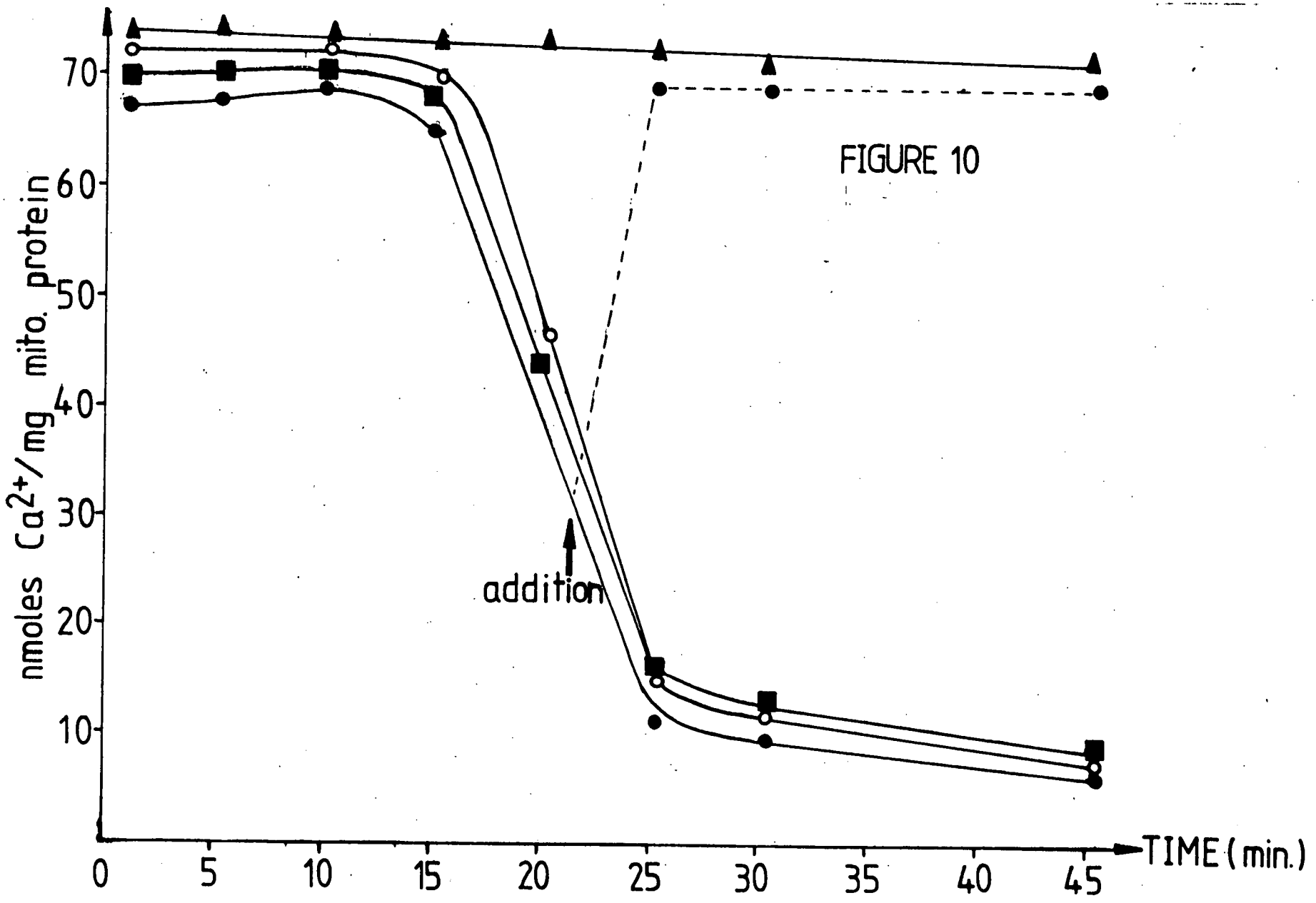

nmoles Ca^{2+}/mg mito. protein
70
60
50
40
30
20
10
addition
0
5
10
15
20
25
30
35
40
45
TIME (min.)

FIGURE 10

polyvinyl pyrrolidone had no significant effect on Ca^{2+} uptake and release by mitochondria. On adding 0.2 µmole BSA at 21 min to the control tube (i.e. at the time of Ca^{2+} release), Ca^{2+} reuptake was observed as shown in fig 10.

2.3.6.3 Ca-binding activity of rat albumin and bovine serum albumin as determined by the Ca^{2+}-electrode

It is also possible that defatted BSA (or rat albumin) helps Ca^{2+} retention in the mitochondria by binding to the free Ca^{2+} in the medium. Therefore, the Ca-binding activity of the albumin (added at the concentration used in the previous experiments) was examined. Measurements on the Ca^{2+}-electrode indicated that the Ca^{2+}-binding activity of 10 mg BSA or 10 mg rat albumin was negligible (fig 11). The Ca^{2+} added is shown on a log scale. Thus BSA or rat albumin added to the incubation medium did not lowere the concentration of free Ca^{2+} in the medium.

2.3.7 Chromatography of the rat liver cytosol

Rat liver cytosol was prepared from livers perfused with sucrose to remove blood. The cytosol had been concentrated by membrane filtration and contained molecules greater than MW 10,000. An outline of the fractionation of the rat liver cytosol on the various columns is shown in diagram 1.

2.3.7.1 Fractionation of ^{3}H-palmitate and ^{45}Ca labelled rat cytosol on the AcA 44 column

The distribution of ^{3}H-palmitate and ^{45}Ca between bound and free forms during chromatography presumably depends on the affinity of the relevant binding proteins for these ligands. A broad peak of protein was eluted between 30 to 120 ml of eluate and at 140 ml, a 280 nm peak of adenine nucleotides were eluted (fig 12). A small peak of ^{45}Ca-binding protein of molecular weight greater than 86,000 but less than 364,000 MW (PTO)

FIGURE 11

Ca-binding activity of rat albumin and bovine serum albumin (BSA) as determined by the Ca-electrode.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture and 400 nmoles Ca^{2+}. Incubation temp. = 25°C. The Ca^{2+} added is shown on a log scale. 10 mg BSA (free from fatty acid) and 10 mg charcoal-treated rat albumin were added at the point indicated.

FIGURE 11

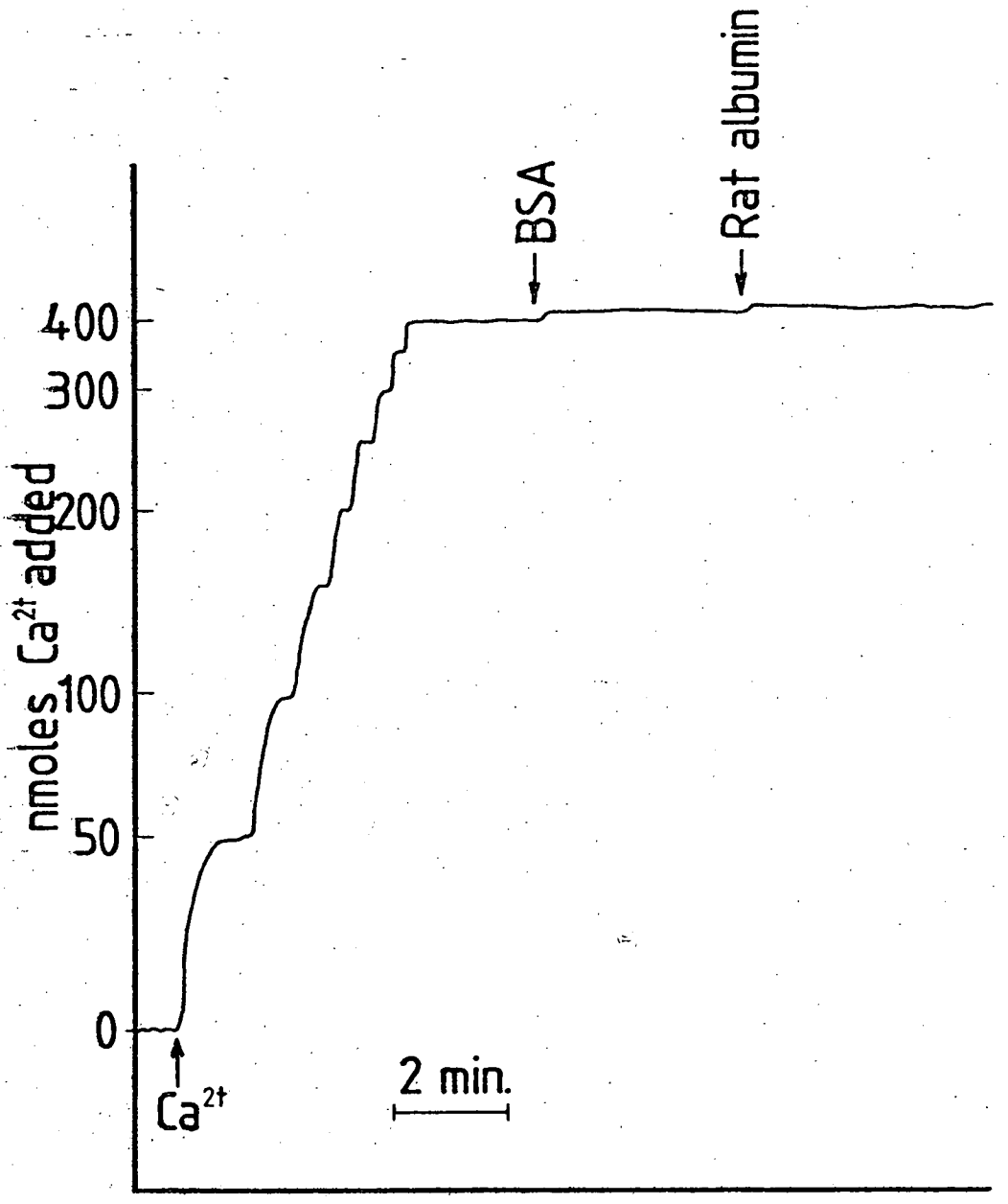


Diagram 1

Fractionation of rat liver cytosol

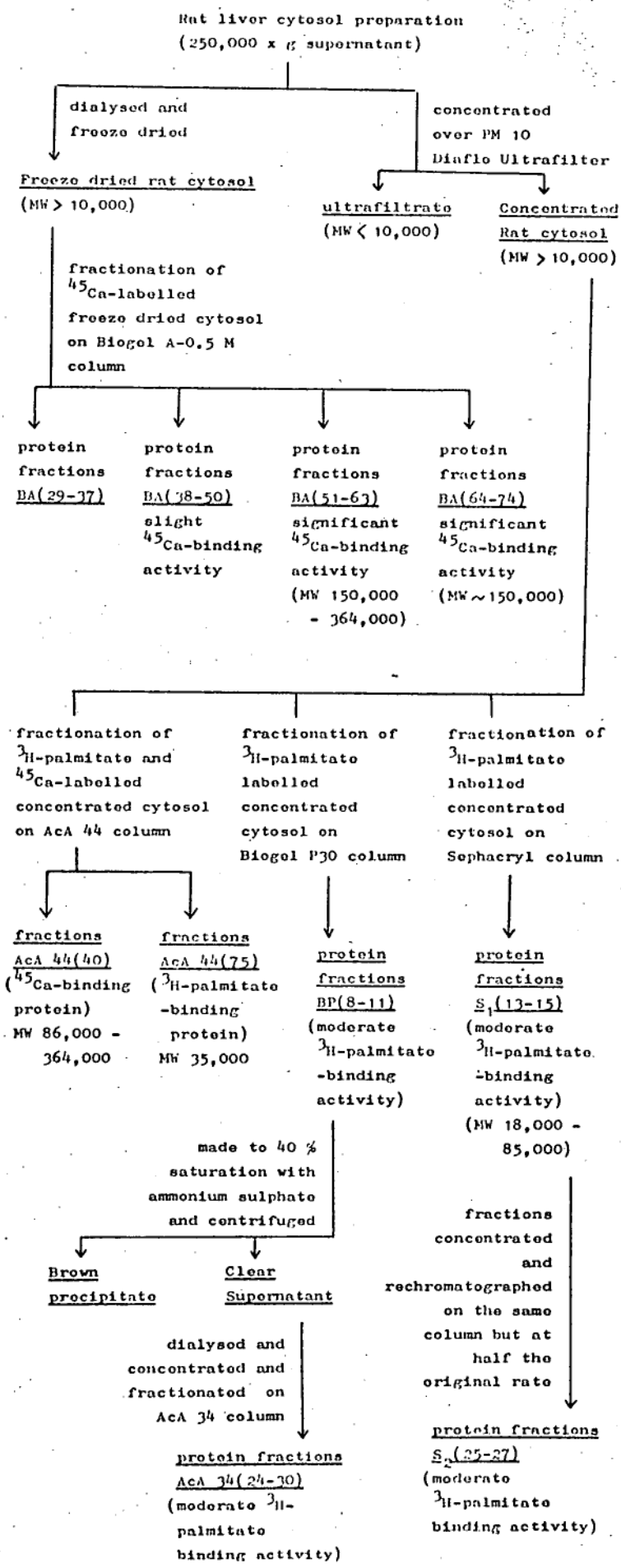


FIGURE 12

Fractionation of ^{3}H-palmitate and $^{45}Ca^{2+}$ labelled concentrated rat liver cytosol on AcA 44 column.

A column of AcA 44 (2 x 50 cm) was used and eluted with 0.1 M Tris-HCl pH 7.6 . 5 ml concentrated cytosol (equivalent to 5 g wet wt. liver) was labelled with 50 µCi (150 µmoles) ^{3}H-palmitic acid plus 10 µCi (2 µmoles) $^{45}CaCl_2$ and passed through the column. Fractionation was carried out at 0 - 4°C. $^{45}Ca^{2+}$ (▭) and ^{3}H (---●---) were counted by liquid scintillation counting. The solid line was a continuous monitoring of absorption at 280 nm.

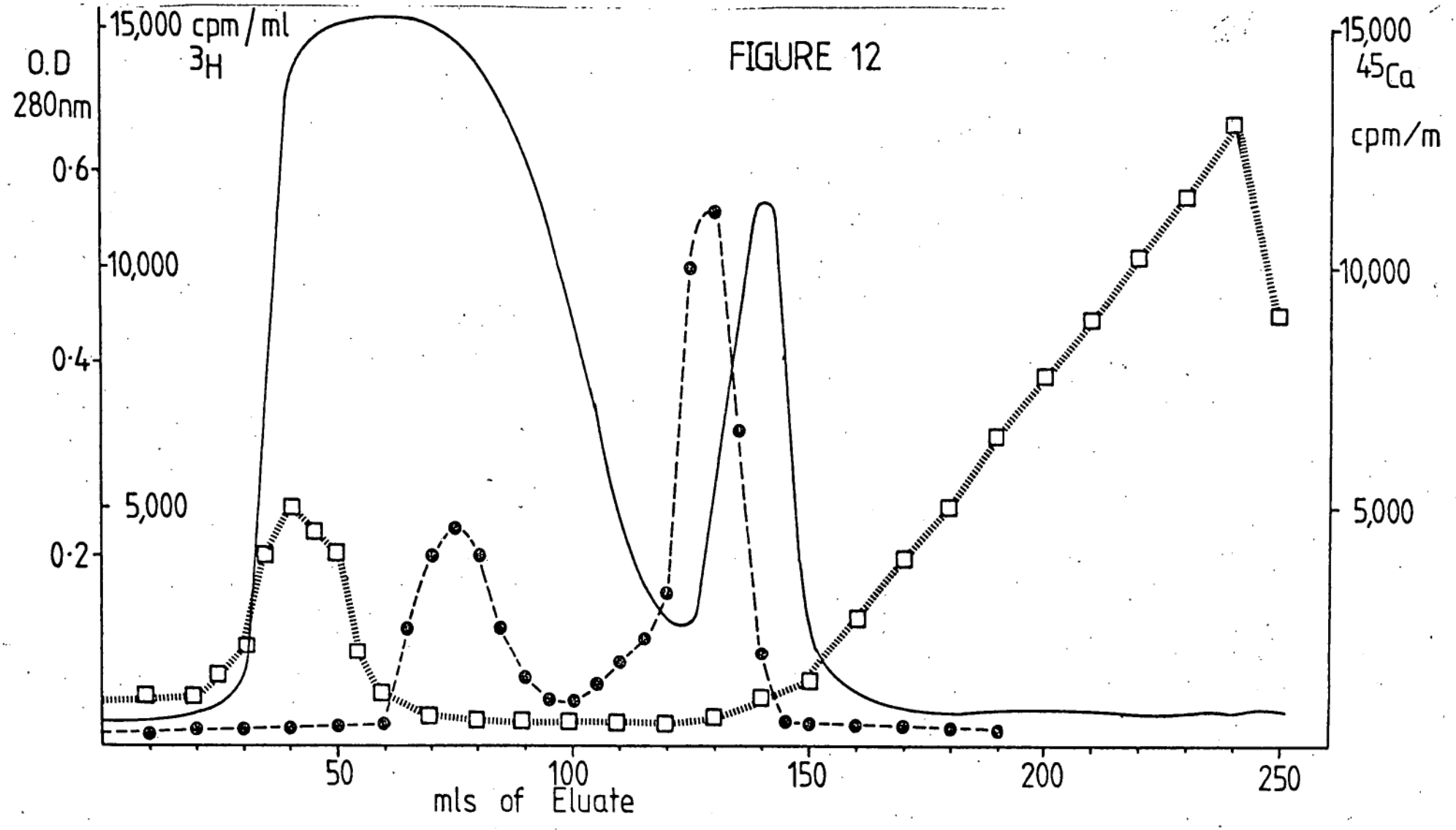


FIGURE 12
O.D 280nm
0·6
0·4
0·2
15,000 cpm / ml ^{3}H
10,000
5,000
15,000 ^{45}Ca cpm/m
10,000
5,000
50
100
150
200
250
mls of Eluate

(corresponding to conalbumin and ferritin respectively on the calibration curve (fig 13)) was eluted at 40 ml and free ^{45}Ca eluted from 150 ml onwards with a peak at 240 ml. Two peaks of ^{3}H-palmitate occurred, one at 75 ml with an approximate molecular weight of 35,000 (corresponding to β-lactoglobulin MW 35,000 on the calibration curve, fig 13). Most of the ^{3}H-palmitate was eluted at 130 ml, which corresponded to a molecular weight of much less than 13,000 (cytochrome C as the reference was eluted at 100 ml (fig 13)) and was probably free ^{3}H-palmitate.

2.3.7.2 Fractionation of ^{3}H-palmitate labelled rat liver cytosol on the Biogel P30 column.

A reddish brown fraction was eluted in tube 6 (fig 14). Fractions in tubes 8 to 11 had a moderate ^{3}H-palmitate count with a molecular weight slightly greater than 30,000 (carbonic anhydrase MW 30,000 was eluted in tube 11 (fig 15)). Maximum ^{3}H-palmitate counts was observed in tube 34, the bulk in tubes 30 - 40. Vitamin B-12 (MW 1,200) was collected in tubes 34 and 35 suggesting that the observed high ^{3}H-palmitate counts in tubes 30 - 40 was probably free ^{3}H-palmitate. Peak 53 had a large symmetrical absorbance and had an absorption spectrum resembling purine nucleotides. Fractions 8 to 11 (denoted as fractions BP(8-11)) were combined (total volume 57 ml) and 1 ml counted for ^{3}H-palmitate and made to 40 % of saturation with ammonium sulphate. The solution was left at 2°C for 30 min, centrifuged and both the clear supernatant and the brown precipitate were dialysed against distilled water in order to remove $(NH_4)_2SO_4$ from the proteins. The dialysed supernatant was concentrated to 5 ml over DIAFLO ultrafilter

FIGURE 13

Calibration of AcA 44 column.

The AcA 44 column was calibrated with 0.4 ml Serva mixture (100 mg/ml) containing ferritin (MW 364,000), conalbumin (MW 86,180), albumin (MW 63,000 - 67,000), β-Lactoglobulin (MW 35,000), myoglobin (MW 18,000), ribonuclease (MW 14,000) and cytochrome c (MW 13,000). Fractions were eluted with 0.1 M Tris-HCl pH 7.6 and collected in tubes at 150 drops/fraction. Calibration was carried out at 0 - 4°C. Eluate was continuously monitored at 280 nm.

FIGURE 13

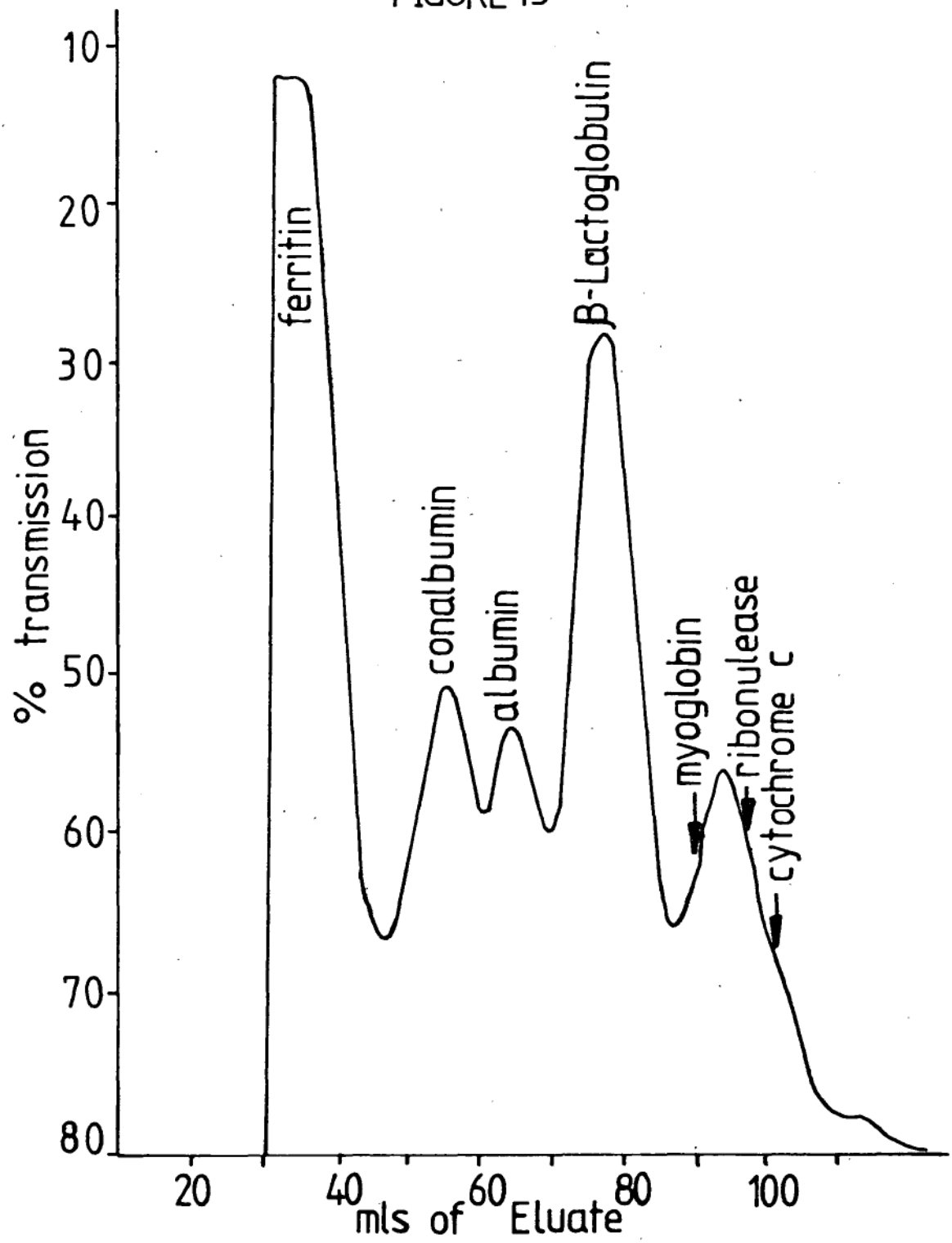

10
20
30
40
50
60
70
80
% transmission
ferritin
conalbumin
albumin
β-Lactoglobulin
myoglobin
ribonulease
cytochrome c
20
40
60
80
100
mls of Eluate

FIGURE 14

Fractionation of ^{3}H-palmitate labelled concentrated rat liver cytosol on Biogel P30 column.

45 ml concentrated cytosol (equivalent to 45 g wet wt. liver) was labelled with 100 µCi ^{3}H-palmitic acid and placed on Biogel P30 column (4 x 60 cm). Fractions were eluted with 50 mM Tris-HCl pH 7.6 and collected in tubes at 250 drops per fraction. Fractionation was carried out at 0 - 4°C. Eluate was continuously monitored at 280 nm.
1 ml of each fraction was counted for ^{3}H by liquid scintillation counting.

FIGURE 14

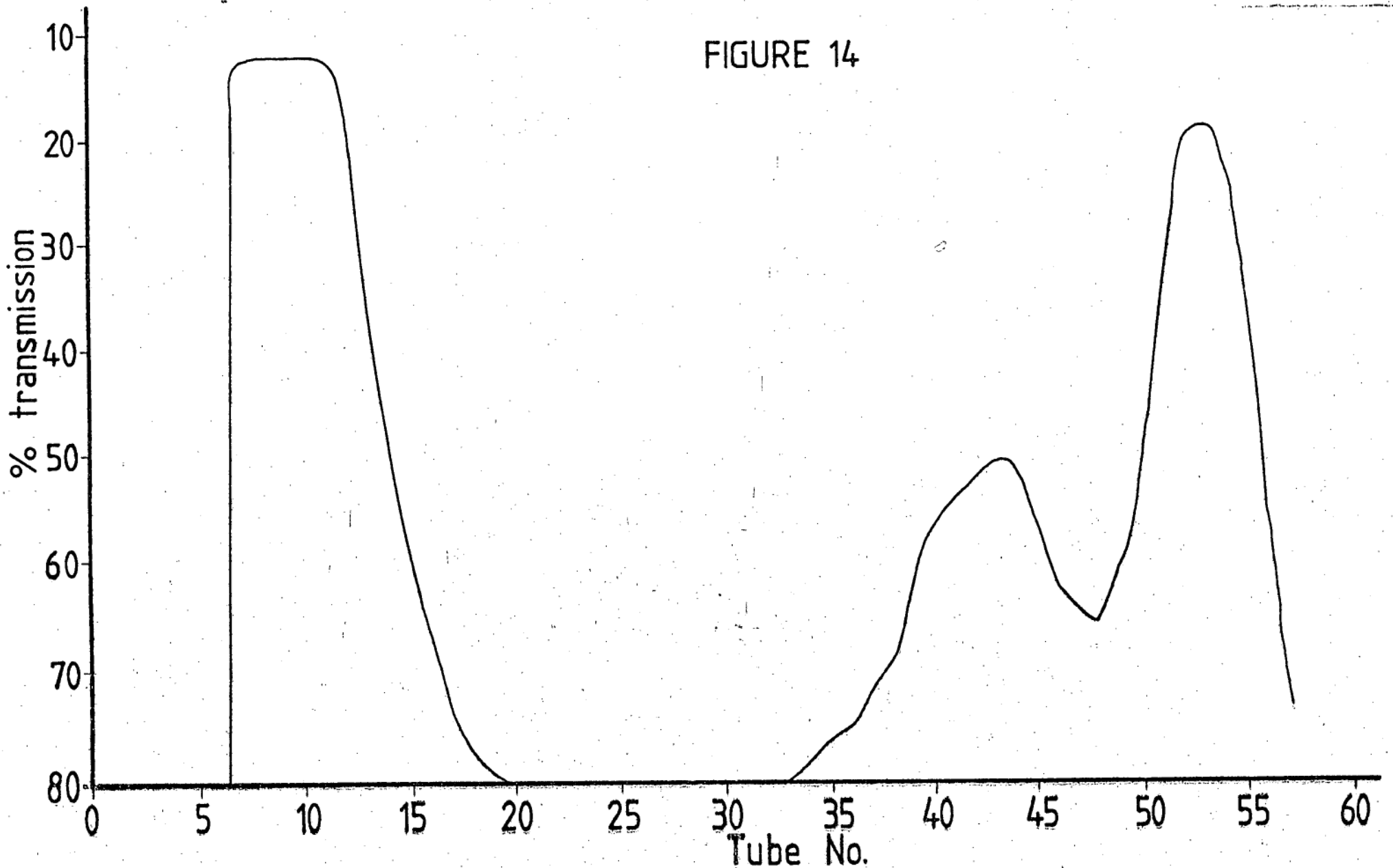
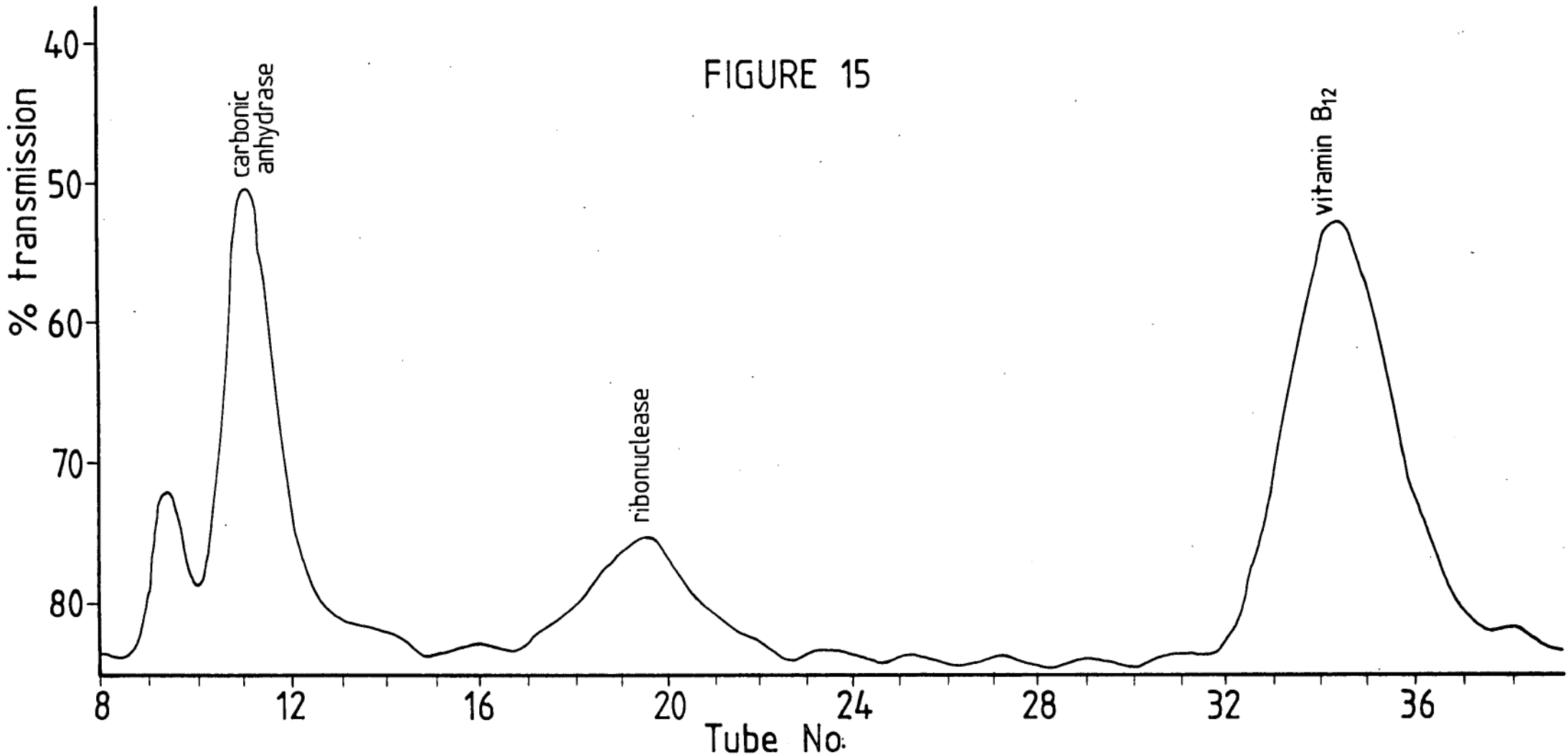

% transmission
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40
45
50
55
60
Tube No.

FIGURE 15

Calibration of Biogel P30 column.

The Biogel P30 column was calibrated with carbonic anhydrase (MW 30,000), ribonuclease (MW 12,600) and vitamin B12 (MW 1,200). Fractions were eluted with 50 mM Tris-HCl pH 7.6 and collected in tubes at 250 drops/fraction. Calibration was carried out at 0 - 4°C. Eluate was continuously monitored at 280 nm.

FIGURE 15


% transmission
40
50
60
70
80
carbonic anhydrase
ribonuclease
vitamin B_{12}
8
12
16
20
24
28
32
36
Tube No.

(MW cut off 10,000, Amincon Corp. Lexington, Mass. U.S.A.). The dialysed brown precipitate most likely contained cytochromes and myoglobin from the cytosol. The ^{3}H-palmitate counts in the various BP(8-11) fractions are as shown in the following scheme:

Clear combined fractions BP(8-11)
Total volume = 57 ml
Total ^{3}H-palmitate = 1.6×10^{6} cpm
Made to 40 % saturation with ammonium sulphate.

↓ centrifuged

Brown precipitate (11 ml)	Clear supernatant (95 ml)
^{3}H-palmitate = 714,956 cpm	^{3}H-palmitate = 593,940 cpm
(most likely contained cytochromes and myoglobin from cytosol)	↓ dialysed against distilled water and concentrated to 5 ml over DIAFLO ultrafilter.
	^{3}H-palmitate = 423,500 cpm.

The dialysed and concentrated clear supernatant obtained was then placed on a AcA 34 column (range 20,000 - 340,000) and eluted with 50 mM Tris HCl pH 7.6 . The brownish fractions in tubes 24 and 25 had moderate ^{3}H-palmitate counts (total ^{3}H-palmitate counts = 85,000 cpm). The remaining ^{3}H-palmitate counts were in the later tubes probably as free ^{3}H-palmitate. The fractions from tubes 24 to 30 (denoted as fractions AcA 34 (24 - 30)) were then concentrated over DIAFLO Ultrafilter

(MW cut off 10,000) to 3 ml and the total ^{3}H-palmitate counts = 90,000 cpm.

An attempt was then made to see whether the concentrated fractions AcA 34(24-30) (i.e. the ^{3}H-palmitate-binding protein) contained rat albumin which is known to bind fatty acids. Two methods were used, namely, (a) double antibody precipitation, (b) Immuno-electrophoresis technique.

2.3.7.2.1 Attempt to show that the ^{3}H-palmitate-binding protein of rat liver cytosol is not rat albumin.

(a) Double Antibody Precipitation.

2 ml of the concentrated fractions AcA 34(24-30) was centrifuged (Eppendorf Centrifuge 5412) for 2 min and 0.1 ml of the clear supernatant was counted for ^{3}H-palmitate. Total counts in 2 ml = 71,640 cpm. 0.1 ml goat antiserum to rat serum albumin (United States Biochem. Co. Cleveland, Ohio) was added to 2 ml of the fractions AcA 34(24-30) and allowed to stand for 16 hr at 2°C. Then 0.1 ml donkey antiserum to goat serum (Wellcome Reagents Ltd. Beckenham England) in 1 ml 0.15 M NaCl solution was added, left for 12 hr at 2°C, then centrifuged at 12,000 x g for 5 min. 0.1 ml of the clear supernatant was counted for ^{3}H-palmitate. The total ^{3}H-palmitate counts was 68,816 cpm. The scheme for the double antibody is shown on the next page.

The double antibody precipitation would remove any rat albumin from the solution. The results obtained showed that most of the ^{3}H-palmitate counts were in the supernatant and not the precipitate suggesting that the ^{3}H-palmitate-binding protein is not rat albumin.

Scheme for the double antibody precipitation:

2 ml fractions AcA 34(24-30)
Total ^{3}H-palmitate counts = 71,640 cpm.

↓

0.1 ml goat antiserum to rat serum albumin added. A faint turbidity observed.

↓

0.1 ml donkey antiserum to goat serum albumin was then added and centrifuged.

precipitate	supernatant (volume 2.2 ml)
(Total ^{3}H-palmitate counts = 1,472 cpm)	Total ^{3}H-palmitate counts = 68,816 cpm.

(b) Immuno-electrophoresis.

The procedure for the electrophoresis on the agarose film is described in section 2.2.7 in this chapter. After electrophoresis a rat albumin and a fraction AcA34(24-30) agarose electrophoresis strip were cut off and stained in Coomasie G 250 in order to examine the protein band. The result is as shown in diagram 2a.

Several protein bands were observed after electrophoresis of the fractions AcA34(24-30) and only a faint band on the agarose electrophoresis strip corresponded to the rat albumin (control). Another of the rat albumin strip and fraction AcA34(24-30) strip were removed and goat antiserum to rat serum albumin was applied to the antibody troughs. The electrophoresis film was kept in a moist chamber for 48 hr and

IMMUNO - ELECTROPHORESIS

DIAGRAM 2a

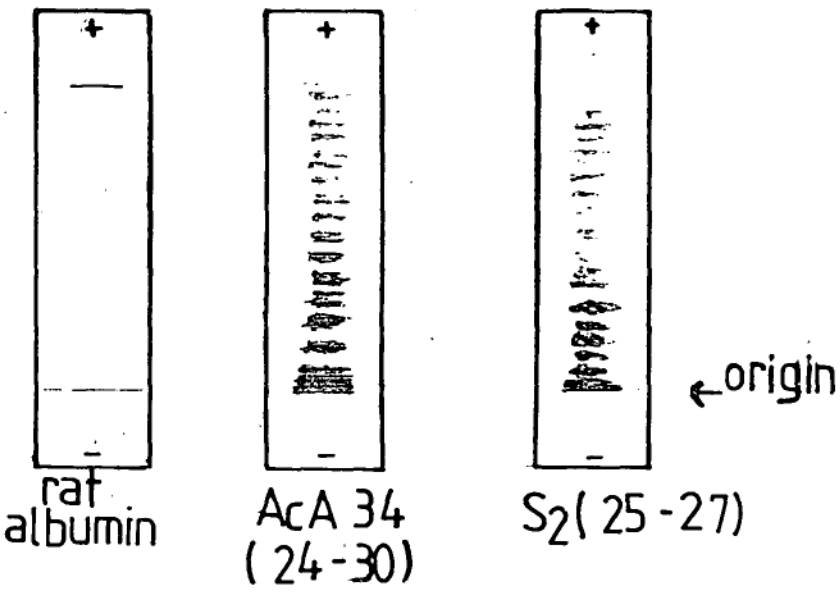
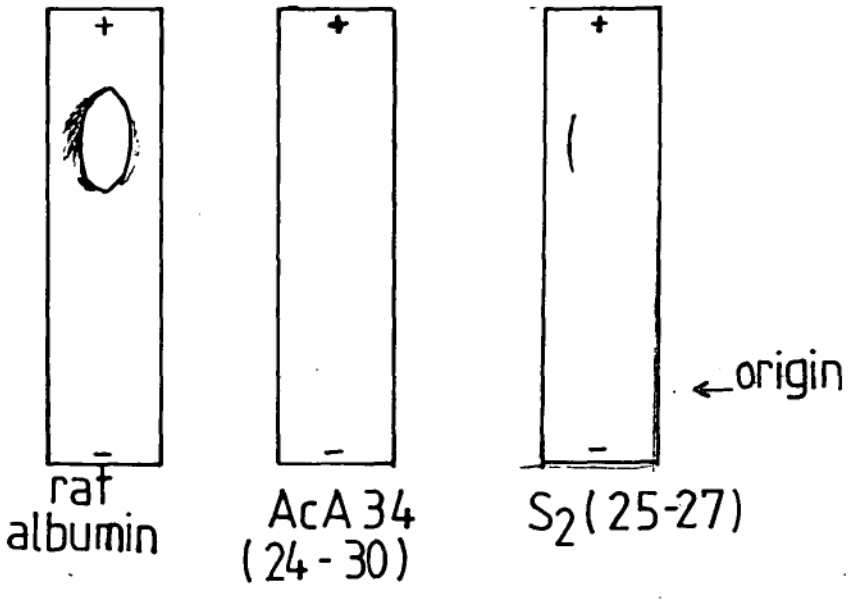


DIAGRAM 2b



the precipitin lines observed, after staining in Coomasie G 250. A distinct goat antiserum to rat serum albumin precipitin was observed with the control rat albumin fraction, however no detectable precipitin was observed with the fractions AcA 34(24-30) (diagram 2b). Thus the result seemed to suggest that there is no detectable rat albumin in the fractions AcA 34(24-30). An attempt was also made to locate the ^{3}H-palmitate on the fractions AcA 34(24-30) electrophoresis strip. After electrophoresis, a further strip of the fractions AcA 34(24-30) was cut into 4 mm strips then dissolved in 0.2 ml NCS solubilizer, 10 ml scintillation fluid and 25 µl acetic acid and counted for ^{3}H-palmitate by liquid scintillation counting. The result from the counts indicated that most of the ^{3}H-palmitate were located around the origin (i.e. from 12 mm above the origin to 8 mm below the origin with most counts at the origin) (Table 3).

The result obtained after Coomasie G 250 staining (diagram 2a) showed that there were proteins at the origin and also above and below it. Since most of the ^{3}H-palmitate counts were around the origin, 12 mm above and 8 mm below origin, it seemed as though the ^{3}H-palmitate-binding protein remained at the origin after electrophoresis and appeared to have a low negative charge.

2.3.7.3 Fractionation of ^{3}H-palmitate-labelled rat liver cytosol on Sephacryl column.

A brownish eluant was observed in tubes 13, 14 and 15. These fractions had moderate ^{3}H-palmitate counts (fig 16) (total ^{3}H counts in the tubes 13, 14, 15 = 632,008 cpm). Maximum ^{3}H-palmitate counts were observed in tube 21 (fig 16), most

TABLE 3

^{3}H-palmitate counts on the AcA 34 (24 - 30) electrophoresis strip.

After electrophoresis, a strip of the fraction AcA 34 (24 - 30) was cut into 4 mm strips, then dissolved in 0,2 ml NCS solubilizer, 10 ml scintillation fluid and 25 µl acetic acid and counted for ^{3}H-palmitate by liquid scintillation counting.

(a) Sample No.	cpm
16	32
15	32
14	25
13	28
12	30
11	30
10	35
9	36
8	36
7	45
6	44
5	46
4	51
3	63
2	65
1 (origin)	138
-1	61
-2	60
-3	29
-4	26
-5	31

(a) Sample No. 1 denotes sample 4 mm above the origin, No. 2 8 mm and so on. The negative sample nos. denote samples below the origin e.g. sample No. -1 shows 4 mm below the origin.

FIGURE 16

Fractionation of ^{3}H-palmitate labelled concentrated rat cytosol on Sephacryl 300 Superfine column.

10 ml concentrated cytosol (equivalent to 10 g wet wt. liver) was labelled with 50 µCi ^{3}H-palmitic acid and placed on the Sephacryl column (2 x 50 cm). Fractions were eluted with 50 mM Tris-HCl pH 7.6 and collected in tubes at 200 drops/tube. Fractionation was carried out at 0 - 4°C. Eluate was continuously monitored at 280 nm. 1 ml of each fraction was counted for ^{3}H by liquid scintillation counting.

——— (solid line) continuous monitoring of eluate at 280 nm

--●-- ^{3}H (cpm/ml)

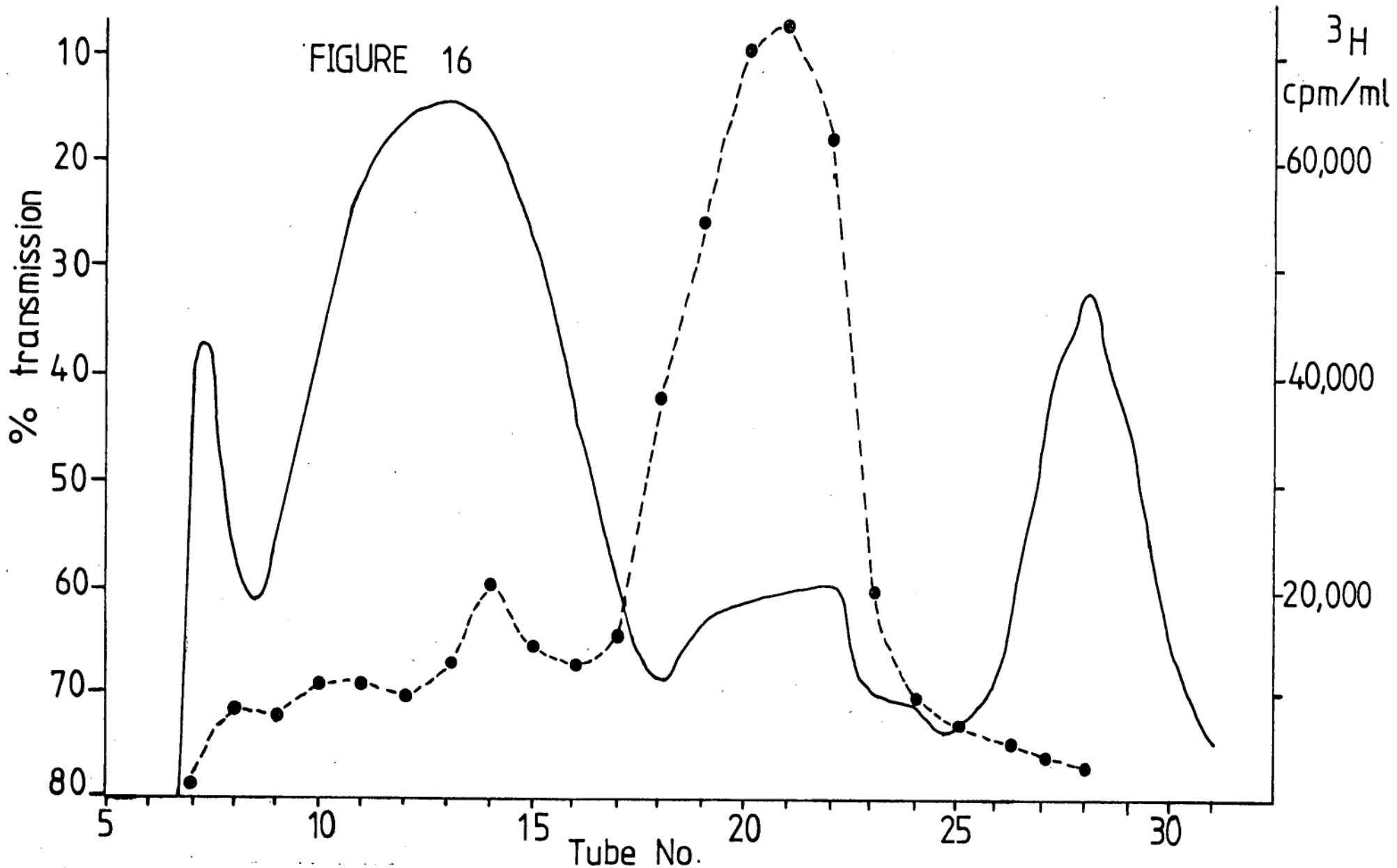


FIGURE 16
% transmission
10
20
30
40
50
60
70
80
5
10
15
20
25
30
Tube No.
^3H
cpm/ml
60,000
40,000
20,000

probably free ^{3}H-palmitate (note that vitamin B-12 was eluted in tube 19 on the calibration curve (fig 17)). The molecular weight of the fractions 13, 14, 15 is between MW 18,000 - 85,000 (corresponding to conalbumin and myoglobin on the calibration curve). Peak 28 had a fairly symmetrical absorbance and had an absorption spectrum resembling purine nucleotides.

The fractions 13-15 (denoted as fractions S_1(13-15)) were combined and concentrated to 1 ml (DIAFLO Ultrafilter) and rechromatographed on the same Sephacryl column with vitamin B-12, and collected in tubes at half the original rate, i.e. 100 drops/fraction (previous run on the Sephacryl column was at 200 drops/fraction). The rechromatographed fractions S_1(13-15) were collected in tubes 25-27 (denoted as fractions S_2(25-27)) and vitamin B-12 (MW 1,200) was collected in tube 39 as shown in fig 18, i.e. MW of the fractions S_2(25-27) is greater than 1,200.

Most of the ^{3}H-palmitate counts were observed in tubes 22 - 28 (total ^{3}H-palmitate counts = 423,499 cpm). Since the molecular weight of the fractions S_2(25-27) is greater than 1,200 , most likely the ^{3}H-palmitate is bound to the protein rather than free ^{3}H-palmitate. The fractions S_2(25-27) were combined and concentrated to 1 ml (DIAFLO Ultrafilter). An examination was made to see whether the ^{3}H-palmitate-binding protein (i.e. fractions S_2(25-27)) was rat albumin. The immunoelectrophoresis technique was used. Several protein bands were observed after Coomasie G 250 staining and only a faint protein band corresponded to the rat albumin (control) as shown in diagram 2a.

FIGURE 17

Calibration of Sephacryl 300 Superfine column.

The Sephacryl column was calibrated with thyroglobulin (MW 669,000), ferritin (MW 364,000), albumin (MW 63,000 - 67,000), conalbumin (MW 86,180), myoglobin (MW 18,000) and vitamin B12 (MW 1,200). Fractions were eluted with 50 mM Tris-HCl pH 7.6 and collected in tubes at 200 drops/tube. Calibration was carried out at 0 - 4°C. Eluate was continuously monitored at 280 nm.

FIGURE 17

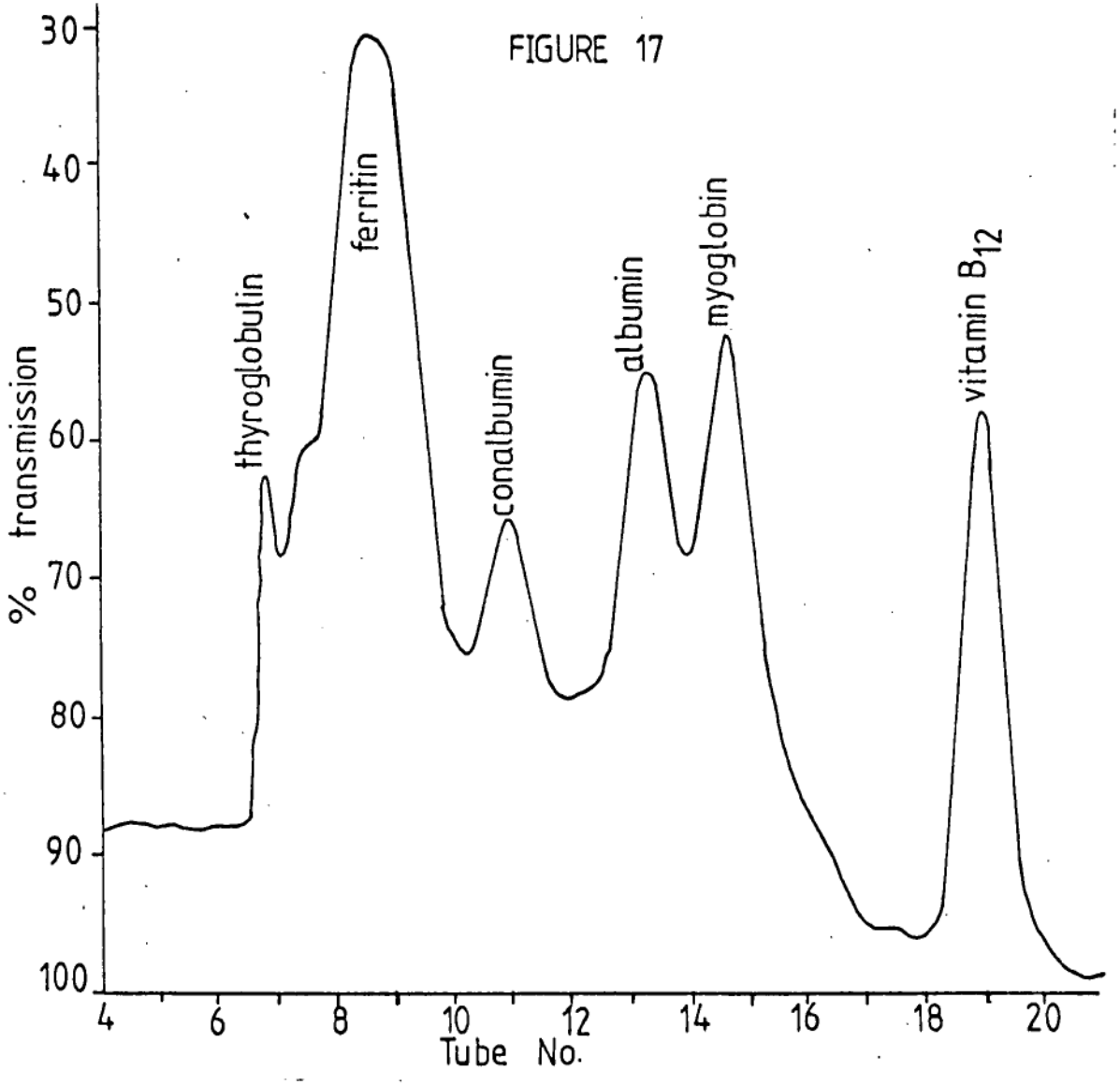

30
40
50
60
70
80
90
100
% transmission
thyroglobulin
ferritin
conalbumin
albumin
myoglobin
vitamin B_{12}
4
6
8
10
12
14
16
18
20
Tube No.

FIGURE 18

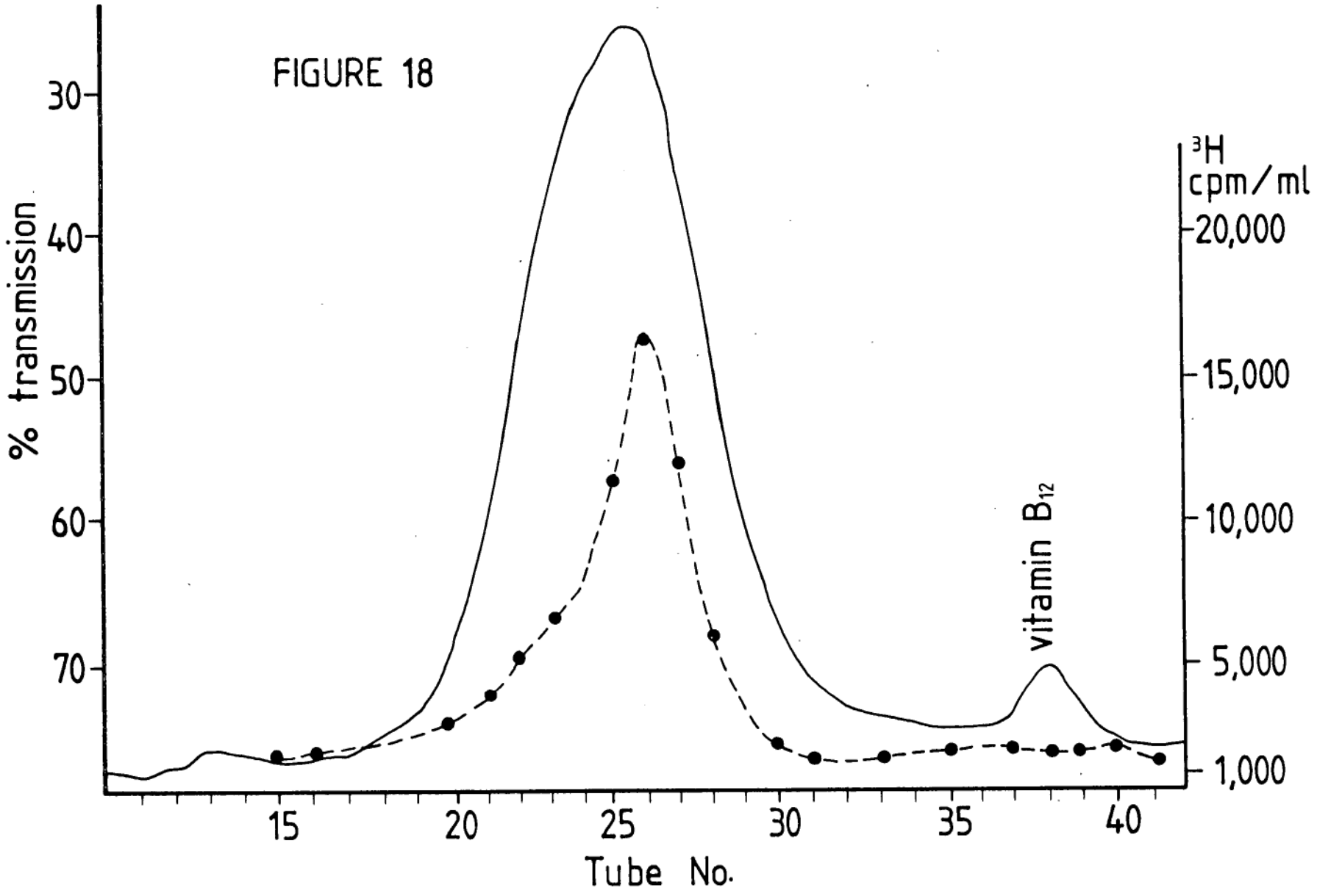
Rechromatography of protein fractions S_1(13 - 15) on Sephacryl 300 Superfine column.

Fractions S_1(13 - 15) were combined and concentrated to 1 ml and rechromatographed on the Sephacryl column with vitamin B12. Fractions were eluted with 50 mM Tris-HCl pH 7.6 and collected in tubes at 100 drops/fraction. Rechromatography was carried out at 0 - 4°C. Eluate was continuously monitored at 280 nm. 1 ml of each fraction was counted for ^3H by liquid scintillation counting.

——— (solid line) continuous monitoring of eluate at 280 nm

--●-- ^3H (cpm/ml)

FIGURE 18


% transmission
30
40
50
60
70
^{3}H cpm/ml
20,000
15,000
10,000
5,000
1,000
vitamin B_{12}
15
20
25
30
35
40
Tube No.

One strip of the fractions S_2(25-27) was also examined for goat antiserum to rat serum albumin precipitin after applying the antibody to the antibody troughs (the procedure similar to that with fractions AcA 34(24-30), section 2.3.7.2.1(b). The result showed a faint precipitin at the position of the control (i.e. with rat serum albumin) as shown in diagram 2b. This suggests that there was trace amounts of rat serum albumin in the fractions S_2(25-27).

After electrophoresis, a further strip of fraction S_2(25-27) was cut into 4 mm strips then dissolved in 0.2 ml °NCS solubilizer, 10 ml scintillation fluid and 25 µl acetic acid and counted for ^{3}H-palmitate by liquid scintillation. A similar result to that of ^{3}H-palmitate-binding protein (fractions AcA 34(24-30)) was observed, i.e. most of the ^{3}H-palmitate counts were located around the origin. This suggests that the ^{3}H-palmitate-binding protein, (fractions S_2(25-27)) is also not rat albumin.

2.3.7.4 Fractionation of ^{45}Ca labelled rat liver cytosol (dialysed and freeze dried) on Biogel A-0.5 M column.

A jelly-like, colourless, protein containing eluant was collected in fraction nos. 29 - 37 (denoted as fractions BA(29-37)) corresponding to a molecular weight greater than 364,000 (ferritin as the reference) (fig 19). In fractions 38 - 50 (denoted as fractions BA(38-50)) (MW $\sim$ 364,000) which had a yellowish coloration, the ^{45}Ca-binding activity was insignificant (maximal ^{45}Ca-binding activity per ml = 25,000 cpm). High ^{45}Ca-binding activity (maximum per ml $\sim$ 200,000 cpm) was noted for fraction nos. 51-63 (denoted as fractions BA(51-63)) (yellowish solution) and fraction nos. 64 - 74

FIGURE 19

Fractionation of $^{45}Ca^{2+}$ labelled freeze dried rat liver cytosol on Biogel A - 0.5 M column.

5 g freeze dried cytosol was dissolved in 20 ml distilled H_2O, labelled with 100 µCi $^{45}CaCl_2$ and placed on Biogel A - 0.5 M column (4 x 60 cm). Fractions were eluted with 100 mM Tris-HCl pH 7.2 and collected in tubes at 150 drops/tube. The column was also calibrated with ferritin (MW 364,000), lactate dehydrogenase (MW 150,000), myoglobin (MW 18,000) and vitamin B12 (MW 1,200). Fractionation and calibration were carried out at 0 - 4°C. Eluate was continuously monitored at 280 nm. 1 ml of each fraction was counted for ^{45}Ca by liquid scintillation counting. Ferritin was collected in tube no. 41; Lactate dehydrogenase tube no. 65; myoglobin tube no. 73 and vitamin B12 in tube no. 86.

—— (solid line) continuous monitoring of eluate at 280 nm

—●— ^{45}Ca (cpm/ml)

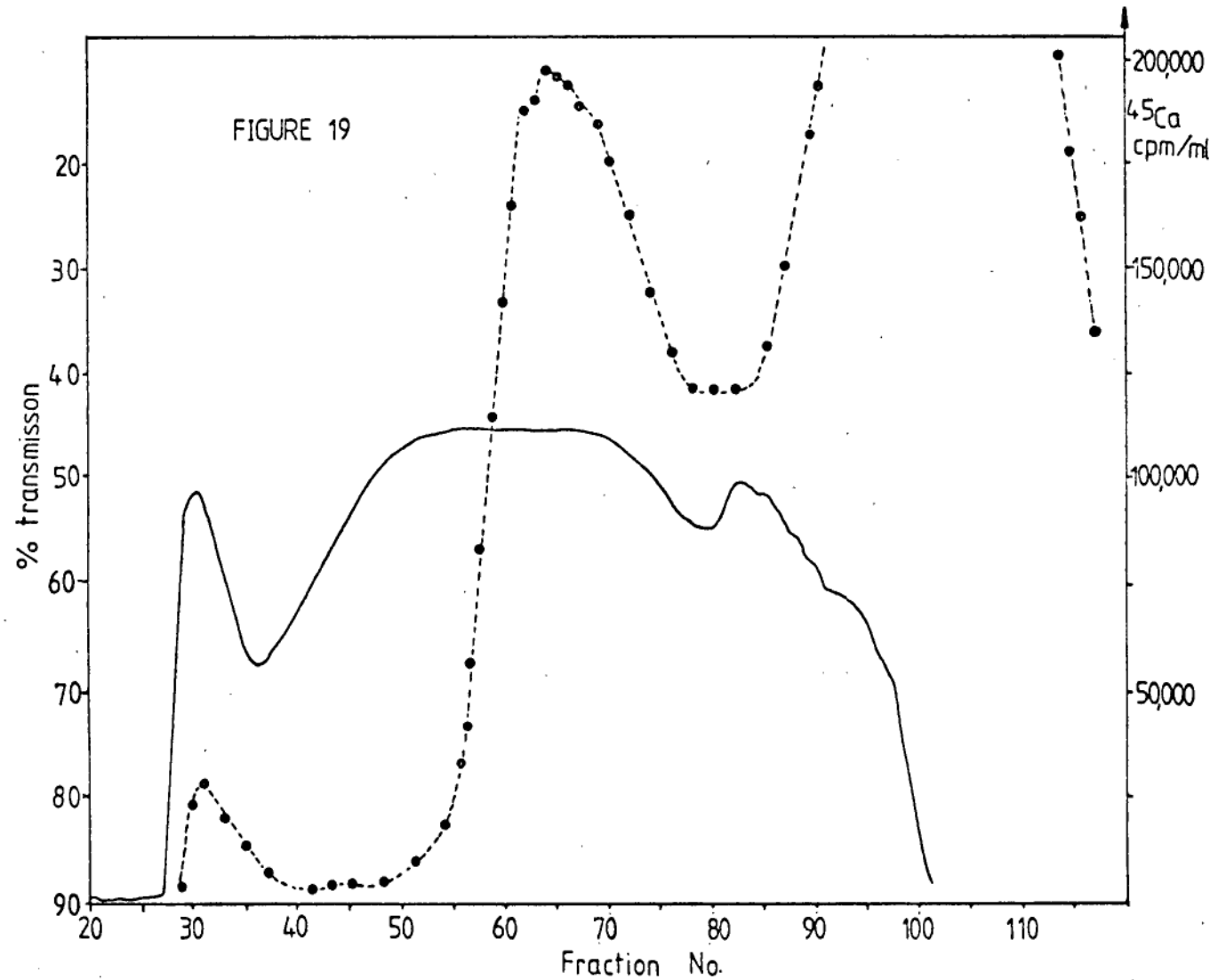


FIGURE 19
% transmisson
20
30
40
50
60
70
80
90
20
30
40
50
60
70
80
90
100
110
Fraction No.
200,000
150,000
100,000
50,000
45Ca
cpm/ml

(denoted as fractions BA(64-74)) (pinkish solution). The molecular weight of the protein fractions BA(64-74) is approximately 150,000 (lactate dehydrogenase as the reference) and protein fractions BA(51-63) is between MW 150,000 - 364,000. A pale orange eluant was observed in fraction nos. 75 - 90. The remaining ^{45}Ca was collected in fraction no. 74 onwards with its peak in fraction no. 101 ($\sim$535, 715 cpm per ml). Since vitamin B 12 was collected in fraction no. 86 when calibrating the column, most likely the ^{45}Ca observed in fraction 101 was free ^{45}Ca. The respective protein fractions was then concentrated (PM 10 DIAFLO Ultrafilter).

2.3.8 Mitochondria Ca^{2+} movement in the presence of ^{3}H-palmitate-binding protein isolated from rat liver cytosol:- studied by means of the radio-isotope technique.

The ^{3}H-palmitate-binding protein was isolated from AcA 34 column (fractions AcA 34(24-30)) and also from Sephacryl column (fractions S_2(25-27)). Its effects on mitochondrial Ca^{2+} transport was then examined in the presence of 1 mM ATP and 2 mM β-hydroxybutyrate as energy sources. The radio-isotope technique described in chapter 1, section 1.4 was used. The control mitochondria accumulated 85 % of the added $^{45}Ca^{2+}$ to the incubation medium. Additions of the ^{3}H-palmitate-binding protein were made at 5 min after adding $^{45}Ca^{2+}$. As shown in fig 20, 50 µl of fractions S_2(25-27) equivalent to the ^{3}H-palmitate-binding protein fractions from 0.25 g wet liver, caused an earlier release compared to the control. On partial removal of free fatty acid with florisil, $^{45}Ca^{2+}$ was retained for 5 min longer than the untreated fractions, but still caused

FIGURE 20

Mitochondrial Ca^{2+} movement in the presence of ^{3}H-palmitate-binding protein isolated from the concentrated rat liver cytosol studied by means of the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (6.4 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 µCi). Incubation temp.= 25°C. Additions of untreated or florisil-treated ^{3}H-palmitate-binding protein fractions were made at 5 min after adding the $^{45}Ca^{2+}$.

—●— control

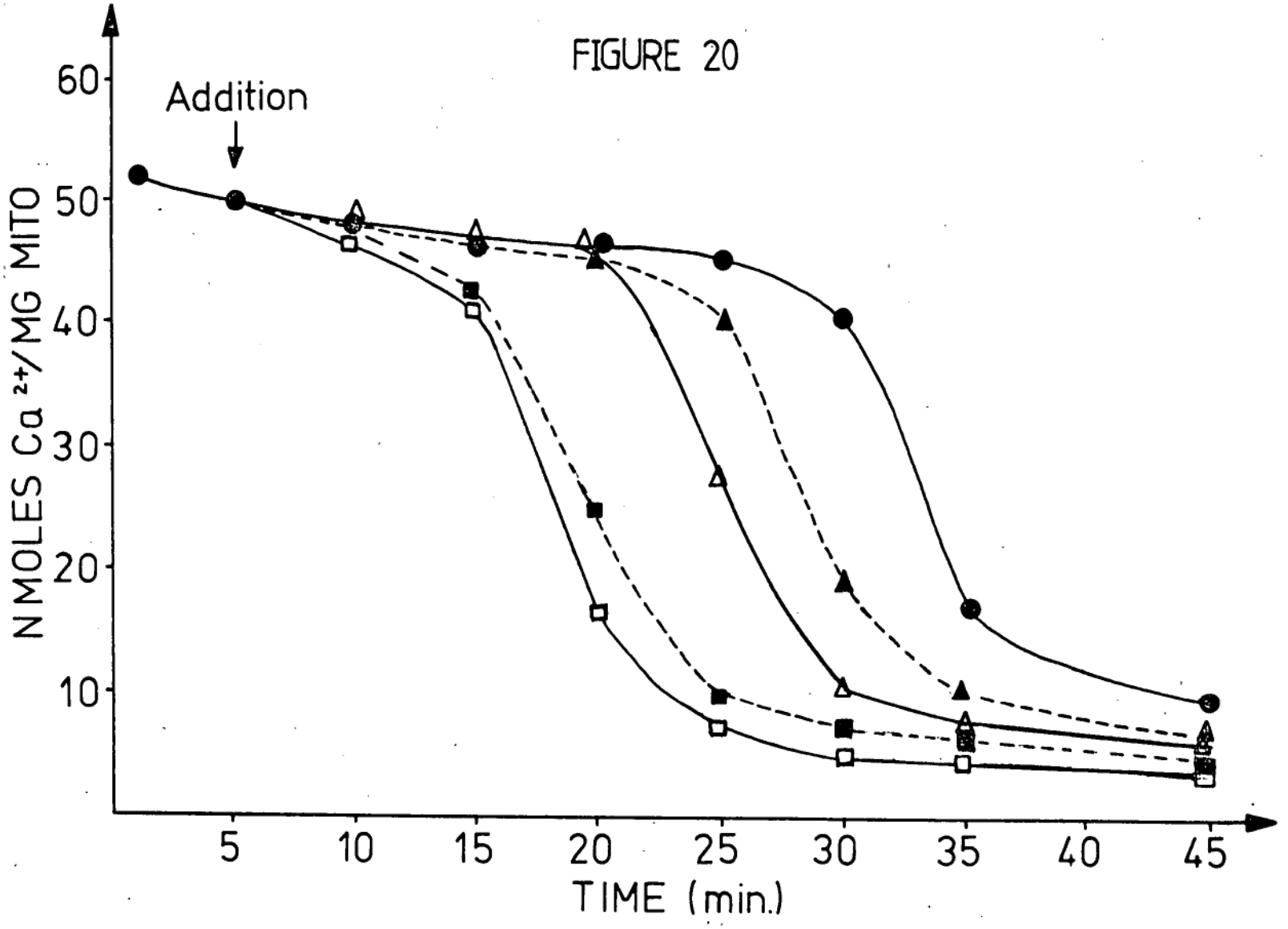
—△— + 50 µl untreated fractions S_2(25 - 27) (≡ 0.25 g wet wt. liver)

--▲-- + 50 µl florisil-treated fractions S_2(25 - 27) (≡ 0.25 g wet wt. liver)

—□— + 0.1 ml untreated fractions AcA 34(24 - 30) (≡ 0.5 g wet wt. liver)

--■-- + 0.1 ml florisil-treated fractions AcA 34 (24 - 30) (≡ 0.5 g wet wt. liver)

FIGURE 20


Addition
60
50
40
30
20
10
N MOLES Ca 2+/MG MITO
5
10
15
20
25
30
35
40
45
TIME (min.)

an earlier release compared to the control experiment. A similar observation was obtained on adding 0.1 ml fractions AcA 34(24-30).

2.3.9 Mitochondrial Ca^{2+} movement in the presence of cytosolic protein and ^{45}Ca-binding cytosolic protein obtained from the Biogel A-0.5 M column.

In order to examine the effects of the ^{45}Ca-binding protein on mitochondrial Ca^{2+} transport, the freeze dried cytosol was fractionated on Biogel A-0.5 M column in the absence of ^{45}Ca.

2.3.9.1 Calcium-binding activity of the protein fractions.

The Ca-binding activity of the various protein fractions was determined based on the method of Wasserman and Taylor described earlier in this chapter, section 2.2.5 . The reslut obtained is shown in table 4. In the presence of 100 nmoles of Ca^{2+} in the medium, approximately 7 nmoles Ca^{2+} were bound per ml of the concentrated protein fractions BA(64-74). Since 400 nmoles Ca^{2+} was added to the incubation medium, and 0.2 ml of the concentrated protein fractions BA(64-74) used to examine its effects on mitochondrial Ca^{2+}-transport, the calculated nmole Ca^{2+} bound/0.2 ml concentrated fractions BA(64-74) was 6 nmoles (table 4). Further estimations showed that 0.2 ml of concentrated fractions BA(51-63) bound 19 nmoles in the presence of 400 nmoles Ca^{2+} and 0.4 ml original freeze dried rat liver cytosol bound 3 nmoles Ca^{2+}. These results were also confirmed, although with less accuracy due to the log scale, with the Ca^{2+}-electrode, and showed less than 50 nmoles Ca^{2+} bound to these protein fractions out of a total of 400 nmoles Ca^{2+} added.

TABLE 4

Calcium binding activity of freeze dried cytosol and protein fractions BA (28 - 34), BA (51 - 63) and BA (64 - 74).

The Ca-binding activity of the various protein fractions was determined based on the method of Wasserman and Taylor described in section 2.2.5.

sample	nmoles Ca^{2+}		
	100	10	1
Freeze dried cytosol	2.907	0.438	0.034
(nmole Ca^{2+} bound/ml)	3.99	0.38	0.03
fractions BA (28 - 34)	1.16	0.1	0.01
(nmole Ca^{2+} bound/ml)	1.49	0.11	0.004
fractions BA (51 - 63)	22.39	1.97	0.212
(nmole Ca^{2+} bound/ml)	26.1	2.54	0.204
fractions BA (64 - 74)	8.9	0.92	0.05
(nmole Ca^{2+} bound/ml)	5.3	0.7	0.07

The Ca-binding activity of 0.2 ml protein fractions in the presence of 400 nmoles Ca^{2+} is as shown

sample	400 nmoles Ca^{2+}
Freeze dried cytosol nmole Ca^{2+} bound/0.4 ml	3 nmoles
fractions BA (28 - 34) nmole Ca^{2+} bound/0.2 ml	1 nmole
fractions BA (51 - 63) nmole Ca^{2+} bound/0.2 ml	19 nmoles
fractions BA (64 - 74) nmole Ca^{2+} bound/0.2 ml	6 nmoles

2.3.9.2 Mitochondrial $^{45}Ca^{2+}$ uptake and release in the presence of the freeze dried rat liver cytosol studied by means of the radio-isotope technique.

0.4 ml freeze dried rat liver cytosol (equivalent to 0.4 g wet weight liver) was added at 4 min after the addition of $^{45}Ca^{2+}$. The standard incubation medium was used, in the presence of 400 nmoles $^{45}Ca^{2+}$, 5 mg mitochondrial protein, and 1 mM ATP, 2 mM sodium succinate as energy sources for Ca^{2+} uptake. An immediate $^{45}Ca^{2+}$ release was observed on adding the 0.4 ml freeze dried cytosol as shown in fig 21. The control experiment on the other hand, accumulated 90 % of the added ^{45}Ca and retained this $^{45}Ca^{2+}$ up to 30 min after which time it released the accumulated $^{45}Ca^{2+}$. The result from the calcium-binding activity of the freeze dried rat liver cytosol (i.e. 0.4 ml binds only 3 nmoles Ca^{2+} in the presence of 400 nmoles Ca^{2+}) suggested that the added sample did not interfere with the free Ca^{2+} concentration in the medium.

2.3.9.3 Mitochondrial $^{45}Ca^{2+}$ uptake and release in the presence of the concentrated protein fractions BA(28-34), BA(35-50), BA(51-63) and BA(64-74) studied by means of the radio-isotope technique.

The effects of concentrated cytosolic proteins eluted from Biogel A-0.5 M column on mitochondrial Ca^{2+} transport were examined in the presence of 1 mM ATP and 2 mM Na succinate as energy sources. 0.2 ml of the concentrated ^{45}Ca-binding proteins BA(51-63) and BA(64-74) chelated only small amounts of Ca^{2+} (4.7 % and 1.5 % respectively) out of the 400 nmoles Ca^{2+} added (section 2.3.9.1) to the standard incubation medium. On adding 0.2 ml of the concentrated ^{45}Ca-binding protein

FIGURE 21

Mitochondrial Ca^{2+} transport in the presence of freeze dried rat liver cytosol studied by means of the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM Na succinate, 1 mM ATP and mitochondria (5 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. The freeze dried cytosol was added at 4 min after the addition of $^{45}Ca^{2+}$.

—●— control

—▲— + 0.4 ml freeze dried cytosol (equivalent to 0.4 g wet wt. liver).

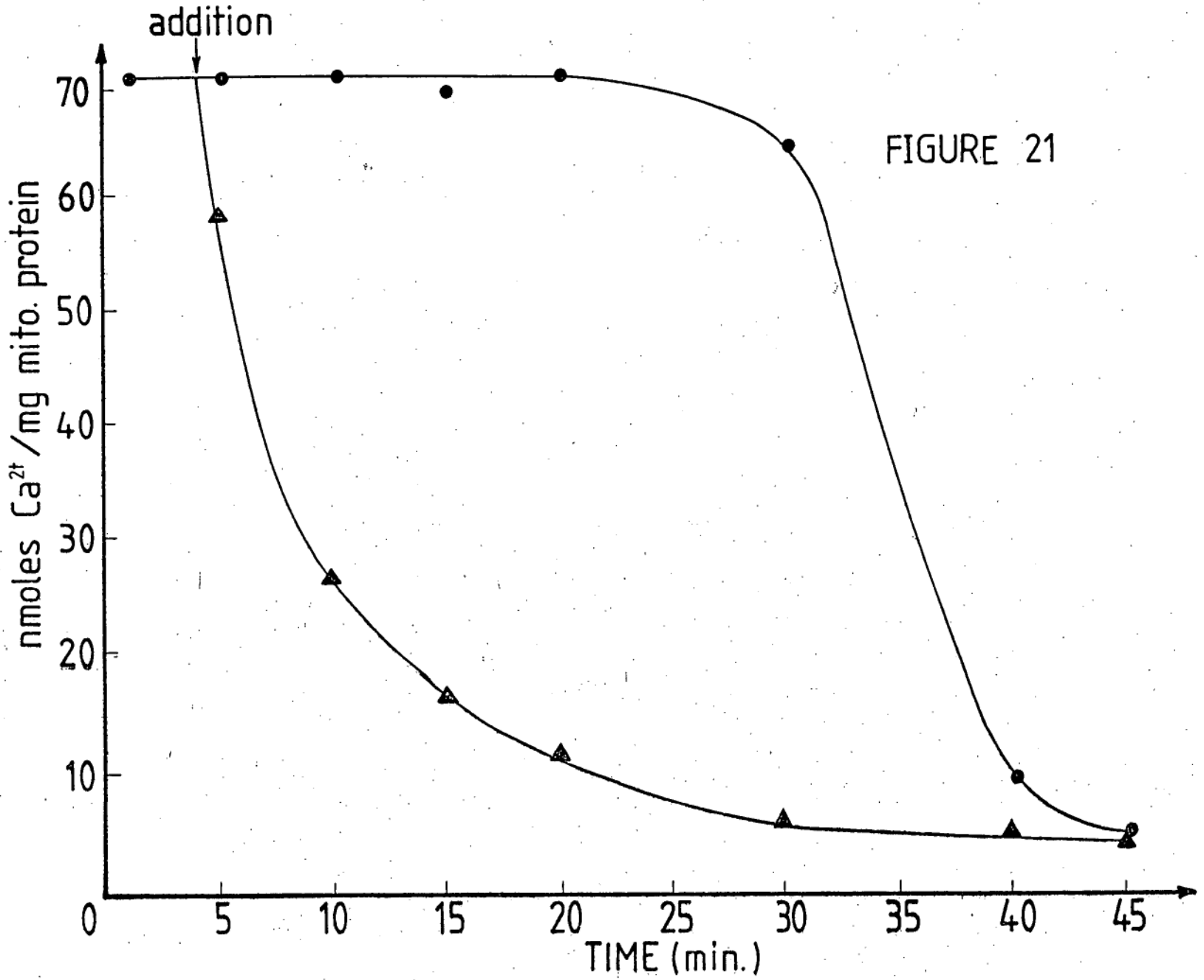

addition
70
60
50
40
30
20
10
0
nmoles Ca^{2+}/mg mito. protein
5
10
15
20
25
30
35
40
45
TIME (min.)

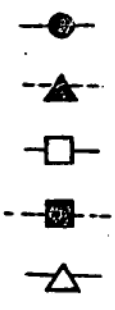
FIGURE 21

fractions BA(51-63) or fractions BA(64-74) to the incubation medium, the mitochondria seemed to retain $^{45}Ca^{2+}$ longer than the control experiment (fig 22). The control mitochondria started to release its accumulated $^{45}Ca^{2+}$ at 30 min and almost complete release was observed at 45 min, whilst the inclusion of the protein fractions BA(51-63) or BA(64-74) caused $^{45}Ca^{2+}$ retention for the 45 min duration of the experiment. The presence of 0.2 ml concentrated protein fractions BA(28-34) had no effect on the mitochondrial $^{45}Ca^{2+}$ transport. Similarly 0.2 ml concentrated protein fractions BA(35-50) did not affect mitochondrial $^{45}Ca^{2+}$ uptake or release (fig 22).

FIGURE 22

Mitochondrial Ca^{2+} transport in the presence of Ca^{2+}-binding fractions isolated from freeze dried cytosol studied by means of radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM Na succinate, 1 mM ATP and mitochondria (5 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 µCi). Incubation temp.= 25°C. 0.2 ml concentrated Ca^{2+}-binding fraction is equivalent to 1 g wet wt. liver. The following additions were made before adding the $^{45}Ca^{2+}$.



control

+ 0.2 ml concentrated fractions BA(28 - 34)

+ 0.2 ml concentrated fractions BA(35 - 50)

+ 0.2 ml concentrated fractions BA(51 - 63)

+ 0.2 ml concentrated fractions BA(64 - 74)

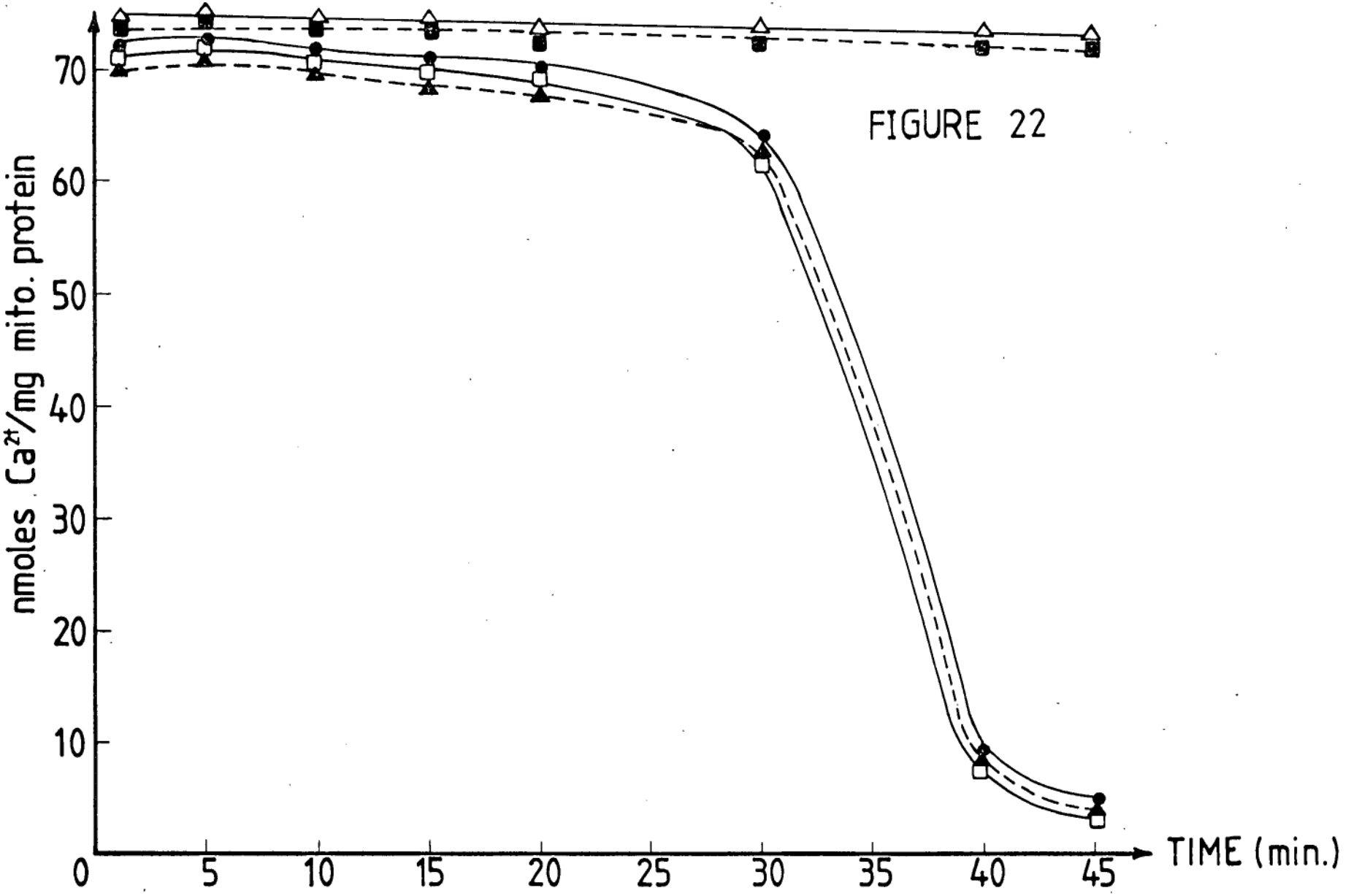


FIGURE 22
nmoles Ca²⁺/mg mito. protein
0
10
20
30
40
50
60
70
5
10
15
20
25
30
35
40
45
TIME (min.)

2.4 DISCUSSION

The main aim of the investigation was to study Ca^{2+} movement in isolated rat liver mitochondria in the presence of rat liver cytosol and also to investigate factors present in the cytosol that might regulate Ca^{2+} transport in the mitochondria.

Characteristics of the cytosol preparation

The concentration of some components of the rat cytosol preparation, the 250,000 x g supernatant is shown in table 1. The cytosolic protein concentration estimated was approximately half the value quoted by Soboll et al. (1976).

The concentration of Mg^{2+} in the rat cytosol preparation was 0.4 mmole/kg wet weight liver. According to Veloso et al (1973), total liver magnesium is 10 mmole/kg wet weight liver but a considerable amount of this occurs in organelles and the free Mg^{2+} was estimated to be buffered at 1 mM Mg^{2+}. The rat cytosol preparation would contain only the Mg^{2+} not bound to organelles.

The K^{+} concentration in the cytosol preparation was 75 mmoles/kg wet weight liver and according to Pieri et al (1977), K^{+} in cytoplasm of liver cells is between 181 - 206 meq/kg intracellular water.

The concentration of adenine 5' nucleotides in the rat cytosol preparation was less than that estimated by Akerboom et al (1978) i.e 2.73 mM total ATP, 0.315 mM total ADP and 0.13 mM total AMP in cytosol of hepatocytes from fasted rate, therefore it appears the cytosol preparation has lost some of these nucleotides.

Determination of inorganic phosphate in the cytosol preparation gave the value of 2.5 mmoles/kg wet weight liver. Akerboom et al (1978) has indicated that the concentration of free Pi in cytosol of hepatocytes from fasted rats is 2.48 mM.

According to Foden and Randle (1978), the extramitochondrial Ca is 0.4 mmoles/kg wet weight hepatocytes (i.e. 20% of the total cell Ca). This value is approx. 2½ times the amount estimated in the rat cytosol preparation of the present studies. The distribution of the extramitochondrial Ca in liver is however not known. Similarly, Hamilton and Holdsworth (1975) observed that when mucosal cells from chick duodenum were homogenized in the presence of inhibitors of mitochondrial calcium uptake such as ruthenium red (i.e. to prevent redistribution of Ca during the isolation process) or when homogenised at 22°- 28°C, 15 - 30 % of the Ca was found in the soluble fraction, the remainder being mainly located in the mitochondria. Since it is generally agreed that the concentration of ionized calcium in the cytoplasm of the liver cells is in the μM concentration range, most of the Ca found in the rat cytosol preparation appears to be as bound Ca. As discussed later, only a small portion appears protein-bound, the majority being chelated to adenine nucleotides.

From consideration of the analyses it would appear that only 50 % of the liver cells were broken in the preparation of rat cytosol. This is due to difficulties in preparing the cytosol and that vigorous homogenation would breakdown organelles.

The iso-osmotic incubation medium used to study the mitochondrial Ca^{2+} transport contained Pi, Mg^{2+} and K^{+} at concentrations almost similar to those found in the rat cytosol. In this medium the mitochondrial P:O ratio obtained using succinate as the substrate ranged between the values 1.6 - 1.8 (section 1.2) indicating that the mitochondria prepared were tightly coupled. The amounts of mitochondria or Ca^{2+} added to the incubation medium were such that no more than 100 nmoles Ca^{2+} were accumulated per mg

mitochondrial protein, in order to avoid massive Ca^{2+} loading which would result in swelling, membrane damage and organelle lysis (Lehninger et al, 1967).

Comparison of Ca-electrode and the radioassay techniques

When using the Ca^{2+}-electrode to study mitochondrial Ca^{2+} movement, in the presence of a respiratory substrate or ATP or both, as energy source/s for uptake, almost all of the added Ca^{2+} in the medium was taken up by the mitochondria such that the concentration of the remaining Ca^{2+} in the medium was $\sim 1.0 \times 10^{-6}$M as calibrated with the Ca-nitrilotriacetic acid buffers (Reed and Bygrave, 1975(a)). (Note : the amounts of Mg^{2+}, K^{+} and Pi used in the incubation medium did not seem to interfere with the sensitivity of the Ca^{2+}-electrode, fig 2). However, when the radioassay technique was employed to monitor mitochondrial movement, only 85 - 90 % of the added Ca^{2+} was accumulated by the mitochondria. A possible explanation of this difference obtained by the two methods is that with the radioassay technique, the Ca^{2+} bound to the low affinity sites on the outer side of the inner mitochondrial membrane was removed by the EGTA-ruthenium red quenching medium thus the Ca^{2+} in the supernatant would also include the Ca^{2+} removed from the mitochondrial low affinity Ca^{2+}-binding sites.

Inhibition of Ca^{2+} uptake by the concentrated rat cytosol

In the presence of 1.5 ml concentrated rat cytosol (equivalent to 1.5 g wet weight liver, MW > 10,000) and using 2 mM Na succinate as the energy source for mitochondrial Ca^{2+} uptake, approximately 70% of the added Ca^{2+} was accumulated and Ca^{2+} release was observed almost immediately (fig 5). For this particular experiment, the amount of mitochondrial protein added was chosen to be

approximately half that of the cytosolic protein present in the concentrated rat cytosol, so as to be similar to the situation in hepatocytes (Soboll et al, 1976). The control mitochondria on the other hand, accumulated almost all of the added Ca^{2+} and retained this Ca^{2+} during the 10 min duration of the experiment (fig 3). A similar result to that of the control was observed in the presence of the ultrafiltrate of the rat cytosol preparation (fig 4). The ultrafiltrate contained substances less than 10,000 MW at only 1/7 the concentration present in the original liver cells. In the presence of the concentrated rat cytosol and succinate as the energy source for Ca^{2+} uptake (table 2), the radiometric method showed no Ca^{2+} uptake.

These results suggest that the concentrated rat liver cytosol contained factor/s that seemed to inhibit mitochondrial Ca^{2+} uptake and/or stimulate Ca^{2+} release.

It was suspected that the inhibitory factor/s could be bound fatty acids or/and their derivatives. Asimakis and Sordahl (1977) found that palmitoyl CoA prevented Ca^{2+} uptake by rat heart mitochondria and also caused the early release of Ca^{2+}. These effects of palmitoyl CoA were prevented when carnitine was present during the incubation. Carnitine in the concentrated rat cytosol would have been lost to the ultrafiltrate during concentration. For this reason, 25 mM carnitine HCl was included in the incubation medium with the concentrated rat cytosol and succinate as the energy source for Ca^{2+} uptake. Under these conditions, it was observed that almost all of the added Ca^{2+} was taken up; however, the mitochondria started to release this Ca^{2+} after 4 min (fig 6). After Ca^{2+} release, further additions of carnitine or succinate did not result in Ca^{2+} reuptake (figs 6,7). (Carnitine was added in order

to remove the inhibitory effects of the remaining fatty acids and/or fatty acyl CoA and succinate to ensure that the mitochondria had sufficient energy for Ca^{2+} uptake).

The addition of BSA, after Ca^{2+} release induced by the concentrated rat cytosol, caused Ca^{2+} reuptake (fig 6). This suggests that the mitochondria may have been uncoupled by bound fatty acids present in the concentrated rat cytosol. BSA is known to restore uncoupled oxidative phosphorylation in mitochondria (Helinski and Cooper, 1960). Excess carnitine (25 mM or 50 mM) was used to ensure complete removal of the inhibitory effects of fatty acids and fatty acyl CoA on mitochondrial Ca^{2+} uptake. It was observed that the high concentration of carnitine (25 mM) alone had no effect on the mitochondrial Ca^{2+} uptake or release (figs 3,4). Borum (1978) estimated that the concentration of carnitine in liver is 2 mM, and according to Christiansen and Bremer (1976), the carnitine content of isolated liver cells can increase to about 15 mM with no apparent harm to the cells.

When ATP was included in the incubation mixture containing the concentrated rat cytosol, carnitine and succinate, the mitochondria retained the accumulated Ca^{2+} up to 35 min, at which time, the control mitochondria had released most of the accumulated Ca^{2+} (fig 8). A possible explanation for the above observation is that in the presence of both ATP and carnitine, the bound fatty acids present in the concentrated rat cytosol form fatty-acylcarnitine which would provide additional source of energy (via β-oxidation) for mitochondrial Ca^{2+} uptake in addition to that from the succinate and ATP.

The results obtained in fig 8 also showed that when the fatty acid content of the concentrated rat cytosol was reduced

from 200 μM to 100 μM Ca^{2+} was retained in the mitochondria up to 55 min, further suggesting that bound fatty acids present in the untreated concentrated cytosol were responsible for the early Ca^{2+} release. For this particular experiment, the radiometric technique was used since long incubation periods were required and furthermore, a number of test situations were examined at the same time. The effect of fatty acids, fatty acyl CoA and fatty acylcarnitine on mitochondrial Ca^{2+} transport is reported in chapter 3.

Effect of albumin on Ca^{2+} retention

Bovine serum albumin (BSA) was found to protect the rat liver mitochondria from the releasing effects of the concentrated cytosol as shown in fig 6. Since BSA is hardly a physiological substrate for rat liver, the effect of rat albumin was also examined. 1 g rat liver is known to be capable of synthesizing approximately 100 mg rat albumin per day (Munro, 1968). Even though most of the rat albumin would be present in the blood plasma, there would be trace amounts present in the liver cytosol. The commercial rat albumin caused a rapid release of Ca^{2+} from the mitochondria; however, if its fatty acid content was reduced by charcoal treatment (fig 9), its presence helped retain Ca^{2+} in the mitochondria. According to Chen (1967), the protein recovery after treating the rat serum albumin with charcoal is usually about 80 %.

At the moment it is not known for certain why albumin helps Ca^{2+} retention in mitochondria. An attempt was made to see whether the ability of BSA to cause mitochondria to retain Ca^{2+} was shared by other high molecular weight polymers such as polyethylene glycol or polyvinylpyrrolidone. The results obtained (fig 10) showed that the high molecular weight polymers examined had no

effect on mitochondrial Ca^{2+} transport, while the high molecular weight protein, BSA, helped Ca^{2+} retention.

Incubation of the mitochondria at 25°C for a period as long as 45 min will probably result in aging. Aged mitochondria generally lose their ability to carry out coupled oxidative phosphorylation, but this ability may be partially restored by the addition of BSA to the incubation medium (Sacktor, 1953). Rossi et al (1964) also showed that aging of the mitochondria in vitro caused a decrease in phosphatidyl compounds (e.g. phosphatidylcholine) with a parallel increase in lyso-compounds (e.g. lysophosphatidylcholine) due to the activity of the mitochondrial phospholipase A that has been shown to be present in rat liver mitochondria (Rossi et al, 1965). In addition, mitochondrial phospholipase is also activated by low concentrations of Ca^{2+} (Nachbaur et al, 1972). By inference, then, aged mitochondria may lose their ability to carry out coupled oxidative phosphorylation as a result of an increase in activity of the phospholipase A situated in the mitochondrial membrane forming lyso-compounds, which in turn would cause Ca^{2+} release from the mitochondria.

Harris (1977) suggested that BSA helps Ca^{2+} retention in mitochondria because it binds to fatty acids and lyso-compounds produced by the mitochondrial phospholipase. Harris (1977) showed that the presence of lysophosphatidylcholine induced Ca^{2+} release from heart mitochondria. On the other hand, Helinski and Cooper (1960), obtained results suggesting that the effect due to fatty acids could be reversed by BSA as a result of its reactivation of the phosphorylating mechanism which had been inactivated by the uncoupling factor/s released by mitochondrial phospholipase. The latter workers showed that merely washing aged mitochondria with albumin does not in itself lead to restoration of oxidative

phosphorylation. Probably this will also explain, why, in the experiment reported in this chapter (fig 10), the addition of BSA after Ca^{2+} release from the control mitochondria caused Ca^{2+} reuptake. If the BSA added after Ca^{2+} release merely binds to the remaining fatty acids, then no further Ca^{2+} uptake or release would be observed. Therefore, most likely, the defatted BSA or rat albumin was able to protect the mitochondria from the uncoupling effects of endogenous fatty acids produced by phospholipase A, during aging for example, or the effects of fatty acids present in the concentrated rat cytosol (fig 6), by reactivating the uncoupled oxidative phosphorylating mechanism. How this is made possible is not known. Due to the high molecular weight of the albumin, it is unlikely that it will penetrate the inner mitochondrial membrane, suggesting that its effect on reversing the uncoupled oxidative phosphorylation is possibly external. Since trace amounts of albumin are present in the rat cytosol <u>in vivo</u>, it may favour mitochondrial Ca^{2+} retention by protecting the mitochondria from the effects of fatty acids initially present in the cytosol or that released by the action of phospholipase.

Another suggestion for the ability of BSA to retain Ca^{2+} in mitochondria would be that BSA lowers the concentration of free Ca^{2+} in the medium. According to Chrambach et al (1961), BSA can bind 10 moles Ca^{2+} per 10^5g (i.e. 10 mg BSA will bind 1,000 nmoles Ca^{2+}). Harris (1977) observed that 1 mg BSA in the presence of K^+, succinate and Pi halved the free Ca^{2+} concentration of 4 µM. However in the present study, it was observed that the Ca-binding activity of 10 mg BSA or rat albumin in the presence of the standard incubation mixtures and 80 µM Ca^{2+} (final concentration) as determined by using the Ca^{2+} -electrode was negligible (fig 11). Similarly, Nielsen et al (1977) observed negligible binding of Ca^{2+} to BSA. They also used the Ca^{2+}-electrode to see whether albumin interfered with the concentration of free Ca^{2+} in the

medium. Therefore, under the conditions examined, it is unlikely that BSA helped mitochondrial Ca^{2+} retention by binding to the free Ca^{2+} in the medium.

The palmitate-binding protein.

When rat livers were thoroughly perfused with 0.25 M sucrose to flush out blood plasma, the cytosol from those livers contained only traces of rat albumin. However, a different protein of molecular weight between 30,000 - 50,000 was isolated from the concentrated rat cytosol which combines with ^{3}H-palmitic acid. This ^{3}H-palmitate-binding protein was eluted from the AcA 44 column corresponding to a molecular weight approximately 35,000 , from the Biogel P30 column with molecular weight slightly greater than 30,000 and from the Sephacryl 300 Superfine column with a molecular weight between 18,000 - 85,000. The ^{3}H-palmitate-binding protein obtained from the Biogel P30 column (total ^{3}H-palmitate counts = 1.6×10^{6} cpm), was fractionated with 40 % ammonium sulphate. The brown precipitate obtained after centrifugation, with a total ^{3}H-palmitate count = 714,956 cpm, most likely contained cytochromes and myoglobin from the concentrated rat cytosol. The clear supernatant (total ^{3}H-palmitate counts = 593,940 cpm) containing the ^{3}H-palmitate-binding protein was dialysed and concentrated using a Diaflo membrane and then fractionated on an AcA 34 column. The brownish fraction containing ^{3}H-palmitate -binding protein was eluted in tubes 24 - 30 (total ^{3}H-palmitate counts = 85,000 cpm). The remaining ^{3}H-palmitate counts were in the later tubes probably as free ^{3}H-palmitate.

The double-antibody precipitation and the immunoelectrophoresis techniques were used in order to show that the ^{3}H-palmitate-binding protein obtained was not rat albumin, which

is also known to bind fatty acids. The double-antibody technique would precipitate any rat albumin from the solution. For this purpose, the goat antiserum to rat serum albumin and the donkey antiserum to goat serum were used. The results obtained showed that most of the ^{3}H-palmitate counts were in the supernatant and not the precipitate suggesting that the ^{3}H-palmitate-binding protein was not rat albumin. The immunoelectrophoresis technique also revealed no detectable goat antiserum to rat serum albumin precipitin. ^{3}H-palmitate counts of the 4 mm strips of the agarose electrophoresis film indicated that most of the ^{3}H-palmitate counts (i.e. probably the ^{3}H-palmitate-binding protein) were located around the origin while the rat albumin as the reference, was located 56 mm from the origin. This further suggests that the ^{3}H-palmitate-binding protein was not rat albumin.

Rustow et al (1978) recently reported a specific fatty acid-binding protein in cytosol. Unfortunately, the report concerned a 'cytosol' prepared from rat livers containing unknown contamination by blood plasma and therefore, three peaks of fatty acids were found during chromatography of the cytosol labelled with radioactive fatty acid, one of which was the rat plasma albumin.

The exact physiological function of the fatty acid-binding protein in the rat liver cytosol is not known, although it might be an intracellular transport protein for fatty acids. New evidence by Barbour and Chan (1979) have indicated that defatted liver fatty acid-binding protein reverses the inhibitory effect of palmitoyl CoA on adenine nucleotide transport in rat liver mitochondria.

The effect of palmitoyl CoA on mitochondrial Ca^{2+} transport will be discussed in the following chapter.

The ^{3}H-palmitate-binding protein isolated from the concentrated rat cytosol in the present study, caused an earlier Ca^{2+} release from the mitochondria compared to the control mitochondria (fig 20) presumably due to a high fatty acid/protein ratio. For this particular experiment, 2 mM β-hydroxybutyrate and 1 mM ATP were the energy sources for mitochondrial Ca^{2+} uptake. After treating the ^{3}H-palmitate-binding protein with florisil, Ca^{2+} was retained for 5 min longer than the untreated fractions. Thus, a slightly lower fatty acid/protein ratio after florisil treatment helped to remove some of the inhibitory effect of the fatty acids on mitochondrial Ca^{2+} transport. Since carnitine was not included in the incubation medium, the Ca^{2+} release observed was possibly due to the uncoupling effects of the ^{3}H-palmitate.

The Ca-binding protein.

A high molecular weight Ca-binding protein (molecular weight between 150,000 - 364,000) was also isolated from the concentrated rat cytosol and the freeze dried rat cytosol. The 250,000 x g supernatant for the preparation of the rat cytosol was either concentrated over a PM 10 Ultrafilter or dialysed against distilled water and freeze-dried. Adding freeze-dried cytosol, equivalent to 0.4 g wet weight liver, to the incubation medium at 5 min caused an immediate release of Ca^{2+} from preloaded mitochondria when 2 mM Na succinate and 1 mM ATP were the energy sources in the absence of carnitine (fig 21). The Ca-binding protein was eluted from the AcA 44 column corresponding to molecular weight greater than 86,000

but less than 364,000 , and also from the Biogel A-0.5 M column with a molecular weight between 150,000 to 364,000. The Ca-binding fractions obtained from the Biogel A-0.5 M column seemed to help Ca^{2+} retention by the mitochondria (fig 22). Out of the 400 nmoles Ca^{2+} added to the incubation medium, 19 nmoles and 50 nmoles Ca^{2+} were bound by the 0.2 ml Ca-binding protein fractions BA(51-63) and BA(64-74) respectively as shown in table 4. Most probably the protein itself helped mitochondrial Ca^{2+} retention not by lowering the concentration of Ca^{2+} in the medium but by other unknown means.

Yamaguchi and Yamamoto (1975) also observed calcium-binding activity in supernatant of the rat liver homogenates based on the competition between Chelex-100 and the supernatant for added calcium. Another calcium-binding protein isolated recently is a low molecular weight $\sim$ 12,000 MW parvalbumin obtained from rabbit (Oryctolagus cuniculus) muscle (Capony et al, 1976). It has a Ca-binding capacity of 2 mole per mole protein, however its physiological function is not really known. A calcium-binding protein was also isolated from the chick intestine and that this protein seemed to cause Ca^{2+} release from the intestinal mitochondria observed in vitro at 30°C (Hamilton and Holdsworth, 1975). The Ca-binding protein (CaBP) was located in the cytoplasm, since after homogenisation of the mucosal cells in 0.25 M sucrose and differential centrifugation, the CaBP was mainly in the 100,000 x g supernatant (Hamilton, 1974).

Another calcium-binding protein, a protein regulator of cyclic nucleotide phosphodiesterase has been reported in rat liver (Smoake et al, 1974; Kakiuchi et al, 1975; Vandermeers

et al, 1977). Recently, MacManus (1979) managed to purify the protein from 100,000 x g rat liver supernatant by using its calcium-binding ability. The protein regulator of the cyclic nucleotide phosphodiesterase is now referred to as calmodulin. In fact it is also recognised as the multifunctional activator of several Ca^{2+}-dependent enzymes including brain adenylate cyclase, erythrocyte membrane Ca^{2+}-dependent ATPase and several protein kinases such as phosphorylase kinase, NAD^{+} kinase. Possibly the Ca-binding protein isolated from the concentrated cytosol and the freeze-dried cytosol is calmodulin. The molecular weight of the protein regulator of cyclic nucleotide phosphodiesterase (i.e. calmodulin) determined by using calibrated polyacrylamide gel is 17,500 daltons (MacManus, 1979). The isolated Ca-binding protein in the present studies, had a molecular weight between 150,000 - 364,000 as determined using a calibrated AcA 44 and Biogel A-0.5 M columns, thus possibly the Ca-binding protein was bound to other high molecular weight protein/s, or it might represent aggregates of the calmodulin on the column. MacManus further calculated the concentration of the calmodulin to be 22 mg per kg weight liver. He isolated the Ca-binding protein from perfused rat liver by centrifugation of the homogenate at 600 x g for 15 min followed by centrifugation at 100,000 x g for 60 min. The Ca-binding protein isolated in the present studies was obtained in an almost similar manner, using perfused liver and centrifugation at 20,000 x g for 20 min, 40,000 x g for 20 min and finally at 250,000 x g for 60 min. Interestingly, MacManus included EDTA in his homogenising medium to liberate any calmodulin bound to other proteins, however no EDTA or EGTA

was added to our homogenising medium. This may explain why a high molecular weight Ca-binding protein was observed in this study as possibly the Ca-binding protein was bound to other high molecular weight proteins. On the other hand the Ca-binding protein could also be the high MW "actin" from microtubular structure.

In this chapter the effects of rat cytosol and some of its components such as the palmitate-binding protein, Ca-binding protein on mitochondrial Ca^{2+} transport were examined. The effects of the other components of the cytosol such as palmitate, palmitoyl CoA, palmitoylcarnitine, Mg^{2+}, K^{+}, Na^{+}, various respiratory substrates, adenine nucleotides and inorganic phosphate on mitochondrial Ca^{2+} transport is reported in the following chapters.

2.5 SUMMARY

1. The rat liver cytosol as prepared contained approximately half the concentration of cytosolic protein, K^+ and Ca^{2+}, suggesting that only 50 % of the cells were broken by the method of preparation.

2. For *in vitro* studies of calcium movement in isolated mitochondria, an iso-osmotic sucrose medium was used containing 1 mM Mg^{2+}, 2 mM Pi and 72 mM K^+, amounts almost identical to those found in the cytosol preparation.

3. Ca^{2+} uptake by the mitochondria was inhibited in the presence of the concentrated cytosol. The presence of both carnitine and concentrated cytosol, however, enabled Ca^{2+} uptake but release was much earlier than the control experiment, i.e. with carnitine but no cytosol. BSA caused reuptake of the Ca^{2+} released by the concentrated rat cytosol. The inhibitory factor present in the concentrated rat cytosol could be bound fatty acids.

4. The ultrafiltrate of the rat cytosol preparation containing substances less than 10,000 MW at only 1/7 of the concentration present in the original liver cells, had no effect on mitochondrial Ca^{2+} uptake.

5. Addition of 1 mM ATP, 50 mM carnitine and 2 mM succinate to the incubation mixtures containing the concentrated rat cytosol caused Ca^{2+} to be retained for longer periods by the mitochondria. Presumably the inhibitory effect of the bound fatty acid present in the concentrated rat cytosol was removed by the formation of fatty acylcarnitine. Reducing the

fatty acid content of the concentrated rat cytosol by half the original amount resulted in the mitochondria retaining the Ca^{2+} for an even longer period.

6. An attempt was made to see whether the property of BSA to cause retention of mitochondrial Ca^{2+} for longer periods, was also shared by high molecular weight polymers polyethylene glycol or polyvinylpyrrolidone. The result suggests that these polymers had no effect on mitochondrial Ca^{2+} transport.

7. A ^{3}H-palmitate-binding protein (MW 30,000 - 50,000), different from the fatty acid-binding rat serum albumin was isolated from the concentrated rat cytosol. Its physiological function is not known, although it may be an intracellular transport protein for fatty acids. Examinations in vitro suggest that the protein saturated with ^{3}H-palmitate caused Ca^{2+} release from Ca^{2+} preloaded mitochondria.

8. A high molecular weight Ca-binding protein (MW between 86,000 - 364,000) was also isolated from the concentrated cytosol and from the freeze dried cytosol. Its physiological function is also not known, although when examined in vitro, it seemed to cause Ca^{2+} to be retained for longer periods by the mitochondria.

CHAPTER 3

CALCIUM MOVEMENT IN RAT LIVER MITOCHONDRIA IN THE PRESENCE OF CYTOSOLIC COMPONENTS: (PALMITATE AND DERIVATIVES).

3.1 AIM

Results in chapter 2 suggest that the protein-bound fatty acids in the rat liver cytosol prevented Ca^{2+} uptake and caused release from rat liver mitochondria. It was therefore necessary to examine the effects of free fatty acid and derivatives on calcium movement in rat liver mitochondria. In this chapter, the effects of palmitate, palmitoyl CoA and palmitoyl carnitine on mitochondrial Ca^{2+} transport were examined.

3.2 METHODS AND MATERIALS.

Calcium movement in rat liver mitochondria was determined using the radio-isotope technique as described in chapter 1, section 1.3 . The preparation of the mitochondria in order to see the effects of chloroquine was as described in chapter 1, section 1.1 with the following modifications:- 2 rats were used for this particular experiment. Liver from one of the rats were homogenized in 0.25 M sucrose, 1 mM $MgCl_2$, 2.5 mM HEPES adjusted to pH 7.4 with Tris base, plus 2.0×10^{-5}M chloroquine sulphate. Chemical formula of chloroquine is as shown in appendix A. The liver from the other rat was homogenised in the above medium minus chloroquine.

MATERIALS

A 50 mM potassium palmitate solution in 30 % ethanol was prepared. S-palmitoyl coenzyme A (MW 1,006) and palmitoyl-L-

carnitine chloride (anhydrous MW 436) were from SIGMA Chemical Company, St. Louis, Mo. U.S.A.; DL-carnitine HCl from Koch - Light Laboratories Ltd., Colnbrook, Bucks, England; bovine serum albumin from Calbiochem, La Jolla, Ca. Chloroquine obtained from May and Baker Ltd.

3.3 RESULTS

3.3.1 The effect of 250 μM palmitate on mitochondrial Ca^{2+} transport.

The estimated concentration of free fatty acid in the rat cytosol preparation examined in chapter 2 was 200 μM. In the presence of the concentrated rat cytosol, no Ca^{2+} was taken up by the isolated mitochondria, even in the presence of ATP and β-hydroxybutyrate as energy sources (table 5), unless carnitine was present. The results in fig 23 show that in the presence of 250 μM potassium palmitate with 1 mM ATP and 2 mM β-hydroxybutyrate as energy sources, only a small amount of the total Ca^{2+} added to the medium was taken up by the mitochondria. Out of a total of 400 nmoles $^{45}Ca^{2+}$ only approximately 10 nmoles $^{45}Ca^{2+}$ were accumulated per mg mitochondrial protein whilst the control mitochondria (without added palmitate) accumulated approximately 73 nmoles $^{45}Ca^{2+}$/mg mitochondrial protein.

The $^{45}Ca^{2+}$ content in the test mitochondria (i.e. in the presence of 250 μM potassium palmitate) seemed to increase slightly during the 45 min incubation period (fig 23). The control mitochondria on the other hand, took up most of the $^{45}Ca^{2+}$ in the medium but started to release this rapidly at 25 min. In fact the $^{45}Ca^{2+}$ content in the control mitochondria at 45 min was less than that in the mitochondria exposed to 250 μM potassium palmitate as shown in fig 23.

3.3.2 Mitochondrial Ca^{2+} transport in the presence of lower concentrations of potassium palmitate.

Fig 24 indicates that even 5 μM potassium palmitate added at

TABLE 5

Effect of concentrated rat cytosol on mitochondrial Ca^{2+} uptake and release studied by means of the radioassay technique.

Mitochondria (20 mg protein), were added to 5.0 ml incubation medium containing the standard incubation mixture, 1.5 ml concentrated rat cytosol, 2 mM β-hydroxybutyrate, 1 mM ATP and 1500 nmoles $^{45}Ca^{2+}$.

Sampling Time (min)	nmoles $^{45}Ca^{2+}$/mg mitochondrial protein
1	6
5	4
10	5
15	4
20	3
25	5

FIGURE 23

Mitochondrial Ca^{2+} transport in the presence of 250 μM potassium palmitate studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (4.6 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. The following addition was made before adding the $^{45}Ca^{2+}$.

—●— control

—▲— + 250 μM potassium palmitate

FIGURE 23

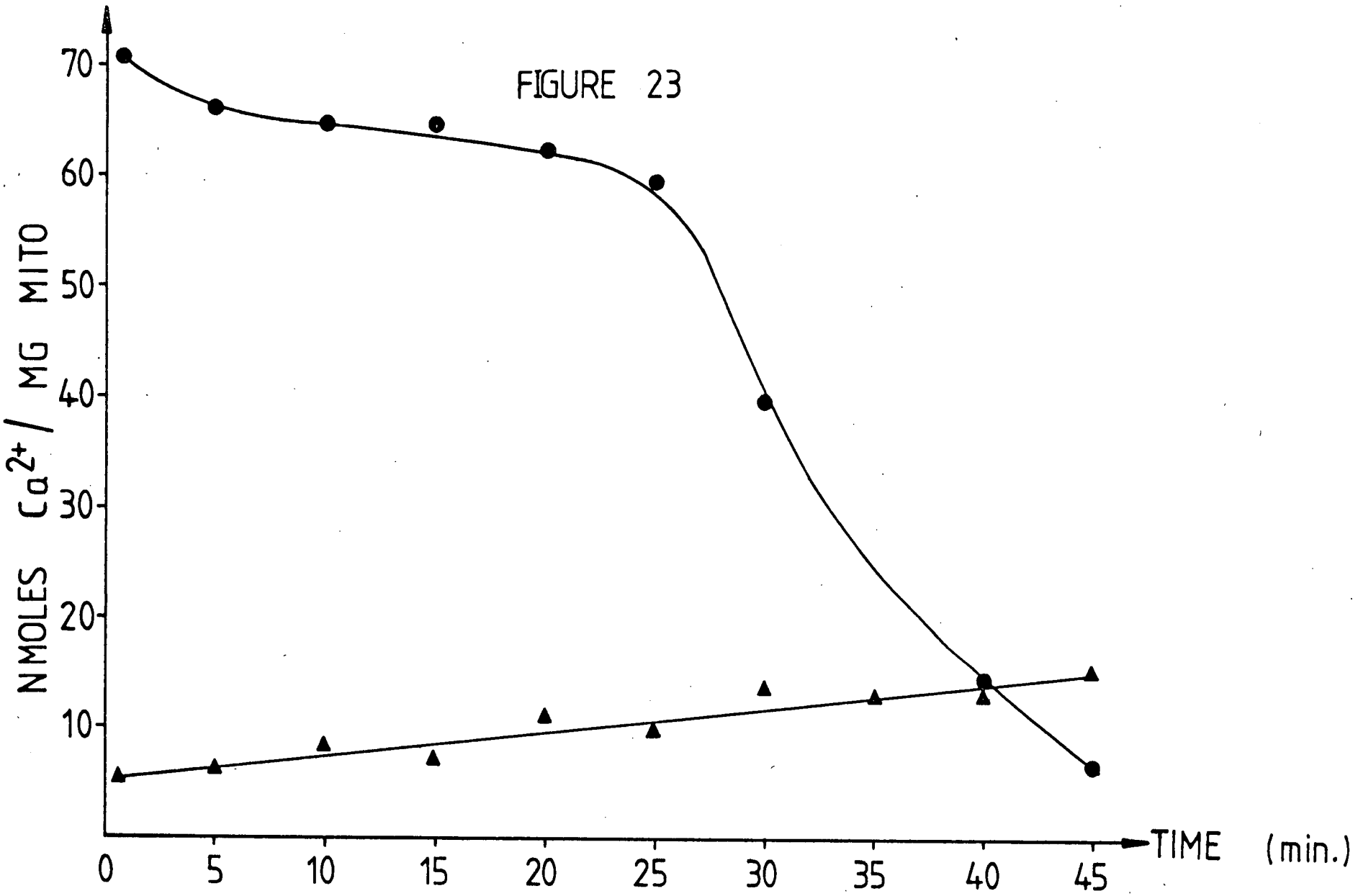
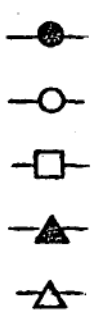

NMOLES Ca^{2+} / MG MITO
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
45
TIME (min.)

FIGURE 24

The effects of bovine serum albumin (BSA) and carnitine on Ca^{2+} release induced by potassium palmitate studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (4.8 mg protein). The reaction was started by addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. The following additions were made before adding the $^{45}Ca^{2+}$ unless stated otherwise.



control

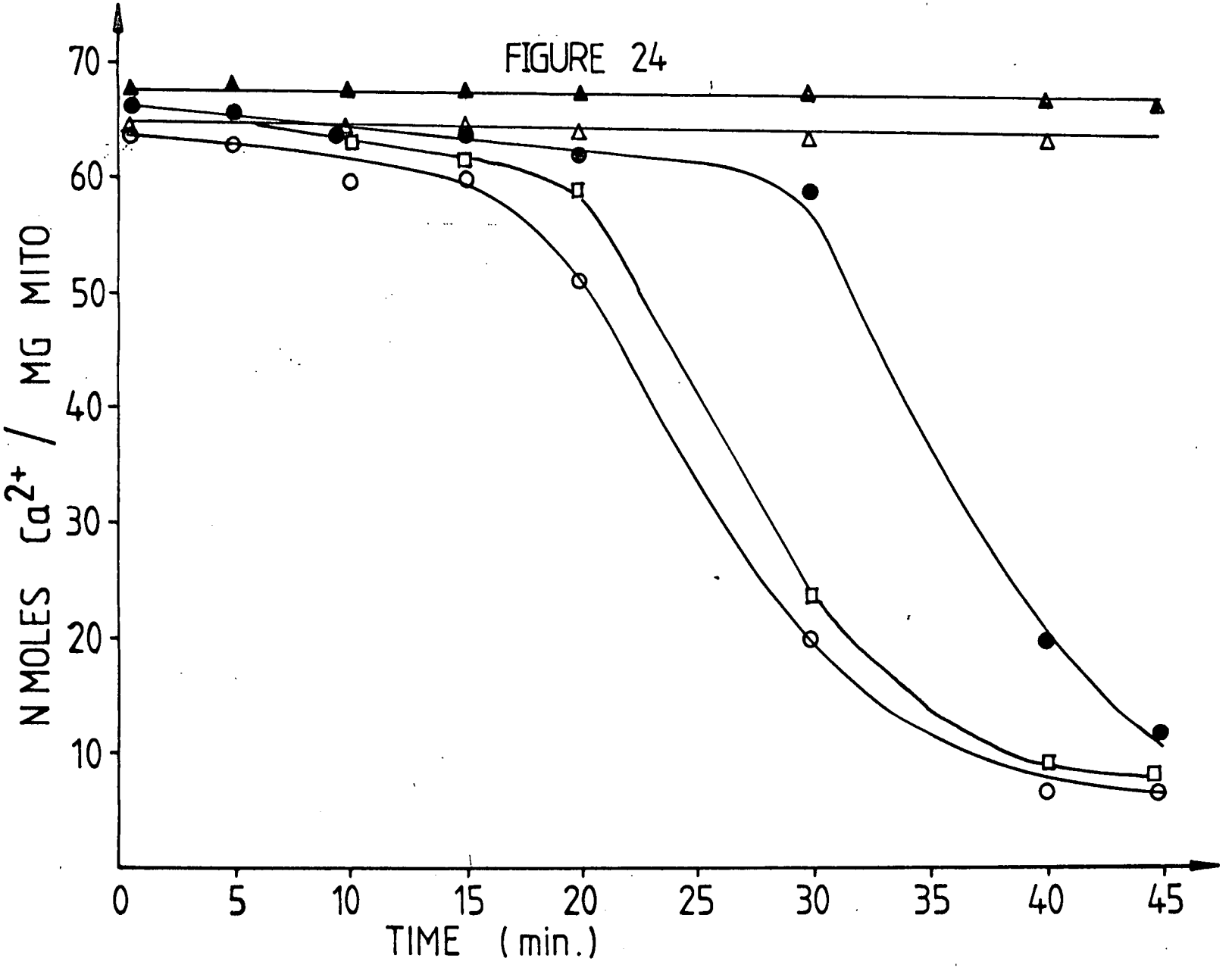
+ 10 μM potassium palmitate

+ 5 μM potassium palmitate (added at 5 min)

+ 10 μM potassium palmitate + 30 μM BSA

+ 10 μM potassium palmitate + 1 mM DL-carnitine HCl

FIGURE 24


70
60
50
40
30
20
10
N MOLES Ca^{2+} / MG MITO
0
5
10
15
20
25
30
35
40
45
TIME (min.)

5 min when $^{45}Ca^{2+}$ had been accumulated caused an earlier release of $^{45}Ca^{2+}$. The capacity of mitochondria to take up $^{45}Ca^{2+}$ was not affected by 10 µM potassium palmitate when added to the incubation medium before the mitochondrial suspension. Ca^{2+} release in the presence of 10 µM palmitate was observed at 15 min.

The presence of 30 µM BSA or 1 mM carnitine HCl in the incubation medium prevented 10 µM potassium palmitate from causing the release of Ca^{2+}; with both substances $^{45}Ca^{2+}$ was retained in the mitochondria during the 45 min duration of the experiment.

3.3.3 Ca^{2+} transport by chloroquine-treated and untreated mitochondria

As shown in fig 25, the inclusion of 1 mM DL-carnitine HCl in the incubation medium, even in the absence of added palmitate, helped $^{45}Ca^{2+}$ retention in the mitochondria, suggesting fatty acid contamination in the mitochondrial suspension. An attempt was then made to see whether the lysosome fraction (which might be present in the 12,000 x g supernatant) were responsible for this fatty acid contamination

Mellor et al (1967) have reported on the capacity of lysosomes to uncouple oxidative phosphorylation and caused mitochondrial swelling (possibly due to free fatty acids released by the action of lipolytic enzymes of the lysosomes). In this experiment, chloroquine was included during the preparation of mitochondria to protect mitochondrial membranes against the effects of lysosomes (Brown et al, 1975; Goldstein et al, 1975). According to the latter workers, chloroquine prevent the release of lipolytic enzymes from lysosomes. It is interesting to note that Allison et al (1964) have shown by fluorescence microscopy that chloroquine is concentrated in lysosomes of mammalian cells probably due to the

FIGURE 25

Effect of carnitine on mitochondrial Ca^{2+} transport studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (4.8 mg protein). The reaction was started by addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. Carnitine was included in the incubation medium before adding the $^{45}Ca^{2+}$.

—●— control

—▲— + 1 mM DL-carnitine HCl

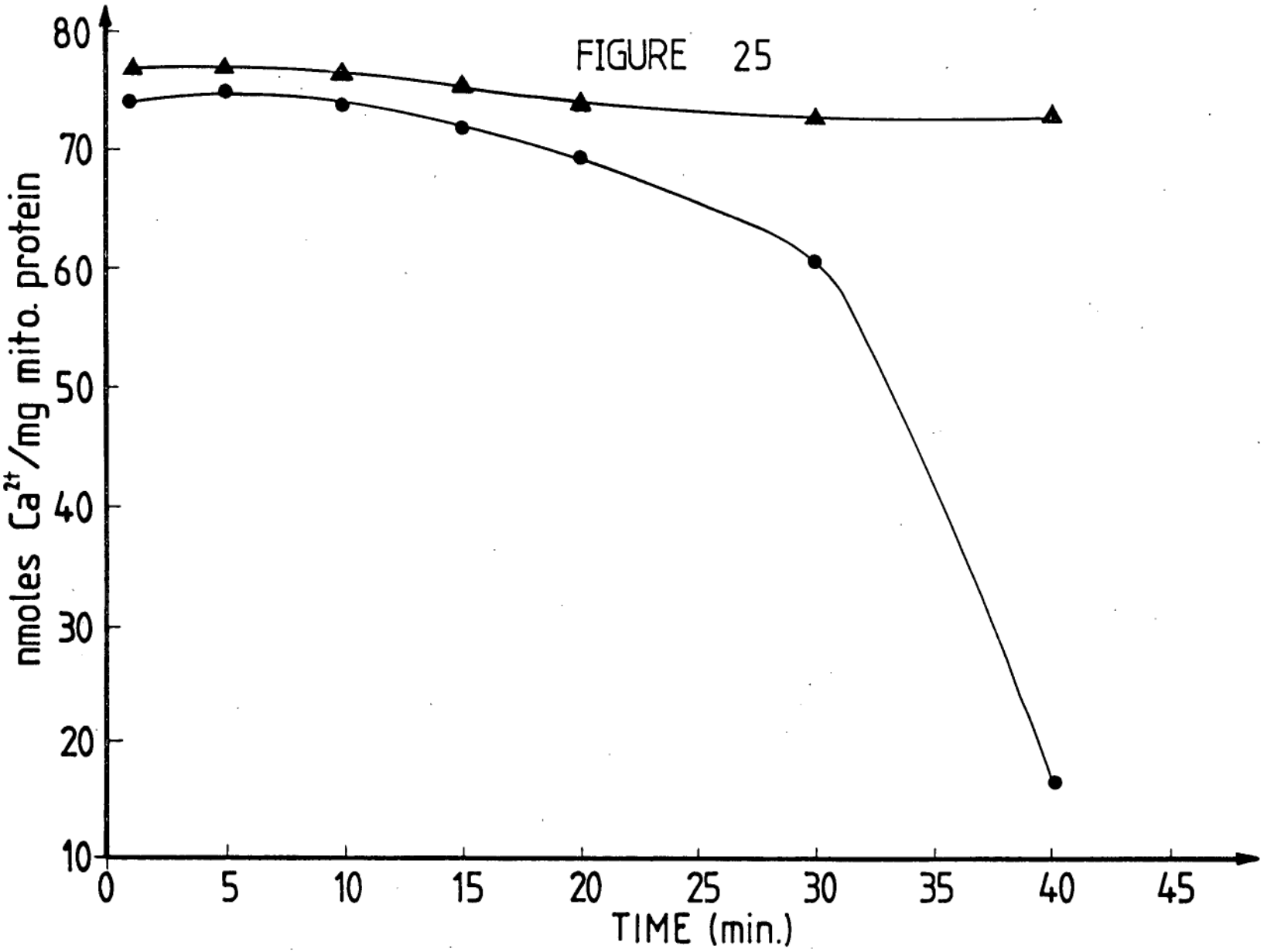


FIGURE 25
nmoles Ca^{2+}/mg mito. protein
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
45
TIME (min.)

FIGURE 26

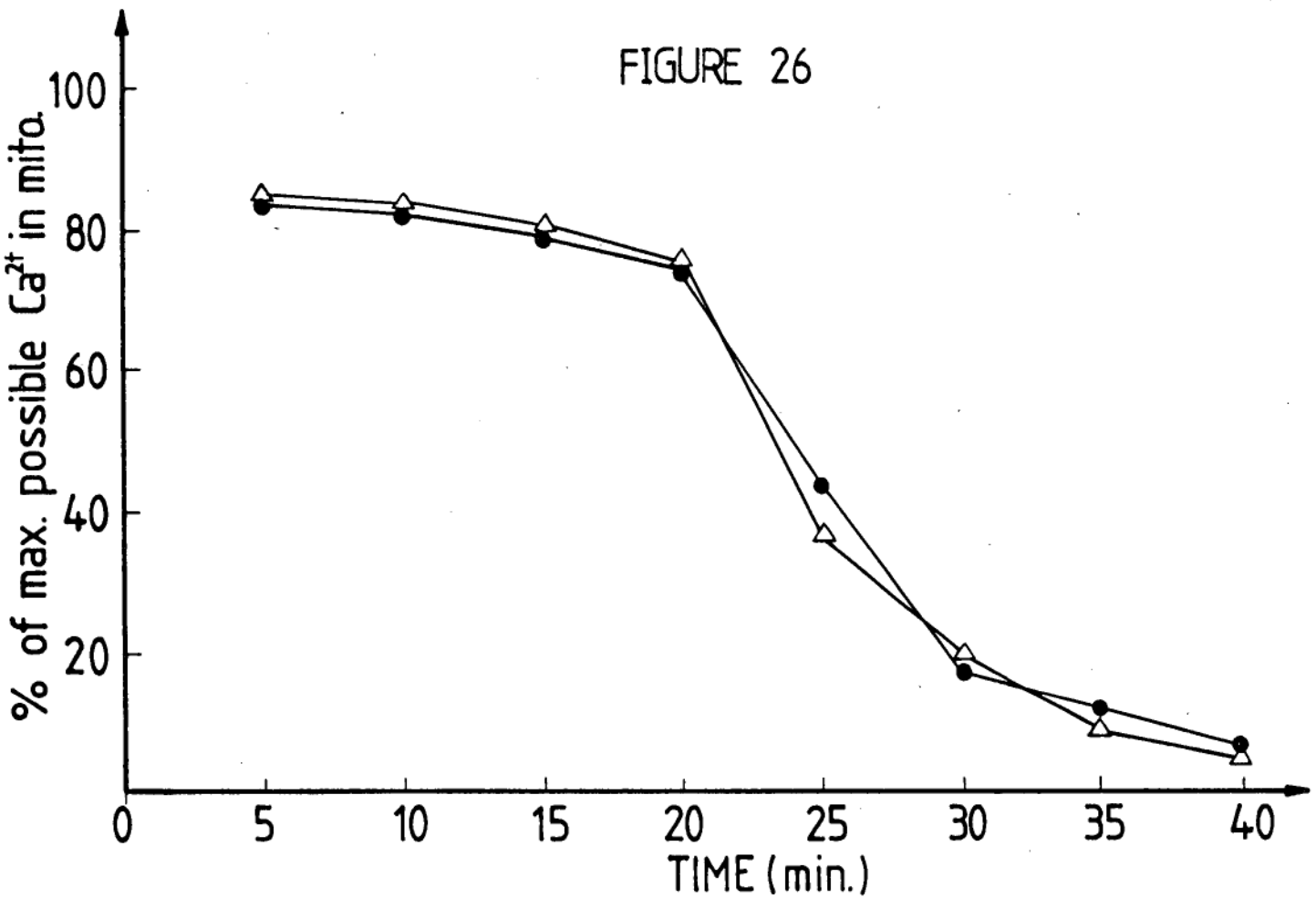
Ca^{2+} transport by chloroquine-treated and untreated mitochondria studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and the untreated mitochondria (5 mg protein) or the chloroquine-treated mitochondria (5.4 mg protein) (the preparation is described in section 3.2). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C.

-△- chloroquine-treated mitochondria

-●- untreated mitochondria

FIGURE 26


% of max. possible Ca^{2+} in mito.
100
80
60
40
20
0
5
10
15
20
25
30
35
40
TIME (min.)

more acidic environment of lysosomes with respect to the cytoplasm.

As shown in fig 26, the capacity to take up $^{45}Ca^{2+}$ and the time of $^{45}Ca^{2+}$ release were almost identical, whether the mitochondria were isolated in the presence or absence of chloroquine.

3.3.4 Mitochondrial Ca^{2+} transport in the presence of potassium palmitate using different respiratory substrates as the energy sources for Ca^{2+} uptake.

Results in fig 27 show that, regardless of the respiratory substrate used, 20 μM potassium palmitate caused Ca^{2+} release from the mitochondria; however, the time of release depended on the respiratory substrate used. (Note : When using 2 mM pyruvate, 0.2 mM malate was included in order to "spark" the reaction). The addition of palmitate was made at 5 min. With the ATP/ succinate system, the control mitochondria retained $^{45}Ca^{2+}$ even at 45 min, whereas complete $^{45}Ca^{2+}$ release was induced by 20 μM potassium palmitate at 30 min. On the other hand the control mitochondria incubated with substrates β-hydroxybutyrate and ATP, started to release $^{45}Ca^{2+}$ at 30 min, while the test mitochondria released their accumulated $^{45}Ca^{2+}$ almost immediately after the addition of 20 μM palmitate (fig 27).

3.3.5 The effect of palmitoyl CoA and palmitoylcarnitine on mitochondrial Ca^{2+} transport

Fig 28 shows that palmitoyl CoA at concentration as low as 5 μM caused an earlier $^{45}Ca^{2+}$ release from the mitochondria than in the control experiment. Additions for this experiment were made at 5 min after the start of the reaction. The results

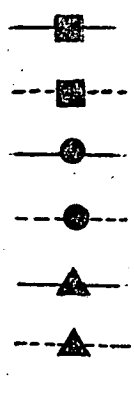
obtained also suggest that palmitoyl CoA is more potent than potassium palmitate in causing release of $^{45}Ca^{2+}$ from mitochondria. When the concentration of palmitoyl CoA was increased to 10 μM and 20 μM, the time required to release the accumulated $^{45}Ca^{2+}$ was decreased.

On the other hand, the presence of 10 μM palmitoyl carnitine favoured $^{45}Ca^{2+}$ retention (fig 28).

FIGURE 27

Mitochondrial Ca^{2+} transport in the presence of potassium palmitate using different energy substrates studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 1 mM ATP and mitochondria (4.8 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. Addition of respiratory substrate was made before adding the $^{45}Ca^{2+}$. Potassium palmitate was added at 5 min.



+ 2 mM succinate

+ 2 mM succinate + 20 μM K palmitate

+ 2 mM β-hydroxybutyrate

+ 2 mM β-hydroxybutyrate + 20 μM K palmitate

+ 2 mM pyruvate (plus 0.2 mM L-malate)

+ 2 mM pyruvate (plus 0.2 mM L-malate) + 20 μM K palmitate

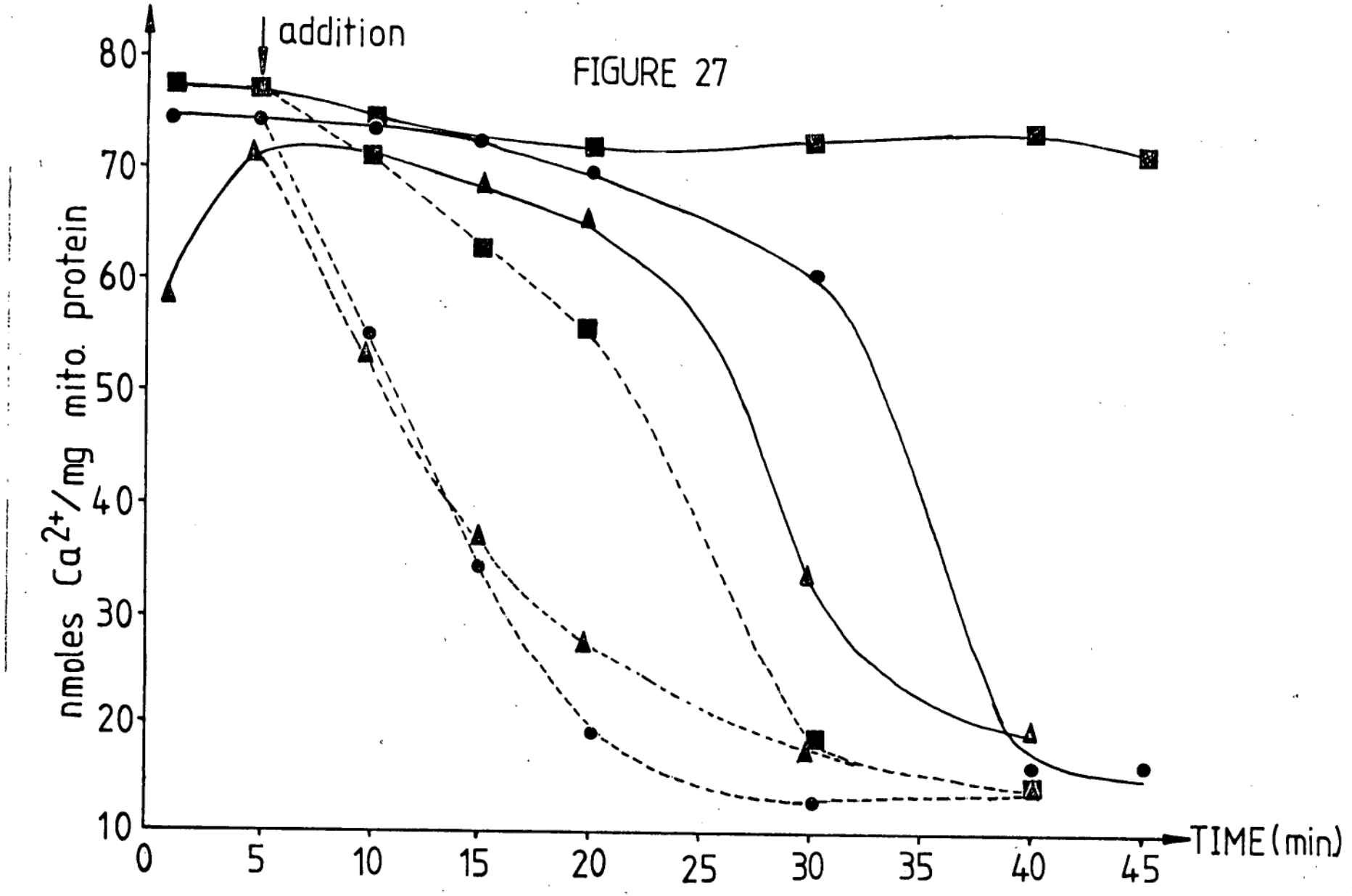

addition
80
70
60
50
40
30
20
10
nmoles Ca^{2+}/mg mito. protein
0
5
10
15
20
25
30
35
40
45
TIME (min)

FIGURE 27

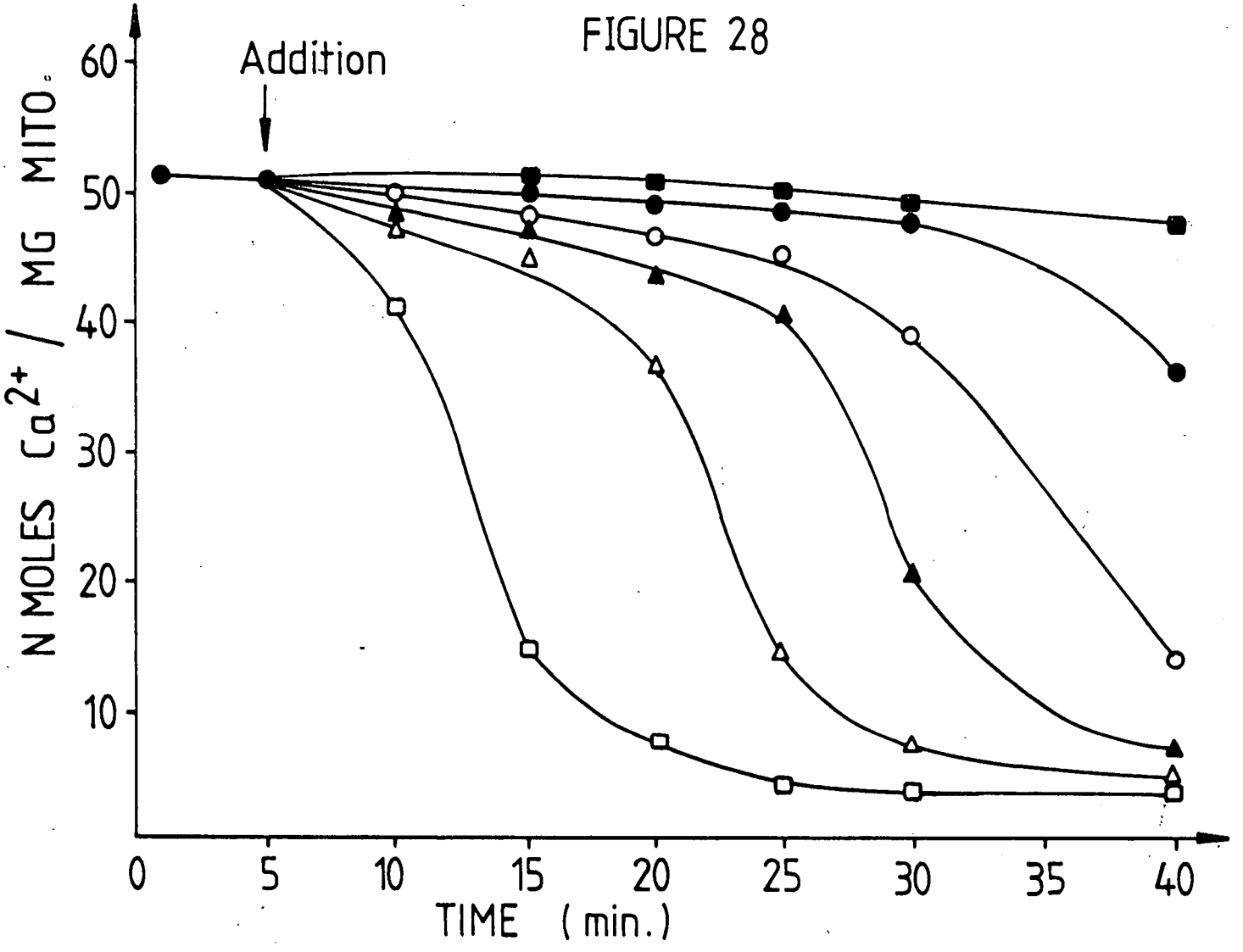
FIGURE 28

Effects of potassium palmitate, palmitoylcarnitine and varying concentrations of palmitoyl CoA on Ca^{2+} transport studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (6 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. The following additions were made at 5 min.

- control
- + 20 μM potassium palmitate
- + 5 μM palmitoyl CoA
- + 10 μM palmitoyl CoA
- + 20 μM palmitoyl CoA
- + 10 μM palmitoylcarnitine

FIGURE 28


Addition
N MOLES Ca^{2+} / MG MITO.
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
TIME (min.)

3.4 DISCUSSION

The Effect of palmitate on Ca^{2+} transport.

No calcium uptake was observed in the presence of the concentrated rat cytosol, using β-hydroxybutyrate and ATP as energy sources (table 5). The incubation medium included 200 μM free fatty acid derived from the concentrated cytosol.

Inclusion of 250 μM potassium palmitate in the standard incubation medium with β-hydroxybutyrate and ATP as energy sources showed that 5 - 10 % of the added Ca^{2+} was taken up by the mitochondria (fig 23). The control mitochondria, on the other hand, accumulated approximately 84 % of the added Ca^{2+} (fig 23). The result also indicates that mitochondrial Ca^{2+} levels in the presence of 250 μM potassium palmitate seemed to increase slightly over the 45 min duration of the experiment. The control mitochondria took up most of the added $^{45}Ca^{2+}$ in the medium within 1 min but started to release this rapidly at 25 min. In fact, the $^{45}Ca^{2+}$ content in the control mitochondria at 45 min was less than that in the mitochondria exposed to the 250 μM potassium palmitate (as shown in fig 23). It is not known why the above phenomenon was observed; nevertheless, the presence of 250 μM potassium palmitate seemed to inhibit the rapid uptake of $^{45}Ca^{2+}$ into mitochondria.

At the lower concentration of 10 μM potassium palmitate, the capacity of mitochondrial Ca^{2+} uptake was not affected; however, an earlier release of Ca^{2+} from the mitochondria than in the control experiment was observed (fig 24). Even 5 μM potassium palmitate added at 5 min caused early Ca^{2+} release (fig 24). It is a well known fact that free fatty acids are uncouplers of oxidative phosphorylation; this would explain

why they caused Ca^{2+} release from mitochondria.

The Ca^{2+}-releasing effect of 10 μM potassium palmitate was prevented by including 30 μM BSA in the incubation medium (fig 24). BSA possibly protects mitochondria from the inhibitory effects of fatty acids by counteracting the uncoupling of oxidative phosphorylation mechanisms caused by the fatty acids (see section 2.4).

1 mM DL-carnitine HCl abolishes the Ca^{2+}-releasing effect of 10 μM potassium palmitate, and in fact causes Ca^{2+} retention in the mitochondria. (The preliminary experiments with the concentrated rat cytosol used high concentrations of carnitine (25 mM and 50 mM) (chapter 2, sections 2.3.4.1 and 2.3.5.1); however for this investigation, the physiological concentration of DL-carnitine HCl (1 mM) was used). Carnitine is not known to protect mitochondria from uncouplers of oxidative phosphorylation, but possibly it helps lower the concentration of free fatty acids in the medium by forming palmitoylcarnitine. The formation of palmitoylcarnitine would result from

(1) the formation of palmitoyl CoA from the coenzyme A present in mitochondria, catalysed by fatty acyl CoA synthetase located on the inner surface of the outer mitochondrial membrane (Nimmo, 1979); and

(2) the reaction of the carnitine in the medium with palmitoyl CoA, catalysed by palmitoylcarnitine transferase A located on the outer surface of the inner membrane (Hoppel & Tomec, 1972). The palmitoylcarnitine would then be transported into the mitochondrial matrix where it would be metabolised.

The effect of 10 μM potassium palmitate on mitohondrial

Ca^{2+} release was also examined using different respiratory substrates as the energy source. In all cases, potassium palmitate caused Ca^{2+} release compared with their respective controls. The time of Ca^{2+} release from the mitochondria seemed to depend on the substrates used. The results obtained showed that with succinate, Ca^{2+} was retained for a longer time than when pyruvate or β-hydroxybutyrate were the substrates as shown in fig 27. The effects of various respiratory substrates on mitochondrial Ca^{2+} transport will be examined in the following chapter.

The effects of palmitoyl CoA and palmitoylcarnitine on Ca^{2+} transport.

The effects of fatty acid derivatives, such as palmitoyl CoA and palmitoylcarnitine, on mitochondrial calcium transport were also examined. Asimakis and Sordahl (1977) showed that 6.7 μM palmitoyl CoA slowed the rate of Ca^{2+} uptake by rabbit heart mitochondria and caused rapid calcium release. In this thesis, it was shown that palmitoyl CoA at concentration as low as 5 μM added at 5 min after the start of reaction caused calcium release from rat liver mitochondria (fig 28). Palmitoyl CoA was more potent than a similar concentration of potassium palmitate in causing release of Ca^{2+} from the isolated mitochondria. With heart mitochondria, Asimakis and Sordahl (1977) observed that 1 mM carnitine was able to prevent the effects of 6.7 μM palmitoyl CoA, and it was suggested that this was due to the formation of palmitoylcarnitine which rapidly entered the mitochondria. This suggestion is supported by the results shown in fig 28, where palmitoylcarnitine at 10 μM concentration enabled the mitochondria to retain calcium

longer than the control mitochondria. A possible explanation for the observed Ca^{2+} retention and release by palmitoylcarnitine and palmitoyl CoA respectively, will be discussed in the final chapter.

It was found that palmitoyl CoA did not interfere significantly with the uptake of Ca^{2+} but induced rapid release of calcium from the mitochondria, and a similar observation was made by Asimakis and Sordahl (1977) with rabbit heart mitochondria. They suggested that the observed effect results from the orientation of adenine nucleotide translocase in the inner mitochondrial membrane as postulated by Klingenberg (1976) (note : palmitoyl CoA was a potent inhibitor of the enzyme, Asimakis and Sordahl, 1977). Prior to Ca^{2+} accumulation, the adenine nucleotide translocase is primarily in the m-state (i.e. oriented to the matrix side of the inner mitochondrial membrane); thus the enzyme is inaccessible to the binding of palmitoyl CoA. After Ca^{2+} accumulation, a new steady state of the membrane is reached and the translocase is primarily in the c-state (oriented to the cytosolic side of the inner membrane), and therefore accessible to the palmitoyl CoA in the medium.

Thus when examined in vitro, palmitate and palmitoyl CoA concentrations as low as 5 μM, induced mitochondrial calcium release ; Palmitoylcarnitine at 10 μM concentration on the other hand caused the retention of Ca^{2+} in mitochondria. The inhibitory effect of palmitoyl CoA can be prevented by the addition of 1 mM carnitine plus 1 mM ATP and the uncoupling effect of palmitate is prevented by 30 μM BSA. It is not known whether palmitoyl CoA or palmitoyl carnitine plays any role at all in regulating the mitochondrial calcium transport under physiological situation.

Attempts to preserve the integrity of organelles by chloroquine

The rationale for using chloroquine in the study was stated in section 3.3.3. The results obtained (fig. 26) showed that inclusion of chloroquine during the preparation of mitochondria had no effect on mitochondrial Ca^{2+} transport under the conditions examined, possibly indicating that if lysosomes were present in the mitochondrial preparation they had no significant influence on mitochondrial calcium transport.

3.5 SUMMARY

1. The effects of potassium palmitate, palmitoyl CoA and palmitoylcarnitine on mitochondrial calcium transport were examined *in vitro*. β-hydroxybutyrate and 1 mM ATP were used as the energy sources.

2. 250 μM potassium palmitate inhibited $^{45}Ca^{2+}$ uptake by the isolated mitochondria. 10 μM potassium palmitate did not affect capacity of calcium uptake but caused an earlier Ca^{2+} release. Even 5 μM potassium palmitate caused Ca^{2+} release.

3. The releasing effect of 10 μM potassium palmitate on mitochondrial $^{45}Ca^{2+}$ was prevented by 30 μM BSA, or by 1 mM carnitine + 1 mM ATP.

4. The effect of 10 μM potassium palmitate on mitochondrial calcium release was also examined using different respiratory substrates such as succinate, pyruvate plus malate and β-hydroxybutyrate.

5. Palmitoyl CoA at concentration as low as 5 μM caused an early calcium release from mitochondria. Palmitoyl CoA was more potent at causing calcium release than a similar concentration of potassium palmitate. On the other hand, palmitoylcarnitine at the same concentration enabled mitochondria to retain calcium for a longer time than in the control experiments.

6. Isolation of mitochondria in the presence or absence of chloroquine did not affect the capacity of calcium transport

or the retention time. Chloroquine has been postulated to help retain the integrity of mitochondrial and lysosomal membranes, and might prevent the release of lipolytic enzymes from lysosomes that would damage the mitochondria.

CHAPTER 4

CALCIUM MOVEMENT IN RAT LIVER MITOCHONDRIA IN THE PRESENCE OF CYTOSOLIC COMPONENTS: (MAGNESIUM, POTASSIUM, SODIUM AND RESPIRATORY SUBSTRATES).

4.1 AIM.

Cations present in the rat cytosol include Mg^{2+}, K^+ and Na^+. This chapter describes the effects of these ions using the incubation medium containing 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM Pi pH 7.2 and in the presence of 1 mM ATP and 2 mM β-hydroxybutyrate or succinate as the energy source/s for Ca^{2+} uptake. As discussed elsewhere (chapter 8), the effects of these ions on Ca^{2+} metabolism have often been studied in the absence of an important substrate such as Pi or ATP (Drahota and Lehninger, 1965; Akerman et al, 1977; Sordahl, 1974), or in the absence of a cation such as Mg^{2+} when studying the effects of Na^+ and K^+ (Drahota and Lehninger, 1965); such results may not reflect the events taking place *in vivo*.

The effect of varying the concentration of Mg^{2+} in the medium on mitochondrial Mg^{2+} and Ca^{2+} accumulation was examined in the presence of ATP plus β-hydroxybutyrate, ADP plus β-hydroxybutyrate and succinate plus rotenone. The influence of Mg^{2+} on Ca^{2+} chelation to ATP and ADP in the incubation medium was also examined.

Various respiratory substrates are present in the cytosol; therefore the influence of some of these respiratory substrates on Ca^{2+} movement into and out of the mitochondria was investigated.

4.2 METHODS AND MATERIALS.

Ca^{2+} movement in rat liver mitochondria was determined using the radioassay technique described in chapter 1, section 1.3 or studied by means of the Ca-electrode (chapter 1, section 1.4). Calcium binding to ATP and ADP was also studied by means of the Ca-electrode. Mg^{2+} in mitochondria was estimated by atomic absorption spectrophotometry of the acid extracts described in chapter 1, section 1.5.4.2 . To ensure that most of the Mg^{2+} in the mitochondrial pellets was actually in the acid extract, the washed mitochondrial pellets were digested with HNO_3 and $HClO_4$ and estimated for Mg^{2+}. Less than 5 nmoles Mg^{2+} remained in the mitochondrial pellets regardless of the total concentration of Mg^{2+} in the mitochondria.

Sodium succinate was from Hopkin and Williams, Searle Company, Chadwell Heath, Essex, England; DL-β-hydroxybutyrate from SIGMA Chemical Co., St. Louis, Missouri, U.S.A. ATP and ADP from Calbiochem, La Jolla, Ca.

4.3 RESULTS

4.3.1 Ca^{2+} movement in mitochondria in the presence of 1 mM ATP and various respiratory substrates, studied by the radioassay technique.

In the presence of 2 mM succinate as the respiratory substrate, the mitochondria accumulated 92 % of the total $^{45}Ca^{2+}$ in the medium and retained this $^{45}Ca^{2+}$ for the 45 min duration of the experiment (fig 29). With 2 mM β-hydroxybutyrate or a similar concentration of glutamate, the mitochondria started to release the accumulated $^{45}Ca^{2+}$ at 15 min and rapid release occurred at 30 min. In the presence of pyruvate (2 mM) plus 0.2 mM malate (to "spark" the reaction), the mitochondria accumulated a maximum of 86 % of the added $^{45}Ca^{2+}$ after 5 min from the start of the reaction, and $^{45}Ca^{2+}$ release from the mitochondria occurred at 20 min as shown in fig 29.

4.3.2 Effect of respiratory inhibitors on mitochondrial Ca^{2+} transport studied by the radioassay technique.

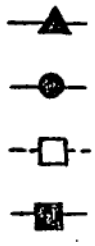
The effect of antimycin A and rotenone, both inhibitors of the respiratory chain, on Ca^{2+} movement into and out of the mitochondria was examined in the presence of either ATP or β-hydroxybutyrate as the energy source for $^{45}Ca^{2+}$ uptake.

In the presence of 1 mM ATP, 87 % of the added $^{45}Ca^{2+}$ was taken up by the mitochondria and this $^{45}Ca^{2+}$ was released at approximately 10 min (fig 30). Antimycin A did not affect Ca^{2+} uptake or release. Similarly in the presence of rotenone C a^{2+} uptake was not affected but the mitochondria retained the accumulated $^{45}Ca^{2+}$ longer than that in the presence of ATP alone (fig 30).

FIGURE 29

Mitochondrial Ca^{2+} transport in the presence of various respiratory substrates studied by the radioassay technique.

The incubation system (final vol.= 5 ml) contained the standard incubation mixture, 1 mM ATP and mitochondria (4.8 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 µCi). Incubation temp. = 25°C. Addition of respiratory substrate was made before adding the $^{45}Ca^{2+}$.



+ 2 mM succinate

+ 2 mM β-hydroxybutyrate

+ 2 mM glutamate

+ 2 mM pyruvate plus 0.2 mM L-malate

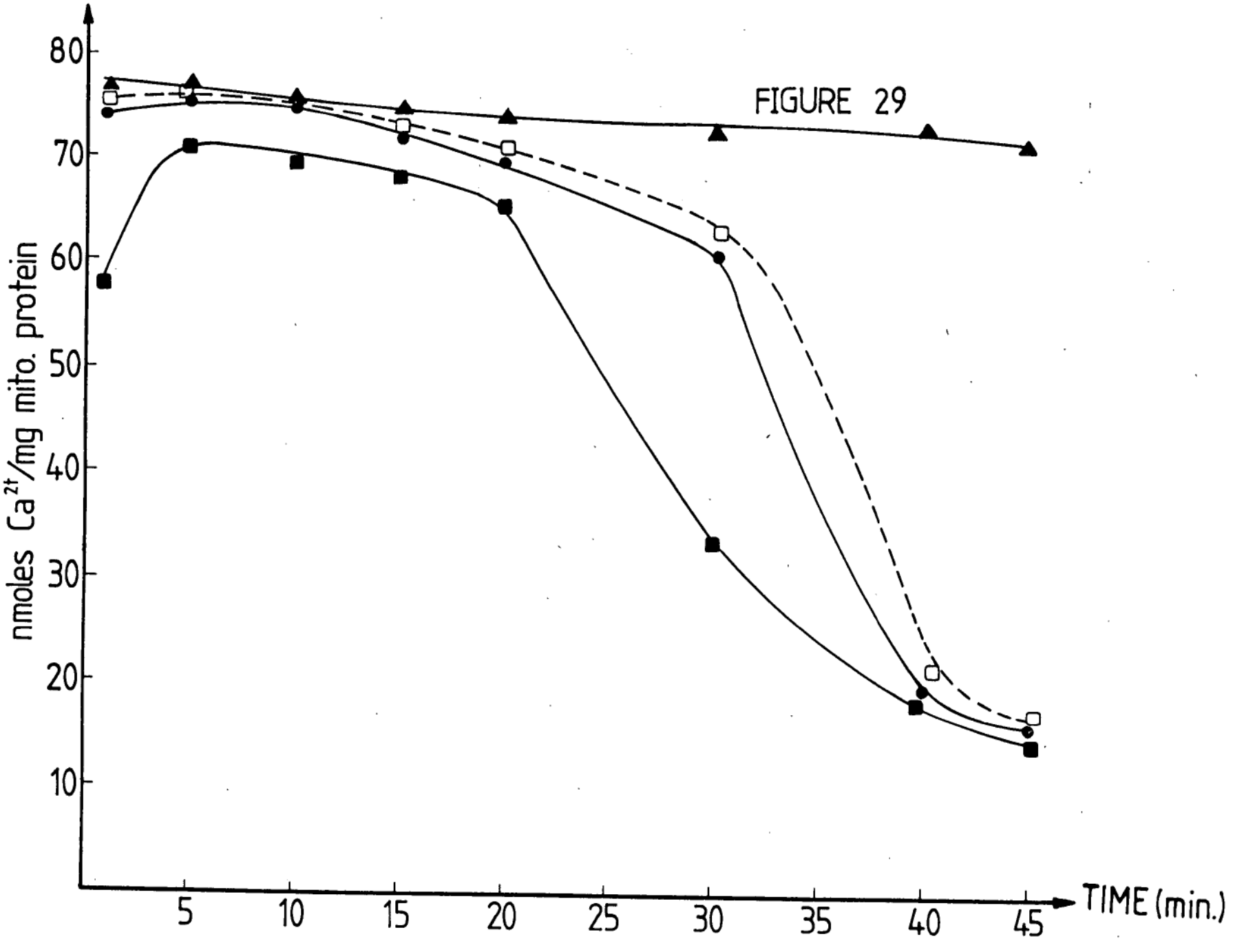
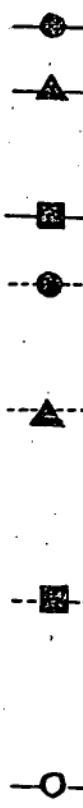


FIGURE 29
nmoles Ca2+/mg mito. protein
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
TIME (min.)

FIGURE 30

Effects of antimycin A and rotenone on mitochondrial Ca^{2+} transport studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 1 mM ATP (solid lines) or 2 mM β-hydroxybutyrate (dashed lines) as the energy substrate and 400 nmoles $^{45}Ca^{2+}$ (1 μCi). The reaction was started by the addition of mitochondria (7 mg protein). Incubation temp. = 25°C. The following additions were made before adding the mitochondria.



+ 1 mM ATP

+ 1 mM ATP + 0.28 μg antimycin A/mg mitochondrial protein

+ 1 mM ATP + 10^{-5}M rotenone

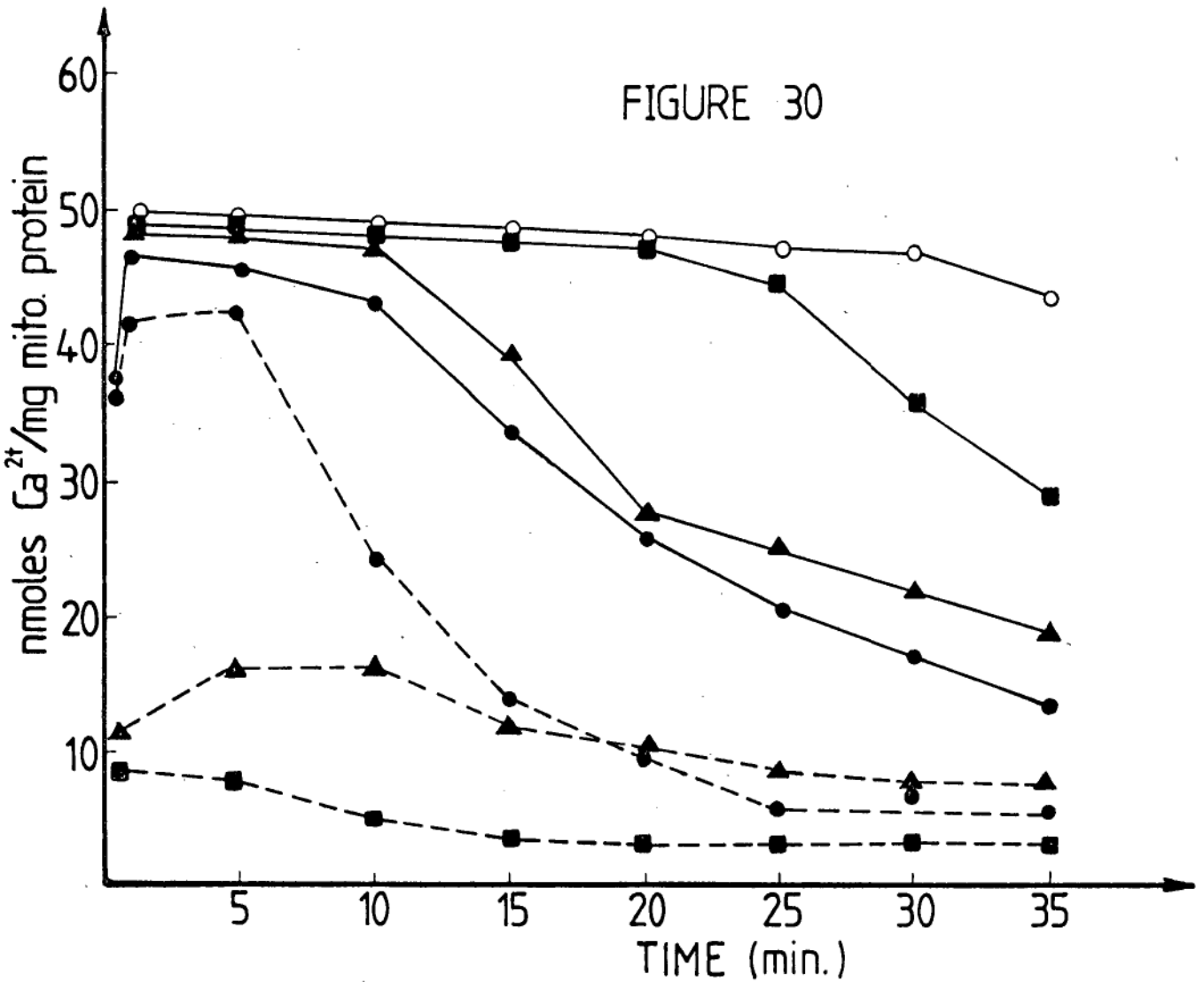
+ 2 mM β-hydroxybutyrate + 2.6 μg oligomycin/mg mitochondrial protein

+ 2 mM β-hydroxybutyrate + 2.6 μg oligomycin/mg mitochondrial protein + 0.28 μg antimycin A/mg protein

+ 2 mM β-hydroxybutyrate + 2.6 μg oligomycin/mg mitochondrial protein + 10^{-5}M rotenone

+ 1 mM ATP + 2 mM β-hydroxybutyrate

FIGURE 30


nmoles Ca2t/mg mito. protein
60
50
40
30
20
10
5
10
15
20
25
30
35
TIME (min.)

Mitochondria respiring on β-hydroxybutyrate (in the absence of external ATP), accumulated 78 % of the $^{45}Ca^{2+}$ in the medium. (For this experiment oligomycin was present to inhibit hydrolysis of endogenous ATP via ATPase.) At 5 min incubation, the mitochondria started to release this $^{45}Ca^{2+}$ rather rapidly. Inclusion of antimycin A or rotenone inhibited $^{45}Ca^{2+}$ uptake by the mitochondria (fig 30).

In the presence of both 1 mM ATP and 2 mM β-hydroxybutyrate, the mitochondria accumulated 87 % of the added $^{45}Ca^{2+}$, and Ca^{2+} release was noted at 35 min (fig 30).

4.3.3 The influence of external Mg^{2+} on the uptake and release of Ca^{2+} and Mg^{2+} by rat liver mitochondria studied by means of the radioassay technique.

The effect of varying the concentration of Mg^{2+} in the medium on Mg^{2+} and Ca^{2+} accumulation by the mitochondria was examined. The Mg^{2+} content in the mitochondria was also examined during Ca^{2+} release and retention. An attempt was also made to see whether Mg^{2+} was transported into the mitochondria via the adenine nucleotide translocase as Mg-ATP or Mg-ADP complex. For this reason, Mg^{2+} accumulation into the mitochondria was examined in the presence of the following substrates (i.e assuming that the concentration of endogenous ATP or ADP in the mitochondria is negligible).

1. ATP + β-hydroxybutyrate
2. ADP + β-hydroxybutyrate
3. Respiratory substrate (in the absence of externally added ATP and ADP). Succinate was chosen as the respiratory substrate instead of β-hydroxybutyrate since the former substrate helped prolong Ca^{2+} retention in the mitochondria.

The Mg^{2+} content in the mitochondria during Ca^{2+} retention was also investigated. To ensure Ca^{2+} retention during the 45 min duration of the experiment, the substrates succinate and ATP were used as the energy sources for Ca^{2+} uptake.

4.3.3.1 The influence of external Mg^{2+} on the uptake and release of Ca^{2+} and Mg^{2+} in the presence of 1 mM ATP and 2 mM β-hydroxybutyrate.

Fig 31 shows that in the absence of externally added Mg^{2+}, the rat liver mitochondria contained 31 nmoles Mg^{2+}/mg mitochondrial protein. The mitochondria were able to accumulate an additional 22 nmoles Mg^{2+}/mg mitochondrial protein in the presence of 1 mM $MgCl_2$ and 32 nmoles Mg^{2+} when 3 mM $MgCl_2$ was present (fig 31). Out of the 5,000 nmoles Mg^{2+} in the medium (i.e. 1 mM $MgCl_2$) and 15,000 nmoles Mg^{2+} (3 mM $MgCl_2$) only 7 % and 3.4 % Mg^{2+} respectively were accumulated by the mitochondria (i.e. excluding the endogenous mitochondrial Mg^{2+}).

As shown in fig 31, 51 nmoles $^{45}Ca^{2+}$ was taken up per mg mitochondrial protein (i.e. approximately 91 % of the total $^{45}Ca^{2+}$ added to the incubation medium) at each concentration of Mg^{2+} used. The extra Mg^{2+} accumulated by the mitochondria did not increase or inhibit $^{45}Ca^{2+}$ uptake, however when extra Mg^{2+} had been taken up the mitochondria were able to retain the $^{45}Ca^{2+}$ for a longer period; for example at 30 min incubation, in the absence of exogenous Mg^{2+}, the mitochondria contained 5 nmoles $^{45}Ca^{2+}$/mg mitochondrial protein but in the presence of 1 mM Mg^{2+} and 3 mM Mg^{2+}, the mitochondria contained 24 nmoles and 41 nmoles $^{45}Ca^{2+}$/mg mitochondria

FIGURE 31

The influence of external Mg^{2+} on the uptake and release of Ca^{2+} and Mg^{2+} in the presence of ATP and β-hydroxybutyrate studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM Pi pH 7.4, 72 mM KCl, 2 mM β-hydroxybutyrate, 1 mM ATP, varying concentrations of Mg^{2+} and mitochondria (17.8 mg protein). The reaction was started by the addition of 1,000 nmoles $^{45}Ca^{2+}$ (1 μCi). 0.2 ml of the incubation mixture was sampled for $^{45}Ca^{2+}$ estimation and 0.5 ml removed for Mg^{2+} estimation (see section 1.5.4.2). Incubation temp. = 25°C.

—●— $^{45}Ca^{2+}$/mg mito. protein in the absence of externally added Mg^{2+}

—▲— $^{45}Ca^{2+}$/mg mito. protein in the presence of 1 mM $MgCl_2$

—■— $^{45}Ca^{2+}$/mg mito. protein in the presence of 3 mM $MgCl_2$

--●-- Mg^{2+}/mg mito. protein in the absence of externally added Mg^{2+}

--▲-- Mg^{2+}/mg mito. protein in the presence of 1 mM $MgCl_2$

--■-- Mg^{2+}/mg mito. protein in the presence of 3 mM $MgCl_2$

mito. = mitochondrial.

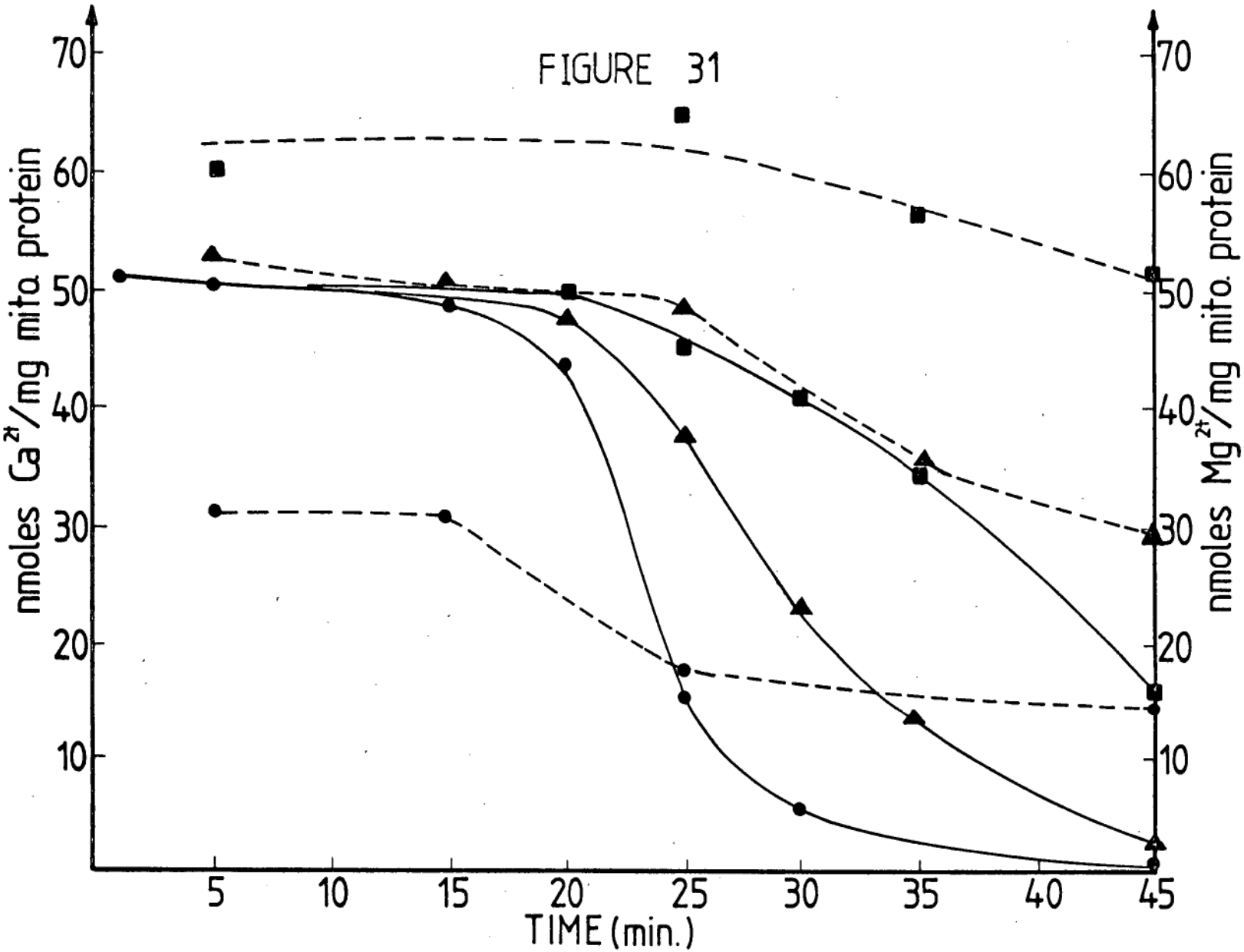


FIGURE 31
nmoles Ca2+/mg mito protein
nmoles Mg2+/mg mito. protein
TIME (min.)
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45

respectively. The results also indicate that during $^{45}Ca^{2+}$ release from the mitochondria there was a corresponding release of mitochondrial Mg^{2+} (fig 31).

4.3.3.2 The influence of external Mg^{2+} on the uptake and release of Ca^{2+} and Mg^{2+} in the presence of 1 mM ADP and 2 mM β-hydroxybutyrate.

Results shown in fig 32 suggest that approximately 12 nmoles and 30 nmoles Mg^{2+} was accumulated per mg mitochondrial protein in the presence of 1 mM and 3 mM $MgCl_2$ respectively. Approximately 4 % of the total Mg^{2+} added was accumulated by the mitochondria. The endogenous Mg^{2+} in the mitochondria was 38 nmoles Mg^{2+} per mg mitochondrial protein.

The results shown in fig 32 also suggest that the accumulated Mg^{2+} caused the mitochondria to retain Ca^{2+} for a longer period of time but had no significant effect on Ca^{2+} uptake by the mitochondria. Approximately 91 % of the total $^{45}Ca^{2+}$ added was taken up by the mitochondria at each concentration of Mg^{2+} present in the incubation medium. Similarly, the results indicate that Mg^{2+} release from the mitochondria seemed to occur simultaneously with Ca^{2+} release.

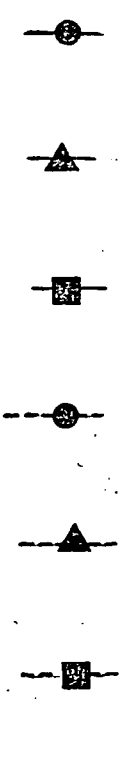
4.3.3.3 The influence of external Mg^{2+} on uptake and release of Ca^{2+} and Mg^{2+} in the presence of 2 mM succinate plus 10^{-5}M rotenone.

Succinate was used as the source of energy for $^{45}Ca^{2+}$ uptake by the mitochondria (i.e. in the absence of ATP or ADP but in the presence of Pi). Rotenone was included in the incubation medium to inhibit respiration via NAD^+ linked substrates.

FIGURE 32

The influence of external Mg^{2+} on the uptake and release of Ca^{2+} and Mg^{2+} in the presence of ADP and β-hydroxybutyrate studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM Pi pH 7.4, 72 mM KCl, 2 mM β-hydroxybutyrate, 1 mM ADP, varying concentrations of Mg^{2+} and mitochondria (17.8 mg protein). The reaction was started by the addition of 1,000 nmoles $^{45}Ca^{2+}$ (1 μCi). 0.2 ml of the incubation mixture was sampled for $^{45}Ca^{2+}$ estimation and 0.5 ml removed for Mg^{2+} estimation as described in section 1.5.4.2 . Incubation temp. = 25°C.



—●— $^{45}Ca^{2+}$/mg mito. protein in the absence of externally added Mg^{2+}

—▲— $^{45}Ca^{2+}$/mg mito. protein in the presence of 1 mM $MgCl_2$

—■— $^{45}Ca^{2+}$/mg mito. protein in the presence of 3 mM $MgCl_2$

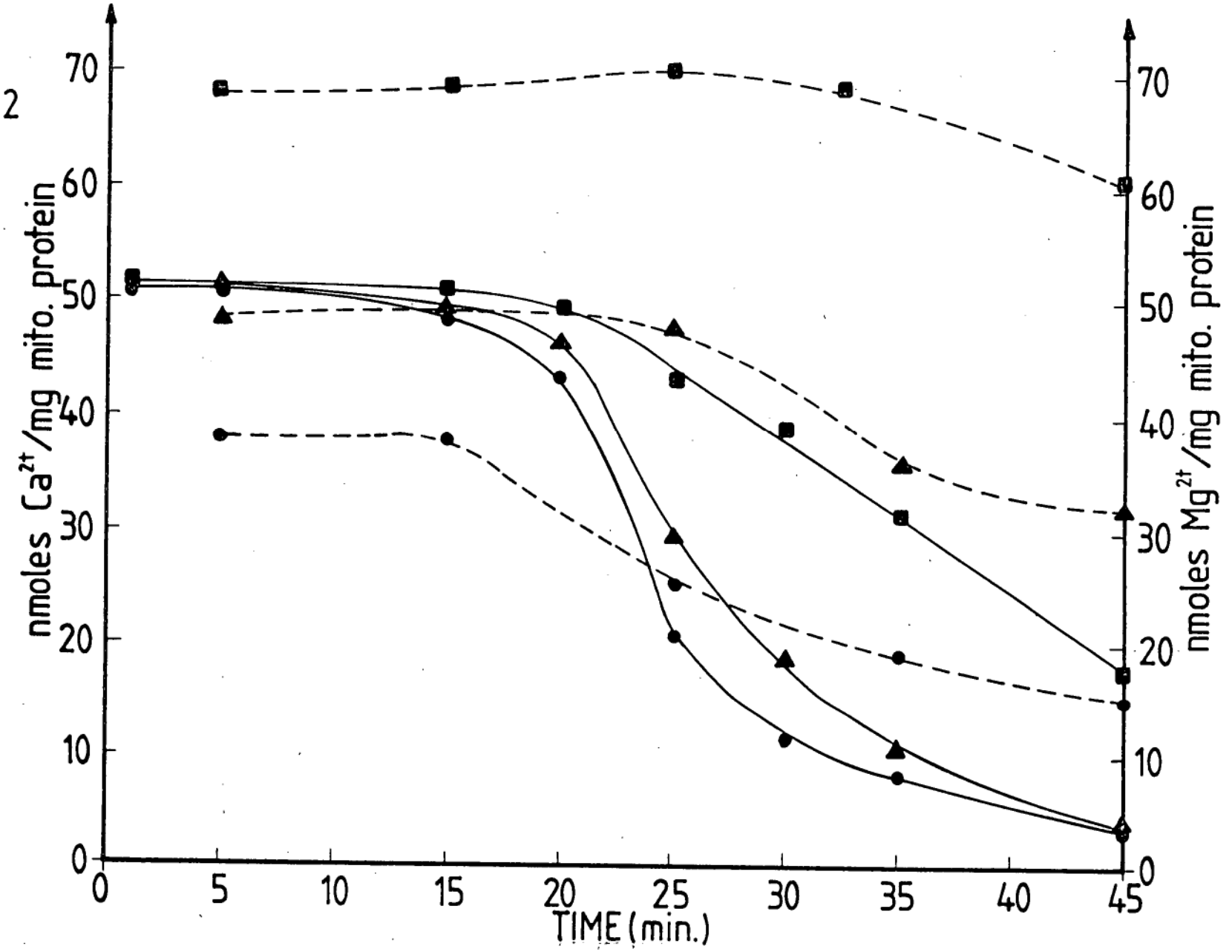
---●--- Mg^{2+}/mg mito. protein in the absence of externally added Mg^{2+}

---▲--- Mg^{2+}/mg mito. protein in the presence of 1 mM $MgCl_2$

---■--- Mg^{2+}/mg mito. protein in the presence of 3 mM $MgCl_2$

mito. = mitochondrial.

FIGURE 32


nmoles Ca^{2+}/mg mito. protein
nmoles Mg^{2+}/mg mito. protein
TIME (min.)
0
5
10
15
20
25
30
35
40
45
0
10
20
30
40
50
60
70

As shown in fig 33, the endogenous mitochondrial Mg^{2+} was approximately 32 nmoles Mg^{2+}/mg mitochondrial protein. In the presence of 1 mM $MgCl_2$, 2 mM and 3 mM $MgCl_2$ an additional 13 nmoles, 42 nmoles and 59 nmoles Mg^{2+} were accumulated by the mitochondria respectively, i.e. between 5 - 9 % of the total Mg^{2+} added.

The extra Mg^{2+} accumulated by the mitochondria caused $^{45}Ca^{2+}$ retention but had no effect on Ca^{2+} uptake by the mitochondria. The mitochondria started to release the accumulated $^{45}Ca^{2+}$ at 7 min, the rate of release appeared to decrease with an increasing amount of Mg^{2+} present in the mitochondria (fig 33). Mg^{2+} release was observed during Ca^{2+} release from the mitochondria (fig 33).

4.3.3.4 The influence of external Mg^{2+} on the uptake and release of Ca^{2+} and Mg^{2+} in the presence of 2 mM succinate and 1 mM ATP.

For this particular experiment, both succinate and ATP were present in the incubation medium as energy sources to ensure mitochondrial Ca^{2+} retention for the 45 min duration of the experiment.

Approximately 90 % of the total Ca^{2+} added was accumulated by the mitochondria and Ca^{2+} retention was observed even at 45 min, at each concentration of $MgCl_2$ present in the incubation medium (fig 34). The endogenous mitochondrial Mg^{2+} was 32 nmoles/mg mitochondrial protein and the mitochondria accumulated an additional 13 nmoles, 31 nmoles and 40 nmoles Mg^{2+} per mg mitochondrial protein in the presence 1 mM, 2 mM and 3 mM $MgCl_2$ in the medium respectively (fig 34), i.e. between 4 - 6 % of the total Mg^{2+} added were accumulated by

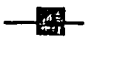
FIGURE 33

The influence of external Mg^{2+} on uptake and release of Ca^{2+} and Mg^{2+} in the presence of succinate plus rotenone.

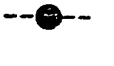
The incubation system (final vol. = 5 ml) contained 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM KH_2PO_4 pH 7.4, 72 mM KCl, 2 mM Na succinate, 10^{-5}M rotenone, varying concentrations of Mg^{2+} and mitochondria (21.5 mg protein). The reaction was started by the addition of 1,000 nmoles $^{45}Ca^{2+}$ (1 µCi). 0.2 ml of the incubation mixtures were sampled for $^{45}Ca^{2+}$ estimation and 0.5 ml removed for Mg^{2+} estimation (see section 1.5.4.2). Incubation temp. = 25°C.

$^{45}Ca^{2+}$/mg mito. protein in the absence of externally added Mg^{2+}.

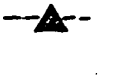
$^{45}Ca^{2+}$/mg mito. protein in the presence of 1 mM $MgCl_2$.



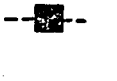
$^{45}Ca^{2+}$/mg mito. protein in the presence of 3 mM $MgCl_2$.



Mg^{2+}/mg mito. protein in the absence of externally added Mg^{2+}.



Mg^{2+}/mg mito. protein in the presence of 1 mM $MgCl_2$.



Mg^{2+}/mg mito. protein in the presence of 3 mM $MgCl_2$.

mito. = mitochondrial.

FIGURE 33

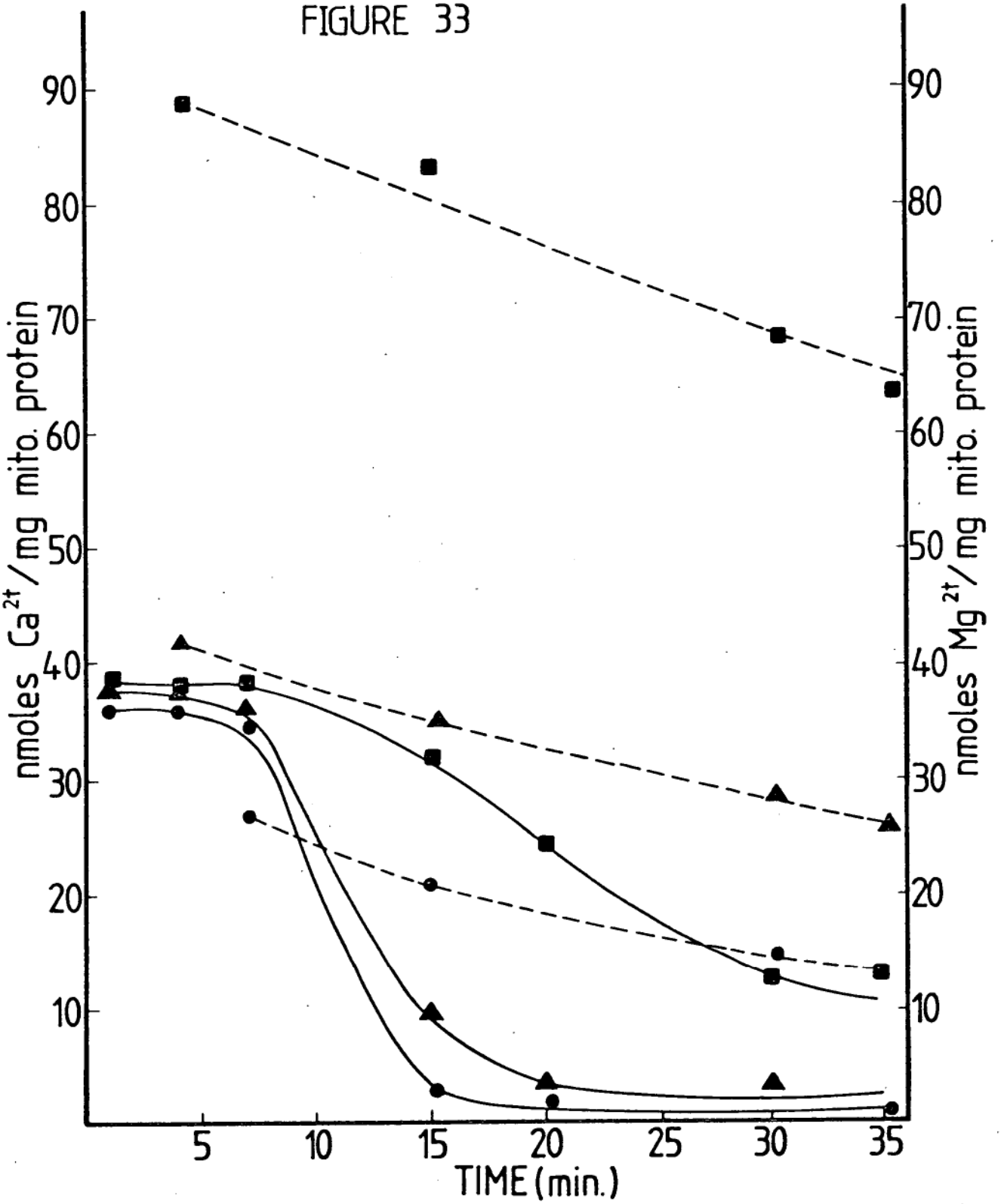

90
80
70
60
50
40
30
20
10
nmoles Ca2+/mg mito. protein
nmoles Mg2+/mg mito. protein
5
10
15
20
25
30
35
TIME (min.)

FIGURE 34

The influence of external Mg^{2+} on uptake and release of Ca^{2+} and Mg^{2+} in the presence of succinate and ATP.

The incubation system (final vol. = 5 ml) contained 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM KH_2PO_4 pH 7.4, 72 mM KCl, 2 mM Na succinate, 1 mM ATP, varying concentrations of Mg^{2+} and mitochondria (20 mg protein). The reaction was started by the addition of 1000 nmoles $^{45}Ca^{2+}$ (1 μCi). 0.2 ml of the incubation mixtures was removed for $^{45}Ca^{2+}$ estimation and 0.5 ml removed for Mg^{2+}. Incubation temp. = 25°C.

--●-- Mg^{2+}/mg mito. protein in the absence of externally added Mg^{2+}.

-▲-- Mg^{2+}/mg mito. protein in the presence of 1 mM $MgCl_2$.

--○-- Mg^{2+}/mg mito. protein in the presence of 2 mM $MgCl_2$.

-■-- Mg^{2+}/mg mito. protein in the presence of 3 mM $MgCl_2$.

——— (solid line) mitochondrial $^{45}Ca^{2+}$ transport in the presence of 0, 1, 2 or 3 mM $MgCl_2$.

mito. = mitochondrial.

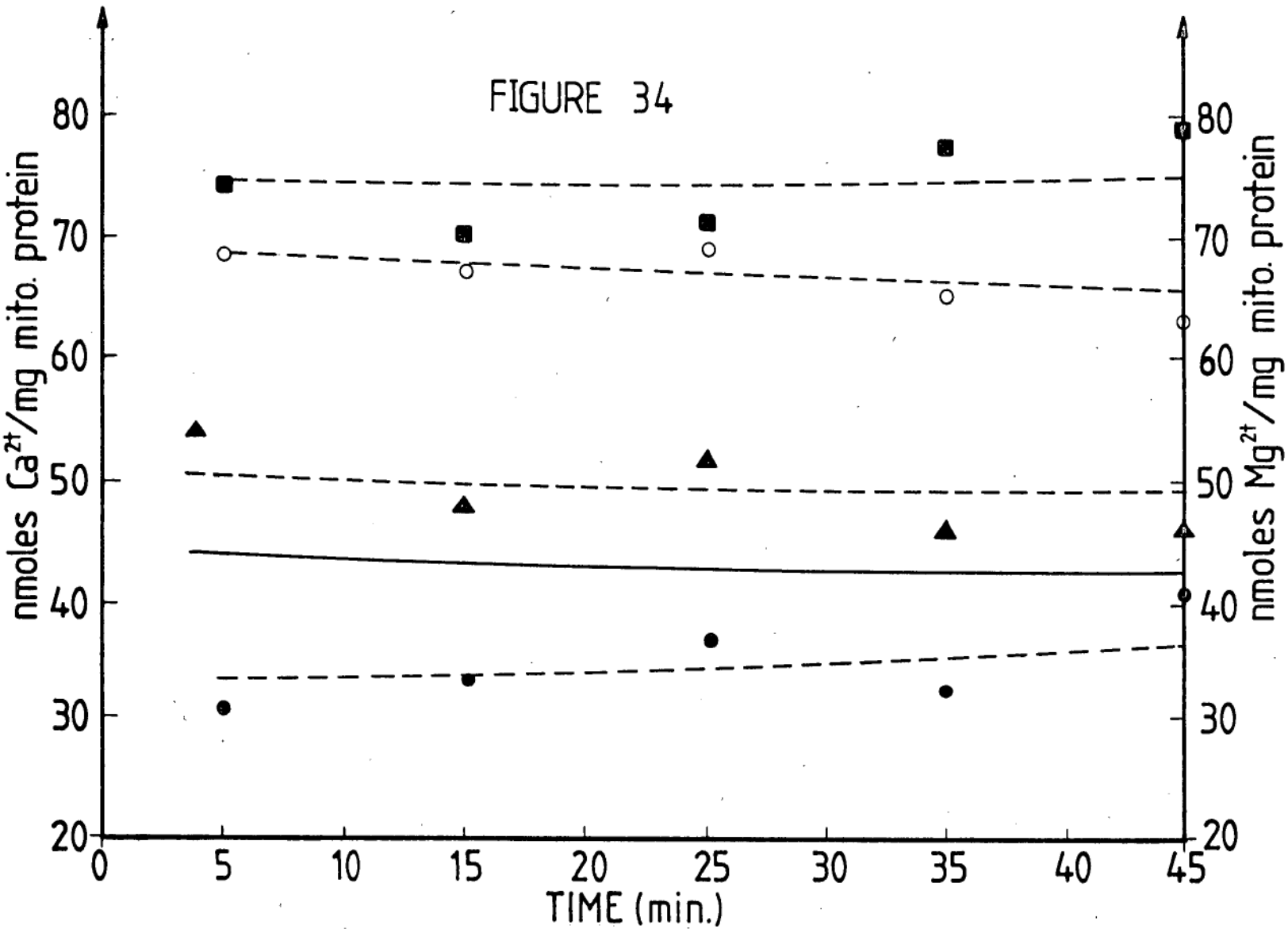


FIGURE 34
nmoles Ca2+/mg mito. protein
nmoles Mg2+/mg mito. protein
TIME (min.)
80
70
60
50
40
30
20
0
5
10
15
20
25
30
35
40
45

the mitochondria. The levels of the mitochondrial Mg^{2+} remained fairly steady during the mitochondrial Ca^{2+} retention as shown in fig 34. In the absence of added Ca^{2+} and Mg^{2+} in the incubation medium, the mitochondrial Mg^{2+} (i.e. endogenous Mg^{2+}) fell slightly during the incubation.

4.3.4 Effect on varying Mg^{2+} concentration on Ca^{2+} chelation to ATP and ADP.

ATP and ADP have a higher affinity for Mg^{2+} than Ca^{2+}. An attempt was made to find out the effect of varying Mg^{2+} concentration in the medium on Ca^{2+} chelation to 1 mM ATP or 1 mM ADP. Ca^{2+} chelation to ATP was estimated by means of the Ca^{2+} electrode in the presence of the 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM KH_2PO_4 pH 7.2, 72 mM KCl, 400 nmoles Ca^{2+}, 1 mM ATP and varying concentrations of $MgCl_2$. The electrode was calibrated by adding small amounts of Ca^{2+} (50 nmoles at a time). The electrode was also calibrated with Ca-NTA buffers (chapter 1, section 1.4).

Fig 35 shows the effect on varying the concentration of Mg^{2+} on Ca^{2+} chelation to 1 mM ATP. A graph of the percentage of the total Ca^{2+} chelated to 1 mM ATP versus the concentration of Mg^{2+} in mM is as shown in fig 36. The results suggest that in the absence of added Mg^{2+}, all the Ca^{2+} (99 %) in the medium was complexed to the 1 mM ATP and the percentage decreased non-linearly with increasing concentration of Mg^{2+} (fig 36), i.e. 68 %, 37 %, and 12 % in the presence of 1 mM, 2 mM and 10 mM Mg^{2+} respectively were complexed to ATP. A similar observation was noted in the presence of 1,000 nmoles Ca^{2+} ~~(fig 38)~~, 99 % Ca^{2+} chelation to 1 mM ATP in the absence of Mg^{2+}, 60 % and 37 % in the presence of 1 mM and 2 mM Mg^{2+}

FIGURE 35

Effect on varying Mg^{2+} concentration on Ca^{2+} chelation to ATP.

ATP (final concentration = 1 mM) was added to the incubation system (final vol. = 5 ml) containing 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM KH_2PO_4 pH 7.2, 72 mM KCl, varying concentrations of $MgCl_2$ and 400 nmoles Ca^{2+}. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C.

(a) 0 Mg^{2+} added to the incubation medium

(b) + 1 mM $MgCl_2$

(c) + 2 mM $MgCl_2$

(d) + 4 mM $MgCl_2$

(e) + 8 mM $MgCl_2$

(f) + 10 mM $MgCl_2$

FIGURE 35

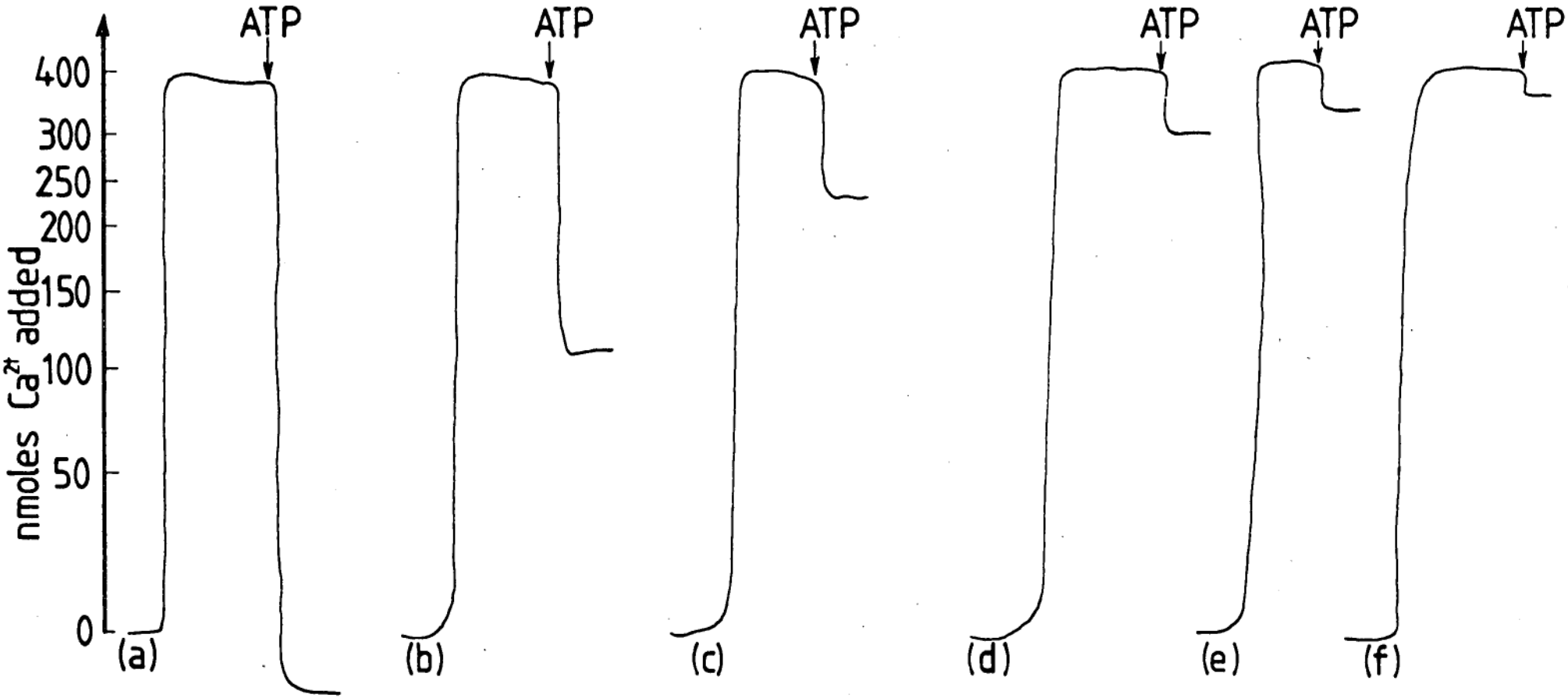
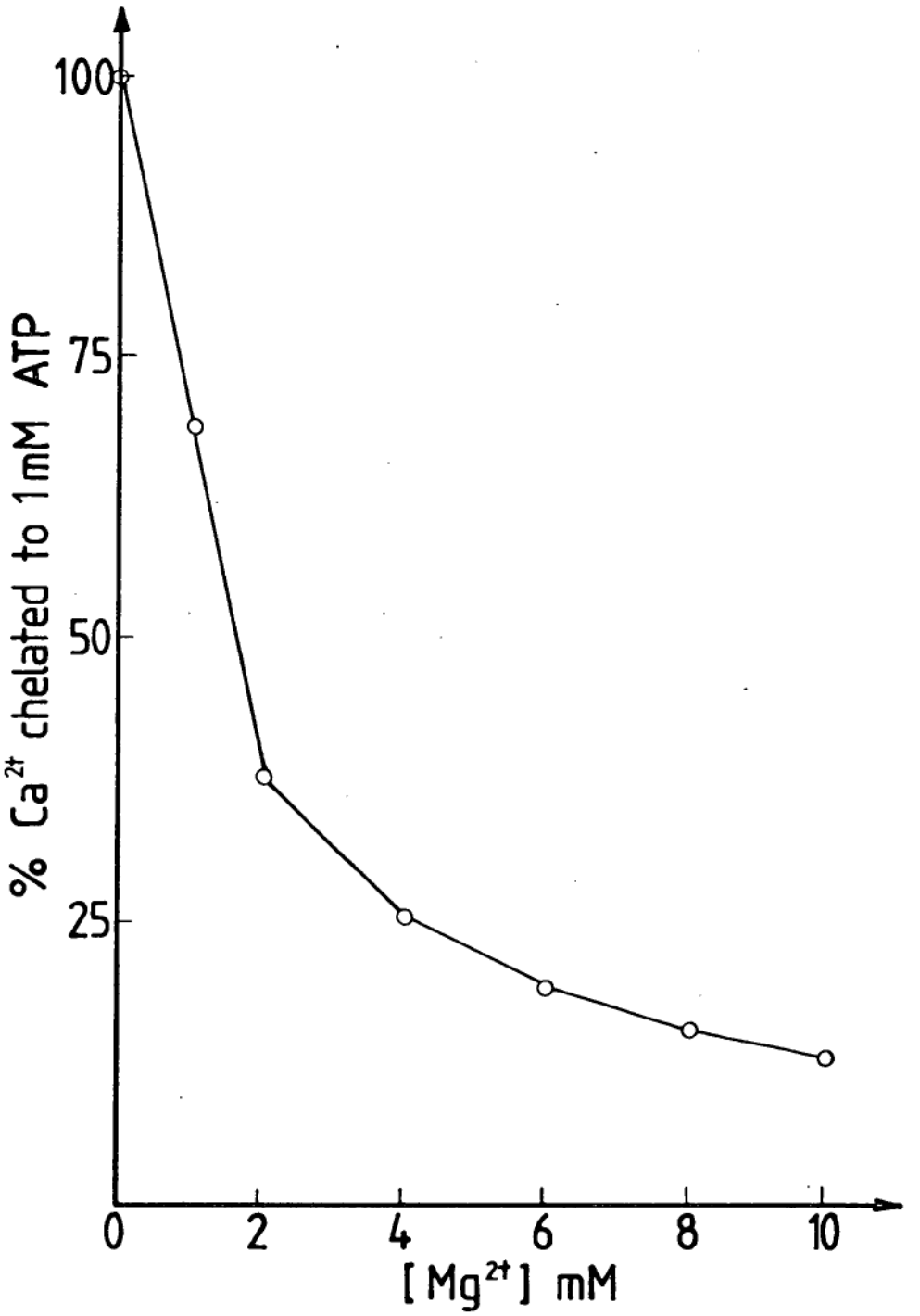

ATP
ATP
ATP
ATP
ATP
ATP
400
300
250
200
150
100
50
0
nmoles Ca²⁺ added
(a)
(b)
(c)
(d)
(e)
(f)

FIGURE 36

% of total Ca^{2+} chelated to ATP versus the concentration of Mg^{2+}.

The legend to figure 36 is similar to that in figure 35. The % of the total Ca^{2+} chelated to 1 mM ATP was calculated and plotted against the concentration of added $MgCl_2$.

FIGURE 36



respectively.

Ca^{2+} chelation to 1 mM ADP, on varying Mg^{2+} concentration, was also examined. Fig 37 shows the percentage of Ca^{2+} chelated to ADP versus Mg^{2+} concentration. In the absence of Mg^{2+}, approximately 62.5 % of the total Ca^{2+} in the medium were complexed to the ADP and the percentage decreased non-linearly with an increasing concentration of Mg^{2+} in the medium.

4.3.5 Effect of Na^+ and K^+ on mitochondrial Ca^{2+} transport.
The radioassay technique was used to monitor Ca^{2+} movement into and out of mitochondria. 2 mM β-hydroxybutyrate and 1 mM ATP were the added energy sources for Ca^{2+} uptake.

4.3.5.1 Effect of Na^+ on mitochondrial Ca^{2+} transport.
For this experiment, the potassium salt of ATP was used instead of the Na^+ salt. As shown in fig 38, Na^+ at concentration between 0 to 10 mM showed no significant effect on mitochondrial Ca^{2+} uptake or release. Maximal Ca^{2+} release from the mitochondria occurred at 25 min.

4.3.5.2 Effect of K^+ on mitochondrial Ca^{2+} transport.
As shown in fig 39, the presence of K^+ at concentration 2 mM, 74 mM and 146 mM did not affect Ca^{2+} uptake or release from mitochondria during the 30 min duration of the experiment.

FIGURE 37

% Ca^{2+} chelated to ADP versus the concentration of Mg^{2+}.

ADP (final concentration = 1 mM) was added to the incubation system (final vol.= 5 ml) containing 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM Pi pH 7.2, 72 mM KCl, varying concentrations of $MgCl_2$ (0, 1 or 3 mM) and 400 nmoles Ca^{2+}. Incubation temp. = 25°C . The % of the total Ca^{2+} chelated to 1 mM ADP was calculated and plotted against the concentration of added $MgCl_2$.

FIGURE 37

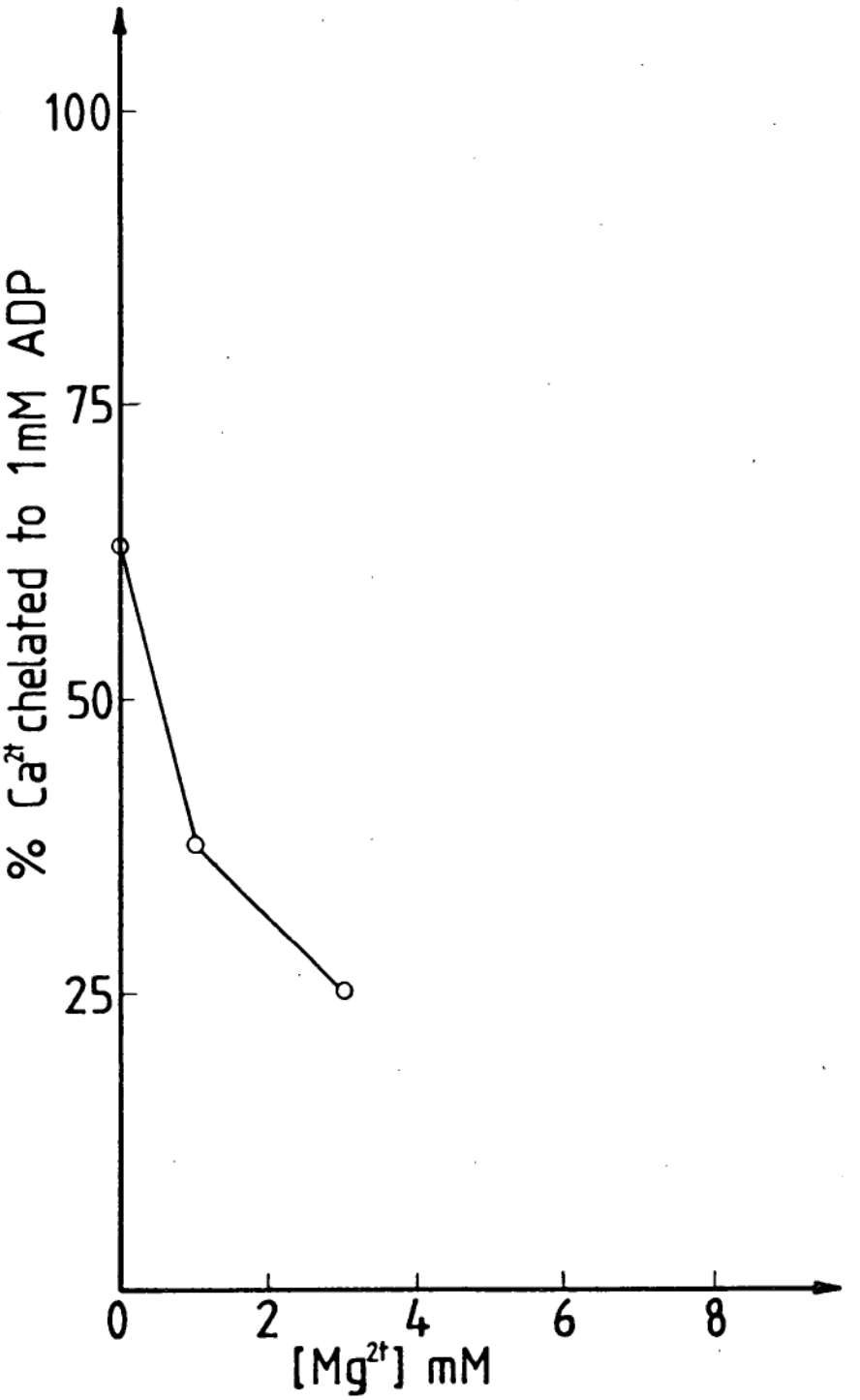
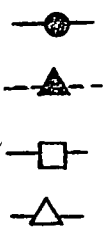


FIGURE 38

Effect of Na^+ on mitochondrial Ca^{2+} transport studied by the radioassay technique.

The incubation system (final vol.= 5 ml) contained the standard incubation mixture, varying concentrations of Na^+, 2 mM β-hydroxybutyrate, 1 mM ATP (potassium salt) and mitochondria (5.6 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi).

Incubation temp. = 25°C



0 mM NaCl

2.5 mM NaCl

5 mM NaCl

10 mM NaCl

FIGURE 38

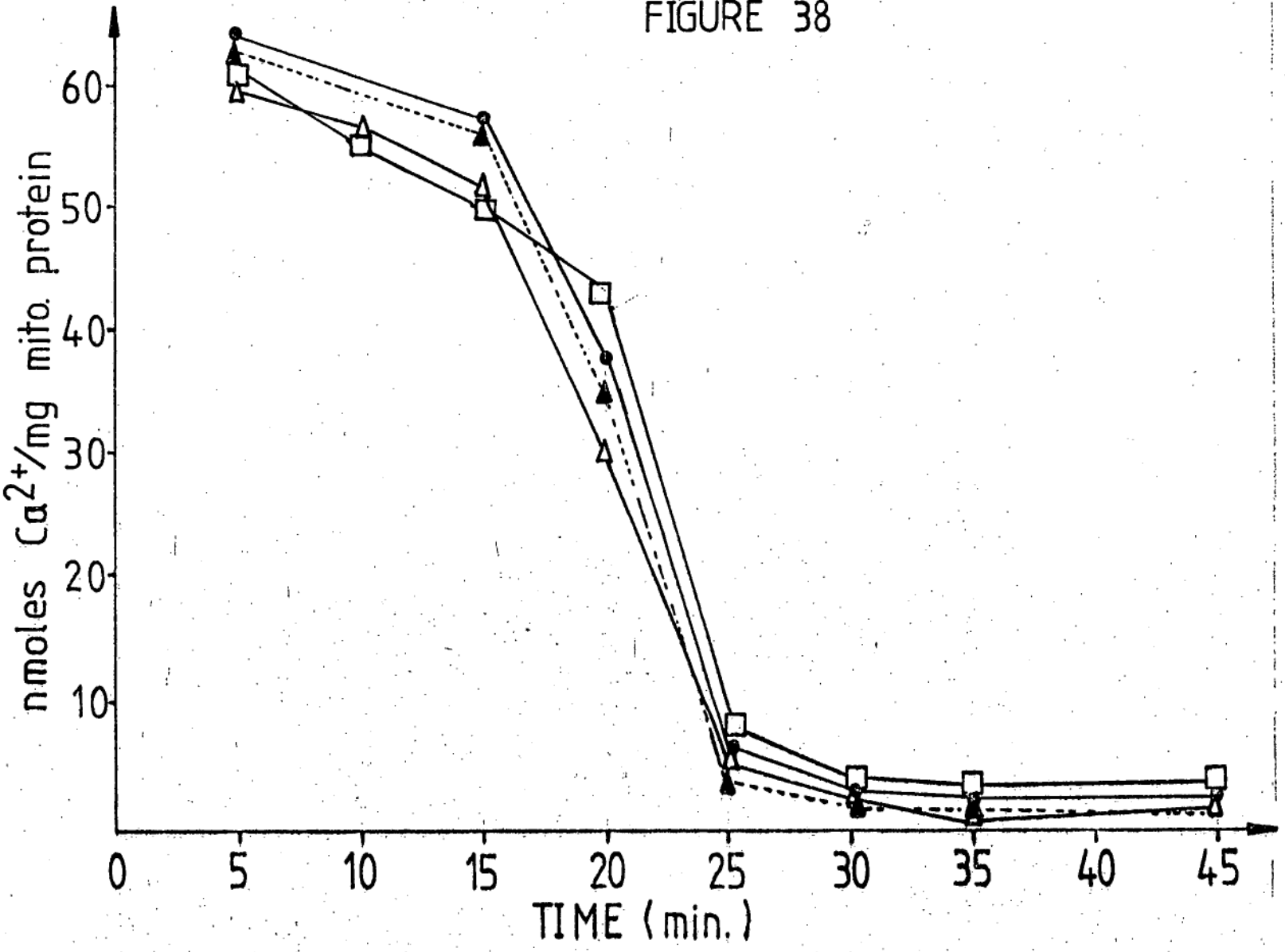

nmoles Ca^{2+}/mg mito. protein
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
45
TIME (min.)

FIGURE 39

Effect of K^+ on mitochondrial Ca^{2+} transport studied by the radioassay technique.

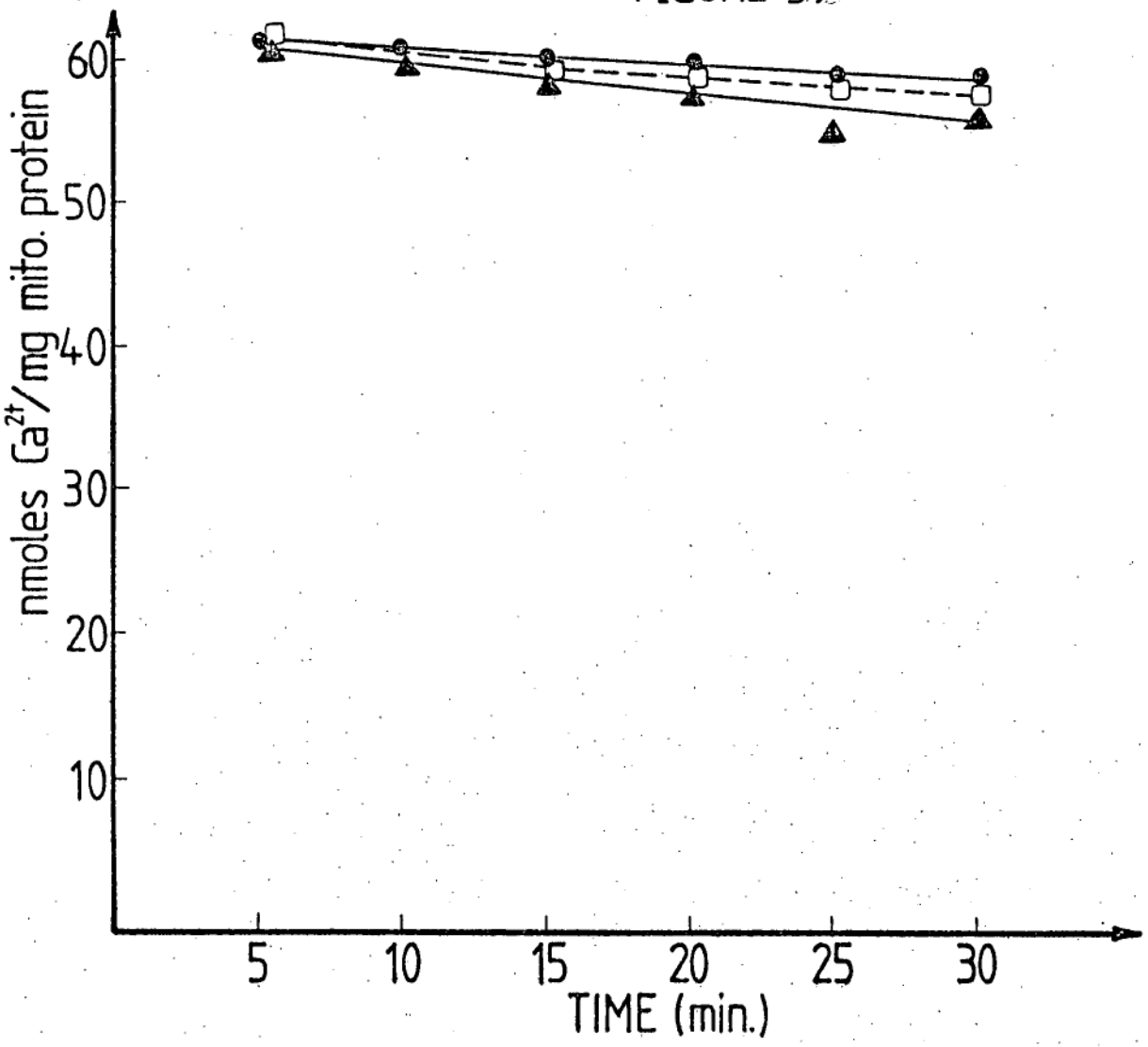
The incubation system (final vol.= 5 ml) contained 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 2 mM Pi, 1 mM $MgCl_2$, varying concentrations of K^+, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (6 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C

2 mM KCl

74 mM KCl

146 mM KCl

FIGURE 39


nmoles Ca^{2+}/mg mito. protein
60
50
40
30
20
10
5
10
15
20
25
30
TIME (min.)

4.4 DISCUSSION

Effect of respiratory substrates on Ca^{2+} transport.

The influence of various respiratory substrates such as succinate, β-hydroxybutyrate, pyruvate (in the presence of malate) and glutamate on Ca^{2+} movement in mitochondria was investigated. The results obtained showed that the mitochondria retained Ca^{2+} longer in the presence of succinate compared to the NAD^+-linked substrates (fig 29). A similar observation was noted by Lehninger et al (1978(b)). A possible explanation for the above effect is that the increased ratio of mitochondrial $NADH/NAD^+$ that occurs in the presence of succinate via reversal of electron transport driven by ATP (Chance and Williams, 1956; Chance and Hollunger, 1961), favours Ca^{2+} retention in mitochondria. The mitochondria respiring on pyruvate accumulated Ca^{2+} at a slower rate and released the accumulated Ca^{2+} earlier than the other substrates examined. It is not known why this occurred but it was possibly related to a slower rate of metabolism of pyruvate in the mitochondria, which in turn increased the $NADH/NAD^+$ ratio only slightly compared with the other respiratory substrates examined.

As shown in fig 30, either ATP or β-hydroxybutyrate alone as the energy substrates could support $^{45}Ca^{2+}$ uptake; however, the mitochondria released their $^{45}Ca^{2+}$ much earlier than that when both ATP and β-hydroxybutyrate were present. It was noted that the $^{45}Ca^{2+}$ release from the mitochondria respiring on β-hydroxybutyrate plus oligomycin was earlier than that in the presence of ATP plus antimycin A, a finding possibly related to the rate of exhaustion of β-hydroxybutyrate and ATP in the mitochondria. Antimycin A and rotenone, both inhibitors of the

respiratory chain, prevented Ca^{2+} uptake by the mitochondria respiring on β-hydroxybutyrate in the absence of added ATP (fig 30). However, the ATP-supported Ca^{2+} accumulation by the mitochondria was not affected by the inclusion of antimycin A or rotenone; in fact, the presence of rotenone helped prolong Ca^{2+} retention in the mitochondria. Presumably, NADH oxidation at the expense of the endogenous NAD^+ -linked substrates, is prevented by rotenone, which would result in a relatively more reduced state of mitochondrial pyridine nucleotides. The results obtained therefore further confirmed that Ca^{2+} accumulation by the mitochondria can be supported by either oxidation of respiratory substrate (Brierley et al, 1964) or from ATP hydrolysis (Bielawski and Lehninger, 1966; Chapter 5, section 5.3.1). Respiratory inhibitors, such as antimycin A and rotenone, abolished the uptake of Ca^{2+} by mitochondria using respiratory substrate but not those using ATP. In addition, it was also shown that $^{45}Ca^{2+}$ retention in mitochondria was favoured in the presence of both a respiratory substrate such as β-hydroxybutyrate and ATP.

Effect of cations on Ca^{2+} transport

K^+, Mg^{2+} and Na^+ are some of the cations present in a rat liver cytosol. The effect of these ions on Ca^{2+} uptake and release by mitochondria was investigated using the iso-osmotic sucrose medium containing 2 mM KH_2PO_4, with 1 mM ATP and 2 mM β-hydroxybutyrate as the energy sources for Ca^{2+} uptake.

The results obtained in the present study indicated that Na^{2+} at concentration between 0 - 10 mM did not affect Ca^{2+} uptake or release by the rat liver mitochondria (fig 38) even when all the necessary 'ingredients' for Ca^{2+} uptake and retention were present. This observation is in agreement with the findings of

Crompton et al (1978) that Na^+ did not induce Ca^{2+} efflux from rat liver mitochondria. However, according to Carafoli et al (1974), Crompton et al (1976), Na^+ caused Ca^{2+} release (presumably via a Na^+/Ca^{2+} exchange system) from mitochondria isolated from heart, bovine adrenal cortex and rat brain.

It was observed in this investigation that K^+ at the concentrations 2, 74 and 146 mM did not affect Ca^{2+} uptake and release by the mitochondria during the 30 min duration of the experiment. According to Drahota and Lehninger (1965), neither Na^+ nor K^+ affected the rate and stoichiometry of energy linked Ca^{2+} accumulation and H^+ extrusion by mitochondria, nor was the process of Ca^{2+} accumulation accompanied by stoichiometric movement of Na^+ and K^+ between mitochondria and the medium.

The investigation on the influence of varying the concentration of Mg^{2+} in the medium on Ca^{2+} and Mg^{2+} accumulation into mitochondria revealed that in the absence of externally added Mg^{2+}, the mitochondria contained between 31 - 38 nmoles Mg^{2+} per mg mitochondrial protein, most likely the endogenous Mg^{2+} content of the isolated rat liver mitochondria. This value agrees well with the previous estimations by Lee et al, 1971 (29 - 35 nmoles per mg mitochondrial protein), Thiers et al, 1960 (42 nmoles per mg mitochondrial protein) and Bogucka and Wojtczak, 1971 (20 - 30 nmoles per mg mitochondrial protein). Distribution of Mg^{2+} in the isolated rat liver mitochondria is as follows:- outer membrane, 4 %; intermembrane compartment, 50 %; inner membrane, 5 %; matrix, 41 % (Bogucka and Wojtczak, 1971).

Results obtained from the experimental work for this

thesis indicated that the mitochondria were able to accumulate a small proportion (4 - 9 %) of the added Mg^{2+} when incubated in the presence of energy substrates, whereas 85 - 90 % of the added Ca^{2+} was taken up by the mitochondria. (The estimated amount of Mg^{2+} accumulated by the mitochondria did not exclude that adsorbed to the outer surface of the inner membrane and the inner surface of the outer membrane. The Mg^{2+} actually transported into the mitochondrial matrix is possibly lower than that estimated.) The observed result is in accordance with the previous observations that Mg^{2+} "does not appear" to be accumulated by liver mitochondria (Sallis et al, 1963; Selwyn et al, 1970), i.e. relative to the large capacity of liver mitochondria to accumulate Ca^{2+}. Since the experimental work for this chapter was completed, a recent paper by Diwan et al (1979) reported that the amount of Mg^{2+} accumulated by rat liver mitochondria (corrected for contaminating extra-mitochondrial Mg^{2+}), increased with increasing concentration of externally added Mg^{2+}, within the range studied, i.e. 0.14 - 5.27 mM. Unlike liver mitochondria, heart mitochondria have been shown to accumulate considerable amounts of Mg^{2+} Brierley et al, 1963a) In the presence of 17 mM $MgCl_2$ in the incubation medium, the latter workers showed that beef heart mitochondria accumulated 1,500 nmoles Mg^{2+} per mg mitochondrial protein.

It was also observed in this investigation that inclusion of 0 - 3 mM $MgCl_2$ did not inhibit or increase Ca^{2+} uptake by the mitochondria. Regardless of the concentration of Mg^{2+} in the incubation medium, the mitochondria accumulated approximately 90 % of the added Ca^{2+}. Similarly, Jacobus et al

(1975) observed that Mg^{2+} did not inhibit Ca^{2+} accumulation by rat liver mitochondria. Akerman et al (1977) however, reported that Mg^{2+} decreased the initial rate of Ca^{2+} uptake by rat liver mitochondria, however, their system did not contain Pi and incubation was at a lower temperature, i.e. 5°C. On the other hand, Jacobus et al (1975) reported that 5 mM Mg^{2+} inhibited Ca^{2+} accumulation by rat heart mitochondria. Similarly, Sordahl ~~et al~~ (1974) noted that the initial rate of Ca^{2+} uptake by rabbit heart mitochondria respiring on succinate, was inhibited by Mg^{2+} at concentration as low as 0.33 mM.

In the present study it was found that 4 - 9 % of the Mg^{2+} in the medium was accumulated by the rat liver mitochondria, and also that the extra Mg^{2+} accumulated prolonged Ca^{2+} retention in the mitochondria (figs 31, 32, 33). The probable reasons for the observed effect are:-

(1) Mg^{2+} inhibits structural and permeability changes induced by Ca^{2+} (Hunter et al, 1976).

(2) Mg^{2+} facilitate "protective" effect on mitochondrial oxidative phosphorylation during Ca^{2+} accumulation (Sordahl, 1974).

(3) Mg^{2+} inhibits Ca^{2+}-induced swelling of rat liver mitochondria (Siliprandi et al, 1975).

The results obtained in the present study suggested that during $^{45}Ca^{2+}$ release from the mitochondria there was a corresponding release of the mitochondrial Mg^{2+} (figs 31, 32, 33). Similarly, Hunter et al (1976), reported that Mg^{2+} loss accompanies Ca^{2+} loss in beef heart mitochondria. Binet and Volfin (1974), on the other hand, claimed that Ca^{2+} uptake by

rat liver mitochondria caused total efflux of mitochondrial Mg^{2+}, and that efflux of Mg^{2+} always precedes the release of the accumulated Ca^{2+}. However their incubation mixtures did not contain either Pi or energy substrate for Ca^{2+} uptake by mitochondria. According to Siliprandi et al (1977), endogenous Mg^{2+} and Ca^{2+} released from liver mitochondria are not necessarily correlated events. A release of 66 % of the endogenous Mg^{2+} was noted when rat liver mitochondria were incubated in the presence of 2 mM Pi and 5 mM succinate, but no release of endogenous Ca^{2+} occurred. Similarly no release of endogenous Mg^{2+} accompanied the release of endogenous Ca^{2+} from uncoupled or non-respiring mitochondria. It should be noted however that the results reported by Siliprandi et al (1977) were obtained by suspending the mitochondria in a sucrose-Tris medium at pH 6.5 in the absence of externally added Mg^{2+} and Ca^{2+}. Apparently, the release of endogenous Mg^{2+} from liver mitochondria in the presence of Pi is respiration dependent (Siliprandi et al, 1977) and rat liver mitochondria take up Mg^{2+} by an energy dependent process (Judah et al, 1965; Johnson and Pressman, 1969). In fact, the rates of both influx and efflux of Mg^{2+} in rat liver mitochondria have been reported to decrease when respiration is inhibited in the presence of 1 mM NaCN (Diwan et al, 1979).

The Mg^{2+} content in the mitochondria during Ca^{2+} retention was also investigated. To ensure Ca^{2+} retention in the 45 min duration of the experiment, the substrates succinate and ATP were used as the energy sources for Ca^{2+} uptake, because Ca^{2+} retention is favoured in the presence of succinate and ATP. The levels of mitochondrial Mg^{2+} (i.e.

endogenous plus accumulated Mg^{2+}) remained fairly steady during Ca^{2+} retention in mitochondria as shown in fig 34. This result further supported the previous observation that Mg^{2+} released from mitochondria accompanied Ca^{2+} release, i.e. the two events are linked.

It was observed in this study that 4 - 7 % of the externally added Mg^{2+} was accumulated by the mitochondria in the presence of 1 mM ATP or ADP (plus β-hydroxybutyrate) and 5 - 9 % accumulation when succinate (plus rotenone) was the only energy substrate for Ca^{2+} uptake. The results suggest the possibility that Mg^{2+} is not transported into mitochondria via adenine nucleotide translocase, as Mg-ATP or Mg-ADP complex. A more specific experiment by Diwan et al (1979) showed that atractyloside, an inhibitor of adenine nucleotide translocation (Klingenberg, 1970), did not affect Mg^{2+} influx into rat liver mitochondria indicating that the adenine nucleotide translocator is not significantly involved in promoting Mg^{2+} uptake. The possibility of a specific carrier for Mg^{2+} transport has been suggested (Kun, 1976). Apparently Mg^{2+} and Ca^{2+} are transported into mitochondria by separate mechanisms, since La^{3+}, an inhibitor of Ca^{2+} uptake by mitochondria, does not prevent Mg^{2+} uptake and also there are differences in the shape of concentration dependence curves for Mg^{2+} and Ca^{2+} fluxes (Diwan et al, 1979).

The stability constant for $MgATP^{2-}$ complex = $7.3 \times 10^4\ M^{-1}$ and that of the $CaATP^{2-}$ complex = $3.2 \times 10^4 M^{-1}$ (O'Sullivan and Perrin, 1964). Since ATP has a higher affinity for Mg^{2+} than Ca^{2+}, the presence of Mg^{2+} in the incubation medium would reduce the amount of Ca^{2+} chelated to ATP. For example,

in the absence of externally added Mg^{2+}, it was demonstrated by means of the Ca^{2+}-electrode that all the Ca^{2+} in the medium combined with 1 mM ATP. In the presence of 1 mM Mg^{2+} (i.e. the concentration normally used in the standard incubation medium), 400 or 1000 nmoles Ca^{2+} and 1 mM ATP, 60 - 68 % of the Ca^{2+} combined to the ATP. Approximately 50 nmoles Ca^{2+} out of the 400 still combined to ATP when 10 mM Mg^{2+} was included in the medium. It should be noted that the results above was obtained in an iso-osmotic sucrose medium at pH 7.4 (incubated at 25°C) containing K^{+}, Pi and β-hydroxybutyrate in addition to ATP, Ca^{2+} and Mg^{2+}. The results thus suggest that, under the conditions examined (which were intended to simulate <u>in vivo</u> cytosolic conditions), the 400 nmoles Ca^{2+} in the medium was mainly chelated to 1 mM ATP.

4.5 SUMMARY

1. Ca^{2+} retention in mitochondria was favoured when succinate was the respiratory substrate compared with NAD^+-linked respiratory substrates such as β-hydroxybutyrate, pyruvate (plus malate) and glutamate.

2. Mitochondria were able to accumulate Ca^{2+} in the presence of β-hydroxybutyrate or ATP, however Ca^{2+} retention was prolonged when both of these substrates were present.

3. Ca^{2+} release from the mitochondria was rapid when β-hydroxybutyrate was the energy source and more gradual with ATP.

4. ATP-supported Ca^{2+} uptake by the mitochondria was not affected by antimycin A or rotenone. Rotenone caused mitochondria to retain Ca^{2+} for a longer period compared with ATP alone.

5. Na^+ at concentration 0 - 10 mM did not affect Ca^{2+} uptake or release by the rat liver mitochondria.

6. K^+ at concentration 2 - 146 mM did not affect Ca^{2+} movement in the mitochondria during the 30 min duration of the experiment.

7. In the absence of externally added Mg^{2+}, the mitochondria contained 31 - 38 nmoles Mg^{2+}/mg mitochondrial protein; possibly the physiological concentration of endogenous Mg^{2+}.

8. Rat liver mitochondria were able to accumulate 4 - 9 % of the added Mg^{2+} when incubated in the presence of $MgCl_2$, Pi and

energy sources for Ca^{2+} uptake such as β-hydroxybutyrate + ATP, β-hydroxybutyrate + ADP, succinate plus rotenone or succinate plus ATP.

9. Inclusion of 0 - 3 mM $MgCl_2$ did not inhibit or increase Ca^{2+} uptake by the mitochondria ; however, the extra Mg^{2+} accumulated helped prolong Ca^{2+} retention in the mitochondria.

10. During Ca^{2+} release from the mitochondria, there was a corresponding release of the mitochondrial Mg^{2+}. During Ca^{2+} retention in mitochondria, the level of mitochondrial Mg^{2+} (i.e. endogenous plus accumulated Mg^{2+}) remained fairly steady.

11. When 400 nmoles or 1,000 nmoles Ca^{2+} and 1 mM ATP were included in the incubation medium, approximately 60 - 68 % of the added Ca^{2+} combined with the ATP in the presence of 1 mM Mg^{2+} (i.e. the concentration generally used in the standard incubation medium). Even at a high Mg^{2+} concentration (10 mM), approximately 50 nmoles Ca^{2+} out of the 400 nmoles combined with 1 mM ATP.

CHAPTER 5

CALCIUM MOVEMENT IN RAT LIVER MITOCHONDRIA IN THE PRESENCE OF CYTOSOLIC COMPONENTS - (ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATES)

5.1 AIM

Adenine nucleotides and inorganic phosphates are components of the cytosol and the aim of this particular investigation was to examine Ca^{2+} movement in rat liver mitochondria in the presence of these substances. Previous studies on the requirements of adenine nucleotides for Ca^{2+} uptake have used inhibitors (Drahota et al, 1965). In the present study, this was examined in the absence of inhibitor and using the Ca^{2+} -sensitive electrode.

It is known that the presence of added ATP favours Ca^{2+} retention in the mitochondria (Drahota et al, 1965). This fact is further confirmed in the study by examining the effect of regenerating and trapping ATP in the incubation medium on Ca^{2+} movement.

Since there has not been any reports on the changes in adenine nucleotide levels during Ca^{2+} release and ratention, this was also examined.

It was also of interest, to investigate the accumulation of radioactive labelled ATP and ADP during Ca^{2+} uptake by the mitochondria.

5.2 METHODS AND MATERIALS

Preparation of the rat liver mitochondria was as described in chapter 1, section 1.1. Ca^{2+} movement in rat liver mitochondria was studied by means of the Ca-electrode (chapter 1, section 1.4) or using the radioassay technique described in chapter 1, section 1.3. Total and mitochondrial adenine nucleotides were estimated enzymatically as described in chapter 1, section 1.6. The total adenine nucleotides in the incubation medium was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotides (mitochondrial plus extra-mitochondrial) present in the incubation medium containing 1 mg mitochondrial protein. The mitochondrial (PTO)

adenine nucleotide was expressed as μmoles/mg mitochondrial protein. At the desired incubation time, the mitochondrial pellets were separated by centrifugation through silicone oil as described in chapter 1, section 1.6 . Adenine nucleotide concentrations during Ca^{2+} transport into mitochondria in the presence of EHDP (ethane-1-hydroxy diphosphonic acid) was determined by High Performance Liquid Chromatography (HPLC). The method is described in chapter 1, section 1.7 . The concentration of the adenine nucleotides was expressed as absorbance units (proportional to the height of the peak, AUFS = 0.05).

5.2.1 ^{14}C-ATP in mitochondria during Ca^{2+} transport.

Rat liver mitochondria, 4 - 5 mg protein were added to 2.5 ml incubation medium containing the standard incubation mixture, 2 mM β-hydroxybutyrate, 200 nmoles $^{45}Ca^{2+}$ (final concentration 80 μM), 1 mM [8 -^{14}C] ATP (2.5 μmole, 2.5 μCi). At 5, 10, 40 and 45 min incubation, 0.5 ml samples of the incubation mixtures was layered on 0.4 ml silicone oil and centrifuged at 12,000 x g for 4 min through the oil into a layer of 0.25 ml 2N $HClO_4$/12.5 % w/v sucrose. Total ^{14}C-ATP and ^{45}Ca in 25 μl of the supernatant above the silicone oil was measured by scintillation counting (i.e. counts in the C-channel). ^{45}Ca in the supernatant was determined after digestion with 0.5 ml concentrated HNO_3 and 0.2 ml H_2O_2 (100 vol A R grade) in order to oxidise the ^{14}C to $^{14}CO_2$. The white residual ash was dissolved in 0.1 ml glacial acetic acid and the solution left overnight. 15 ml scintillation fluid was added and ^{45}Ca determined by scintillation counting. The difference between the total ^{45}Ca + ^{14}C-ATP counts and the counts obtained after

digestion of the sample gave the ^{14}C-ATP counts in the supernatant. 0.1 ml of the lower perchloric/sucrose layer was also estimated for total ^{45}Ca + ^{14}C-ATP and for ^{45}Ca.

5.2.2 ^{3}H-ADP in mitochondria during Ca^{2+} transport.

Rat liver mitochondria, 6.4 mg protein, were added to 2.5 ml incubation medium containing the standard incubation mixture 2 mM β-hydroxybutyrate, 200 nmoles Ca^{2+} (final concentration 80 μM) and 1 mM [2 - ^{3}H] ADP (2.5 μmoles, 1.25 μCi). At 1, 6, 40 and 45 min incubation, 0.5 ml samples of the incubation mixtures were layered on 0.4 ml silicone oil and centrifuged at 12,000 x g for 4 min through the oil into a layer of 0.25 ml 2N $HClO_4$/12.5 % w/v sucrose. [2 -^{3}H] ADP and ^{45}Ca in 25 μl of the supernatant above the silicone oil and in 0.1 ml of the lower perchloric layer was measured by scintillation counting. Counts in the C-channel = 100 % ^{3}H + 100 % ^{45}Ca, B-channel = no ^{3}H + 73 % ^{45}Ca and in A-channel = 100 % ^{3}H + 27 % ^{45}Ca. ^{3}H and ^{45}Ca were calculated from these figures.

5.2.3 MATERIALS.

The [8 -^{1} ^{4}C] ATP.sodium salt (5 μCi/ml, 1.04 mCi/mmole) and [2 -^{3}H] ADP.ammonium salt (15 Ci/mmole) were from Radiochem. Centre, Amersham Bucks, U.K. αB methylene ATP (Li salt), anhydrous MW = 529, Bγ methylene ATP (Na salt) anhydrous MW = 549 and oligomycin were from SIGMA Chem. Co. St. Louis, Mo., U.S.A, Ap_5A (P^1P^5-BIS (5'-adenosyl pentaphosphate)) and hexokinase (EC 2.7.1.1) were from Boehringer Mannheim (Australia Pty. Ltd., Hardner Rd., Mt. Waverley, Victoria). Pyruvate kinase (EC 2.7.1.40) and N-ethylmaleimide (NEM) were from Calbiochem. San Diego, Calif., U.S.A. Rat albumin was

Pentex fraction V (Miles Lab. Inc., Eckhart, Indiana, U.S.A.). Quinidine sulphate was from Chemistry Department, University Tasmania. EHDP as the disodium salt was prepared by Procter and Gamble Company (Cincinnati, Ohio).

5.3 RESULTS

5.3.1 Initial Ca^{2+} uptake by rat liver mitochondria in the presence of adenine nucleotides studied by means of the Ca-electrode

A new approach was employed to show that ATP, ADP but not AMP can support Ca^{2+} uptake by the mitochondria. The study used the Ca-sensitive electrode whereby Ca^{2+} movement in mitochondria can be monitored continuously. Mitochondria (= 4 mg protein) were added to the incubation medium containing the standard incubation mixture and 400 nmoles Ca^{2+} but in the absence of added energy source for Ca^{2+} uptake. Under the stated conditions, the mitochondria accumulated 250 nmoles Ca^{2+} (as estimated on a log scale, fig 40), i.e. approximately 63 nmoles Ca^{2+}/mg mitochondrial protein whilst the maximum possible Ca^{2+} accumulation was 100 nmoles Ca^{2+}/mg mitochondrial protein. After 4 min, Ca^{2+} release was observed from the mitochondria suggesting the exhaustion of endogenous substrates. At this point adenine nucleotides were added.

5.3.1.1 Initial Ca^{2+} uptake in the presence of ATP

Adding ATP (final concentration 1 mM) at the point of Ca^{2+} release resulted in an immediate fall in ionic Ca^{2+} due to chelation of Ca^{2+} with ATP and this was followed by a rapid uptake of 100 nmoles Ca^{2+} per mg mitochondrial protein (fig 40 (a)).

5.3.1.2 Initial Ca^{2+} uptake in the presence of ATP analogues

Bᵞmethylene ATP (final concentration 1 mM) added after

FIGURE 40

Initial Ca^{2+} uptake by mitochondria in the presence of adenine nucleotide studied by means of the Ca-electrode.

Mitochondria, (4 mg protein) were added to 5 ml incubation medium containing the standard incubation mixture and 400 nmoles Ca^{2+}. The Ca^{2+} added is shown on a log scale. The following adenine nucleotide (final concentration = 1 mM) was added to the respective experiments (expt.) at the point of Ca^{2+} release. (Incubation temp. = 25°C).

Expt. (a) + ATP

Expt. (b) + Bγ methylene ATP

Expt. (c) + αB methylene ATP

Expt. (d) + ADP

Expt. (e) + AMP

FIGURE 4b

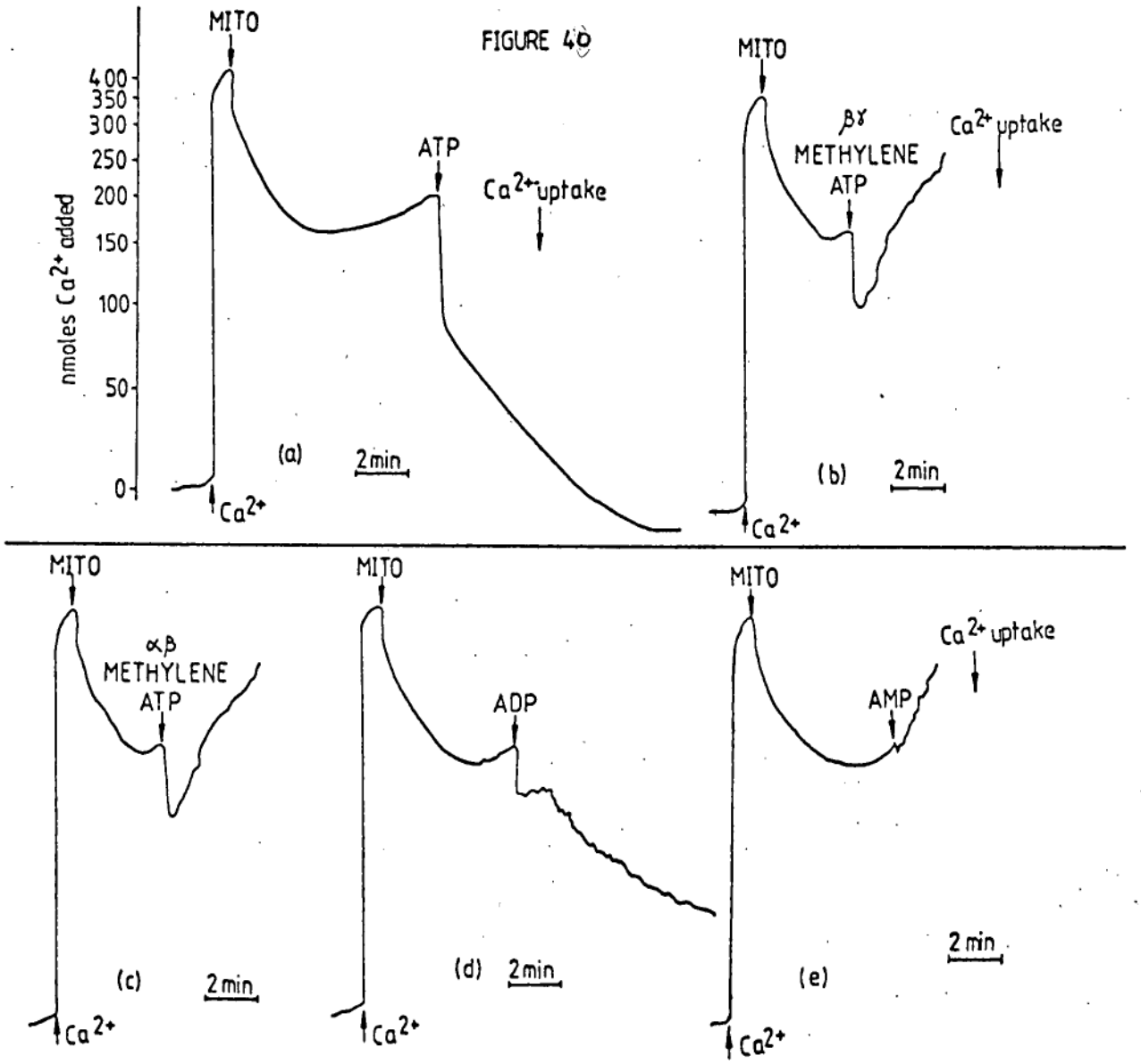

MITO
ATP
Ca2+ uptake
nmoles Ca2+ added
400
350
300
250
200
150
100
50
0
(a)
2min
Ca2+
MITO
βγ
METHYLENE
ATP
Ca2+ uptake
(b)
2min
Ca2+
MITO
αβ
METHYLENE
ATP
(c)
2min
Ca2+
MITO
ADP
(d)
2min
Ca2+
MITO
Ca2+ uptake
AMP
2 min
(e)
Ca2+

TABLE 6

Ca^{2+}-chelating ability of adenine nucleotides determined by means of the Ca-electrode.

Adenine nucleotide (final concentration = 1 mM) was added to 5 ml incubation medium containing the standard incubation mixture and 400 nmoles Ca^{2+}. Ca^{2+} added was recorded on a log scale.

Adenine nucleotides	nmoles Ca^{2+} chelated
ATP	275
$\beta\gamma$ methylene ATP	175
$\alpha\beta$ methylene ATP	175
ADP	150
AMP	<50

depletion of endogenous substrates in the mitochondria resulted in an immediate decrease in ionic Ca^{2+} in the medium due to combination with the ATP analogue, but Ca^{2+} uptake into mitochondria did not take place (fig 40(b)). Similar results were obtained with αB methylene ATP (fig 40(c)).

5.3.1.3 Initial Ca^{2+} uptake in the presence of ADP.

Mitochondria accumulated Ca^{2+} when 1 mM ADP was substrate but the rate of uptake was slow compared to that with ATP as shown in fig 40(d). Combination of ionic Ca^{2+} in the medium with the ADP was also observed.

5.3.1.4 Initial Ca^{2+} uptake in the presence of AMP.

No Ca^{2+} uptake by the mitochondria was observed when 1 mM AMP was substrate as shown in fig 40(e).

5.3.2 Calcium binding activity of adenine nucleotides as determined by means of the Ca-electrode.

To measure the affinity of adenine nucleotide for Ca^{2+}, the Ca-electrode experiments were done under similar conditions to the above, i.e. section 5.3.1 but in the absence of mitochondria. The calibration of the electrode was by addition of small amounts of Ca^{2+} (log scale). The electrode was also calibrated with Ca-NTA buffers.

Table 6 shows that under the stated conditions, the affinity for Ca^{2+} is in the following order:-

ATP > Bγmethylene ATP, αB methylene ATP > ADP > AMP.

5.3.3 Mitochondrial Ca^{2+} transport in the presence of an ATP regeneration system and an ATP trapping system studied by the radioassay technique.

In order to form an ATP regeneration system, 4 mM phosphoenol-

pyruvate (PEP) and 15 U pyruvate kinase (EC 2.7.1.40) were included in the incubation medium. Aliquots of PEP and pyruvate kinase were added at 5 min and also at 25 min to ensure constant regeneration of ATP during incubation. Fig 41 shows that in the presence of 1 mM ATP and 2 mM β-hydroxybutyrate as energy sources, the control mitochondria started to release their accumulated $^{45}Ca^{2+}$ at 25 min. However when PEP and pyruvate kinase were included in the incubation mixtures, $^{45}Ca^{2+}$ was retained in the mitochondria up to 45 min, at which time $^{45}Ca^{2+}$ release commenced.

In order to generate an ATP trapping system, 5 mM D-glucose and 15 U hexokinase (EC 2.7.1.1) were included in the incubation medium. For this particular experiment 1 mM ATP and 2 mM β-hydroxybutyrate were substrates. As shown in fig 42, in the presence of the ATP trapping system, the mitochondria started to release the accumulated $^{45}Ca^{2+}$ at 15 min. The control mitochondria retained the $^{45}Ca^{2+}$ at that time and only started to release their $^{45}Ca^{2+}$ at 30 min (fig 42).

5.3.4 Adenine nucleotide concentrations during mitochondrial Ca^{2+} release and retention.

At different time intervals, the incubation mixtures were assayed for Ca^{2+} and adenine nucleotides. Enzymatic determination of adenine nucleotides is as described in chapter 1, section 1.6, Ca^{2+} movement in mitochondria determined by the radioassay technique described in chapter 1, section 1.3 .

FIGURE 41

Mitochondrial Ca^{2+} transport in the presence of an ATP regenerating system studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (8 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. Aliquots of phosphoenolpyruvate (PEP; final concentration = 4 mM) and pyruvate kinase (15 U) were added at 5 min and 25 min.

—●— control

—▲— + (PEP plus pyruvate kinase)

DIAGRAM 3

1 ml of the above incubation medium was removed at 15 and 45 min for the estimation of total adenine nucleotide as described in section 1.6 (note: total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein).

▨ μmoles ADP/mg mito. protein

□ μmoles AMP/mg mito. protein

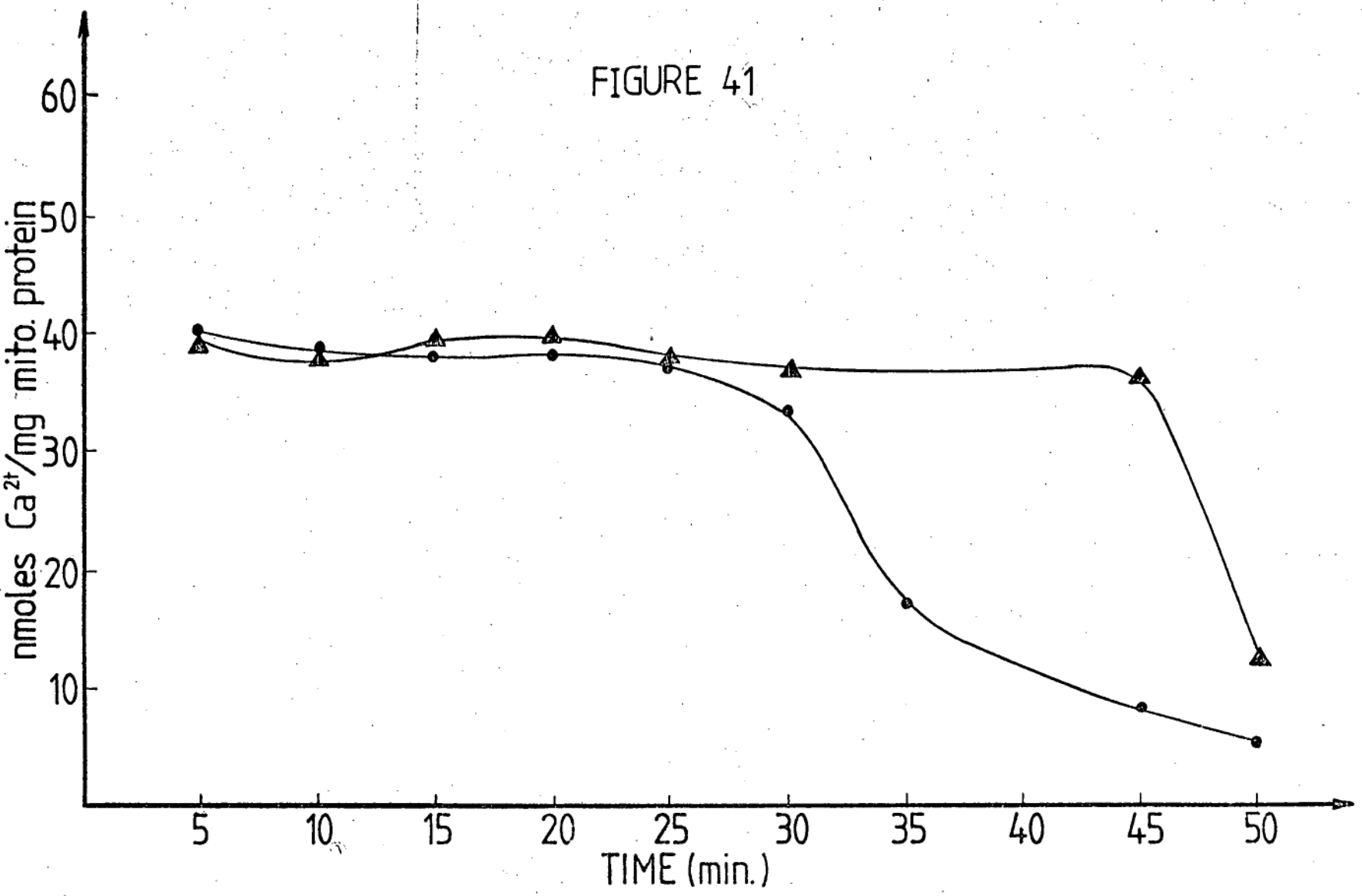
▩ μmoles ATP/mg mito. protein

PK = pyruvate kinase

ad. = adenine

mito. = mitochondrial

FIGURE 41


nmoles Ca2+/mg mito. protein
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50
TIME (min.)

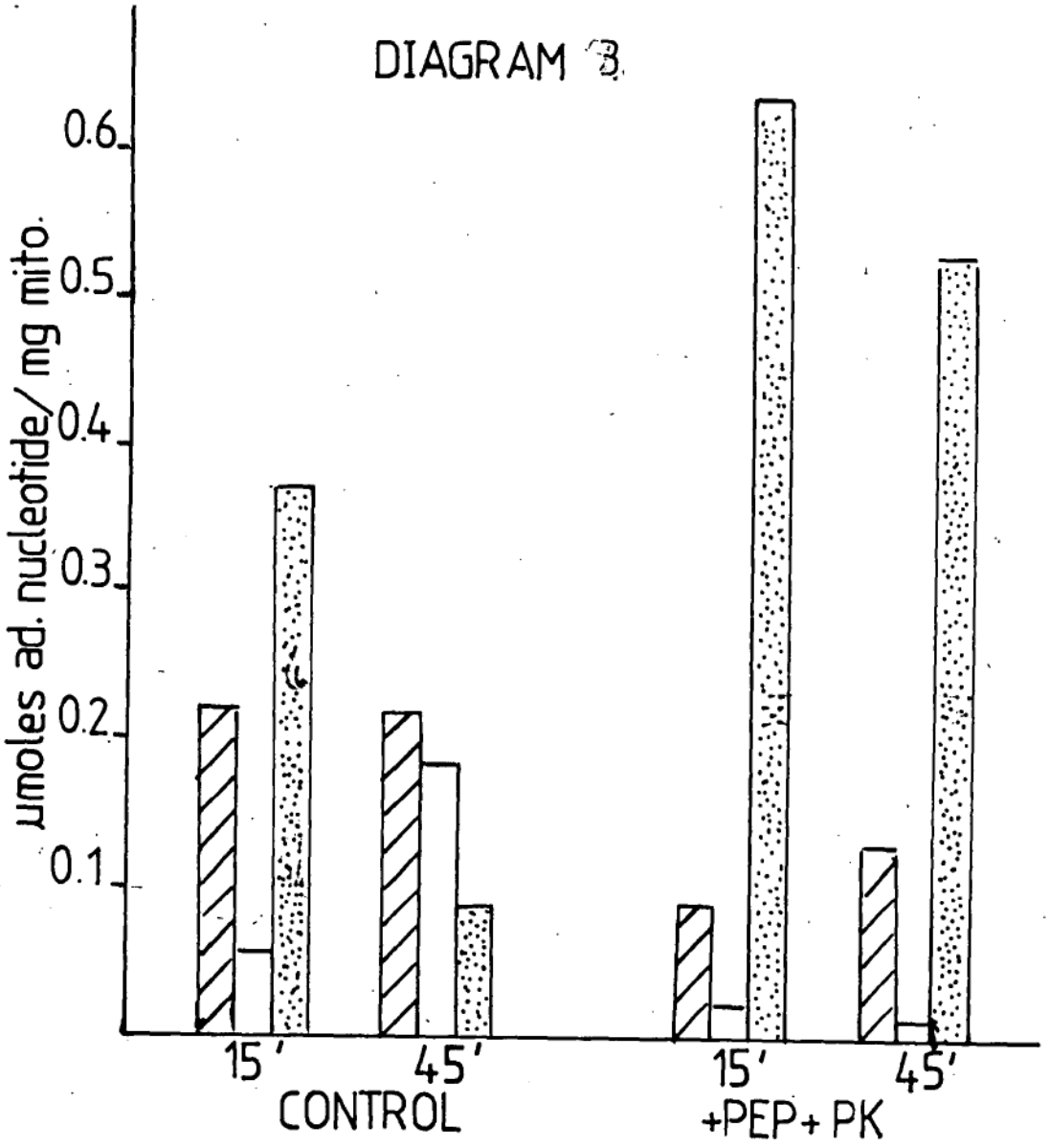


DIAGRAM 3
μmoles ad. nucleotide/ mg mito.
0.6
0.5
0.4
0.3
0.2
0.1
15'
45'
15'
45'
CONTROL
+PEP+ PK

FIGURE 42

Mitochondrial Ca^{2+} transport in the presence of ATP trapping system studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (5.4 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. D-glucose (final concentration = 5 mM) and 15 U hexokinase were included in the incubation medium before adding the $^{45}Ca^{2+}$.

—●— control

—▲— + (glucose plus hexokinase)

DIAGRAM 4

1 ml of the above incubation medium was removed at 15 and 30 min for the estimation of total adenine nucleotide (described in section 1.6). The total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein.

▨ μmoles ADP/mg mito. protein

□ μmoles AMP/mg mito. protein

▩ μmoles ATP/mg mito. protein

HK = hexokinase

ad. = adenine

mito. = mitochondrial

FIGURE 42

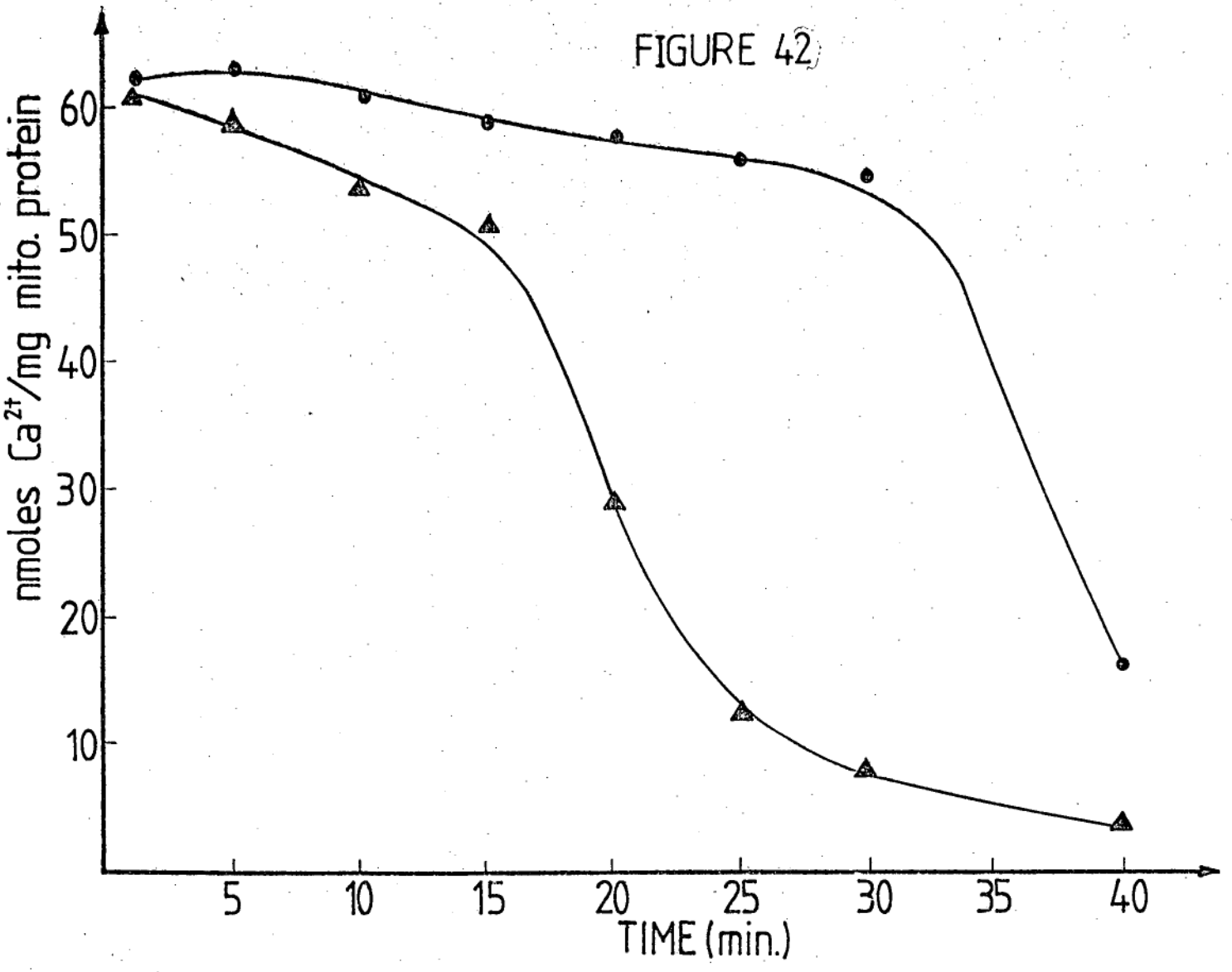
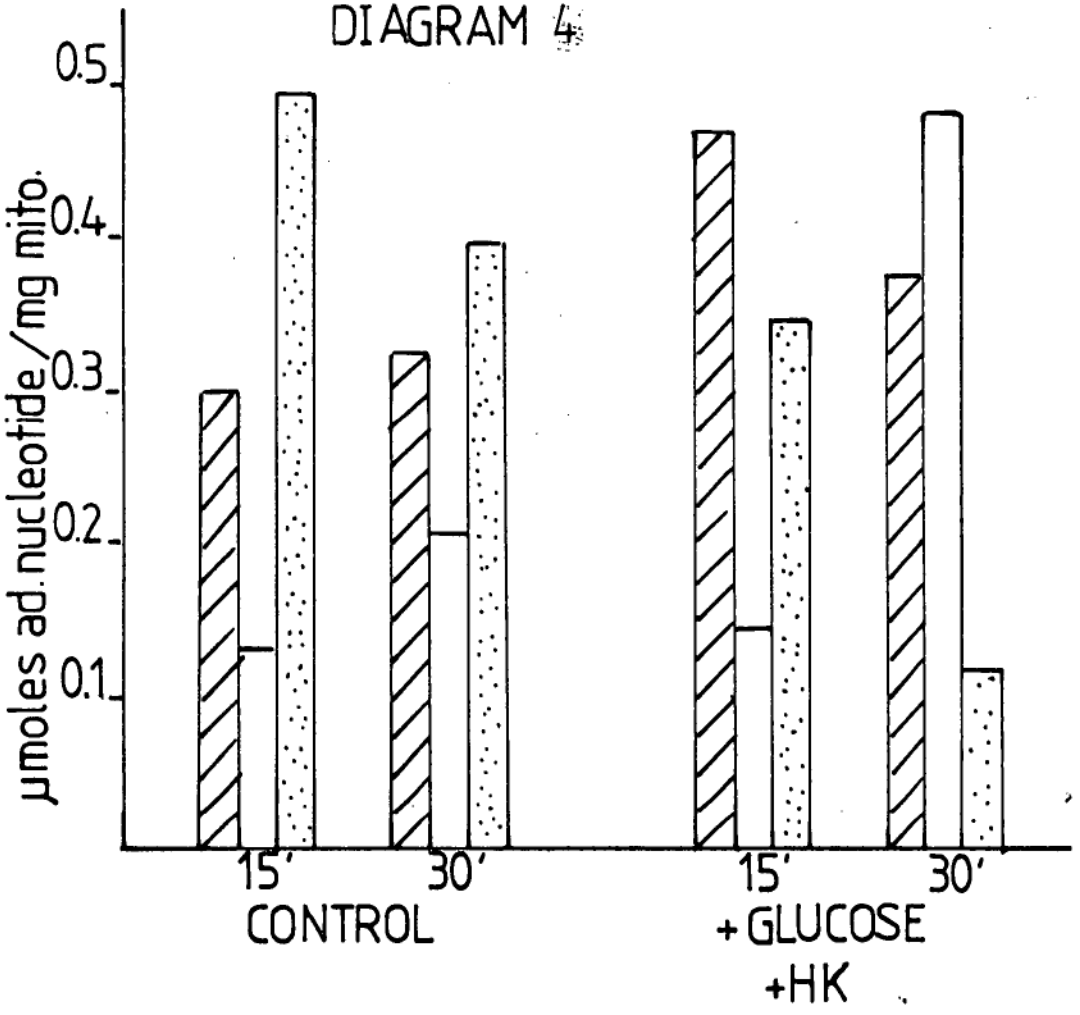

nmoles Ca^{2+}/mg mito. protein
60
50
40
30
20
10
5
10
15
20
25
30
35
40
TIME (min.)

DIAGRAM 4


0.5
0.4
0.3
0.2
0.1
µmoles ad.nucleotide/mg mito.
15'
30'
15'
30'
CONTROL
+GLUCOSE
+HK

5.3.4.1 Adenine nucleotide concentrations during Ca^{2+} release from mitochondria caused by phosphoenolpyruvate.

The control mitochondria, in the presence of substrates 1 mM ATP and 2 mM β-hydroxybutyrate, accumulated 60 nmoles $^{45}Ca^{2+}$ per mg mitochondrial protein (i.e. approximately 80 % of the added $^{45}Ca^{2+}$) and started to release this $^{45}Ca^{2+}$ at 25 min. However when 1 mM PEP was added at 5 min, $^{45}Ca^{2+}$ release was observed at 20 min, i.e. 5 min earlier than the control mitochondria as shown in fig 43. At 30 min incubation, a release of 80 % of the accumulated $^{45}Ca^{2+}$ was noted in the presence of PEP, while only 20 % of the accumulated $^{45}Ca^{2+}$ was released from the control mitochondria.

Mitochondrial and total adenine nucleotides were estimated at 16 min and 30 min. As shown in diagram 5, at 30 min, i.e. during the earlier $^{45}Ca^{2+}$ release induced by 1 mM PEP, the total ATP and AMP concentrations were 0.13 μmole and 0.47 μmole per mg mitochondrial protein respectively. The control experiment showed a total ATP concentration of 0.29 μmole/mg and a total AMP concentration of 0.33 μmole/mg at 30 min. The total ADP concentration remained almost constant during $^{45}Ca^{2+}$ uptake and release.

Estimation of adenine nucleotides in the mitochondria indicated that only a small percentage of the total adenine nucleotides (i.e. between 5 - 10 %) were present in the mitochondria (diagram 5). During Ca^{2+} release from the mitochondria (i.e. at 30 min) in the presence of 1 mM PEP, the mitochondrial ATP concentration and AMP concentration were 0.009 and 0.021 μmole/mg mitochondrial protein respectively. The ATP and AMP concentration in the control mitochondria

FIGURE 43

Ca^{2+} release from mitochondria in the presence of phosphoenolpyruvate (PEP).

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (5 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. Addition of PEP (final concentration = 1 mM) was made at 5 min.

—●— control

—▲— + PEP

DIAGRAM 5

1 ml of the above incubation medium was removed at 16 and 30 min for the estimation of mitochondrial adenine nucleotide and total adenine nucleotide as described in section 1.6. The total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein.

▨ μmoles ADP/mg mito. protein

□ μmoles AMP/mg mito. protein

▩ μmoles ATP/mg mito. protein

m = mitochondrial adenine nucleotide

t = total adenine nucleotide

PEP = phosphoenolpyruvate

ad. = adenine

mito. = mitochondrial

FIGURE 43

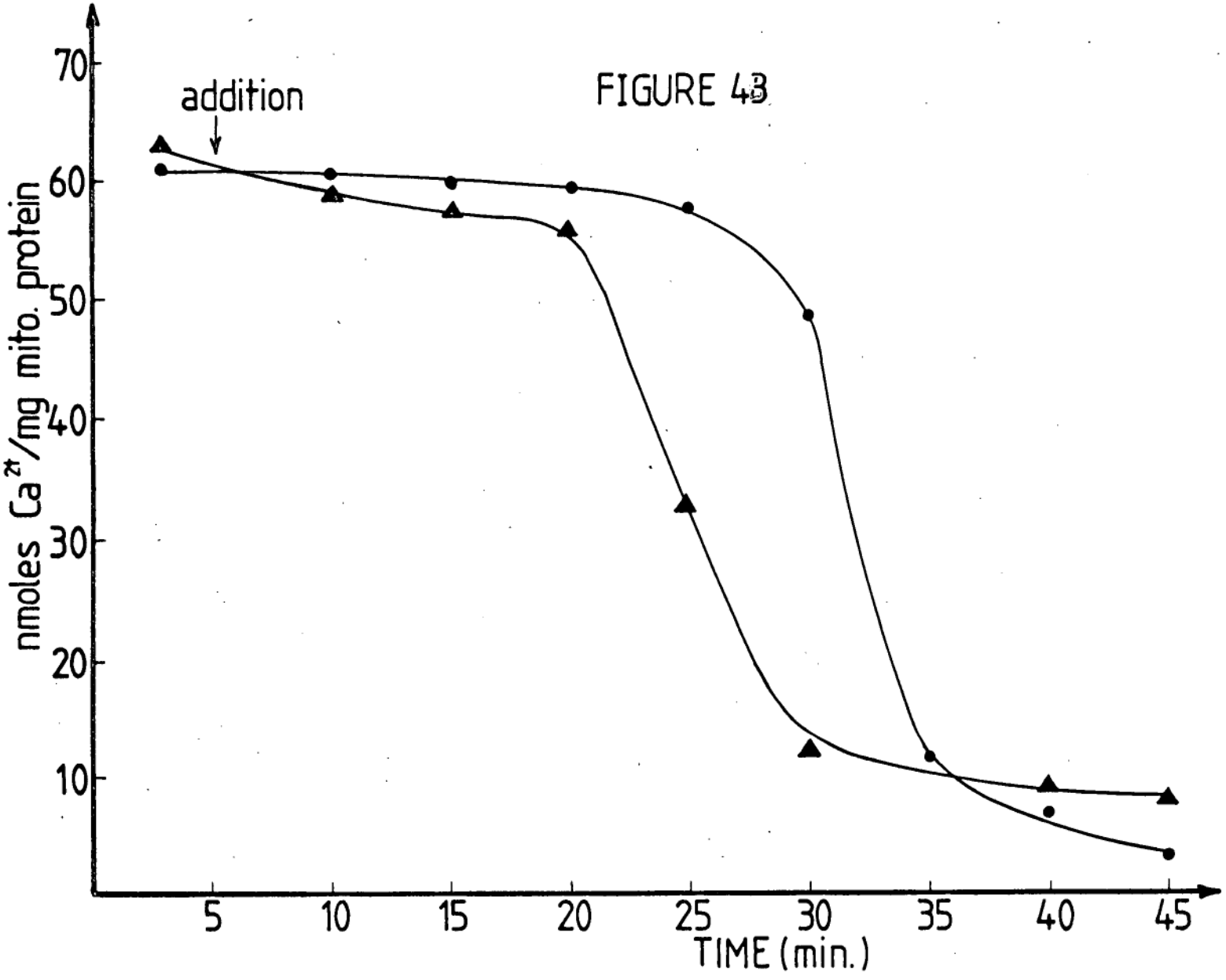
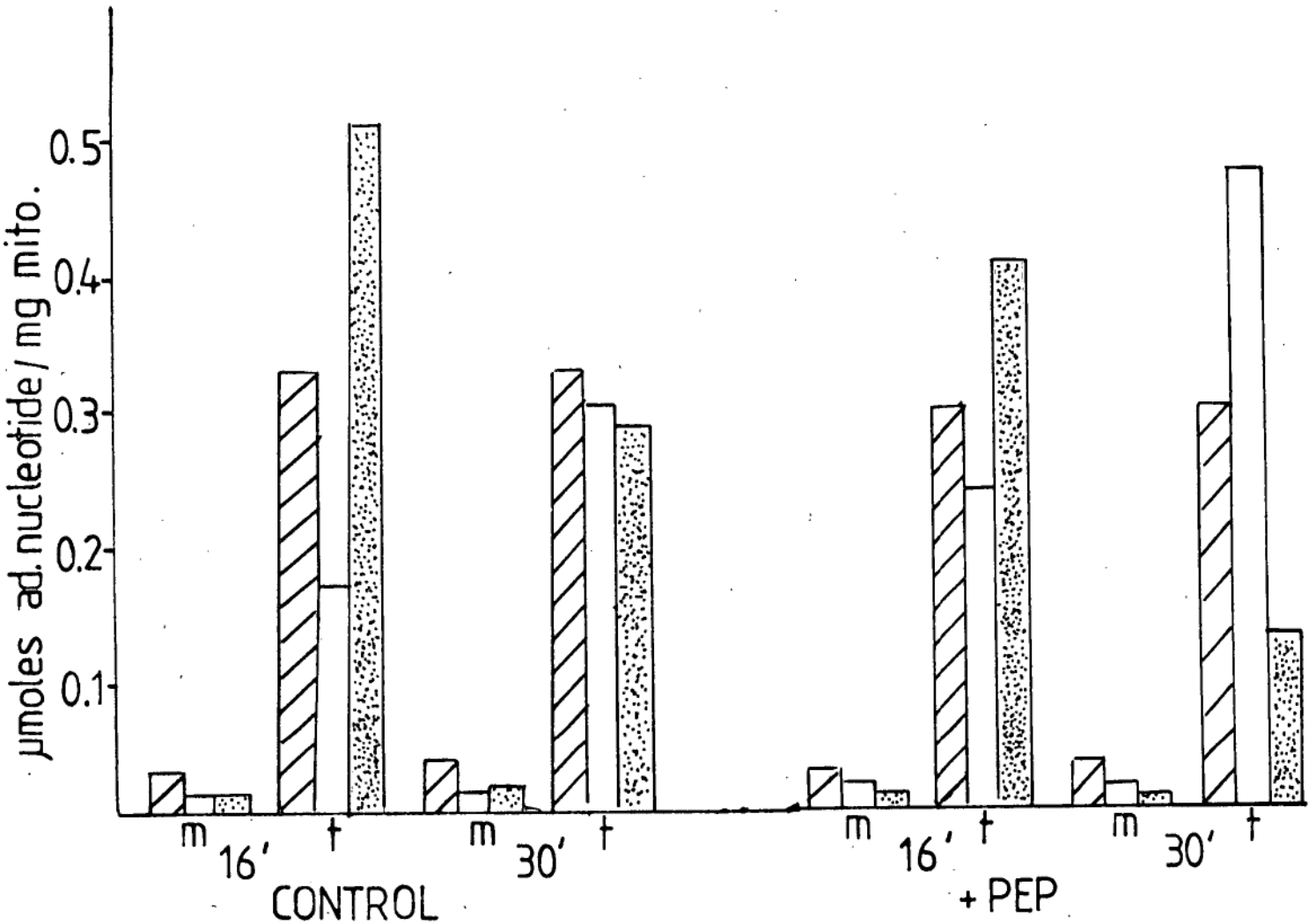

addition
nmoles Ca²⁺/mg mito. protein
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
TIME (min.)

DIAGRAM 5


µmoles ad. nucleotide / mg mito.
0.5
0.4
0.3
0.2
0.1
m
16'
t
m
30'
t
CONTROL
m
16'
t
m
30'
t
+ PEP

being 0.018 and 0.015 respectively.

5.3.4.2 Adenine nucleotide concentrations during Ca^{2+} retention in mitochondria in the presence of an ATP regeneration system.

Inclusion of 4 mM PEP and 15 U pyruvate kinase in the incubation medium favoured $^{45}Ca^{2+}$ retention at 45 min, at which time the control mitochondria had released their $^{45}Ca^{2+}$ (fig 41).

As shown in diagram 3, the total adenine nucleotides in the presence of PEP and pyruvate kinase (i.e. an ATP regeneration system), determined at 15 min and 45 min were maintained at a high level of ATP and a low level of AMP. During $^{45}Ca^{2+}$ release at 45 min, the total ATP of the control samples had decreased from 0.37 μmole to 0.09 μmole and total AMP concentration increased from 0.055 μmole to 0.19 μmole. The total ADP concentration in the presence of PEP and pyruvate kinase was lower than the control (diagram 3).

5.3.4.3 Adenine nucleotide concentrations during Ca^{2+} release from mitochondria caused by an ATP trapping system.

Results in section 5.3.3 suggested that, in the presence of an ATP trapping system (inclusion of 5 mM D-glucose plus 15 U hexokinase), the mitochondria started to release their $^{45}Ca^{2+}$ at 15 min. The control mitochondria on the other hand started to release the accumulated $^{45}Ca^{2+}$ at 30 min.

Total adenine nucleotides were determined at 15 min incubation, at which time, the control mitochondria still retained the accumulated $^{45}Ca^{2+}$ while a release of

approximately 17 % of the accumulated $^{45}Ca^{2+}$ was observed in the presence of glucose and hexokinase.

Total adenine nucleotides were also determined at 30 min during $^{45}Ca^{2+}$ release induced by the ATP trapping system. At this time, 87 % of the accumulated $^{45}Ca^{2+}$ was released from the test mitochondria, the control mitochondria still retaining their $^{45}Ca^{2+}$ (fig 42). The total adenine nucleotides estimated at 30 min in the trapping system showed a marked decrease in the ATP concentration and an increase in AMP concentration. The total ADP concentration at 15 min was 0.47 µmole/mg mitochondrial protein in the presence of glucose plus hexokinase while the control experiment had 0.30 µmole total ADP per mg mitochondrial protein (diagram 4).

5.3.4.4 Adenine nucleotide concentrations during Ca^{2+} release from mitochondria caused by palmitoyl CoA.

Addition of 10 µM palmitoyl CoA at 5 min to the standard incubation medium containing 2 mM β-hydroxybutyrate, 1 mM ATP as substrates caused an immediate $^{45}Ca^{2+}$ release from the mitochondria (fig 44). The control mitochondria started to release their $^{45}Ca^{2+}$ at 25 min.

At 10 min incubation, a release of approximately 66 % of the accumulated $^{45}Ca^{2+}$ was observed in the presence of palmitoyl CoA, the total AMP and ATP concentrations were 0.16 µmole per mg mitochondrial protein and 0.58 µmole/mg mitochondria respectively (diagram 6). The control sample at that particular time, contained approximately 0.05 µmole AMP per mg mitochondria and 0.66 µmole ATP per mg mitochondria. The total ADP concentrations for the test and control experiments were similar.

FIGURE 44

Mitochondrial Ca^{2+} transport in the presence of palmitoyl CoA and palmitoylcarnitine.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (5 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. Addition of palmitoyl CoA or palmitoylcarnitine was made at 5 min.

- control
- + palmitoyl CoA (10 μM final concentration)
- + palmitoylcarnitine (10 μM final concentration)

DIAGRAM 6

1 ml of the above incubation medium was removed at 10 and 30 min for total adenine nucleotide estimation as described in section 1.6 . The total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein.

- μmoles ADP/mg mito. protein
- μmoles AMP/mg mito. protein
- μmoles ATP/mg mito. protein

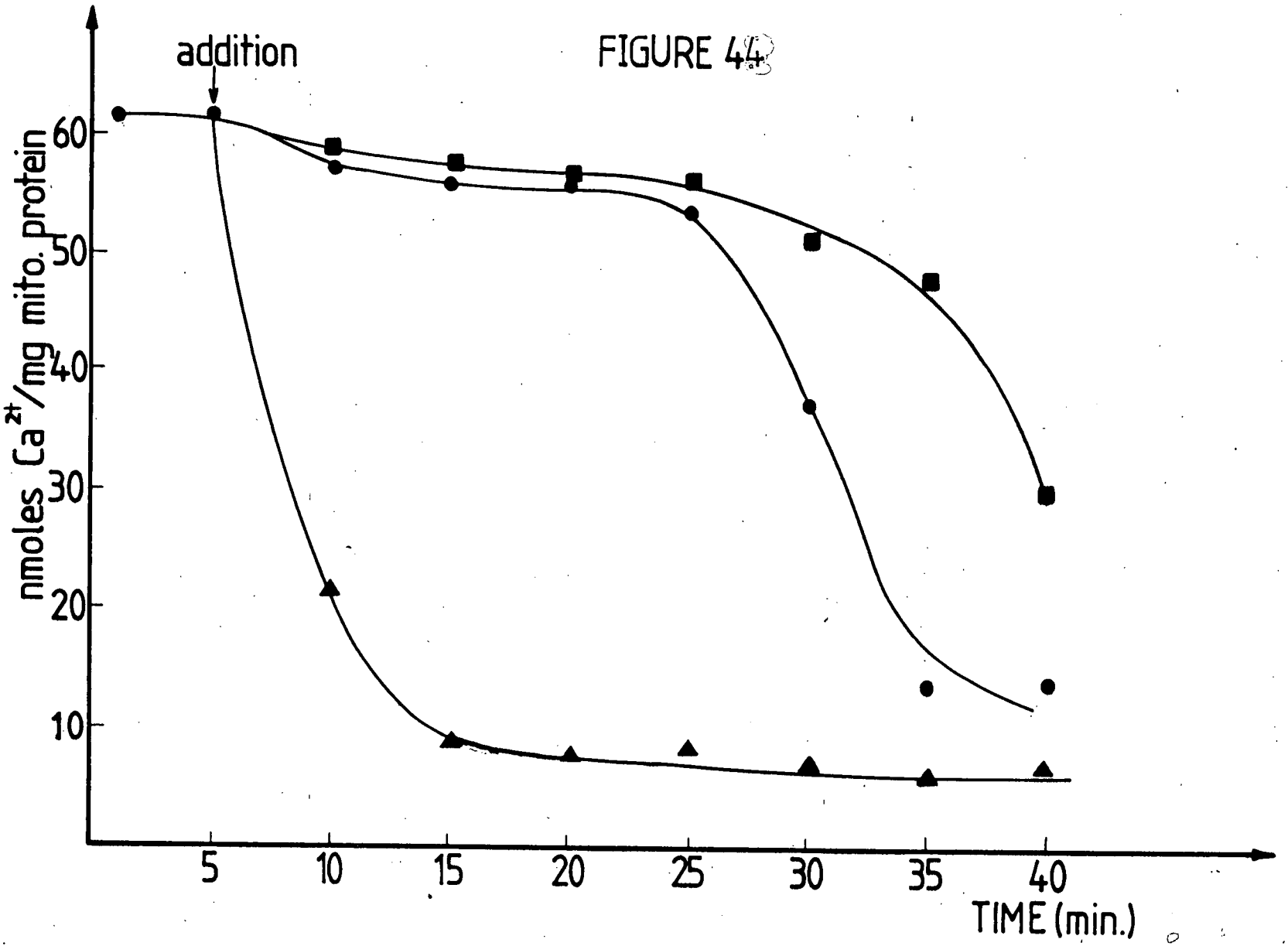
PAL.CoA = palmitoyl CoA

PAL.CARNITINE = palmitoylcarnitine

ad. = adenine

mito. = mitochondrial

FIGURE 43


addition
nmoles Ca^{2+}/mg mito. protein
60
50
40
30
20
10
5
10
15
20
25
30
35
40
TIME (min.)

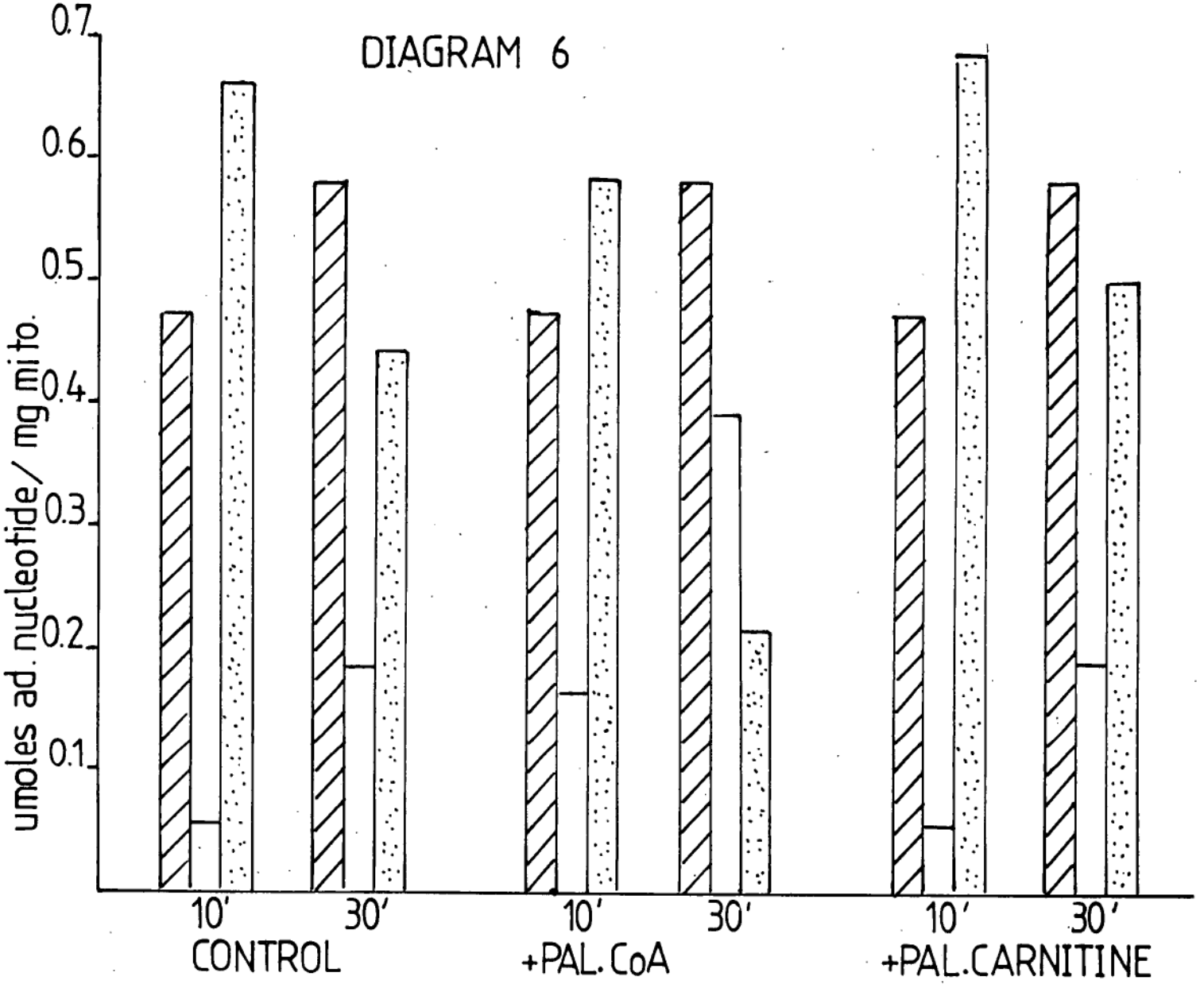


DIAGRAM 6
umoles ad. nucleotide/ mg mito.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10'
30'
10'
30'
10'
30'
CONTROL
+PAL. CoA
+PAL. CARNITINE

At 30 min incubation, 89 % of the accumulated $^{45}Ca^{2+}$ was in the supernatant of the test experiment, while only 39 % was in the supernatant of the control experiment. The total AMP concentration in the test sample had increased to 0.39 µmole/mg mitochondria while the control sample contained 0.19 µmole AMP/mg mitochondria. The total ATP concentration on the other hand, had decreased to 0.21 µmole/mg mitochondria in the test sample, compared with 0.44 µmole/mg mitochondria in the control sample.

5.3.4.5 Adenine nucleotide concentrations during Ca^{2+} retention in mitochondria in the presence of palmitoyl carnitine.

10 µM palmitoylcarnitine added at 5 min after Ca^{2+} uptake helped mitochondria to retain $^{45}Ca^{2+}$ longer than the control mitochondria (fig 44).

At 10 min during Ca^{2+} retention in control and test mitochondria, the total AMP concentration was 0.055 µmole/mg mitochondrial protein and the total ATP was approximately 0.68 µmole/mg mitochondrial protein (diagram 6).

At 30 min, 39 % of the accumulated $^{45}Ca^{2+}$ had released from the control mitochondria and only 15 % from the test mitochondria (fig 44). At that time, the AMP levels had increased to 0.19 µmole/mg for both test and control samples and the ATP level had decreased by 0.18 µmole and 0.24 µmole in test and control samples respectively (diagram 6).

5.3.4.6 Adenine nucleotide concentrations during Ca^{2+} retention in mitochondria in the presence of palmitate, carnitine and ATP.

An alternative to using the substrate palmitoylcarnitine was

to use palmitate, carnitine and ATP. Inclusion of 10 μM palmitate and 1 mM carnitine in the incubation mixtures containing Pi, Mg^{2+}, K^{+} and 1 mM ATP plus 2 mM β-hydroxybutyrate resulted in $^{45}Ca^{2+}$ retention in mitochondria up to 50 min (fig 45) after which time the experiment was terminated. A high ATP concentration and low AMP were maintained as shown in diagram 7. The control mitochondria released the $^{45}Ca^{2+}$ gradually and rapid release occurred at 30 min. Adenine nucleotides in control samples determined at 10, 30 and 50 min showed that the AMP concentrations increased and ATP concentrations decreased with time (diagram 7).

5.3.4.7 Adenine nucleotide concentrations during Ca^{2+} release from mitochondria caused by rat albumin.

The energy sources for Ca^{2+} uptake by mitochondria were 1 mM ATP and 2 mM β-hydroxybutyrate. 10 mg rat albumin (30 μM) as purchased induced $^{45}Ca^{2+}$ release from the mitochondria at 20 min. The control mitochondria retained the accumulated $^{45}Ca^{2+}$ during the duration of the experiment (fig 46). The total AMP concentration in the presence of rat albumin at 10 min and 30 min was higher than control. The total ATP concentration on the other hand was lower than control (diagram 8).

5.3.4.8 Adenine nucleotide concentrations during Ca^{2+} retention in mitochondria in the presence of palmitate and bovine serum albumin (BSA).

As shown in fig 45, in the presence of 2 mM β-hydroxybutyrate and 1 mM ATP as substrates for Ca^{2+} uptake, the control mitochondria accumulated approximately 80 % of the added $^{45}Ca^{2+}$. The mitochondria started to release this $^{45}Ca^{2+}$ at

FIGURE 45

Ca^{2+} retention in mitochondria in the presence of (palmitate + carnitine) or (palmitate + BSA).

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (4 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. Addition of palmitate plus carnitine (final concentrations = 10 μM and 1 mM respectively) or palmitate plus BSA (final concentrations = 10 μM and 30 μM respectively) were made at 5 min.

—●— control

—▲— + (palmitate plus carnitine)

—■— + (palmitate plus BSA)

DIAGRAM 7

1 ml of the above incubation medium was removed at 10, 30 and 50 min for total adenine nucleotide estimation as described in section 1.6 . The total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein.

▨ μmoles ADP/mg mito. protein

□ μmoles AMP/mg mito. protein

▩ μmoles ATP/mg mito. protein

P + C = palmitate + carnitine

P + BSA = palmitate + BSA

ad. = adenine mito. = mitochondrial

FIGURE 45

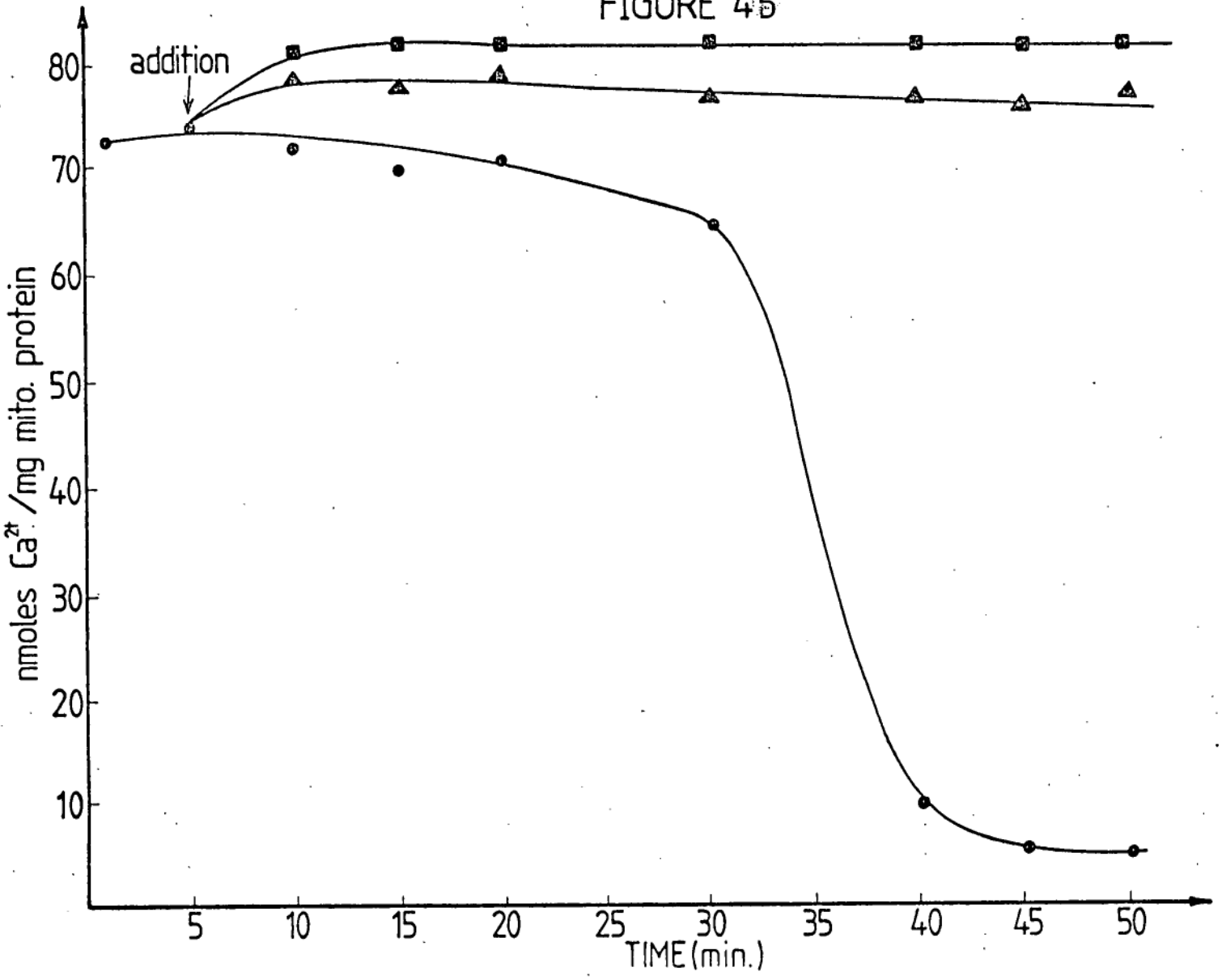

addition
80
70
60
50
40
30
20
10
nmoles Ca2+/mg mito. protein
5
10
15
20
25
30
35
40
45
50
TIME (min.)

DIAGRAM 7

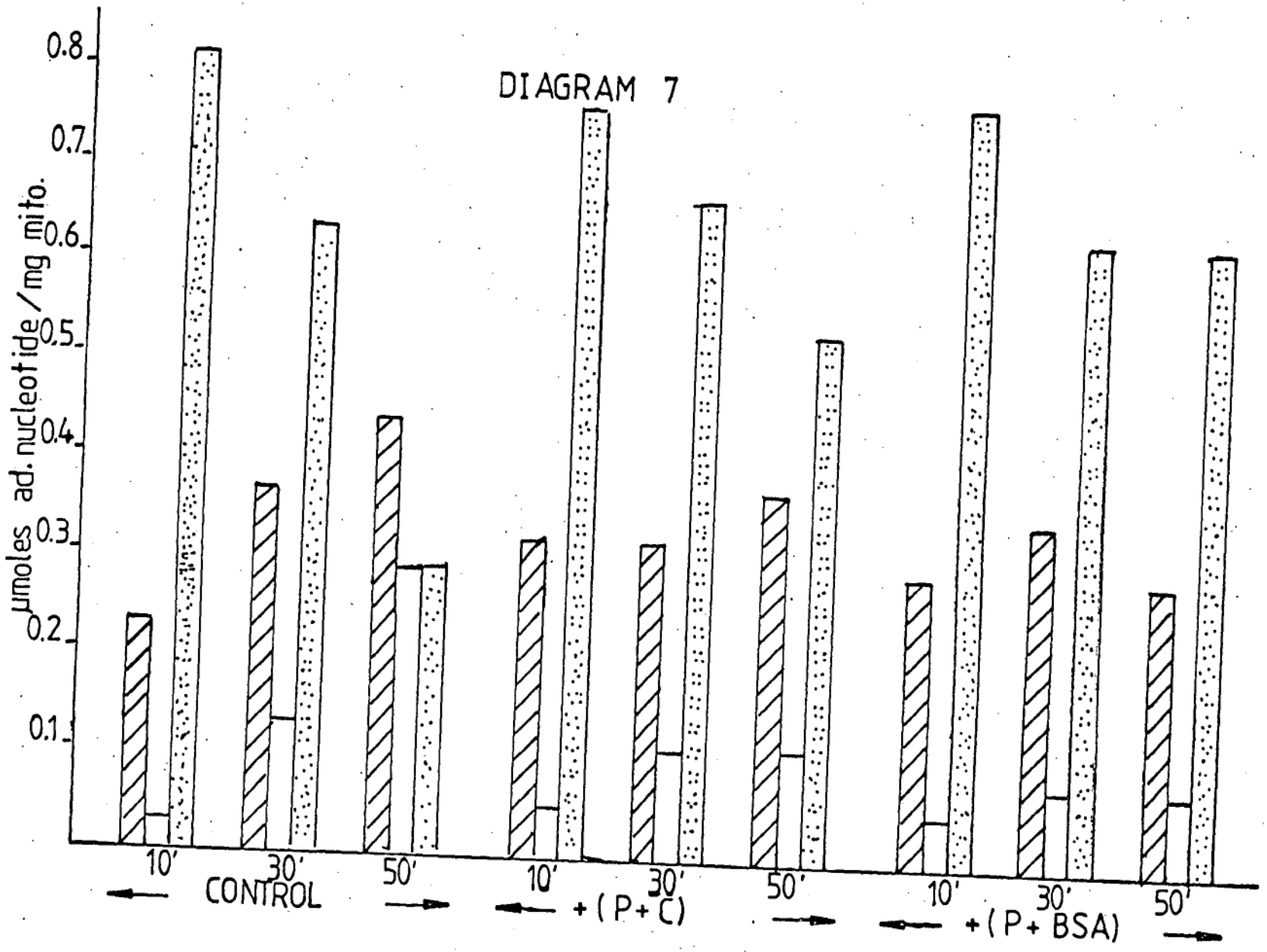

µmoles ad. nucleotide / mg mito.
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10'
30'
50'
CONTROL
10'
30'
50'
+ (P+C)
10'
30'
50'
+ (P+ BSA)

FIGURE 46

Ca^{2+} release from mitochondria in the presence of rat albumin.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (4.6 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. The commercially available rat albumin (final concentration = 30 μM) was included in the incubation medium before adding the $^{45}Ca^{2+}$.

control

+ rat albumin

DIAGRAM 8

1 ml of the above incubation medium was removed at 10 and 30 min for total adenine nucleotide estimation (described in section 1.6). The total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein.

μmoles ADP/mg mito. protein

μmoles AMP/mg mito. protein

μmoles ATP/mg mito. protein

ad. = adenine

mito. = mitochondrial

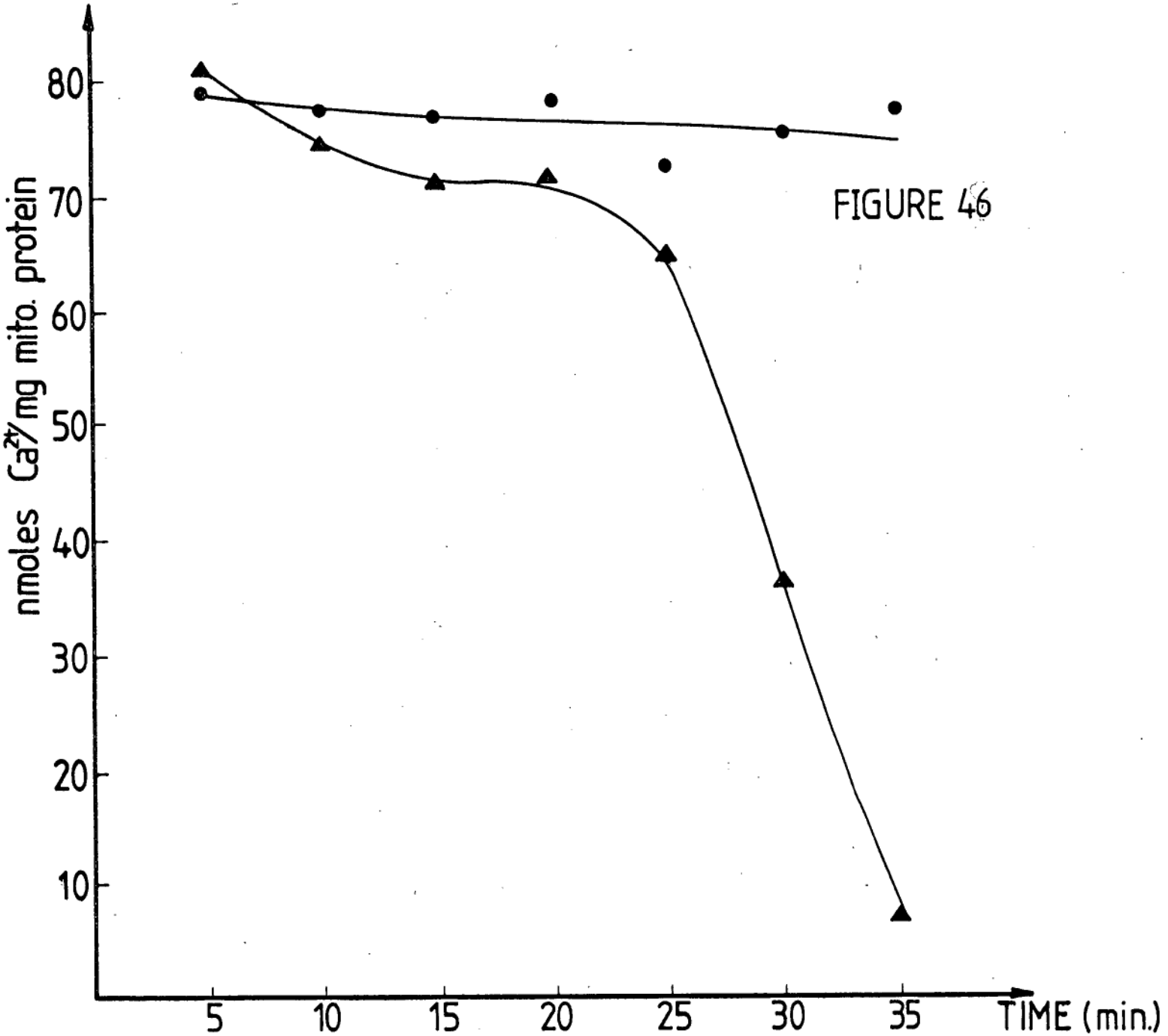
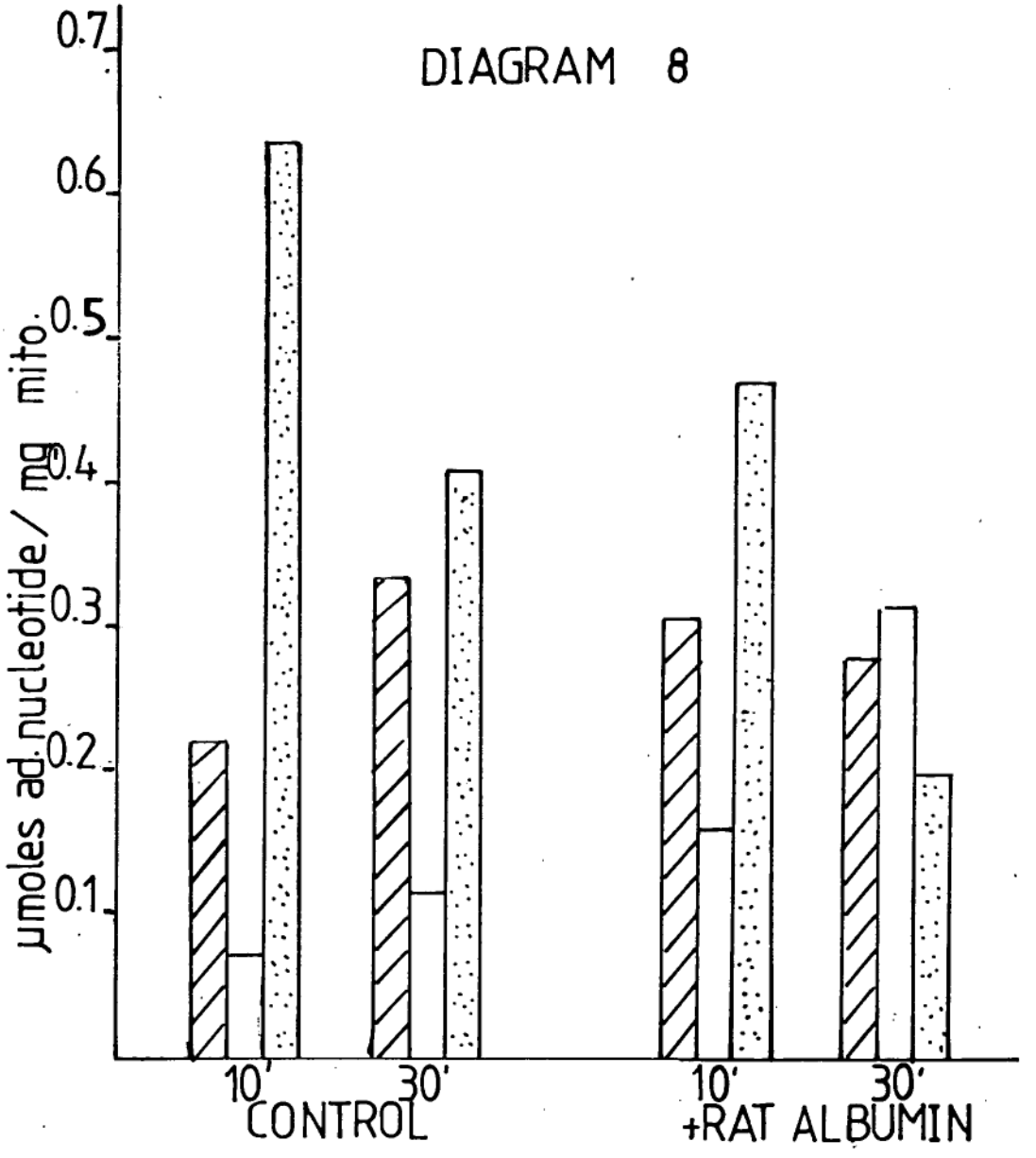

nmoles Ca²⁺/mg mito. protein
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
TIME (min.)

FIGURE 46

DIAGRAM 8


0.7
0.6
0.5
0.4
0.3
0.2
0.1
µmoles ad. nucleotide / mg mito.
10'
30'
CONTROL
10'
30'
+RAT ALBUMIN

30 min. However, inclusion of 10 μM potassium palmitate and 30 μM BSA at 5 min after $^{45}Ca^{2+}$ uptake resulted in $^{45}Ca^{2+}$ retention with respect to the control mitochondria (fig 45). A high ATP concentration and low AMP were maintained during the $^{45}Ca^{2+}$ retention as shown in diagram 7.

5.3.4.9 Adenine nucleotide concentrations during Ca^{2+} release from mitochondria caused by quinidine sulphate

Quinidine sulphate caused marked release of Ca^{2+} from frog muscle mitochondria (Batra, 1976). This study examined the effect of quinidine sulphate on Ca^{2+} release from rat liver mitochondria and on adenine nucleotide concentrations during Ca^{2+} release. 0.5 mM quinidine sulphate added at 5 min after $^{45}Ca^{2+}$ uptake by mitochondria caused an immediate release of this $^{45}Ca^{2+}$ as shown in fig 47. The control mitochondria released the accumulated $^{45}Ca^{2+}$ at 30 min.

An investigation was also made to see whether the sulphate moiety was causing this release and as shown in fig 47, 0.5 mM K_2SO_4 did induce an earlier $^{45}Ca^{2+}$ release from mitochondria compared to the control however not significant enough to account for the immediate release caused by 0.5 mM quinidine sulphate.

At 15 min incubation, a release of 82 % of the accumulated $^{45}Ca^{2+}$ was noted when 0.5 mM quidine sulphate was present while the control mitochondria still retained their $^{45}Ca^{2+}$. The total AMP concentration at that instance was only 0.05 μmole greater and the ATP 0.12 μmole lesser than control.

At 30 min, the test mitochondria had released 87 % of the accumulated $^{45}Ca^{2+}$, the control mitochondria still retaining their $^{45}Ca^{2+}$. The total AMP concentration at that time was only 0.04 μmole greater and ATP 0.11 μmole lesser than control (diagram 9).

FIGURE 47

Ca^{2+} release from mitochondria in the presence of quinidine sulphate.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (5.4 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. Addition of quinidine sulphate or K_2SO_4 was made at 5 min.

- control
- + K_2SO_4 (final concentration = 0.5 mM)
- + quinidine sulphate (final concentration = 0.5 mM)

DIAGRAM 9

1 ml of the above incubation medium was removed at 15 and 30 min for total adenine nucleotide estimation (described in section 1.6). The total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein.

- μmoles ADP/mg mito. protein
- μmoles AMP/mg mito. protein
- μmoles ATP/mg mito. protein

ad. = adenine

mito. = mitochondrial

FIGURE 47

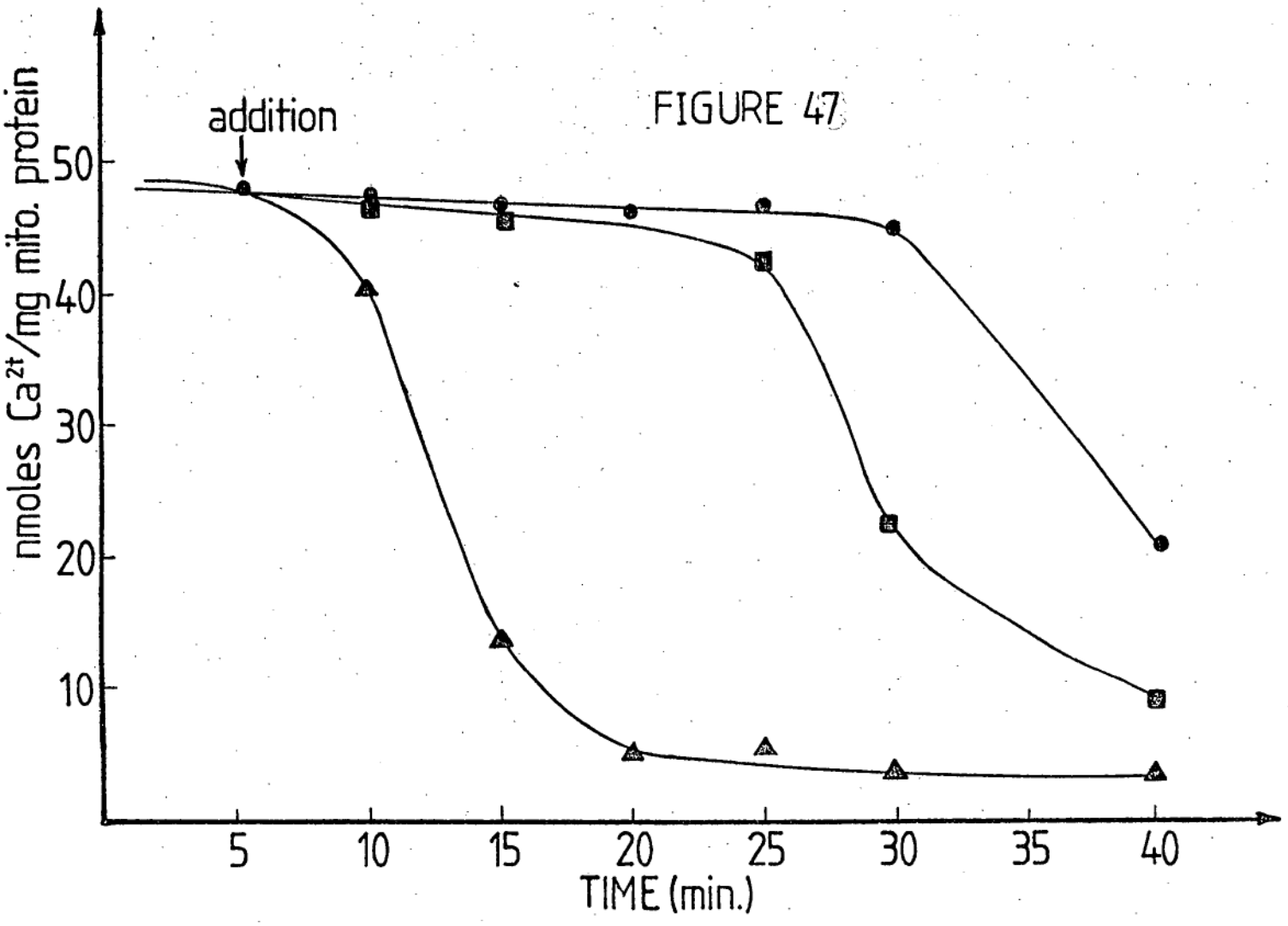
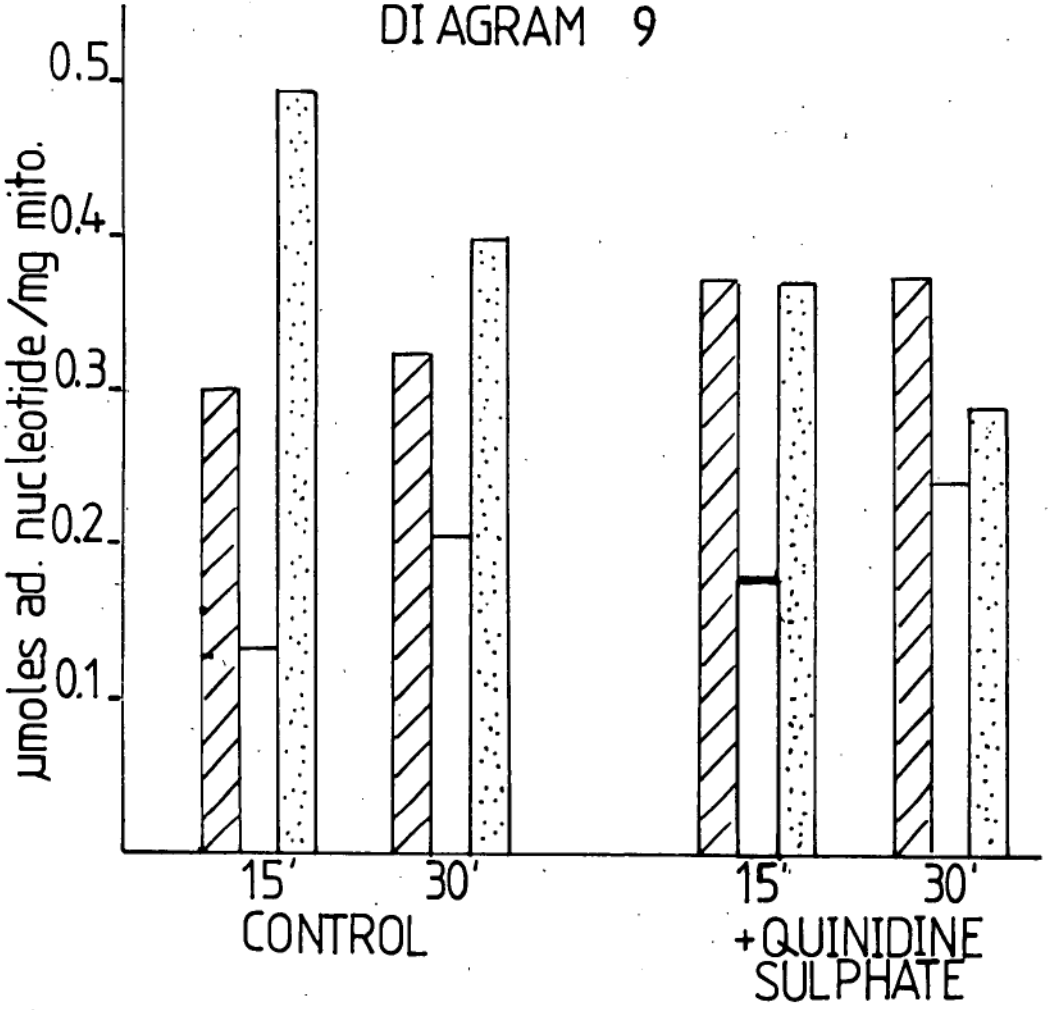

addition
nmoles Ca2t/mg mito. protein
50
40
30
20
10
5
10
15
20
25
30
35
40
TIME (min.)

DIAGRAM 9


0.5
0.4
0.3
0.2
0.1
µmoles ad. nucleotide/mg mito.
15'
30'
15'
30'
CONTROL
+QUINIDINE SULPHATE

5.3.5 Adenine nucleotide concentrations during mitochondrial Ca^{2+} transport in the presence of ethane-1-hydroxy-diphosphonic acid (EHDP) studied by High Performance Liquid Chromatography (HPLC).

EHDP is known to help Ca^{2+} retention in rat kidney mitochondria (Guillard et al, 1974). The concentrations of adenine nucleotide during Ca^{2+} retention in rat liver mitochondria in the presence of 0.2 mM EHDP was examined. For this experiment, total adenine nucleotides were estimated at 30 s, 10, 15, 20, 25, 30 and 45 min, and $^{45}Ca^{2+}$ in mitochondria estimated at 10 and 45 min. Since there was a large number of samples for adenine nucleotide estimations, instead of using the relatively slow and laborious enzymatic assay, high performance liquid chromatography was used. The method was described in chapter 1, section 1.7 . The concentration of adenine nucleotides in 25 µl of the 1.5 ml TCA extract was expressed as absorbance units (abs. units) proportional to the height of the peak on the chart recorder.

The results in fig 48(b) show that in the presence of EHDP (final concentration = 0.2 mM) the total ATP, ADP and AMP concentrations remained steady, i.e. total ATP concentration between 0.03 to 0.04 abs. units, ADP 0.015 - 0.022 abs. units and AMP 0.002 - 0.008 abs. units, even at 45 min at which time $^{45}Ca^{2+}$ was still retained in the mitochondria. The $^{45}Ca^{2+}$ content in the control and test mitochondria at 10 min incubation were the same, i.e. approximately 80 % of the added $^{45}Ca^{2+}$ accumulated in the mitochondria.

The control mitochondria however, had released the accumulated $^{45}Ca^{2+}$ at 45 min and adenine nucleotides estimated

FIGURE 48

Adenine nucleotide concentrations during mitochondrial Ca^{2+} transport in the presence of ethane-1-hydroxy-diphosphonic acid (EHDP) studied by High Performance Liquid Chromatography (HPLC).

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (5.4 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. Addition of EHDP (final concentration = 0.2 mM) was made before adding the $^{45}Ca^{2+}$. 0.5 ml of the incubation medium was removed at 30 s, 10, 15, 20, 25, 30 and 45 min for the estimation of total adenine nucleotide by means of HPLC described in section 1.7 .

Fig 48(a) control experiment

Fig 48(b) + EHDP

-□- ATP (abs. unit)/25 μl TCA extract

-●- ADP (abs. unit)/25 μl TCA extract

-○- AMP (abs. unit)/25 μl TCA extract

$^{45}Ca^{2+}$ in the mitochondria was also estimated at 10 and 45 min and the results shown below:-

SAMPLING TIME	nmoles $^{45}Ca^{2+}$/mg mitochondrial protein	
	CONTROL	+ EHDP
10 min	57	59
45 min	5	56

FIGURE 48a

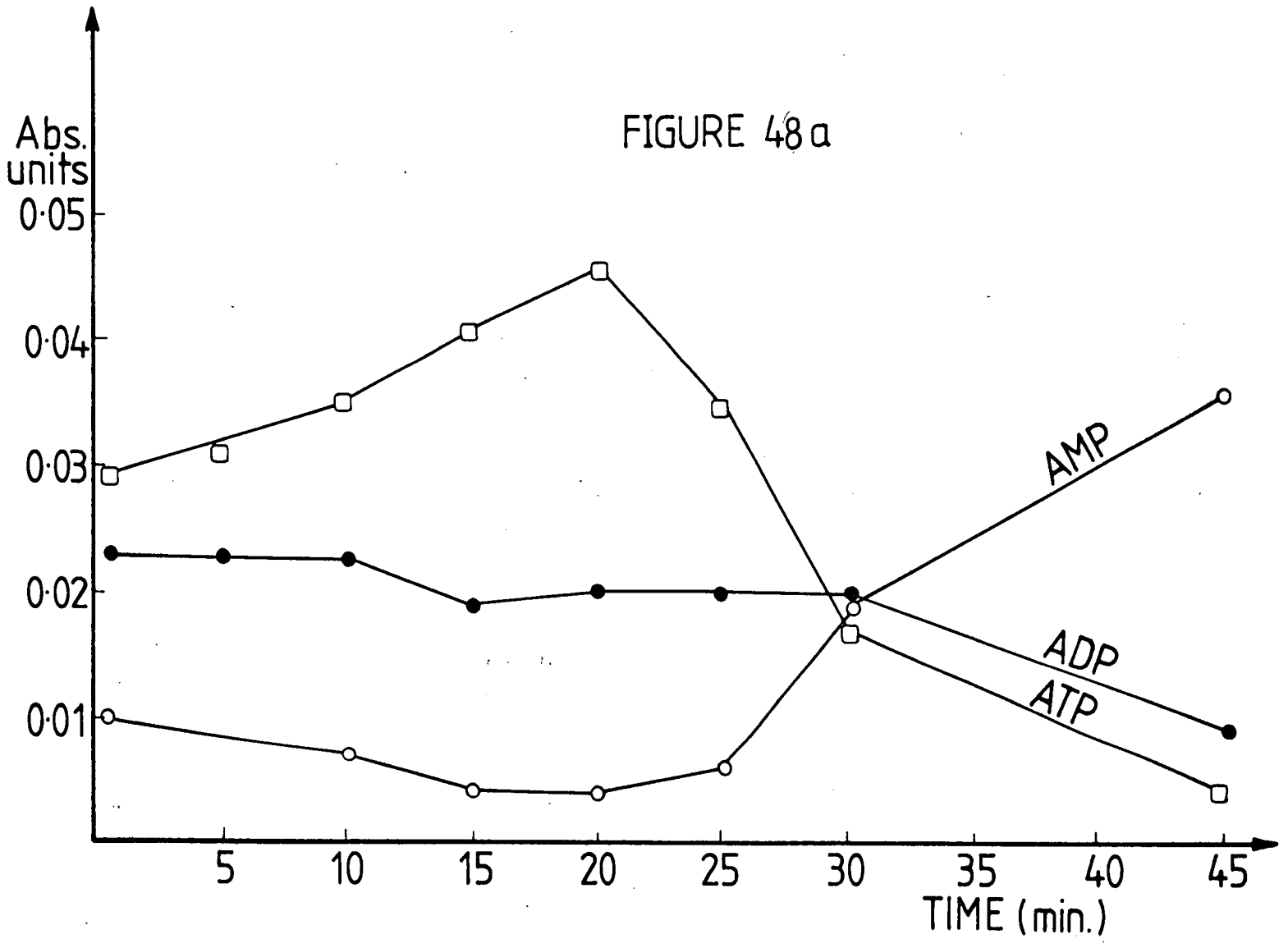
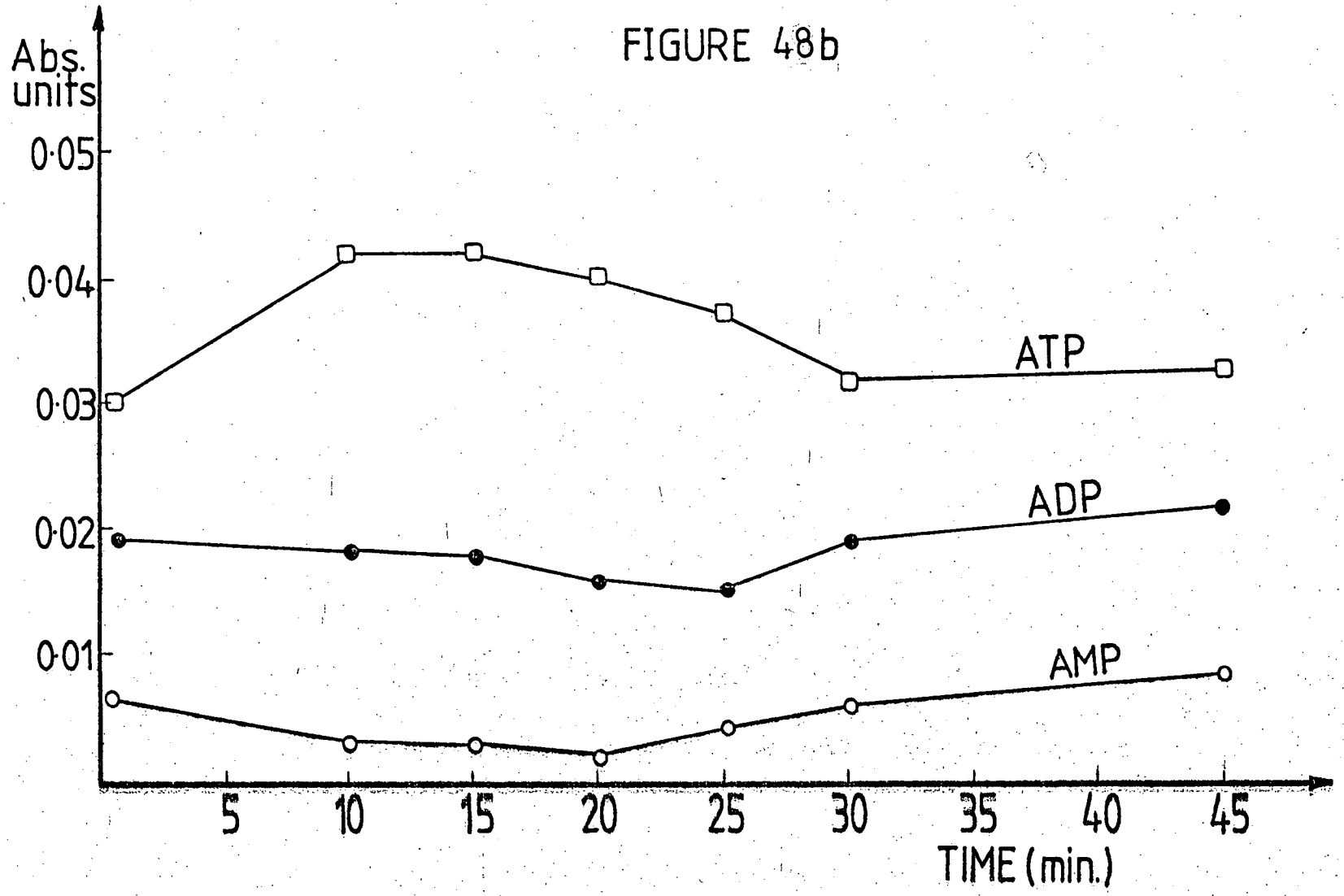

Abs. units
0·05
0·04
0·03
0·02
0·01
AMP
ADP
ATP
5
10
15
20
25
30
35
40
45
TIME (min.)

FIGURE 48b


Abs. units
0·05
0·04
0·03
0·02
0·01
ATP
ADP
AMP
5
10
15
20
25
30
35
40
45
TIME (min.)

at that particular time revealed that the ATP concentration had decreased from approximately 0.04 abs. units to 0.005 and the AMP concentration had increased from 0.005 abs. units to 0.035 abs. units (fig 48 (a)).

5.3.6 Mitochondrial Ca^{2+} transport in the presence of Ap_5A, an inhibitor of adenylate kinase, studied by the radioassay technique

An attempt was made to inhibit the activity of adenylate kinase (EC 2.7.4.3) which catalyses the reversible reaction :-

$$2\ ADP \rightleftharpoons ATP + AMP$$

Ap_5A (P^1P^5-BIS (5'-adenosyl penta phosphate)) is known to inhibit adenylate kinase (Köhrle et al, 1977). In the presence of 1 mM ATP and 2 mM B-hydroxybutyrate as the energy sources for $^{45}Ca^{2+}$ uptake, the control mitochondria released the accumulated $^{45}Ca^{2+}$ at 40 min (fig 49). The inclusion of 0.1 mM Ap_5A did not appear to affect $^{45}Ca^{2+}$ uptake or release, but resulted in negligible concentrations of AMP estimated at 10 and 45 min as shown in diagram 10. During $^{45}Ca^{2+}$ release at 45 min, the total ATP concentration had decreased in a similar manner to the control experiment but the ADP concentration was approximately 1½ times greater than the control.

5.3.7 Distribution of ATP and ADP in mitochondria during Ca^{2+} uptake

ATP, ADP and Ca^{2+} in mitochondria were estimated by using $[8 - {}^{14}C]$ ATP, $[2 - {}^{3}H]$ ADP and ^{45}Ca respectively. The procedure is described in chapter 5, section 5.2.1 and 5.2.2. The results presented assumed that the radioactively labelled ATP and ADP are metabolised at a slow rate under the conditions examined.

FIGURE 49

Mitochondrial Ca^{2+} transport in the presence of Ap_5A, an inhibitor of adenylate kinase.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM ATP and mitochondria (7 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp.= 25°C. Addition of Ap_5A (final concentration = 0.1 mM) was made before adding the $^{45}Ca^{2+}$.

—●— control

—▲— + Ap_5A

DIAGRAM 10

1 ml of the above incubation medium was removed at 10 and 45 min for total adenine nucleotide estimation (described in section 1.6). The total adenine nucleotide was expressed as μmoles/mg mitochondrial protein, i.e. the total adenine nucleotide present in the incubation medium containing 1 mg mitochondrial protein.

▨ μmoles ADP/mg mito. protein

□ μmoles AMP/mg mito. protein

▩ μmoles ATP/mg mito. protein

mito. = mitochondrial

ad. = adenine

FIGURE 49

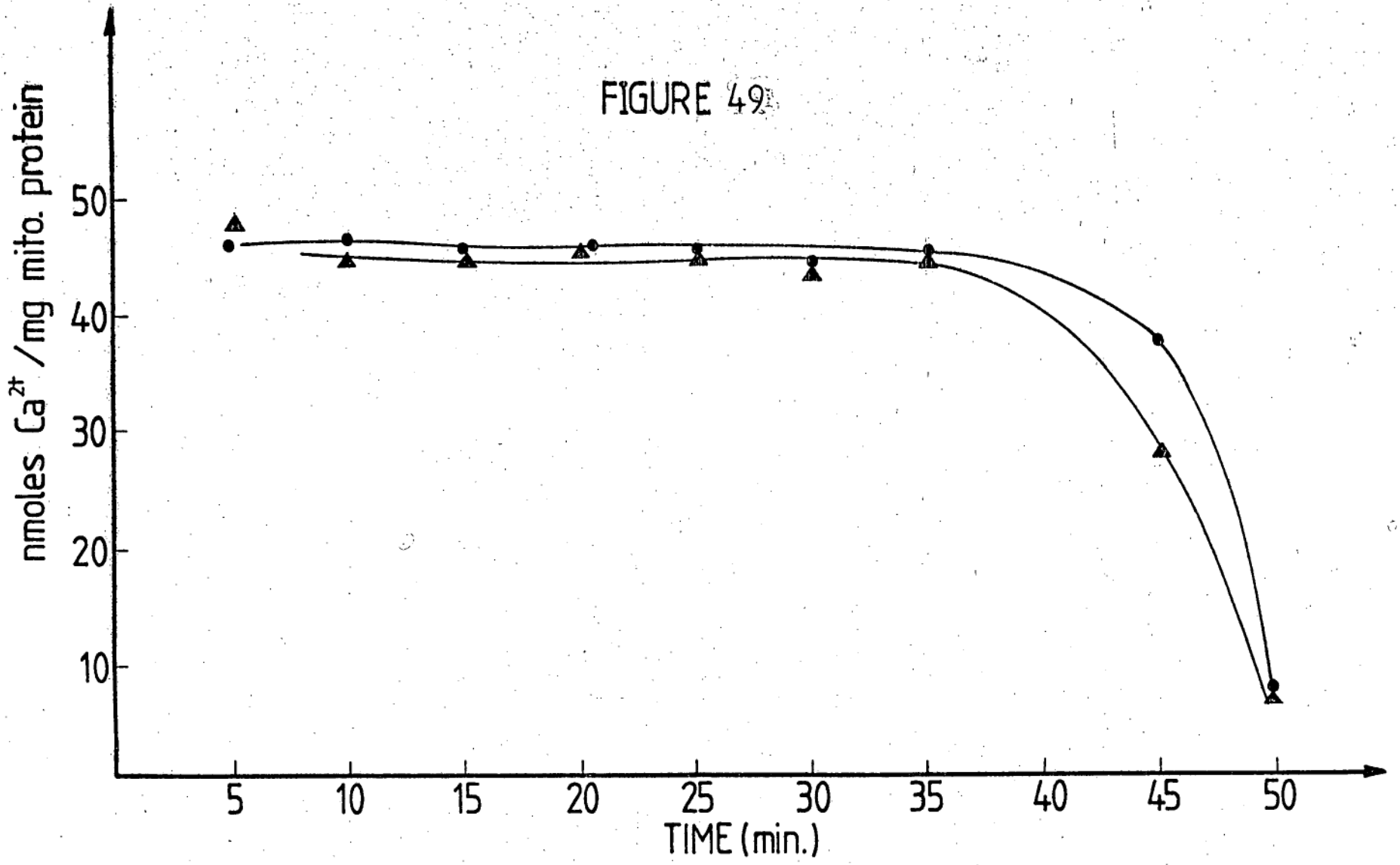
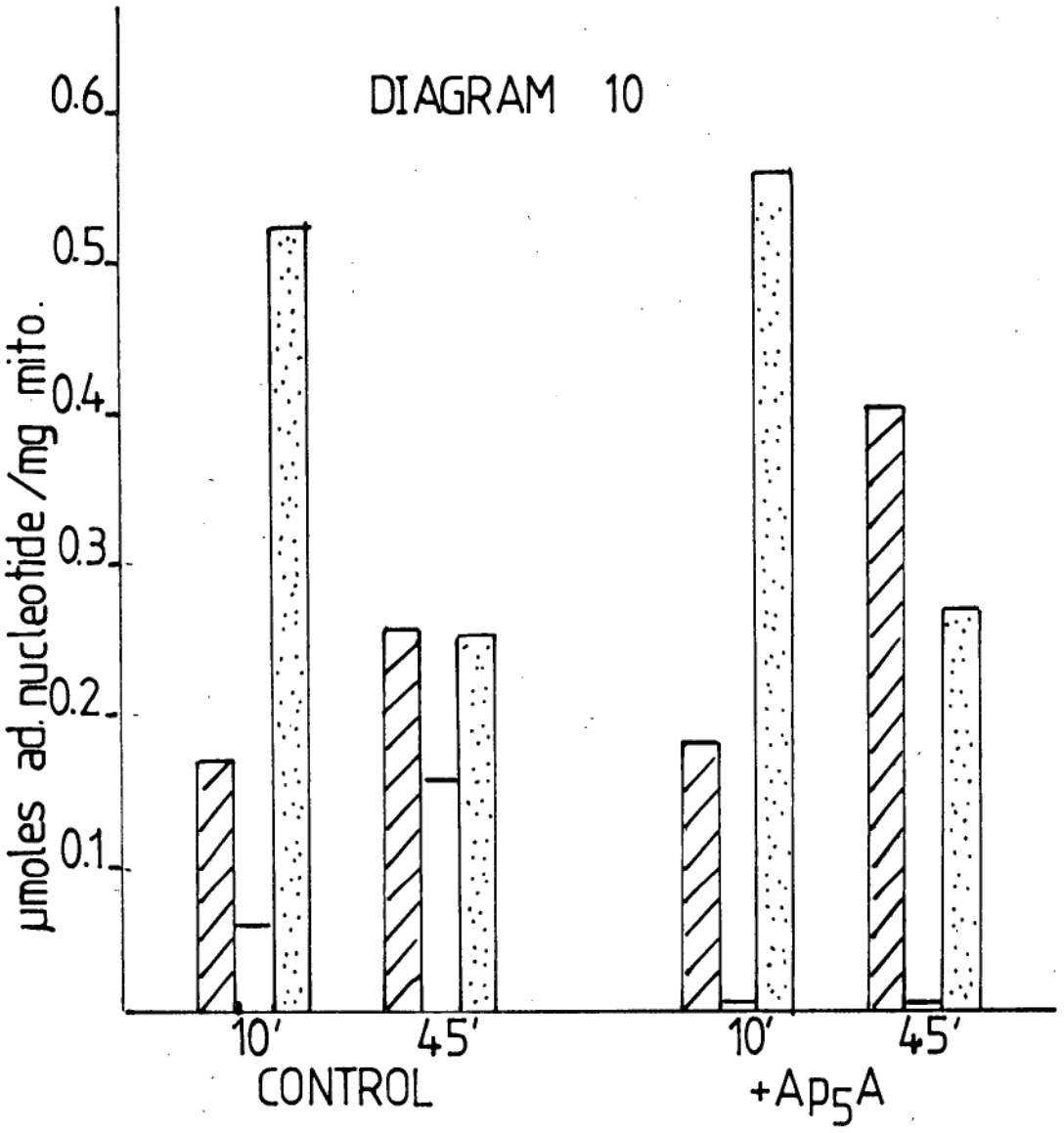

nmoles Ca^{2+} /mg mito. protein
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50
TIME (min.)

DIAGRAM 10


0.6
0.5
0.4
0.3
0.2
0.1
µmoles ad. nucleotide /mg mito.
10'
45'
10'
45'
CONTROL
+Ap5A

5.3.7.1 Distribution of [8 -^{14}C] ATP in mitochondria during Ca^{2+} uptake in the presence of 2 mM β-hydroxybutyrate and 1 mM [8 -^{14}C] ATP.

In the presence of 1 mM [8 -^{14}C] ATP, but without added Ca^{2+} in the incubation medium, between 6 - 8 nmoles [8 -^{14}C] ATP was found per mg mitochondrial protein. Estimations were made at 5, 10, 40 and 45 min incubation (table 7). 2 % of the total added ^{14}C-ATP was observed in the lower perchloric/sucrose medium while the remaining ^{14}C-counts were in the supernatant above the silicone oil layer.

When 200 nmoles $^{45}Ca^{2+}$ was included in the incubation medium, 67 % of the added $^{45}Ca^{2+}$ was observed in the mitochondria at 5 and 10 min. However only 17 % and 10 % of the added $^{45}Ca^{2+}$ remained in the mitochondria at 40 and 45 min respectively, an indication of $^{45}Ca^{2+}$ release from the mitochondria (table 8). As shown in table 8, during $^{45}Ca^{2+}$ uptake and release, between 6 - 8 nmoles [8 -^{14}C] ATP was found per mg mitochondrial protein. The amount of [8 -^{14}C] ATP in the mitochondria in the presence or absence of externally added Ca^{2+} was similar.

5.3.7.2 Distribution of [2 -^{3}H] ADP in mitochondria during Ca^{2+} uptake in the presence of 2 mM β-hydroxybutyrate and 1 mM [2 -^{3}H] ADP.

The mitochondria contained between 4 - 9 nmoles [2 -^{3}H] ADP per mg mitochondrial protein when 1 mM [2 -^{3}H] ADP was included in the incubation medium without added $^{45}Ca^{2+}$ (table 9).

As shown in table 10, in the presence of 200 nmoles $^{45}Ca^{2+}$, the mitochondria accumulated the $^{45}Ca^{2+}$ and 71 % of

TABLE 7

^{14}C-ATP concentration in mitochondria in the absence of added $^{45}Ca^{2+}$ in the incubation medium.

Rat liver mitochondria (4.5 mg protein), were added to 2.5 ml incubation medium containing the standard incubation mixture, 2 mM β-hydroxybutyrate and 1 mM [8-^{14}C] ATP. Sampling times were at 5, 10, 40 and 45 min.

Sampling Time (min)	nmoles ^{14}C-ATP/mg mitochondrial protein	(a) nmoles ^{14}C-ATP above silicone oil after centrifugation
5	7 (6-8)	454 (450-459)
10	8 (7-9)	459 (458-460)
40	6 (5-7)	468 (467-470)
45	8 (7-9)	447 (443-454)

(a) Estimation of ^{14}C-ATP in the supernatant when an equivalent of 1 mg mitochondrial protein were centrifuged through the oil into the lower perchloric/sucrose layer.

Note: The results are given as mean values for 3 samples. The range is as indicated in brackets

TABLE 8

^{14}C-ATP concentration in mitochondria during $^{45}Ca^{2+}$ uptake and release.

Rat liver mitochondria (5 mg protein), were added to 2.5 ml incubation medium containing the standard incubation mixture, 2 mM β-hydroxybutyrate, 200 nmoles $^{45}Ca^{2+}$ (final concentration = 80 μM) and 1 mM [8-^{14}C] ATP. $^{45}Ca^{2+}$ and [8-^{14}C] ATP in the lower perchloric/sucrose solution were estimated at 5, 10, 40 and 45 min.

Sampling Time (min)	nmoles $^{45}Ca^{2+}$/mg mitochondrial protein	nmoles ^{14}C-ATP/mg mitochondrial protein
5	25 (24.7, 23.9)	6 (5-7)
10	26 (27.2, 24.7)	7 (6-8)
40	7 (7.6, 5.8)	7 (6-8)
45	4 (5.4, 3.7)	8 (7-9)

Note: The results are given as mean values for 3 samples. The range is as indicated in brackets.

TABLE 9

^{3}H-ADP concentration in mitochondria in the absence of added $^{45}Ca^{2+}$ in the incubation medium.

Rat liver mitochondria (6.4 mg protein), were added to 2.5 ml incubation medium containing the standard incubation mixture, 2 mM β-hydroxybutyrate and 1 mM [2-^{3}H] ADP. At, 1, 6, 40 and 45 min. after adding the mitochondria, 0.5 ml of the incubation mixtures was removed for silicone oil centrifugation.

Sampling Time (min)	nmoles ^{3}H-ADP/mg mitochondrial protein	(a) nmoles ^{3}H-ADP above silicone oil after centrifugation
1	4 (3-5)	348 (340-356)
6	6 (5-7)	335 (323-347)
40	7 (6-8)	343 (340-346)
45	9 (8-10)	341 (338-344)

(a) Estimation of ^{3}H-ADP in the supernatant when an equivalent of 1 mg mitochondrial protein were centrifuged through the oil into the lower perchloric/sucrose layer.

Note: The results are given as mean values for 3 samples. The range is as indicated in brackets.

the added $^{45}Ca^{2+}$ was found in the mitocondria at 1 and 6 min. 8 - 9 nmoles $[2\text{-}^{3}H]$ ADP was present per mg mitochondrial protein at the stated times.

However at 40 and 45 min, when the mitochondria contained 29 % and 19 % of the added $^{45}Ca^{2+}$ respectively, the $[2\text{-}^{3}H]$ ADP content was 11 nmoles per mg mitochondrial protein (table 10).

Only 1 - 3 % of the total $[2\text{-}^{3}H]$ ADP added was present in the lower perchloric/sucrose layer (i.e. had been accumulated by the mitochondria).

5.3.8 Ca^{2+} accumulation by mitochondria in the presence of varying concentrations of KH_2PO_4 studied by means of the Ca-electrode

In the presence of 2 mM β-hydroxybutyrate as the energy source for Ca^{2+} uptake, 5 µg oligomycin/mg mitochondrial protein, 1 mM Mg^{2+}, 72 mM KCl and 2 mM KH_2PO_4 the mitochondria (4 mg protein) accumulated all the added Ca^{2+} (400 nmoles). Similar results were obtained in the presence of 4 mM and 6 mM KH_2PO_4.

5.3.9 Ca^{2+} uptake by mitochondria in the absence of KH_2PO_4 studied by means of the Ca-electrode

The control experiment is as described above. However, omitting KH_2PO_4 from the incubation medium resulted in approximately 75 % uptake of the added Ca^{2+} (i.e. 75 nmoles Ca^{2+}/mg mitochondrial protein).

The mitochondria accumulated 40 nmoles Ca^{2+}/mg mitochondrial protein in the presence of 200 nmoles N-ethylmaleimide (NEM)/mg mitochondrial protein, 5 µg oligomycin per mg mitochondrial protein (without KH_2PO_4). When the mitochondria were preincubated at $0^{o}C$ with NEM and oligomycin for 5 min, only 15 nmoles Ca^{2+} was accumulated per mg protein.

TABLE 10

^{3}H-ADP concentration in mitochondria during Ca^{2+} uptake and release.

Rat liver mitochondria (6.4 mg protein), were added to 2.5 ml incubation medium containing the standard incubation mixture, 2 mM β-hydroxybutyrate, 1 mM [2-^{3}H] ADP and 200 nmoles $^{45}Ca^{2+}$ (final concentration = 80 μM). $^{45}Ca^{2+}$ and ^{3}H-ADP in the lower perchloric/sucrose solution were estimated at 1, 6, 40 and 45 min.

Sampling Time (min)	nmoles $^{45}Ca^{2+}$/mg mitochondrial protein	nmoles ^{3}H-ADP/mg mitochondrial protein
1	22 (23, 22.5)	9 (8-10)
6	22 (23, 21)	8 (7-9)
40	9 (8, 9.5)	12 (11-13)
45	6 (5, 7)	11 (10-12)

Note: The results are given as mean values for 3 samples. The range is as indicated in brackets.

5.3.10 Ca^{2+} uptake by mitochondria in the presence of 1-aminoethylphosphonic acid and 2-aminoethylphosphonic acid studied by means of the Ca-electrode

Note : 5 µg oligomycin/mg mitochondrial protein was included in the incubation medium to inhibit the generation of Pi via mitochondrial ATPase. Control mitochondria respiring on 2 mM β-hydroxybutyrate (and in the presence of 2 mM KH_2PO_4) rapidly accumulated all the added Ca^{2+}, i.e. approximately 100 nmoles per mg mitochondrial protein. The mitochondria still retained the accumulated Ca^{2+} at 10 min. In the absence of KH_2PO_4, only about 73 nmoles Ca^{2+} was taken up per mg mitochondrial protein and the Ca^{2+} started to release at 8 min.

Similar results to that when KH_2PO_4 was excluded from the medium were obtained when 2 mM 1-aminoethylphosphonic acid and 2-aminoethylphosphonic acid was substituted for 2 mM KH_2PO_4 .

5.4 DISCUSSION

The aim of this particular investigation was to examine mitochondrial Ca^{2+} transport in the presence of adenine nucleotides and inorganic phosphates, which would be present in the cytosol.

Requirement of adenine nucleotides for Ca^{2+} uptake.

In order to examine whether externally added ATP, ADP or AMP can support ~~initial~~ Ca^{2+} uptake by mitochondria, it was essential to firstly deplete the endogenous energy source, since according to Brand and Lehninger (1975), Ca^{2+} transport in mitochondria used their endogenous ATP before utilising the energy from added energy source. For this particular experiment it was appropriate to use the Ca-electrode for continuous monitoring of Ca^{2+} movement into and out of the mitochondria.

As shown in fig 40, in the absence of added energy source, mitochondria were able to transport approximately 60 % of the added Ca^{2+} and this Ca^{2+} was released 4 min after uptake. The energy for Ca^{2+} uptake by the mitochondria was derived from endogenous store. At the point of Ca^{2+} release (i.e. after exhaustion of endogenous substrates), the various adenine nucleotides was added.

Addition of ATP or ADP (final concentration = 1 mM) caused Ca^{2+} reuptake by the mitochondria; however, the rate of reuptake in the presence of ADP was slower than in the presence of ATP (figs 40(a),(c)). Possibly ADP had to be phosphorylated to ATP prior to Ca^{2+} uptake. Ca^{2+} reuptake by the mitochondria did not take place on adding a similar

concentration of β γ methylene ATP, α β methylene ATP or AMP. The ATP analogues are translocated into the mitochondria by the same atractyloside sensitive process as ATP, but these analogues are not metabolised (Duee and Vignais, 1968); this result confirms that ATP has to be metabolised to support Ca^{2+} uptake (Bielawski and Lehninger, 1966). AMP cannot support Ca^{2+} uptake since it is not even transported into the mitochondrial matrix (Klingenberg, 1976). Therefore availability of energy sources either from ATP hydrolysis or from oxidation of respiratory substrates such as β-hydroxybutyrate (Brierley et al, 1964; chapter 4, fig 30) is essential for Ca^{2+} uptake by mitochondria. When both ATP and a respiratory substrate (e.g. β-hydroxybutyrate, succinate) were present, the mitochondria were able to retain Ca^{2+} for a much longer period (chapter 4, fig 30). (It is also worth noting that influx of Ca^{2+} could be coupled to the efflux of K^{+} in the presence of valinomycin in a respiration inhibited mitochondria (Scarpa and Azzone, 1970).

Fig 40 shows that addition of ATP, ATP analogues or ADP resulted in a fall in ionic Ca^{2+} in the medium due to combination with Ca^{2+}. In a separate experiment, with no mitochondria in the medium, it was noted that out of a total of 400 nmoles Ca^{2+} added to the medium, approximately 275 nmoles Ca^{2+} was chelated to 1 mM ATP, 175 nmoles to 1 mM β γ methylene ATP or α β methylene ATP, 150 nmoles to 1 mM ADP and less than 50 nmoles Ca^{2+} combined to 1 mM AMP (table 6). Therefore, with no mitochondria in the incubation medium, the free Ca^{2+} concentration was approximately 25 μM in the presence of 1 mM ATP, 45 μM with 1 mM β γ methylene ATP or α β methylene ATP, 50 μM with 1 mM ADP and 75 μM with 1 mM AMP. In the

presence of mitochondria and 1 mM ATP (or ADP), the free Ca^{2+} concentration was approximately 1 - 2 μM. Using the Ca-electrode, which detects only free Ca^{2+}, it is not possible to establish whether the Ca^{2+} has been chelated to ATP (or ADP) or taken up by the mitochondria.

However, Ca^{2+} movement in mitochondria studied by the radioassay technique revealed that most of the $^{45}Ca^{2+}$ was transported into the mitochondria; out of 400 nmoles $^{45}Ca^{2+}$ added to the medium, approximately 350 nmoles $^{45}Ca^{2+}$ was accumulated (i.e. approximately 85 - 90 % of the total $^{45}Ca^{2+}$). Thus, the mitochondria have a higher affinity for Ca^{2+} in the medium than has ATP. Under the conditions examined, i.e. in the absence of mitochondria, the affinity of the tested adenine nucleotides for Ca^{2+} in the incubation medium is as follows:-

ATP > βγ methylene ATP = αβ methylene ATP > ADP > AMP.

<u>External ATP favours Ca^{2+} retention in mitochondria</u>. It is known that the presence of external ATP favours Ca^{2+} retention by mitochondria (Drahota et al, 1965). This fact is further confirmed in the present study when it was observed that in the presence of 4 mM PEP plus 15 U pyruvate kinase (i.e. an ATP generation system), mitochondria were able to retain $^{45}Ca^{2+}$ for 45 min. The control mitochondria on the other hand started to release their $^{45}Ca^{2+}$ at 25 min (fig 41).

The effect of an ATP "trapping" system on a Ca^{2+} transport in mitochondria was also examined. This was made possible by including 5 mM D-glucose and 15 U hexokinase in the incubation medium. The results obtained showed an earlier $^{45}Ca^{2+}$ release from the test mitochondria compared with the

control mitochondria. The uptake of Ca^{2+} by the test mitochondria was not affected. This may have been due to ATP present in the early stages of the experiment.

Thus Ca^{2+} retention in mitochondria was favoured in an ATP generation system while an ATP "trapping" system caused Ca^{2+} release from mitochondria.

While 4 mM PEP plus 15 U pyruvate kinase favoured $^{45}Ca^{2+}$ retention in mitochondria, it was observed that 1 mM PEP alone (without the enzyme) caused an earlier release of $^{45}Ca^{2+}$ from mitochondria with respect to the control mitochondria (fig 43). Chudapongse and Haugaard (1973) showed that PEP at concentrations as low as 0.1 mM caused Ca^{2+} release from liver and heart mitochondria respiring in the presence of glutamate or pyruvate ~~kinase~~ plus malate. They also showed that the PEP effect was inhibited in the presence of ATP or atractylate, suggesting that adenine nucleotide translocase is involved. Presumably the presence of PEP caused removal of endogenous ATP in mitochondria in exchange with the added PEP via the adenine nucleotide translocase. However, it is unlikely that PEP is a physiological modulator of mitochondrial Ca^{2+} efflux, since the presence of ATP or pyruvate kinase in the cytosol would prevent the PEP effect.

Results obtained from the experiments on the adenine nucleotide concentrations during Ca^{2+} release and retention in mitochondria revealed that the time of Ca^{2+} release from control mitochondria in the presence of 1 mM ATP and 2 mM β-hydroxybutyrate varied from one experiment to the other even though the experimental conditions were kept constant (e.g. using chemicals from the same batch, stirring the

incubation mixtures at the same speed, making sure that the pH of the incubation medium was between pH 7.3 - 7.4). A possible explanation for the observed effects may have been the variation in the mitochondrial preparations, e.g. variation in the possible contamination with fatty acids, the amounts of endogenous ATP or respiratory substrates or even the physiological state of the rat before decapitation. Therefore results obtained were compared with the control of the respective experiments.

Concentrations of adenine nucleotides during Ca^{2+} release. Since external ATP seems to favour Ca^{2+} retention in mitochondria, it was of interest to investigate the concentrations of ATP, ADP and AMP in the mitochondria and in the medium during Ca^{2+} retention and release. Various substances were used to induce an earlier Ca^{2+} release from the mitochondria. Such substances include 1 mM PEP, 5 mM D-glucose plus 15 U hexokinase, 10 µM palmitoyl CoA, 30 µM rat albumin contaminated with fatty acid and 0.5 mM quinidine sulphate. The energy substrates for $^{45}Ca^{2+}$ uptake by the mitochondria were 2 mM β-hydroxybutyrate and 1 mM ATP.

Enzymatic determination of ATP, ADP and AMP indicated that during Ca^{2+} release from the mitochondria the total ATP (i.e. that in the medium and in the mitochondria) decreased, while the total AMP concentration increased significantly with respect to the control experiment. The total ADP concentration appeared to remain fairly constant during Ca^{2+} uptake and release. The mitochondria contained 5 - 10 % of these nucleotides and their concentrations seemed to change in the same direction as the medium when $^{45}Ca^{2+}$ was released (section

5.3.4.1). The results obtained in this thesis, i.e. a decrease in ATP concentration while the ADP concentration remained unchanged in the medium during Ca^{2+} release, agrees with the theory postulated by Lehninger et al (1978b) that whenever the [ATP]/[ADP][Pi] declines in the cytosol, then there is a shift to a more oxidised state of NAD^+ in mitochondria via reversibility of site 1 phosphorylation in the respiratory chain:-

$NADH + H^+ + Pi + ADP + fumarate \rightleftharpoons NAD^+ + ATP + succinate + H_2O$

which in turn favours Ca^{2+} release. The effect of pyridine nucleotides on mitochondrial Ca^{2+} movement will be examined in chapter 6.

Batra (1976) showed that 2 mM quinidine sulphate caused marked release of Ca^{2+} from preloaded mitochondria of frog skeletal muscle. It was shown in this thesis that 0.5 mM quinidine sulphate added at 5 min after $^{45}Ca^{2+}$ uptake by the rat liver mitochondria caused an immediate release of the accumulated $^{45}Ca^{2+}$ (fig 47). Although 0.5 mM SO_4^{2-} caused $^{45}Ca^{2+}$ release 5 min earlier than the control, it was the quinidine moiety that caused the immediate $^{45}Ca^{2+}$ release from the mitochondria (fig 47). The present investigation also indicated that unlike the other "Ca^{2+}-releasing" substances examined, the presence of quinidine sulphate in the medium did not result in a marked increase in AMP or a significant decrease in ATP compared to the control during $^{45}Ca^{2+}$ release from the mitochondria. The table below illustrates this point.

"Ca^{2+}-releasing" substance	% $^{45}Ca^{2+}$ released from mitochondria	Total ATP µmoles/mg mitochondrial protein	Total AMP µmoles/mg mitochondrial protein
Palmitoyl CoA(10µM)	89	0.216	0.392
Control	39	0.446	0.189
(a)Rat albumin (30µM)	56	0.197	0.31
Control	6	0.408	0.113
quinidine sulphate (0.5mM)	82	0.373	0.181
Control	0	0.494	0.133
PEP (1 mM)	80	0.130	0.47
Control	20	0.290	0.33

(a)Rat albumin as purchased, contaminated with fatty acid.

Possibly the mechanism of Ca^{2+} efflux from mitochondria in the presence of quinidine sulphate differs from the other "Ca^{2+}-releasing" substances examined.

Concentrations of adenine nucleotides during Ca^{2+} retention. In order to examine a longer period of Ca^{2+} retention in mitochondria with respect to the control, substances such as palmitoylcarnitine plus ATP, PEP plus pyruvate kinase, or defatted bovine serum albumin were included in the incubation medium. During Ca^{2+} retention, a high ATP concentration and a low AMP were maintained; the control experiment showed a decrease in ATP and an increase in AMP during the earlier Ca^{2+} release with respect to the test experiment.

EHDP is known to help Ca^{2+} retention in rat kidney mitochondria (Guillard et al, 1974). The concentrations of adenine nucleotide during $^{45}Ca^{2+}$ retention in rat liver mitochondria in the presence of 0.2 mM EHDP was examined in

the present study. For this particular investigation, the High Performance Liquid Chromatography (HPLC) was employed to estimate the concentrations of adenine nucleotides. During Ca^{2+} retention in the mitochondria the total ATP, ADP and AMP concentrations remained steady. The control mitochondria had released their $^{45}Ca^{2+}$ at 45 min at which time the test mitochondria still retained their $^{45}Ca^{2+}$. The control experiment showed an increase in AMP concentration and a decrease in ATP concentration (fig 48).

Ca^{2+} transport in the presence of Ap_5A.

Since it was observed that an increase in AMP concentration seemed to be related to Ca^{2+} release from mitochondria, an attempt was therefore made to diminish the concentration of AMP in the medium by inhibiting the activity of adenylate kinase. This enzyme catalyses the reaction $2ADP \rightleftharpoons ATP + AMP$. Ap_5A (0.1 mM), an inhibitor of adenylate kinase (Kohrle et al, 1977) was included in the incubation mixtures. Even though the presence of the inhibitor resulted in negligible concentrations of AMP estimated at 10 and 45 min, however Ca^{2+} uptake and release was not affected compared to the control. Thus the high concentration of AMP in the medium most likely did not cause Ca^{2+} release. Presumably the depletion of external ATP induced Ca^{2+} release from the mitochondria.

Distribution of ATP and ADP in mitochondria during Ca^{2+} uptake.

The distribution of $[8\text{-}^{14}C]$ ATP and $[2\text{-}^{3}H]$ ADP in mitochondria in the presence of 1 mM concentration of the radioactive labelled nucleotide in the incubation medium was also investigated. Only 1 - 3 % of the total added ^{14}C-ATP or

^{3}H-ADP was located in the lower perchloric/sucrose layer after silicone oil centrifugation (i.e. in the mitochondria). This is in close approximation with the results obtained when mitochondrial adenine nucleotides were estimated enzymatically (i.e. between 5 - 10 % of the total adenine nucleotides were present in the mitochondria). In the absence of added Ca^{2+}, 4 nmoles ^{3}H-ADP and 7 nmoles ^{14}C-ATP were found per mg mitochondrial protein at the early stages of the experiment and 9 nmoles ^{3}H-ADP, 6 nmoles ^{14}C-ATP when the mitochondria accumulated approximately 22 - 25 nmoles Ca^{2+} per mg mitochondrial protein. The result indicated only a slight change in the uptake of ^{14}C-ATP but a small increase of ^{3}H-ADP when mitochondria accumulated Ca^{2+}. It is interesting that according to Carafoli et al (1965), when rat liver mitochondria accumulate Ca^{2+} in the presence of Pi, a ratio of 1 molecule of ATP and ADP to about 12 Ca^{2+} ions is accumulated. Thus according to the result obtained in this thesis an accumulation of approximately 24 nmoles Ca^{2+} should result in an accumulation of approximately 2 nmoles ATP or ADP, which would explain why there were only slight changes in the uptake of ^{14}C-ATP or ^{3}H-ADP when mitochondria accumulated Ca^{2+}.

<u>The effect of KH_2PO_4 on Ca^{2+} transport</u>.

The presence of a penetrant anion such as Pi is a prerequisite for Ca^{2+} uptake by mitochondria. In the presence of both N-ethylmaleimide (to prevent entry of Pi into mitochondria via Pi carrier) and oligomycin (to inhibit generation of internal Pi via ATPase), Harris and Zaba (1977) showed that liver and heart mitochondria were able to transport 1 nmole Ca^{2+} per mg mitochondrial protein. Bygrave et al (1977)

reported a higher value, i.e. approximately 11 nmoles/mg mitochondrial protein.

In this thesis, it was shown that in the presence of NEM and oligomycin and in the absence of added Pi, the mitochondria were able to transport 19% of the added Ca^{2+} (note: NEM was added to inhibit uptake of Pi which might be present in the mitochondrial preparation). In the presence of oligomycin but without Pi or NEM, the mitochondria respiring on 2 mM β-hydroxybutyrate accumulated 73% of the added Ca^{2+} ; however, the control (i.e. in the presence of 2 mM Pi) accumulated 100% of the added Ca^{2+}. The result suggested that the mitochondrial suspensions themselves contained sufficient phosphate for Ca^{2+} uptake by the mitochondria.

It was further demonstrated in the present study that substituting 2 mM 2-aminoethylphosphonic acid or 1-aminoethylphosphonic acid for KH_2PO_4 did not result in 100% Ca^{2+} uptake; however, similar results to that in the absence of KH_2PO_4 were obtained (i.e. 73 % uptake). The results obtained in this thesis also indicated that higher concentrations of Pi (i.e. 2 - 6 mM) in the incubation medium did not seem to affect Ca^{2+} accumulation by the mitochondria.

5.5 SUMMARY

1. Studies with the Ca^{2+}-electrode showed that, when endogenous substrates in mitochondria were depleted, ATP or ADP but not AMP supported Ca^{2+} uptake.

2. No Ca^{2+} uptake by the mitochondria was observed in the presence of ATP analogues namely $\beta\gamma$ methylene ATP and $\alpha\beta$ methylene ATP further confirming that ATP had to be metabolised to support Ca^{2+} uptake.

3. The Ca^{2+}-chelating ability of adenine nucleotides in the incubation medium is as follows:-
ATP $>$ $\beta\gamma$ methylene ATP = $\alpha\beta$ methylene ATP $>$ ADP $>$ AMP.

4. Ca^{2+} retention in mitochondria was observed in an ATP regeneration system (PEP + pyruvate kinase) while an ATP trapping system (D-glucose + hexokinase) caused an earlier Ca^{2+} release.

5. During Ca^{2+} release from mitochondria the total concentration of ATP (i.e. mitochondrial ATP plus that in the medium) decreased, while the total AMP concentration increased.

6. In the *in vitro* system the mitochondria contained 5 - 10 % of the total adenine nucleotides. During Ca^{2+} release, mitochondrial ATP decreased and AMP increased.

7. In the presence of Ap_5A (an inhibitor of adenylate kinase) the total AMP concentration was negligible; however, Ca^{2+} uptake and release by mitochondria was not affected. The cause of Ca^{2+} release is not AMP itself, but rather the depletion of external ATP.

8. Quinidine sulphate (0.5 mM) caused an immediate Ca^{2+} release from mitochondria; however, it did not result in marked increase in AMP or a decrease in ATP. Possibly another mechanism of Ca^{2+} release is involved when quinidine is present.

9. During Ca^{2+} retention in mitochondria, a high ATP concentration and low AMP concentration was always observed.

10. Approximately 1 - 3 % of the added ^{14}C-ATP or ^{3}H-ADP was recovered in the mitochondria. Only slight changes in the uptake of ^{14}C-ATP or ^{3}H-ADP was noted when the mitochondria accumulated Ca^{2+}.

11. In the absence of added Pi, but including oligomycin to prevent generation of internal Pi via ATPase, the mitochondria respiring on 2 mM β-hydroxybutyrate accumulated 73 % of the added Ca^{2+} suggesting that the mitochondrial suspensions themselves contained sufficient Pi for considerable Ca^{2+} uptake.

12. In the absence of added Pi and including oligomycin and NEM (to prevent uptake of Pi via Pi/OH carrier), the mitochondria accumulated 15 nmole Ca^{2+} per mg protein. NEM was added to prevent uptake of Pi (which might be present in the mitochondrial preparation) by the mitochondria.

13. Unlike 2 mM KH_2PO_4, a similar concentration of 1-aminoethylphosphonic acid or 2-aminoethylphosphonic acid did not increase the capacity of the mitochondria to accumulate Ca^{2+}.

CHAPTER 6

THE EFFECT OF PYRIDINE NUCLEOTIDES ON MITOCHONDRIAL CALCIUM TRANSPORT

6.1 AIM

While work for this thesis was in progress, Lehninger et al (1978) reported that the redox state of mitochondrial pyridine nucleotides can influence Ca^{2+} movement into and out of the mitochondria. Since the study in chapter 5 indicated that ATP concentration decreased, while AMP concentration increased during Ca^{2+} release caused by "Ca releasing substances" (e.g. PEP, palmitoyl CoA) it was therefore of interest to examine :-

(i) Concentration of adenine nucleotides during Ca release caused by oxidant of mitochondrial pyridine nucleotides.

(ii) Ca cycling and the corresponding change in concentration of mitochondrial adenine nucleotides.

The effect of externally added pyridine nucleotides on Ca^{2+} transport was also examined.

Since Vinogradov et al (1972) proposed the formation of Ca-NADH complex in the non-polar region of the mitochondrial membrane, it was decided to examine <u>in vitro</u> the possibility of a Ca-pyridine nucleotide complex in a non-aqueous phase.

6.2 METHOD AND MATERIALS.

Mitochondria were prepared as described in chapter 1, section 1.1 . Calcium movement in and out of the mitochondria was monitored by means of the Ca-electrode (chapter 1, section 1.4) or using the radioassay technique (chapter 1, section 1.3). The concentrations of pyridine nucleotide were determined by an enzymatic and fluorimetric assay described in chapter 1, section 1.8 . Total and mitochondrial adenine nucleotides were estimated enzymatically as described in chapter 1, section 1.6 .

Lithium acetoacetate tripalmitin and DL-β hydroxybutyrate were from SIGMA Chem. Co. St. Louis, MO, U.S.A. Lithium chloride was from Ajax Chem. Ltd. Sydney, Australia; sodium laurylsulphate from B.D.H. Lab. Chem. Division, Poole, England. Oxaloacetate, NAD^+, $NADP^+$ and NADPH were from Calbiochem, La Jolla, Ca., U.S.A., NADH and $NADP^+$ from Boehringer Mannheim, Australia Pty. Ltd., Hardner Road, Mt. Waverley, Victoria.

6.3 RESULTS

6.3.1 The influence of the redox state of mitochondrial pyridine nucleotide on Ca^{2+} uptake and release by mitochondria studied by the radioassay technique.

Experiments in section 6.3.1.1 to 6.3.1.2 examined the effects of β-hydroxybutyrate (reductant of mitochondrial NAD^+ via β-hydroxybutyrate dehydrogenase) and acetoacetate (oxidant of mitochondrial NADH via β-hydroxybutyrate dehydrogenase) on Ca^{2+} uptake and release by mitochondria. The radioassay technique described in chapter 1, section 1.3 was used. The energy sources for Ca^{2+} uptake by the mitochondria were 0.5 mM ATP plus 0.5 mM ADP, and rotenone (10^{-5}M) was included in the incubation medium to inhibit oxidation of NADH via the respiratory chain. 2 mM Pi was the permeant anion for these experiments.

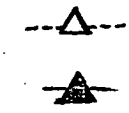
6.3.1.1 The effect of NAD^+-reductant (β-hydroxybutyrate) and NADH-oxidant (acetoacetate).

The control mitochondria (i.e. in the presence of 0.5 mM ATP, 0.5 mM ADP and 10^{-5}M rotenone) contained 80 % of the added $^{45}Ca^{2+}$ at 7 min and started to release this $^{45}Ca^{2+}$ after 16 min (fig 50). When 2 mM lithium acetoacetate was included in the medium, only 38 % of the externally added $^{45}Ca^{2+}$ was found in the mitochondria at 4 min and at this time release of the accumulated $^{45}Ca^{2+}$ was observed. As shown in fig 50, lithium itself (2 mM LiCl) had no effect on Ca^{2+} uptake or release by the mitochondria compared with the control mitochondria. The presence of β-hydroxybutyrate (2 mM) in the incubation mixtures did not affect the capacity of the

FIGURE 50

The effect of NAD^+-reductant (β-hydroxybutyrate) and NADH-oxidant (acetoacetate) on mitochondrial Ca^{2+} transport studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 10^{-5}M rotenone, 0.5 mM ATP, 0.5 mM ADP and mitochondria (4.5 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. The following additions were made before adding the $^{45}Ca^{2+}$.



control

+ Lithium acetoacetate (final concentration = 2 mM)

+ LiCl (final concentration = 2 mM)

+ β-hydroxybutyrate (final concentration = 2 mM)

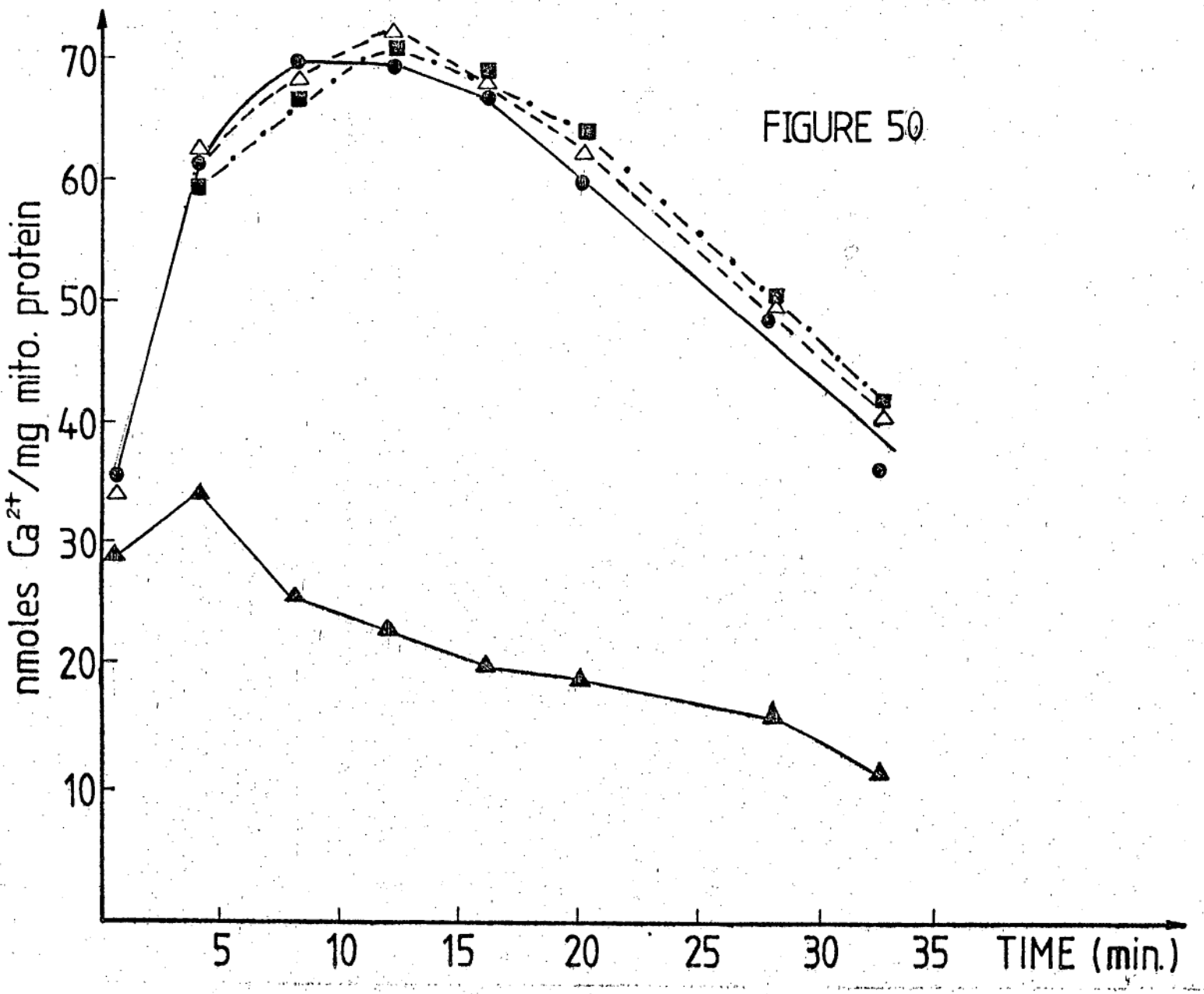

70
60
50
40
30
20
10
nmoles Ca2+/mg mito. protein
5
10
15
20
25
30
35
TIME (min.)

FIGURE 50

mitochondria to accumulate Ca^{2+} nor did it affect release and retention of $^{45}Ca^{2+}$ in the mitochondria, compared to the control.

6.3.1.2 The concentrations of pyridine nucleotide during $^{45}Ca^{2+}$ uptake and release by mitochondria in the presence of acetoacetate and β-hydroxybutyrate.

At 10 and 30 min, samples of the incubation mixtures were removed for $^{45}Ca^{2+}$ and pyridine nucleotide estimations, and the results obtained are in table 11 (note: the maximum possible Ca^{2+} accumulation by the mitochondria was 45 nmoles $^{45}Ca^{2+}$ per mg protein).

In the presence of 2 mM acetoacetate, the medium at 10 min had a higher concentration of NAD^+ and a lower concentration of NADH compared to the control. The NADH/NAD^+ ratio in the presence of acetoacetate was lower than control. At that time, the control mitochondria contained 80 % of the added Ca^{2+} while the mitochondria incubated with acetoacetate contained 62 % of the added Ca^{2+}.

At 30 min the control mitochondria contained 44 % of the added Ca^{2+} while the test mitochondria in the presence of acetoacetate had 22 % of the added Ca^{2+}. There was no significant difference in the NAD^+ or NADH levels in the control mitochondria at 10 min or 30 min. The NAD^+ concentration in the presence of acetoacetate was lower at 30 min than that estimated at 10 min but still relatively higher than the control, whereas the NADH concentration was very low.

Inclusion of 2 mM β-hydroxybutyrate did not affect the Ca^{2+} content in the mitochondria at 10 and 30 min relative to

TABLE 11

Concentration of pyridine nucleotide during Ca^{2+} uptake and release by rat liver mitochondria in the presence of acetoacetate and β-hydroxybutyrate.

Mitochondria (8.9 mg protein) were added to 5.0 ml incubation medium containing the standard incubation mixture, 0.5 mM ATP, 0.5 mM ADP, 10^{-5} M rotenone and 400 nmoles Ca^{2+}. At 10 and 30 min. incubation, samples of the incubation mixtures were removed for $^{45}Ca^{2+}$ and pyridine nucleotide estimations.

	SAMPLING TIME = 10 min				
	nmoles ^{45}Ca/mg mito. protein	nmoles/mg mito protein			NADH / NAD^+
		NAD^+	NADH	NADPH	
control	36	2.3	0.8	3.0	0.35
+ AA (2 mM)	28	3.1	0.3	<0.1	0.10
+ BOH (2mM)	34	2.0	0.9	2.6	0.45
	SAMPLING TIME = 30 min				
control	20	2.3	0.7	0.95	0.3
+ AA (2 mM)	10	2.5	<0.05	<0.1	<0.05
+ BOH (2 mM)	19	1.8	0.8	0.6	0.44

AA = acetoacetate

BOH = β-hydroxybutyrate

mito. = mitochondria

the control, although the NAD^+ concentration was slightly lower and NADH relatively higher. The NADPH concentration for the control mitochondria was slightly higher than the mitochondria incubated with β-hydroxybutyrate. On the other hand, the concentration of NADPH in the mitochondria incubated with acetoacetate was very low.

It should be noted that the total pyridine nucleotide estimated was presumably the mitochondrial pyridine nucleotide, assuming that there was negligible contamination in the washed mitochondrial suspension and negligible leakage of the pyridine nucleotides from the mitochondria during incubation.

6.3.1.3 Total adenine nucleotide concentrations during Ca^{2+} release from mitochondria in the presence of acetoacetate.

Samplings for $^{45}Ca^{2+}$ and total adenine nucleotide determinations were made at 10 and 30 min. The result obtained is as shown in table 12. The maximum possible $^{45}Ca^{2+}$ accumulation by the mitochondria for this particular experiment was 77 nmoles/mg mitochondrial protein.

At 10 min, the control mitochondria contained 79 % of the added $^{45}Ca^{2+}$ in the medium; however, at 10 min, mitochondria in the presence of acetoacetate contained only 25 % of the total $^{45}Ca^{2+}$ in the medium (table 12) suggesting $^{45}Ca^{2+}$ release from the test mitochondria. The total ATP concentration at 10 min in the presence of acetoacetate was lower by 0.06 μmole per mg mitochondrial protein, and the AMP concentration was greater by 0.08 μmole/mg mitochondrial protein, compared with the control experiment as shown in table 12.

TABLE 12

Concentrations of total adenine nucleotide during Ca^{2+} release from mitochondria in the presence of acetoacetate.

Mitochondria (5.2 mg protein), were added to 5.0 ml incubation medium containing the standard incubation mixture, 0.5 mM ATP, 0.5 mM ADP, 10^{-5}M rotenone and 400 nmoles $^{45}Ca^{2+}$. At 10 and 30 min. incubation samples of the incubation mixtures were removed for ^{45}Ca and total adenine nucleotide estimations (see section 1.6).

	nmoles ^{45}Ca/mg mito. protein	μmoles/mg mito. protein		
		ATP	ADP	AMP
SAMPLING TIME = 10 min.				
control	61	0.38	0.28	0.31
+ acetoacetate (2mM)	19	0.32	0.26	0.39
SAMPLING TIME = 30 min.				
control	33	0.23	0.23	0.51
+ acetoacetate (2mM)	6	0.16	0.21	0.60

Note: $^{45}Ca^{2+}$ estimated at 5 min:- control mitochondria (mito.) = 61 nmoles ^{45}Ca/mg protein; test mitochondria = 28 nmoles ^{45}Ca/mg protein.

At 30 min incubation, the control and the test mitochondria contained only 43 % and 8 % of the total $^{45}Ca^{2+}$ added respectively. A decrease of 0.07 μmole ATP and an increase of 0.09 μmole AMP per mg mitochondrial protein was found in the presence of acetoacetate when compared with the control experiment at 30 min.

6.3.2 The influence of redox state of mitochondrial pyridine nucleotides on Ca^{2+} uptake and release by mitochondria studied by means of the Ca-electrode.

The Ca-electrode was used to monitor Ca^{2+} uptake and release by the mitochondria for experiments in sections 6.3.2.1 to 6.3.2.5 . For these experiments, 2 mM sodium succinate was the energy substrate for Ca^{2+} uptake and the permeant anion was either Pi (i.e. the anion used in the standard incubation medium) or sodium acetate (as used by Lehninger et al (1978b)). 10^{-5}M rotenone was also included in the incubation medium to inhibit oxidation of mitochondrial NADH via the respiratory chain. Oxaloacetate was used as the oxidant of mitochondrial NADH via malate dehydrogenase (EC 1.1.1.37) and β-hydroxybutyrate as the reductant of mitochondrial NAD^+ via β-hydroxybutyrate dehydrogenase (EC 1.1.1.30).

6.3.2.1 The effect of oxaloacetate and β-hydroxybutyrate on Ca^{2+} uptake and release by mitochondria.

When 6.6 mg mitochondrial protein were added to the control incubation medium at the point indicated on fig 51(a), all the added Ca^{2+} in the medium was taken up almost immediately by the mitochondria and this Ca^{2+} was retained during the 10 min duration of the experiment. 10 mM acetate was the

FIGURE 51

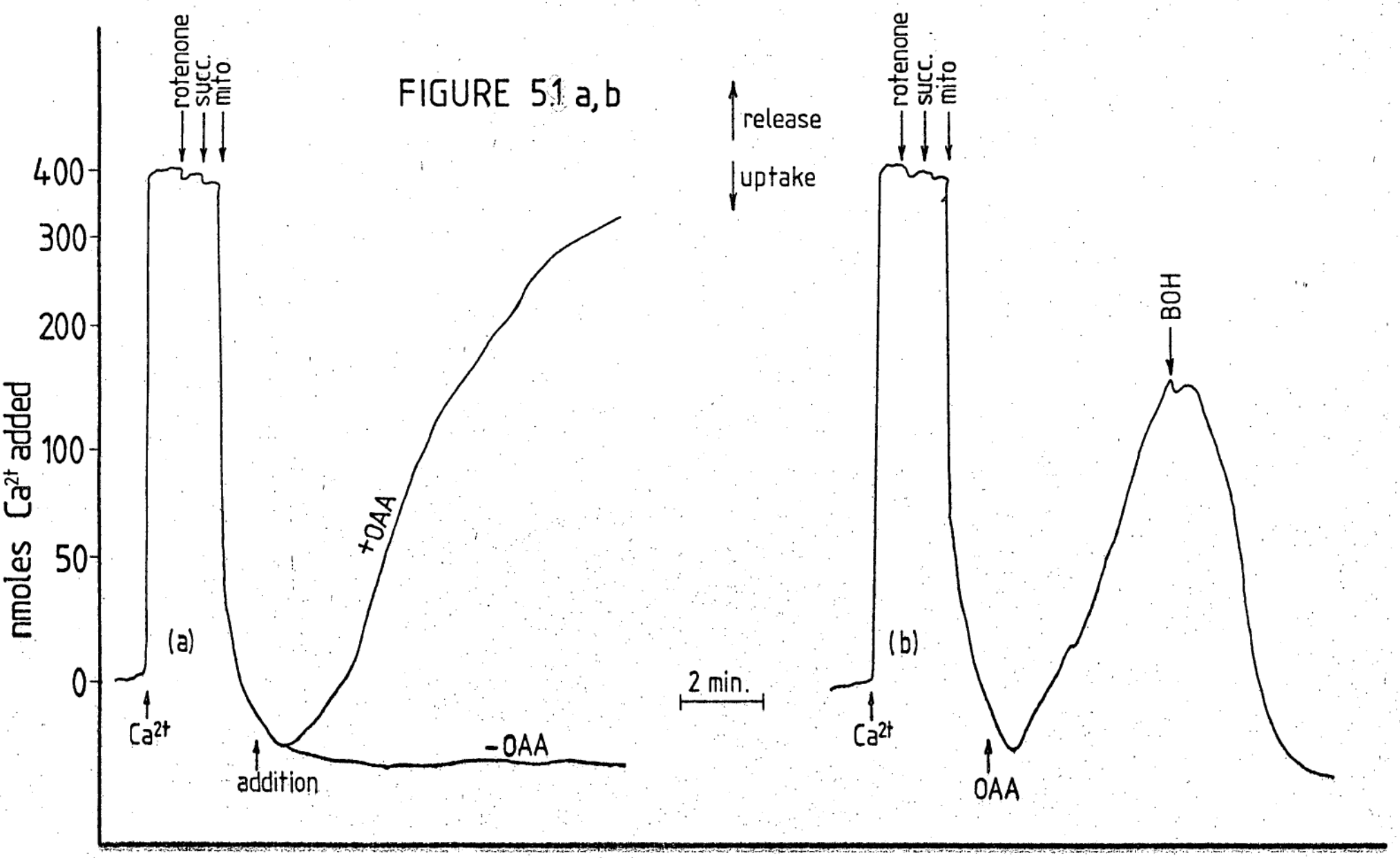
The effect of oxaloacetate and β-hydroxybutyrate on mitochondrial Ca^{2+} transport studied by the Ca-electrode.

Mitochondria (6.6 mg protein), were added to 5 ml incubation medium containing 250 mM sucrose, 2.5 mM HEPES Tris HCl pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 10 mM Na acetate, 400 nmoles Ca^{2+}, 10^{-5}M rotenone and 2 mM Na succinate (succ.). The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Incubation temp. = 25°C.

Fig 51(a): Superimposed tracings of 2 separate experiments. H_2O or oxaloacetate (OAA; final concentration = 0.5 mM) was added at the point shown (addition).

Fig 51(b): After Ca^{2+} uptake by the mitochondria, OAA (final concentration = 0.5 mM) and β-hydroxybutyrate (BOH; final concentration = 4 mM) were added at the points shown.

FIGURE 5.1 a, b


nmoles Ca2+ added
400
300
200
100
50
0
rotenone
succ.
mito
(a)
Ca2+
addition
+OAA
-OAA
release
uptake
2 min.
(b)
BOH
OAA

permeant anion for the Ca^{2+} movement into the mitochondria. Fig 51(a) shows that adding 0.5 mM oxaloacetate at the point of almost maximal Ca^{2+} uptake, caused Ca^{2+} release after a lag period of approximately 1 min. Subsequent addition of 4 mM β-hydroxybutyrate caused the Ca^{2+} released by oxaloacetate to be taken up again after approximately 1 min as shown in fig 51(b).

6.3.2.2 Ca^{2+} release from mitochondria in the presence of oxaloacetate and the effect of adding BSA or EHDP.

It has been reported that BSA and EHDP helped prolong Ca^{2+} retention in mitochondria. For the experiments in this section, 10 mM Na acetate was the permeant anion for Ca^{2+} uptake by the mitochondria. As shown in fig 52, addition of 0.5 mM oxaloacetate at the point indicated caused release of the accumulated Ca^{2+} from the mitochondria after a lag period of 1 min.

The addition of defatted BSA (final concentration = 30 μM) prior to adding oxaloacetate did not prevent Ca^{2+} release but caused reuptake of the released Ca^{2+} at 12 min.

Fig 53 shows that the presence of 0.3 mM EHDP in the incubation medium also helped Ca^{2+} reuptake after release caused by 0.5 mM oxaloacetate. Re-accumulation of approximately 50 % of the released Ca^{2+} occurred at 38 min in the presence of EHDP. EHDP did not prevent Ca^{2+} release induced by oxaloacetate.

6.3.2.3 Ca^{2+} cycling in mitochondria by altering the redox state of mitochondrial pyridine nucleotides.

Fig 54(a), (b) and (c) show that the mitochondria respiring on

FIGURE 52

Ca^{2+} release from mitochondria in the presence of oxaloacetate and the effect of adding bovine serum albumin (BSA).

Mitochondria (8 mg protein) were added to 5 ml incubation medium containing 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 10 mM Na acetate, 400 nmoles Ca^{2+}, 10^{-5}M rotenone and 2 mM Na succinate (succ.). The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Incubation temp. = 25°C. The result shows a superimposed tracing of 2 separate experiments, in the presence or absence of BSA (final concentration = 30 μM) in the incubation medium. Oxaloacetate (OAA; final concentration = 0.5 mM) was added at the point shown for both experiments.

FIGURE 52

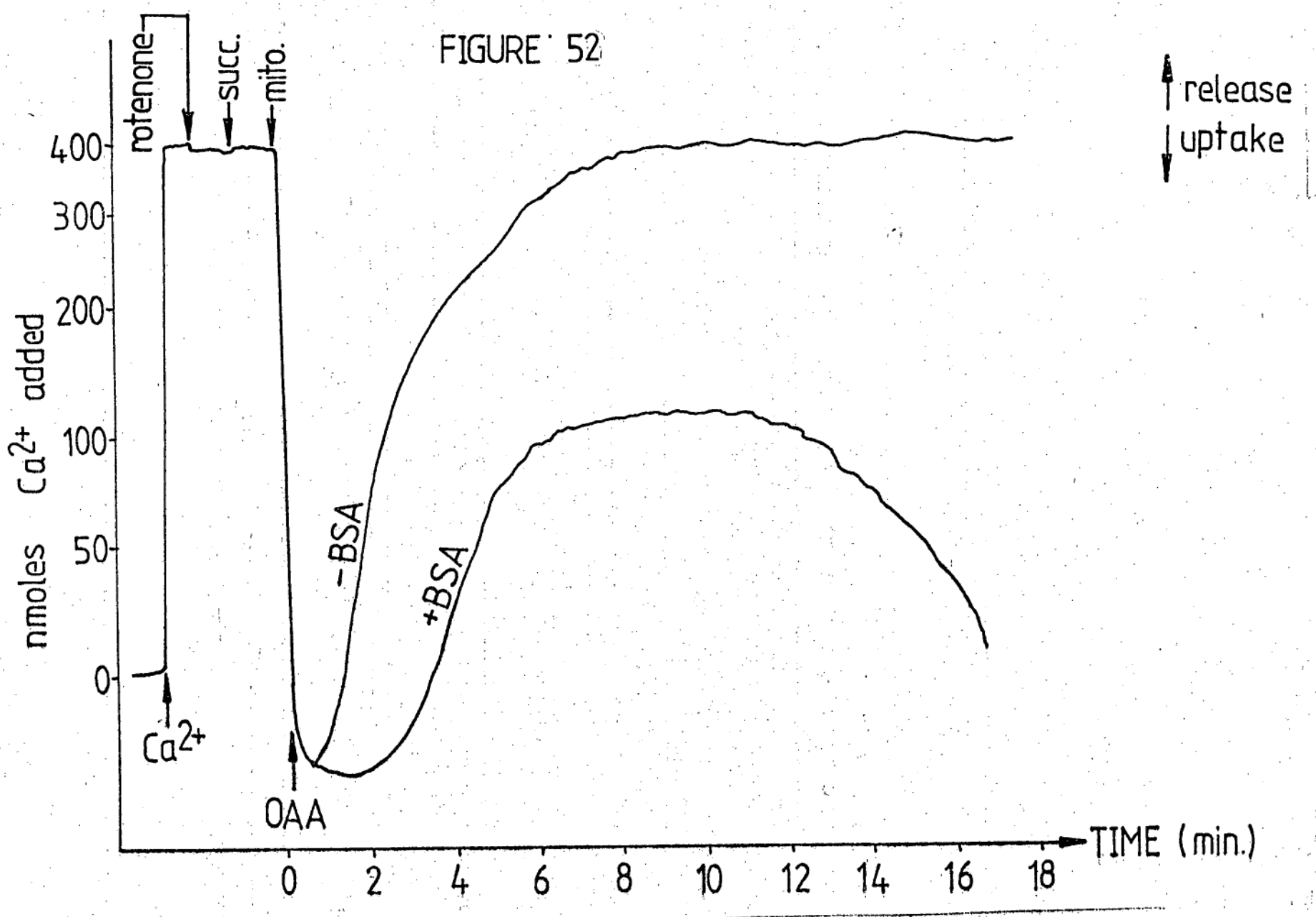

rotenone
succ.
mito.
400
300
200
100
50
0
nmoles Ca^{2+} added
Ca^{2+}
OAA
-BSA
+BSA
release
uptake
0
2
4
6
8
10
12
14
16
18
TIME (min.)

FIGURE 53

Ca^{2+} release from mitochondria in the presence of oxaloacetate and the effect of adding EHDP.

Mitochondria (8 mg protein) were added to 5 ml incubation medium containing 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 10 mM Na acetate, 400 nmoles Ca^{2+}, 10^{-5}M rotenone and 2 mM Na succinate (succ.). The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Incubation temp. = 25°C. The result shows a superimposed tracing of 2 separate experiments; in the presence of EHDP (final concentration = 0.3 mM; dashed line) or without EHDP (solid line) in the incubation medium. Oxaloacetate (OAA; final concentration = 0.5 mM) was added at the point shown for both experiments.

FIGURE 53

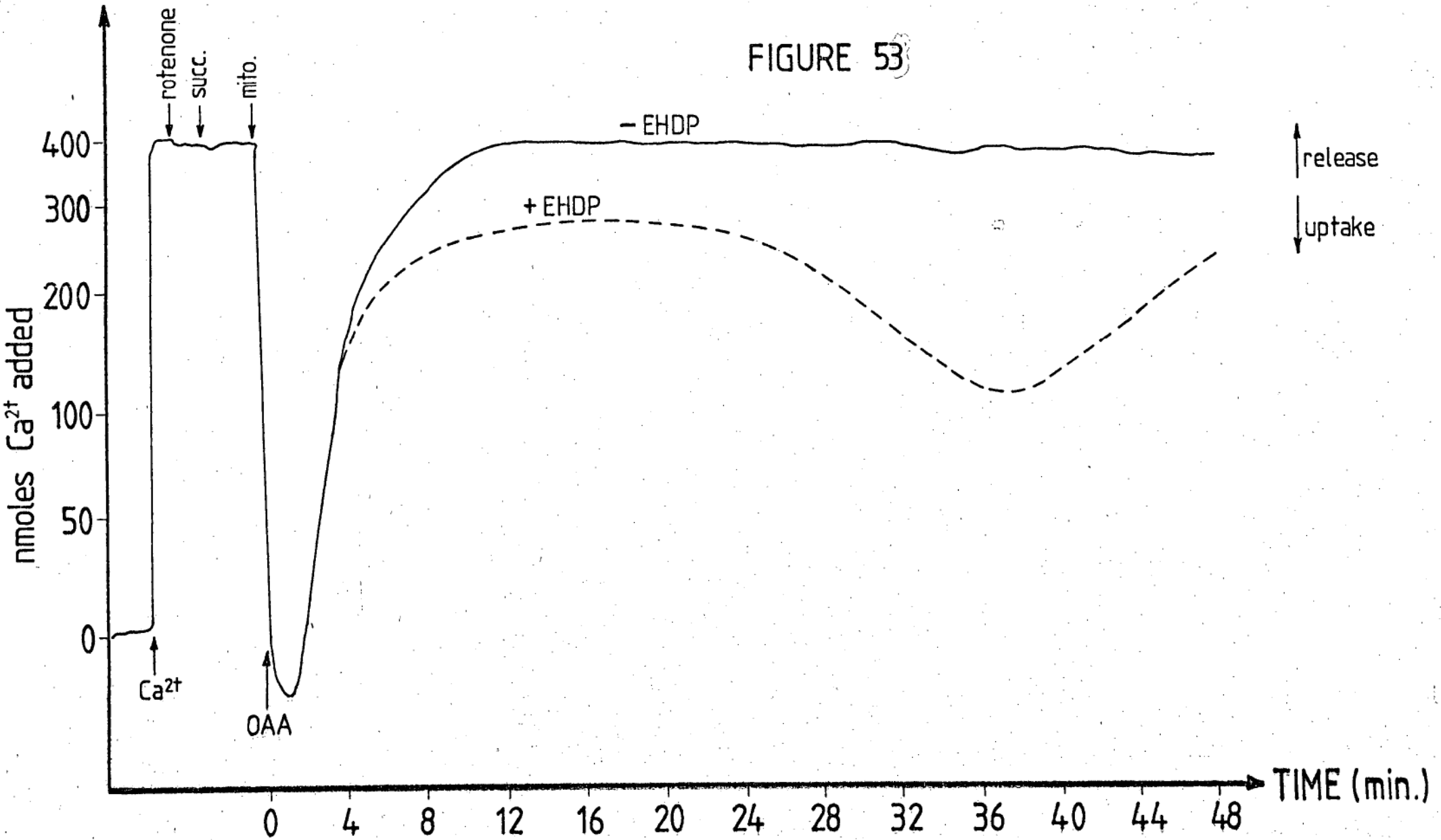

rotenone
succ.
mito.
− EHDP
+ EHDP
release
uptake
nmoles Ca2+ added
400
300
200
100
50
0
Ca2+
OAA
TIME (min.)
0
4
8
12
16
20
24
28
32
36
40
44
48

FIGURE 54

Ca^{2+} cycling in mitochondria by altering the redox state of mitochondrial pyridine nucleotides.

Mitochondria (6.6 mg protein) were added to 5 ml incubation medium containing 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, a permeant anion, 400 nmoles Ca^{2+}, 10^{-5}M rotenone and 2 mM Na succinate (succ.). The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Incubation temp. = 25°C. Addition of oxaloacetate (OAA; final concentration = 0.5 mM) and β-hydroxybutyrate (BOH; final concentration = 4 mM) is as shown.

Fig 54 (a): 0.2 mM KH_2PO_4 pH 7.2 as the permeant anion

Fig 54(b) : 2 mM KH_2PO_4 pH 7.2 as the permeant anion

Fig 54(c) : 10 mM Na acetate as the permeant anion

FIGURE 54a

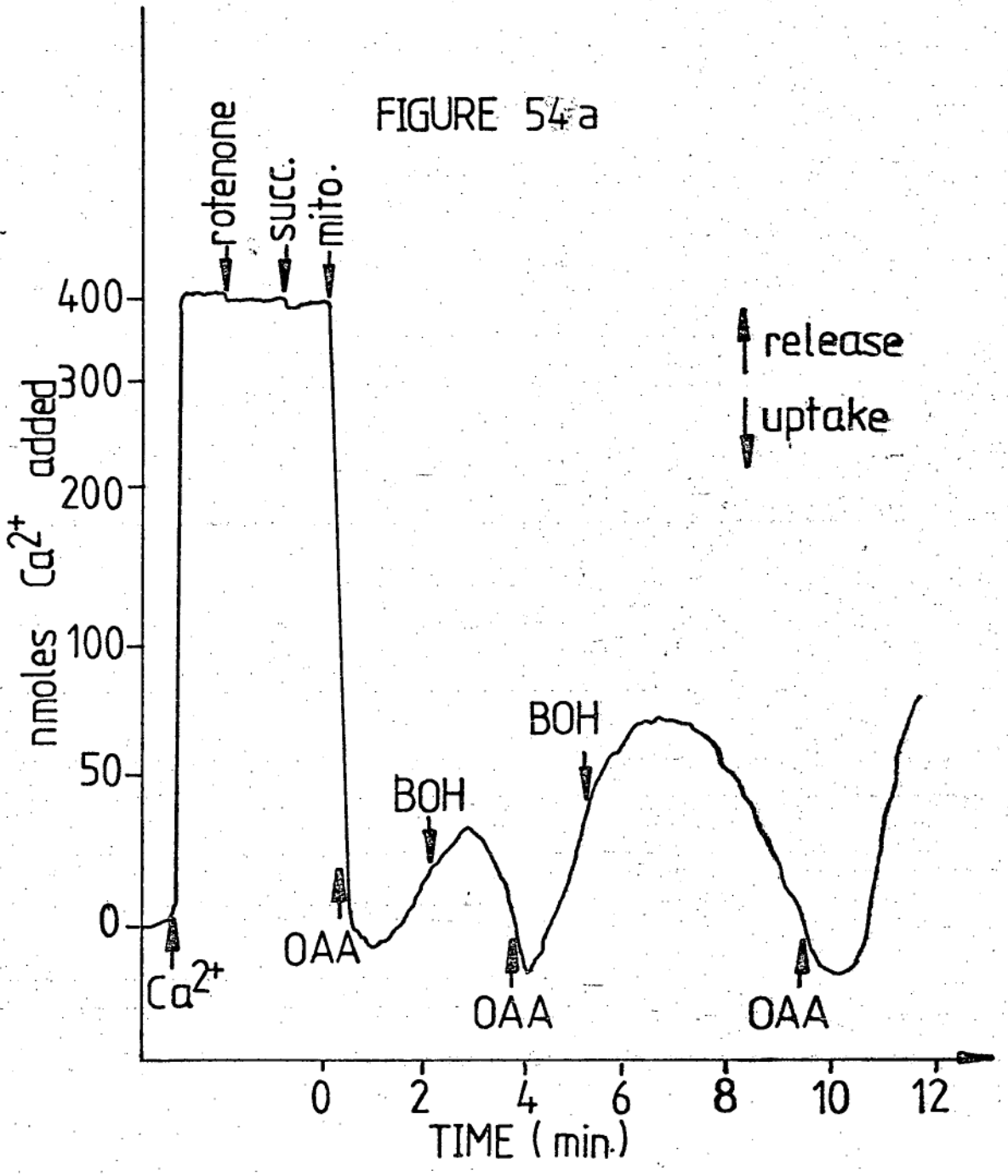

rotenone
succ.
mito.
400
300
200
100
50
0
nmoles Ca^{2+} added
release
uptake
BOH
BOH
Ca^{2+}
OAA
OAA
OAA
0
2
4
6
8
10
12
TIME (min.)

FIGURE 54 b

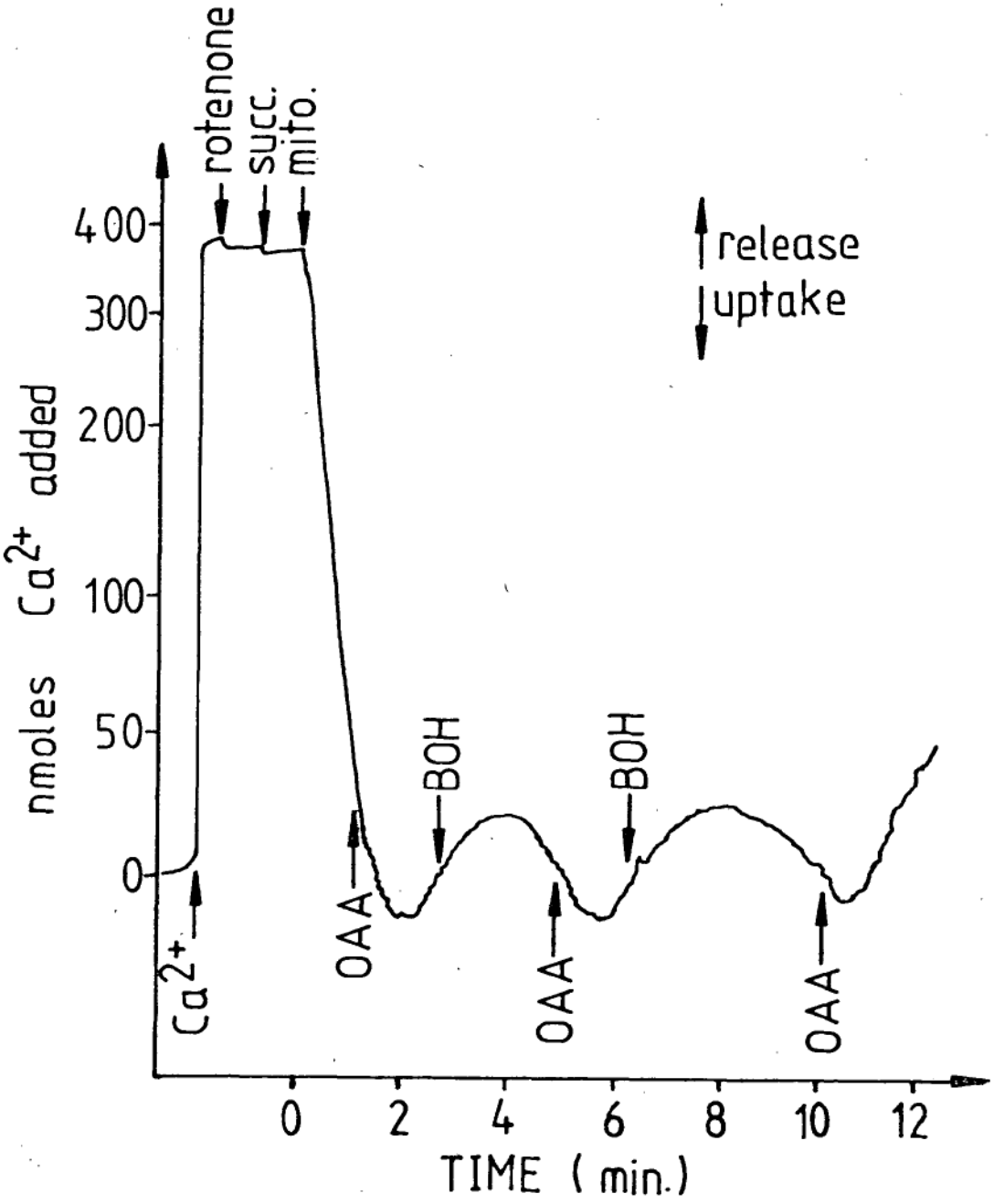
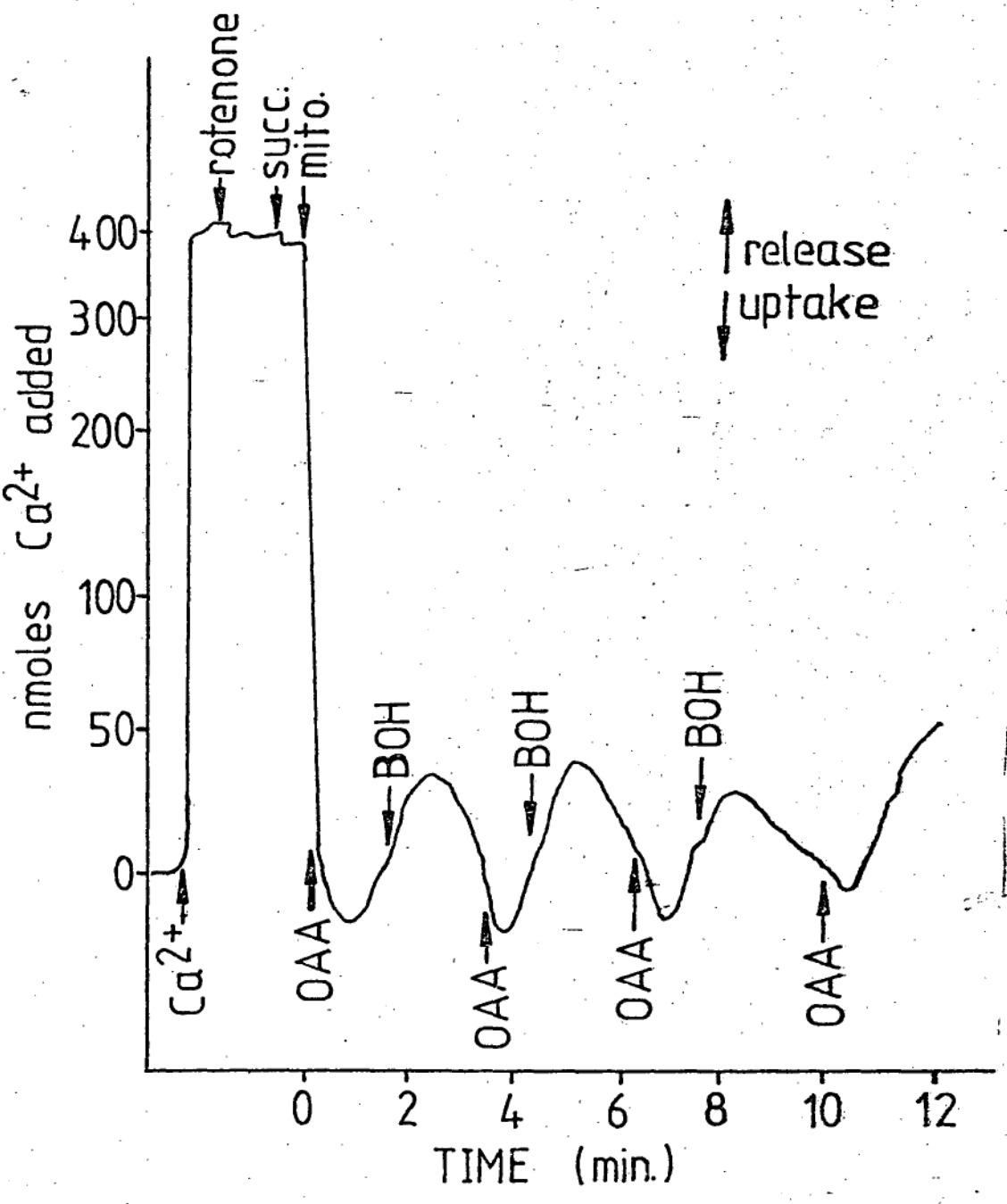

rotenone
succ.
mito.
release
uptake
nmoles Ca^{2+} added
400
300
200
100
50
0
Ca^{2+}
OAA
BOH
OAA
BOH
OAA
0
2
4
6
8
10
12
TIME (min.)

FIGURE 54 c


rotenone
succ.
mito.
release
uptake
nmoles Ca^{2+} added
400
300
200
100
50
0
Ca^{2+}
OAA
BOH
OAA
BOH
OAA
BOH
OAA
0
2
4
6
8
10
12
TIME (min.)

2 mM sodium succinate, exhibited cycles of Ca^{2+} release and reuptake on alternately adding 0.5 mM oxaloacetate and 4 mM β-hydroxybutyrate respectively. Calcium cycling in the mitochondria was observed in the presence of either 0.2 mM Pi (fig 54 (a)), 2 mM Pi (fig 54(b)) or 10 mM sodium acetate (fig 54(c)). It should be noted however, that it was relatively more difficult to obtain more than 1 cycle of Ca^{2+} release and reuptake in the presence of 2 mM Pi compared with 0.2 mM Pi or 10 mM sodium acetate.

6.3.2.4 Ca^{2+} cycling in mitochondria and the corresponding change in the concentrations of mitochondrial adenine nucleotide

At the points of Ca^{2+} release and reuptake during Ca^{2+} cycling in the mitochondria as shown in fig 55, 0.5 ml of the incubation mixtures was removed and concentrations of adenine nucleotide in the mitochondrial pellets were estimated after centrifugation through silicone oil as described in chapter 1, section 1.6.2. Table 13 shows that the concentrations of mitochondrial adenine nucleotides remained fairly steady although Ca^{2+} was lost or gained during the cycles.

6.3.3 The possibility of Ca-pyridine nucleotide complex in non-aqueous phase

Two approaches were attempted namely (i) organic phase extraction of a lipid soluble radioactive labelled $^{45}Ca^{2+}$ complex, (ii) the formation of a Ca-complex was studied using

FIGURE 55

Ca^{2+} cycling in mitochondria and the corresponding change in concentrations of mitochondrial adenine nucleotides.

Mitochondria (20 mg protein) were added to 5 ml incubation medium containing 250 mM sucrose, 2.5 mM HEPES Tris pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 10 mM Na acetate, 400 nmoles Ca^{2+}, 10^{-5}M rotenone and 2 mM Na succinate (succ.). The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Incubation temp. = 25°C. Addition of oxaloacetate (OAA; final concentration = 0.5 mM) and β-hydroxybutyrate (BOH; final concentration = 4 mM) is shown. At the point shown (↯), 0.5 ml of the incubation medium was removed and concentrations of adenine nucleotide in the mitochondrial pellets were estimated after centrifugation through silicone oil as described in section 1.6 . The result is shown in table 13.

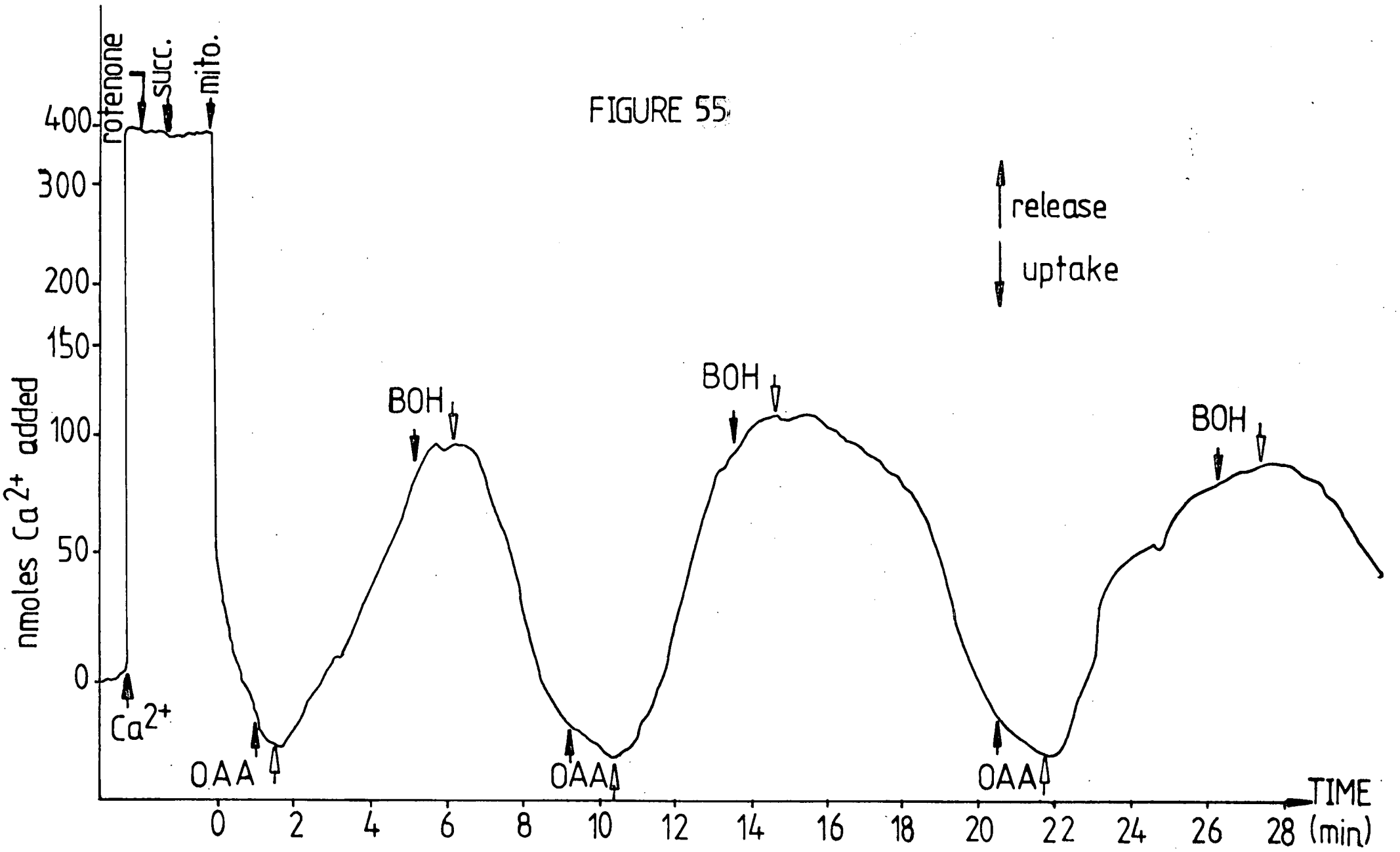


FIGURE 55
rotenone
succ.
mito.
400
300
200
150
100
50
0
nmoles Ca 2+ added
release
uptake
Ca2+
OAA
BOH
OAA
BOH
OAA
BOH
TIME
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
(min)

TABLE 13

Calcium release and re-uptake in rat liver mitochondria as monitored by the redox state of mitochondrial pyridine nucleotides and the corresponding change in mitochondrial adenine nucleotides.

Calcium uptake by rat liver mitochondria using a Ca-sensitive electrode: rat liver mitochondria (20 mg), were added to 5 ml of 0.25M sucrose, 2.5 mM HEPES pH 7.4, 10 mM Na acetate pH 7.2, 1 mM $MgCl_2$, 72 mM KCl, 2 mM Na succinate, 10^{-5}M rotenone and 400 nmoles Ca^{2+}. Calcium release from mitochondria was induced by 0.5 mM oxaloacetate and calcium re-uptake by adding 4 mM β-hydroxybutyrate. 0.5 ml samples at various points during the successive cycles of Ca^{2+} uptake and release were estimated for adenine nucleotides. The mitochondrial pellets were obtained by silicone centrifugation. Maximum possible calcium accumulated in mitochondria = 20 nmoles/mg mitochondrial protein.

	Ca^{2+} nmoles/mg mitochondrial protein	ATP	ADP	AMP
		nmoles/mg mitochondrial protein		
Uptake	20.0	6.8	10.0	10.0
1st release by oxaloacetate	15.5	9.0	10.3	6.0
Re-uptake by β-hydroxybutyrate	20.0	7.0	10.6	10.6
2nd release by oxaloacetate	14.8	8.0	10.2	6.3
Re-uptake by β-hydroxybutyrate	20.0	6.7	9.8	9.8
3rd release by oxaloacetate	16.0	10.0	10.3	12.0

High Performance Liquid Chromatography (HPLC).

6.3.3.1 The possibility of Ca-pyridine nucleotide complex in non-aqueous phase studied by means of organic phase extraction of $^{45}Ca^{2+}$.

A variety of organic solvents was examined for this particular study. They were:-

(i) 6 % tripalmitin dissolved in n-butanol/benzene, 10/40

(ii) 2.8 % tripalmitin dissolved in n-butanol/benzene, 10/40

(iii) n-butanol/benzene, 25/25

(iv) benzene/olive oil/H_2O, 20/20/20

(v) hexanol

2 ml of the organic solvent was pipetted to 2 ml of aqueous solution containing 0.1 mM $^{45}CaCl_2$ (200 nmoles $^{45}CaCl_2$, approximately 1 x 10^6cpm) and 0.5, 1 or 2 mM pyridine nucleotide. The negative control consisted of 2 ml organic solvent + 2 ml of 0.1 mM $^{45}CaCl_2$. For a positive control:- 2 ml organic solvent + 2 ml of 0.1 mM $^{45}CaCl_2$ and 0.5 mM sodium lauryl sulphate (to ensure that ^{45}Ca complexes can be detected in the organic phase (Webling and Holdsworth, 1966)). The organic and aqueous solution was whirl-mixed, and the mixture was centrifuged to separate the two layers. 0.5 ml of the top layer (i.e. the organic phase) was then pipetted and ^{45}Ca estimated by scintillation counting.

The results in table 14 show that under the conditions tested, the ^{45}Ca count in the respective organic phase was not significantly different from the negative control (i.e. in the presence of ^{45}Ca alone). Most of the radioactivity would be in the aqueous layer. With sodium lauryl sulphate as the positive control, significant ^{45}Ca counts was noted in all the

TABLE 14

Organic phase extraction of $^{45}Ca^{2+}$ in the presence of pyridine nucleotide.

The method is described in section 6.3.3.1

Samples present in the aqueous phase	$^{45}Ca^{2+}$ cpm in 0.5 ml organic phase				
	6 % tripalmitin in n-butanol/benzene	2.8 % tripalmitin in n-butanol/benzene	n-butanol/benzene	benzene/olive oil/H_2O	hexanol
$^{45}Ca^{2+}$ alone (negative control)	136	121	105	1,322	109
$^{45}Ca^{2+}$ + Na Lauryl SO_4 (positive control)	63,453	38,515	42,199	20,971	2,218
$^{45}Ca^{2+}$ + NAD^+	118 (0.5 mM)	128 (1 mM) 107 (2 mM)	88 (1 mM) 75 (2 mM)	not tested	not tested
$^{45}Ca^{2+}$ + NADH	378 (0.5 mM)	129 (1 mM) 127 (2 mM)	80 (1 mM) 91 (2 mM)	1,009 (1 mM) 3,900 (2 mM)	99 (1 mM)
$^{45}Ca^{2+}$ + $NADP^+$	179 (0.5 mM)	100 (1 mM) 86 (2 mM)	101 (1 mM) 69 (2 mM)	not tested	not tested
$^{45}Ca^{2+}$ + NADPH	not tested	116 (1 mM) 110 (2 mM)	94 (1 mM)	not tested	not tested

note: the concentration in brackets indicate the concentration of pyridine nucleotide.

organic phases examined with the exception of hexanol (table 14).

6.3.3.2 The possibility of Ca-pyridine nucleotide complex in non-aqueous phase studied by means of High Performance Liquid Chromatography (HPLC).

An attempt was made to see if Ca^{2+} affects retention of pyridine nucleotides in a non-polar μ-Bondapak C_{18} column since this would indicate interaction between the two substances. The liquid chromatograph used for this study was described in chapter 1, section 1.7 . The reverse phase column (i.e. non-polar stationary phase with polar solvent) contained micro-Bondapak C_{18} with 0.1 M Tris buffer pH 7.4 as the solvent. (Note: the functional group of the Bondapak C_{18} is - $Si(CH_2)_{17}CH_3$.) The study is based on the fact that the more polar sample will be eluted first from the reverse phase column. 10 μl pyridine nucleotide (1 mg/ml) or 10 μl pyridine nucleotide (1 mg/ml) + Ca^{2+} (50 mM) was injected into the column and the elution position of the pyridine nucleotide detected at wavelength 254 nm. The elution position was expressed as distance in cm from the point of injection. The flow rate of eluant = 1 ml/min; AUFS = 0.5 . The elution position of the various pyridine nucleotides with or without Ca^{2+}/Mg^{2+} is as shown in table 15.

A symmetrical peak due to $NADP^+$ was observed at 2.7 cm from the point of injection. When 10 μl solution of $NADP^+$ and Ca^{2+} was injected into the column, the eluant position of $NADP^+$ increased from 2.7 to 3.7 cm, indicating retention of $NADP^+$ in the C_{18} column (fig 56(a)). A shoulder possibly due to free $NADP^+$ was noted at 2.7 cm.

TABLE 15

High Performance Liquid Chromatography of pyridine nucleotide in the absence of presence of Ca^{2+}/Mg^{2+}.

The method is described in section 6.3.3.2.

Samples	elution position (distance from point of injection) (cm)
$NADP^+$	2.8
$NADP^+ + Ca^{2+}$	3.7
$NADP^+ + Mg^{2+}$	3.8
NADPH	3.0
$NADPH + Ca^{2+}$	4.2
$NADPH + Mg^{2+}$	4.0
NAD^+	8.9
$NAD^+ + Ca^{2+}$	9.2
NADH	7.4
$NADH + Ca^{2+}$	7.4
$(NADP^+ + Ca^{2+}) + (NADPH + Ca^{2+})$	4.0

FIGURE 56

High Performance Liquid Chromatography (HPLC) of pyridine nucleotide in the presence of Ca^{2+} (or Mg^{2+}).

The method is described in section 6.3.3.2 . The liquid chromatograph used for this study is described in section 1.7 . 10 µl pyridine nucleotide (1 mg/ml) or 10 µl pyridine nucleotide (1 mg/ml) + Ca^{2+} or Mg^{2+} (final concentration = 50 mM) was injected into the column and the elution position of the pyridine nucleotide detected at wavelength 254 nm.

Experiment (a): (——) NADP
(----) NADP + Ca^{2+}

Experiment (b): (——) NADPH
(----) NADPH + Ca^{2+}

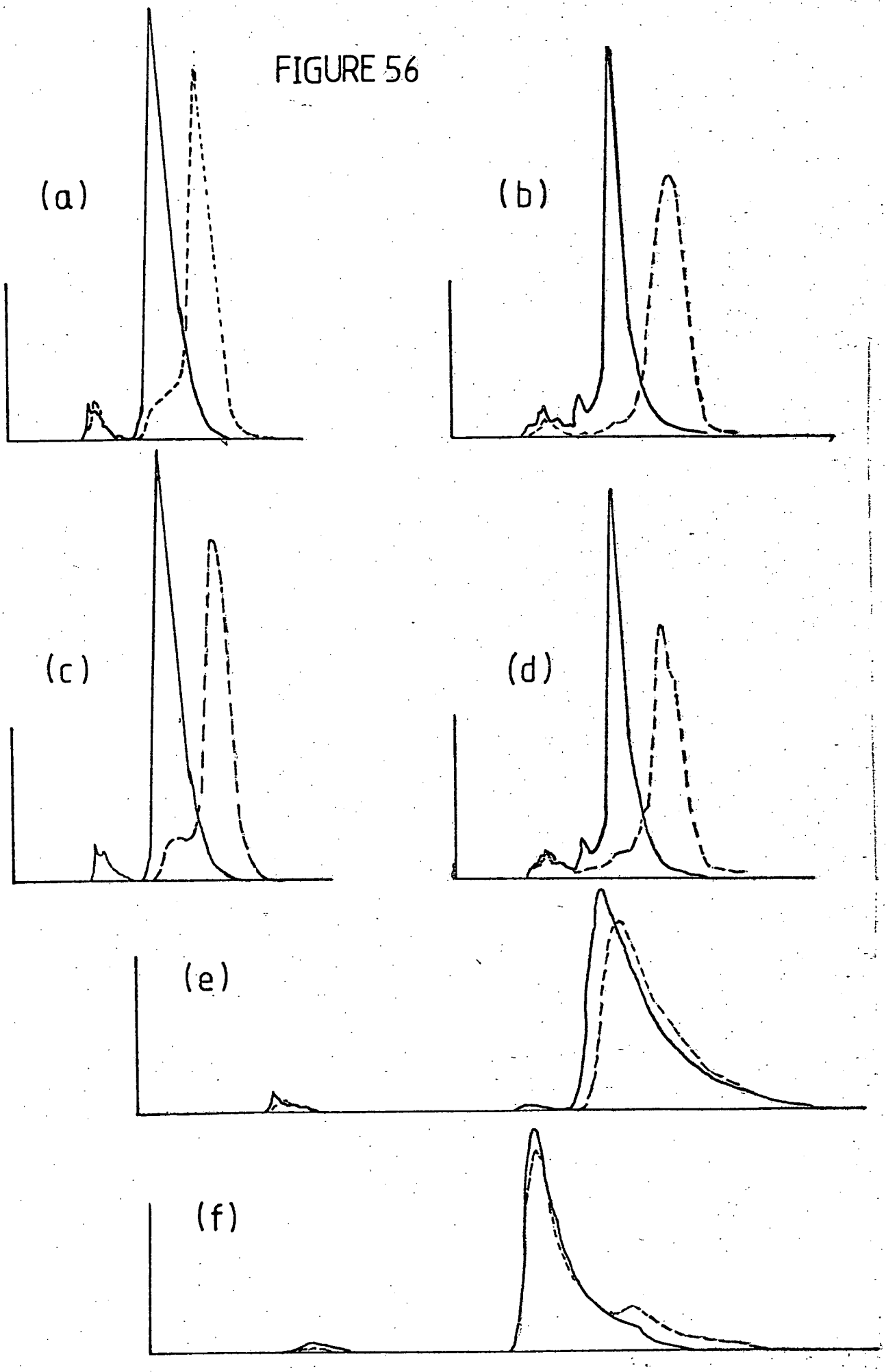
Experiment (c): (——) NADP
(----) NADP + Mg^{2+}

Experiment (d): (——) NADPH
(----) NADPH + Mg^{2+}

Experiment (e): (——) NAD
(----) NAD + Ca^{2+}

Experiment (f): (——) NADH
(----) NADH + Ca^{2+}

FIGURE 56



NADPH was eluted later than $NADP^+$ (i.e. at 3.0 cm) and the presence of Ca^{2+} increased the elution position from 3.0 cm to 4.2 cm (fig 56(b)).

When Ca^{2+} was replaced by a similar concentration of Mg^{2+}, the $NADP^+$ or NADPH was again eluted later than in the absence of cation (fig 56(c) and (d)).

A non-symmetrical peak was detected at 8.9 cm after chromatography of NAD^+. The presence of Ca^{2+} did not significantly affect the retention of NAD^+ in the column (fig 56(e)).

The elution position for NADH in the presence or absence of Ca^{2+} was 7.4 cm. When Ca^{2+} was included, a shoulder was observed at 9.2 cm possibly due to contaminating NAD^+ (fig 56(f)).

Prior to using the 0.1 M Tris buffer pH 7.4 as the solvent, other solvents were tested, e.g. methanol/H_2O, 50/50; methanol/H_2O, 20/80; methanol/H_2O, 5/95. The flow rate was either 2 ml or 4 ml per min. Under the stated conditions, the samples migrated too quickly from the column (i.e. elution position approximately 1 cm from the point of injection). Similarly a quick elution of the samples was observed when using μ Bondapak-CN column with methanol/H_2O (50/50 or 5/95) as solvent.

6.3.4 The effect of externally added pyridine nucleotides on mitochondrial Ca^{2+} transport studied by means of the Ca-electrode.

The Ca-binding activity of the pyridine nucleotide was examined in section 6.3.4.1 and the effect of the pyridine nucleotide on Ca^{2+} uptake and release by the mitochondria in

section 6.3.4.2 . Ca^{2+} movement in and out of the mitochondria was monitored by the Ca-electrode.

6.3.4.1 Ca-binding activity of pyridine nucleotide using the Ca-electrode.

Fig 57 shows an insignificant binding of Ca^{2+} to 0.5 mM NADH, NAD^+ or NADPH in the presence of the standard incubation mixtures and 400 nmoles $CaCl_2$. Addition of 0.5 mM $NADP^+$ from Boehringer did not significantly reduce the concentration of free Ca^{2+} in the medium; however, the addition of 0.5 mM $NADP^+$ from Calbiochem resulted in an increase in free Ca^{2+} in the sucrose medium (fig 58), suggesting Ca^{2+} contamination of the nucleotide. To show that the observed effect was due to Ca^{2+} contamination and not merely due to interference effect on the Ca^{2+}-electrode, 1.25 mM ATP was added to the sucrose medium containing the 0.5 mM $NADP^+$ but without externally added Ca^{2+}. As shown in chapter 5, section 5.3.2, ATP has a high affinity for Ca^{2+} and the Ca^{2+} present in the $NADP^+$ was complexed by 1.25 mM ATP reducing the concentration of Ca^{2+} from 250 nmoles Ca^{2+} to approximately 75 nmoles. For future experiments, $NADP^+$ from Boehringer was used.

6.3.4.2 The effect of pyridine nucleotides on mitochondrial Ca^{2+} transport.

The effect of 0.5 mM NAD^+, NADH, $NADP^+$ and NADPH on mitochondrial Ca^{2+} transport was studied by means of the Ca-electrode. The energy source for mitochondrial Ca^{2+} uptake was 2 mM succinate in the presence of 10^{-5}M rotenone. In the absence of added pyridine nucleotide, the control mitochondria (6.6 mg mitochondrial protein) accumulated all the added

FIGURE 57

Ca^{2+}-binding activity of pyridine nucleotide using the Ca-electrode.

Pyridine nucleotide (final concentration = 0.5 mM) was added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture and 400 nmoles Ca^{2+}. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C.

Experiment (a): + NADH

Experiment (b): + NAD^+

Experiment (c): + NADPH

FIGURE 57

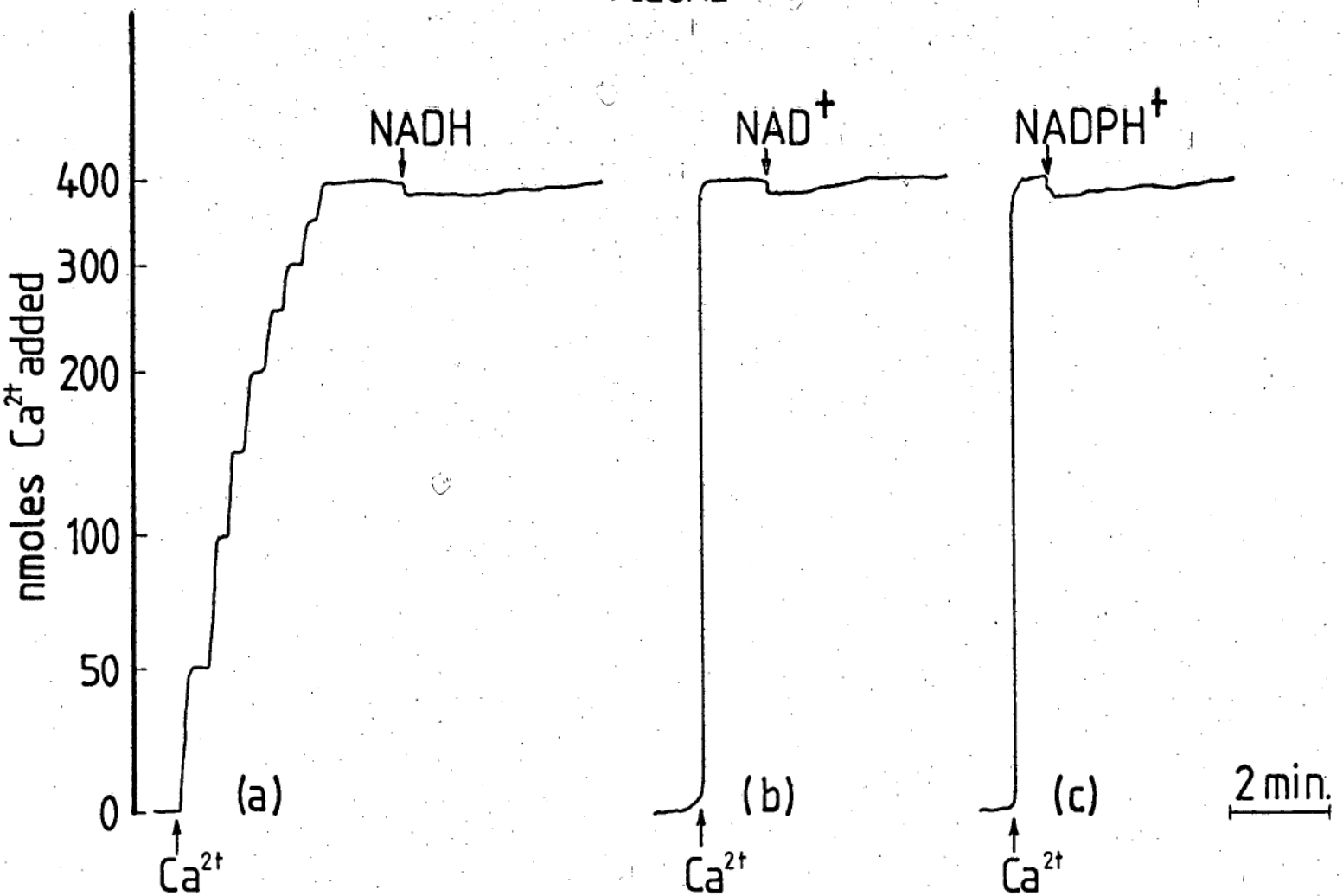

nmoles Ca^{2+} added
400
300
200
100
50
0
NADH
NAD^+
$NADPH^+$
(a)
(b)
(c)
Ca^{2+}
Ca^{2+}
Ca^{2+}
2 min.

FIGURE 58

Ca^{2+}-binding activity of $NADP^+$ using the Ca-electrode.

$NADP^+$ (final concentration = 0.5 mM) was added to incubation medium (final vol. = 5 ml) containing the standard incubation mixture and 400 nmoles Ca^{2+}. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C.

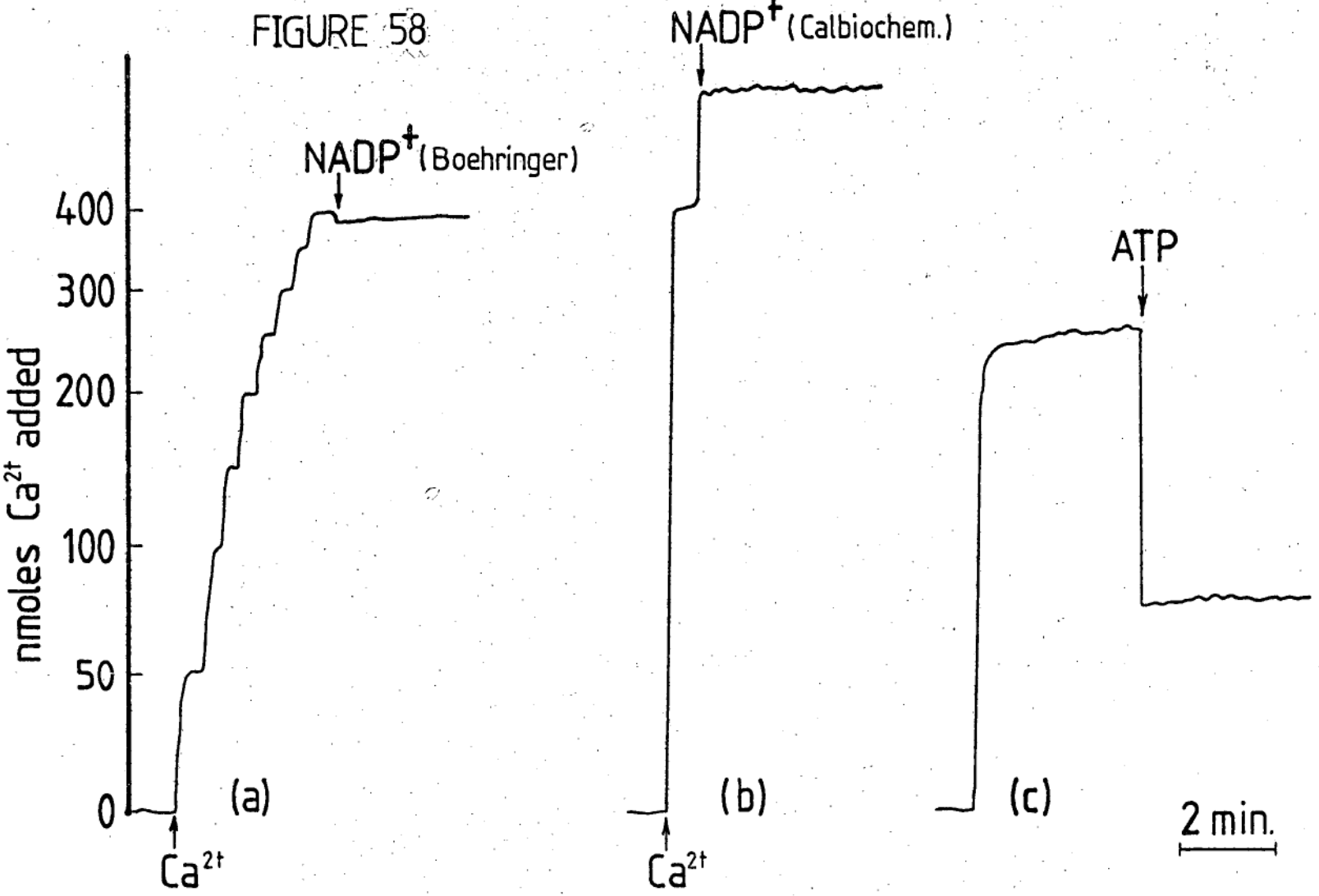
Experiment (a): + $NADP^+$ (from Boehringer)

Experiment (b): + $NADP^+$ (from Calbiochem)

FIGURE 58(c)

ATP (final concentration = 1.25 mM) was added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture and 0.5 mM $NADP^+$ (from Calbiochem).

FIGURE 58


NADP+ (Boehringer)
NADP+ (Calbiochem.)
ATP
nmoles Ca2+ added
400
300
200
100
50
0
Ca2+
Ca2+
(a)
(b)
(c)
2 min.

400 nmoles Ca^{2+} and the mitochondria started to release this Ca^{2+} at 24 min as shown in fig 59(a).

In the presence of NAD^+ (final concentration 0.5 mM), the mitochondria retained the accumulated Ca^{2+} up to 42 min after which time the experiment was terminated (fig 59(b)).

Including NADH (final concentration 0.5 mM) in the incubation medium resulted in Ca^{2+} retention in the mitochondria compared with the control experiment (fig 59(c)). Since some of the NADH will be oxidised to NAD^+ during the long incubation, the concentration of NADH in the medium at 40 min was estimated by determining its absorbance at 340 nm (SP 1800 Spectrophotometer). Molar extinction coefficient of NADH at 340 nm = 6.2×10^3. 2.5 µmoles NADH was initially added to the 5 ml incubation medium. At 40 min incubation, between 2.1 - 1.9 µmole of NADH was estimated in the clear incubation medium.

Fig 59(d) shows that Ca^{2+} uptake and release by the mitochondria was not affected in the presence of $NADP^+$ (Boehringer) (final concentration 0.5 mM) compared with the control experiment. A similar result was obtained in the presence of 0.125 mM $NADP^+$ (fig 59(e)).

In the presence of NADPH (final concentration 0.5 mM), the mitochondria accumulated all the externally added Ca^{2+} and started to release this Ca^{2+} at 15 min (fig 59(f)). The control mitochondria however released their accumulated Ca^{2+} at 24 min (fig 59(a)) suggesting that NADPH caused an earlier Ca^{2+} release from the mitochondria.

FIGURE 59

The effect of externally added pyridine nucleotide on mitochondrial Ca^{2+} transport using the Ca-electrode.

Mitochondria (6.6 mg protein), were added to 5 ml incubation medium containing the standard incubation mixture, 400 nmoles Ca^{2+}, 10^{-5}M rotenone and 2 mM Na succinate (succ.). The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C. Addition of the pyridine nucleotide was made before adding the Ca^{2+}. The results shown are superimposed tracings of 2 to 3 separate experiments.

fig 59 a, b, c

——— (a) control experiment (in the absence of externally added pyridine nucleotide)

– – – – (b) + NAD^+ (final concentration = 0.5 mM)

– – – – (c) + NADH (final concentration = 0.5 mM)

fig 59 a, d, e

——— (a) control experiment

– – – (d) + $NADP^+$ (final concentration = 0.5 mM)

..... (e) + $NADP^+$ (final concentration = 0.125 mM)

fig 59 a, f

——— (a) control experiment

– – – (f) + NADPH (final concentration = 0.5 mM)

FIGURE 59 a, b, c

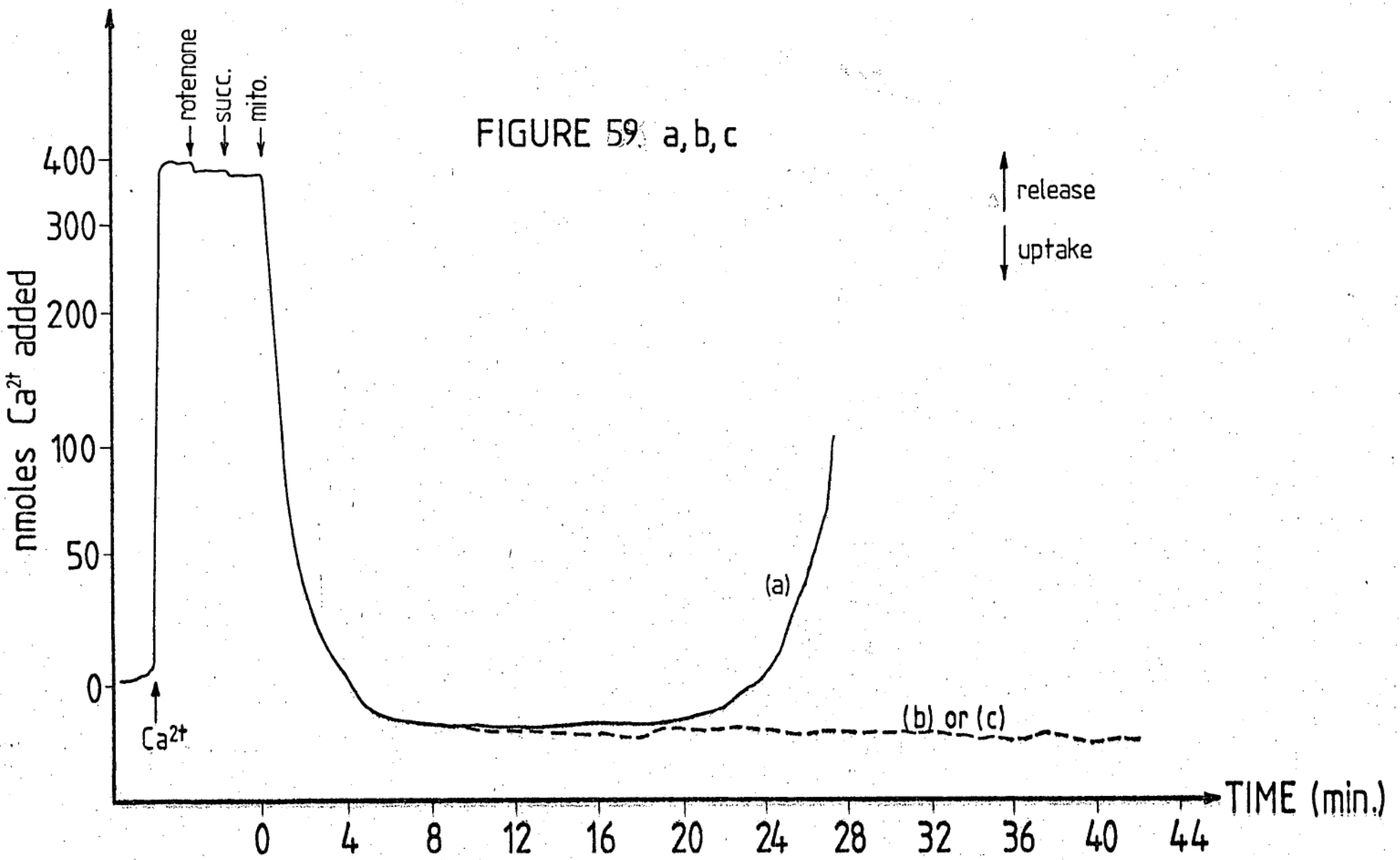


FIGURE 59 a,d,e

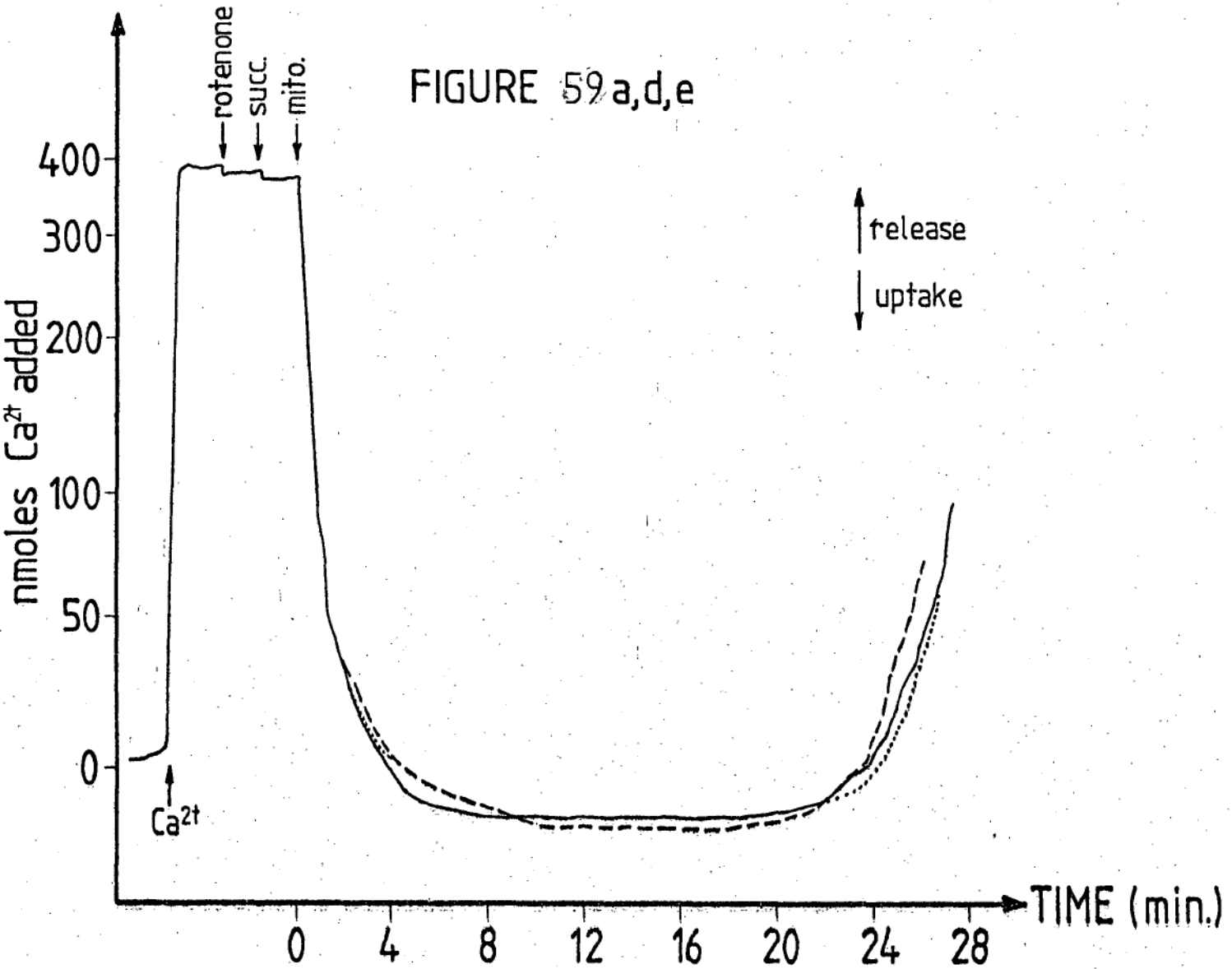
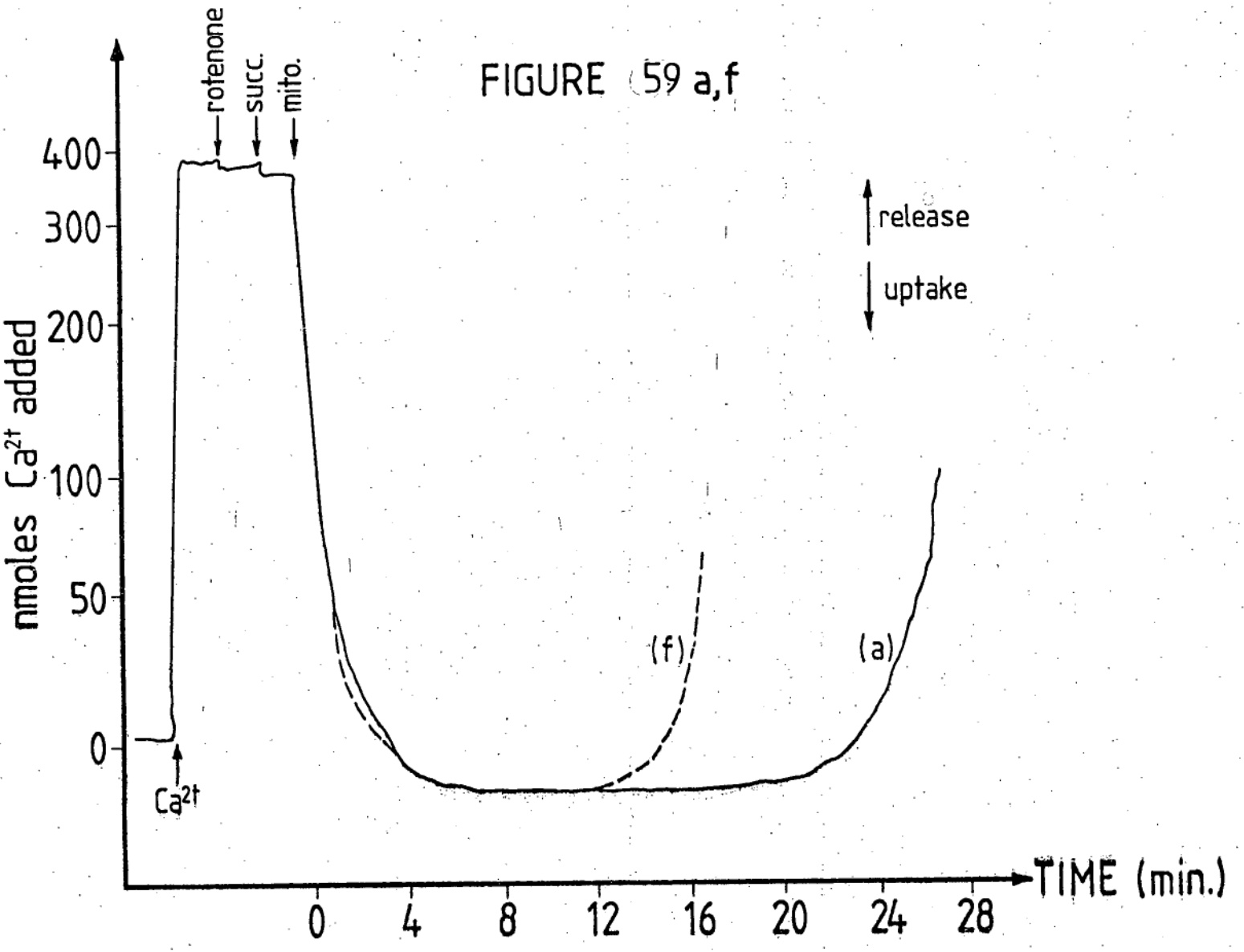


FIGURE 59 a,f


rotenone
succ.
mito.
400
300
200
100
50
0
nmoles Ca^{2+} added
Ca^{2+}
release
uptake
(f)
(a)
0
4
8
12
16
20
24
28
TIME (min.)

6.4 DISCUSSION

Ca^{2+} movement in the presence of oxaloacetate and β-hydroxybutyrate.

The redox state of mitochondrial pyridine nucleotide can influence Ca^{2+} release and retention in mitochondria from rat heart, liver and also Ehrlich tumour cells (Lehninger et al, 1978). It was observed in this thesis that the mitochondria respiring on succinate (2mM), in the presence of 10^{-5}M rotenone, released the accumulated Ca^{2+} after adding 0.5 mM oxaloacetate (oxidant of mitochondrial NADH via malate dehydrogenase), and that the released Ca^{2+} was taken up again after adding 4 mM β-hydroxybutyrate (reductant of mitochondrial NAD^+ via β-hydroxybutyrate dehydrogenase). The observation is in agreement with that of Lehninger et al (1978).

In addition, the present study also showed that BSA or EHDP, i.e. substances known to help Ca^{2+} retention in mitochondria, caused Ca^{2+} reuptake after release caused by 0.5 mM oxaloacetate. However BSA or EHDP did not prevent Ca^{2+} release induced by oxaloacetate. The results suggest that the presence of BSA and EHDP in the incubation medium possibly favours the reduced state of the mitochondrial pyridine nucleotide.

Ca^{2+} cycling in mitochondria by altering the redox state of mitochondrial pyridine nucleotides.

More than 1 cycle of Ca^{2+} release and reuptake by the mitochondria was observed when the redox state of the mitochondrial pyridine nucleotide was altered by alternately adding oxaloacetate and β-hydroxybutyrate (figs 54(a),(b),(c)).

It should be noted that Lehninger et al (1978) reported successive cycles of Ca^{2+} release and reuptake in the presence of 10 mM acetate. In the present study, the Ca^{2+} cycling was observed when either 0.2 mM Pi, 2 mM Pi or 10 mM sodium acetate was the permeant anion. However it was difficult to obtain more than 1 cycle of Ca^{2+} release and reuptake in the presence of 2 mM Pi. A possible explanation is the formation of calcium phosphate precipitate in the matrix when the mitochondria accumulate large amounts of Pi with Ca^{2+}; however calcium acetate in the matrix is soluble (Lehninger et al, 1967; Lehninger, 1970).

Concentrations of adenine nucleotide in mitochondria during Ca^{2+} cycling.

During Ca^{2+} cycling in the presence of 10 mM sodium acetate, samples were removed at the point of Ca^{2+} release and reuptake and adenine nucleotides in the mitochondria were estimated after centrifugation through silicone oil. As shown in table 13, the concentrations of mitochondrial adenine nucleotides during Ca^{2+} release (after adding oxaloacetate) and reuptake (after adding β-hydroxybutyrate) remained fairly steady, i.e. per mg mitochondrial protein, mitochondrial ATP = 7 nmole, ADP = 10 nmole and AMP = 10 nmole. It should be noted that the incubation medium for the above investigation did not contain externally added ATP or ADP (compare with the previous estimation of mitochondrial adenine nucleotide in chapter 5, diagram 5, in the presence of 1 mM ATP, the mitochondrial ATP was 12 nmoles, ADP = 30 nmoles and AMP = 12 nmoles per mg protein).

Total adenine nucleotides (in medium plus mitochondria) during Ca^{2+} release caused by acetoacetate.

The total adenine nucleotides (in medium plus mitochondria) during Ca^{2+} release caused by acetoacetate was also examined in this thesis. For this study, the mitochondria were incubated with 0.5 mM ATP and 0.5 mM ADP as the energy sources in the presence of 10^{-5}M rotenone.

As shown in table 12, at 10 min incubation the control mitochondria contained 79 % of the added $^{45}Ca^{2+}$, while the test mitochondria (i.e. in the presence of 2 mM acetoacetate) contained 25 % of the total Ca^{2+} in the medium. The differences in the total adenine nucleotide concentrations between test and control was much less in the case of acetoacetate than in similar experiments where palmitoyl CoA or rat albumin was used instead (see table below). The result suggests that the Ca^{2+} release observed at 10 min in the presence of acetoacetate is not due to a decrease in the total ATP concentration but due to the oxidised state of the mitochondrial pyridine nucleotides.

At 30 min incubation, the release of Ca^{2+} from the control mitochondria was most likely due to depletion in external ATP, since the total ATP had decreased from 0.38 to 0.23 μmole per mg mitochondrial protein. Interestingly, the total ATP concentration in the presence of acetoacetate was not significantly different from the control experiment at 30 min, although only 8 % of the total $^{45}Ca^{2+}$ was present per mg protein of the test mitochondria, whilst the control mitochondria contained 43 % of the externally added $^{45}Ca^{2+}$. The table below illustrates the marked change in the total ATP

and AMP concentrations in the presence of palmitoyl CoA and rat albumin as compared with acetoacetate.

"Ca^{2+}-releasing" substances.	$^{45}Ca^{2+}$ in mitochondria as % of added $^{45}Ca^{2+}$.	Total ATP (μmoles/mg mitochondrial protein).	Total AMP (μmoles/mg mitochondrial protein).
palmitoyl CoA	8 %	0.216 Δ=0.23	0.392 Δ=0.2
control A	45 %	0.446	0.189
(sampling time: 30 min)			
rat albumin	41 %	0.197 Δ=0.21	0.31 Δ=0.2
control B	86 %	0.408	0.113
(sampling time: 30 min)			
acetoacetate	25 %	0.32 Δ=0.06	0.39 Δ=0.08
control C	79 %	0.38	0.31
(sampling time: 10 min)			
acetoacetate	8 %	0.16 Δ=0.07	0.60 Δ=0.09
control D	43 %	0.23	0.51
(sampling time: 10 min)			

It should be noted that 1 mM ATP was the added adenine nucleotide for the experiments with palmitoyl CoA and rat albumin (not purified). However 0.5 mM ATP and 0.5 mM ADP were added to the incubation medium on examining the effect of acetoacetate, which would explain the relatively lower concentration of total ATP and higher concentration total AMP

for the controls C and D compared with control A or B.

Mitochondrial pyridine nucleotides

The concentrations of pyridine nucleotides in mitochondria during the release of $^{45}Ca^{2+}$ caused by acetoacetate were also determined by the enzymatic and fluorimetric assay. Estimation at 10 min indicated a decrease in NADH and an increase in NAD^+ concentration in the presence of acetoacetate compared with the control, i.e. the $NADH/NAD^+$ ratio was 0.1 in the presence of acetoacetate whilst the control $NADH/NAD^+$ ratio was 0.35 (table 11). This result suggests that the acetoacetate had oxidised the mitochondrial NADH during 10 min incubation. At that time, the control mitochondria contained 80% of the total externally added Ca^{2+} whereas in the presence of acetoacetate, the mitochondria contained 62% of the total Ca^{2+}.

It was reported earlier in this discussion that the concentrations of total and mitochondrial ATP and AMP did not alter significantly (compared with the respective controls) during Ca^{2+} release from the mitochondria in the presence of oxidants of mitochondrial NADH such as acetoacetate or oxaloacetate, suggesting that it is the relatively high ratio of oxidised to reduced mitochondrial pyridine nucleotides that is important in the control of Ca^{2+} release.

Inclusion of 2 mM β-hydroxybutyrate did not affect the Ca^{2+} content in the mitochondria at 10 and 30 min relative to the control. The slightly lower concentration of NAD^+ and a higher NADH observed was due to the presence of the reductant of mitochondrial NAD^+.

The NADPH estimated at 10 min (3.0 nmole/mg mitochondrial protein for the control) in this study seems to agree with the value reported by Vinogradov et al (1972), i.e. NADPH = 2.32 nmole per mg mitochondrial protein. The incubation medium used

by the latter workers contained K^+, Mg^{2+}, Pi, 7 mM succinate and 5 μM rotenone.

In this present study, it was observed that when the mitochondria were incubated at 25°C with 2 mM acetoacetate for 1 min prior to adding the $^{45}Ca^{2+}$, the mitochondria accumulated lesser amount of $^{45}Ca^{2+}$ relative to the control mitochondria (fig 50). This result suggests that the relatively high ratio of oxidised to reduced mitochondrial pyridine nucleotides (due to the presence of acetoacetate) interfered with $^{45}Ca^{2+}$ uptake, in addition to influencing the $^{45}Ca^{2+}$ release from mitochondria.

The possibility of Ca-pyridine nucleotide complex in non-aqueous phase.

Vinogradov et al (1972) proposed the formulation of Ca-NADH complex in the non-polar region of the mitochondrial membrane, based on fluorescence study of NADH. The latter workers reported that additions of Ca^{2+} to solutions of NADH in methanol (90 %) but not in water, produces a large increase in fluorescence intensity and a shift in the emission spectrum to a shorter wavelength. They also observed that Ca^{2+} accumulation by rat liver mitochondria increased the NADH fluorescence in the presence of rotenone.

In this thesis, attempts were made to examine *in vitro* the possibility of Ca-pyridine nucleotide complex in non-aqueous phase. Two approaches were attempted, namely:-

(1) Organic phase extraction of ^{45}Ca in the presence of pyridine nucleotides. A variety of organic phases was examined. A significant ^{45}Ca count in the organic phase in the presence of pyridine nucleotide would suggest

^{45}Ca-pyridine nucleotide complex. Since the ^{45}Ca counts in the respective organic phase was not significantly different from the negative control (i.e. in the presence of ^{45}Ca alone), the result suggests the absence of Ca-pyridine nucleotide complex in the organic phase examined, although the positive control with sodium lauryl sulphate showed a significant $^{45}Ca^{2+}$ counts in all the organic phases examined with the exception of hexanol (100 %) (table 14).

(2) High Performance Liquid Chromatography was employed to see if Ca^{2+} would affect the retention of pyridine nucleotide in a non-polar μ-Bondapak C_{18} reverse-phase column. The results obtained (fig 56) indicated that $NADP^+$ or NADPH, in the presence of Ca^{2+}, was eluted later from the column compared with the respective pyridine nucleotide alone. A possible explanation is the formation of a less polar Ca-NADP or Ca-NADPH complex, which was eluted later than the more polar $NADP^+$ or NADPH from the reverse-phase column. Mg^{2+} also affects the retention of $NADP^+$ and NADPH, suggesting the formation of Mg-NADP and Mg-NADPH complexes in the non-polar μ-Bondapak column. Ca^{2+} did not affect the retention of NAD^+ and NADH in the column (table 15).

The first approach was not effective in showing the possibility of a Ca-NADP or Ca-NADPH complexes, however the second approach suggested that these complexes may be formed in the mitochondrial membrane and may be direct modulators of Ca^{2+} release and retention. A relatively high NADH/NAD^+ ratio favours a relatively high NADPH/$NADP^+$ ratio via mitochondrial pyridine nucleotide transhydrogenase (EC 1.6.1.1) which catalyses the following reaction:

$NADH + NADP^+ \rightleftharpoons NADPH + NAD^+$. Ca-NADP may be the stimulatory regulator of Ca^{2+} efflux while Ca-NADPH could be the inhibitory modulator. Similarly Mg-NADP and Mg-NADPH may also be the modulators of Mg^{2+} release and retention in mitochondria respectively.

The effect of externally added pyridine nucleotides on Ca^{2+} transport.

The effect of externally added pyridine nucleotide on Ca^{2+} movement into and out of the mitochondria was examined in the present study. The concentration of pyridine nucleotide used for this investigation was 0.5 mM, which is the approximate concentration of pyridine nucleotide found in rat cytosol. (According to Tischler et al (1977), the concentrations of pyridine nucleotide in cytosol of hepatocytes from fed rats are as follows:- 0.650 mM NAD^+, 0.270 mM NADH, 0.004 mM $NADP^+$ and 0.367 mM NADPH.) The effect of a lower concentration of $NADP^+$, i.e. 0.125 mM, was also investigated.

No significant binding of Ca^{2+} to 0.5 mM of the pyridine nucleotides could be detected using the Ca-electrode (note: $NADP^+$ from Calbiochem was contaminated with Ca^{2+} at approximately 125 mole Ca^{2+}/mole $NADP^+$) (fig 57, 58).

The mitochondria respiring on 2 mM succinate in the presence of 10^{-5}M rotenone retained the accumulated Ca^{2+} in the presence of externally added NAD^+ (final concentration 0.5 mM) compared with the control (fig 59(a)). NADH (0.5 mM) also caused Ca^{2+} retention in the mitochondria; this was not due to the oxidation of NADH to NAD^+, since 80 % of the NADH remained at the end of the 40 min incubation, suggesting that the observed Ca^{2+} retention was possibly due to NADH rather

than NAD^+. On the other hand, 0.5 mM NADPH caused an earlier Ca^{2+} release from the mitochondria with respect to the control (fig 59(f)), while 0.125 mM and 0.5 mM $NADP^+$ did not affect Ca^{2+} uptake or release (fig 59(d), (e)). It should be noted that the mitochondria suspended in the iso-osmotic sucrose medium were most likely impermeable to the external NAD^+, NADH, $NADP^+$ and NADPH, thus the effects observed were presumably due to the external pyridine nucleotides. This will be discussed further in chapter 8.

The physiological implication of the influence of redox state of the mitochondrial and cytosolic pyridine nucleotide on Ca^{2+} release and retention in mitochondria will be discussed in chapter 8.

6.5 SUMMARY

1. The presence of 2 mM acetoacetate in the incubation medium intefered with Ca^{2+} uptake by the mitochondria and also caused an earlier Ca^{2+} release. The energy sources for Ca^{2+} uptake were 0.5 mM ADP and 0.5 mM ATP. 2 mM acetoacetate decreased the mitochondrial NADH/NAD^{+} ratio from 0.35 (control) to 0.1 . The total ATP and AMP were not significantly different from the control during Ca^{2+} release from the mitochondria in the presence of acetoacetate.

2. The observations of Lehninger et al (1978), i.e. Ca^{2+} release and reuptake after adding oxidant of mitochondrial NADH and reductant of mitochondrial NAD respectively, were confirmed. In addition, BSA or EHDP, known to help Ca^{2+} retention in mitochondria, also caused the Ca^{2+} released by 0.5 mM oxaloacetate to be taken up again. However BSA or EHDP did not prevent Ca^{2+} release induced by oxaloacetate.

3. Successive cycles of Ca^{2+} release and reuptake were observed in the mitochondria on alternately adding oxaloacetate and β-hydroxybutyrate, when either 0.2 mM Pi, 2 mM Pi or 10 mM sodium acetate was the permeant anion. It was difficult to obtain more than 1 cycle with 2 mM Pi.

4. The concentrations of mitochondrial adenine nucleotides remained fairly steady during the Ca^{2+} cycling in the mitochondria.

5. The possibility of Ca-pyridine nucleotide complex in non-aqueous phase was investigated. Results obtained from the organic phase extraction of $^{45}Ca^{2+}$ suggest the absence of

^{45}Ca-pyridine nucleotide complex in the non-aqueous phase examined. Results obtained from HPLC indicated the possibility of Ca-NADP and Ca-NADPH formation in the low polarity μ-Bondapak C_{18} column.

6. Externally added NAD^+ (0.5 mM) and possibly NADH (0.5 mM) prolonged Ca^{2+} retention in mitochondria. A similar concentration of $NADP^+$ did not affect Ca^{2+} uptake or release; however, 0.5 mM NADPH caused an earlier Ca^{2+} release from the mitochondria compared with the control.

CHAPTER 7

STIMULATION OF Ca^{2+} RELEASE FROM MITOCHONDRIA BY CYCLIC ADENOSINE 3'5'-MONOPHOSPHATE

7.1 AIM

The mechanism of Ca^{2+} release from mitochondria is still poorly understood . Borle (1974) reported that cAMP caused Ca^{2+} release from mitochondria in the presence of succinate and ATP as substrates. His claim was withdrawn since the observation was not reproducible (Borle, 1976). In this chapter, the effect of cAMP on Ca^{2+} release was reinvestigated based on the findings of Lehninger et al (1978) and Christiansen (1977). The former workers reported that the redox state of mitochondrial pyridine nucleotides can influence Ca^{2+} release and retention by mitochondria ; the more oxidised steady state favours Ca^{2+} release while a relatively reduced steady state favours Ca^{2+} retention. In an earlier paper, Christiansen (1977) observed that glucagon lowered the ratio of β-hydroxybutyrate/ acetoacetate of rat hepatocytes (i.e. reflecting a lowered ratio of mitochondrial NADH/NAD^+), when palmitate was substrate (note : cAMP mediates many of the effects of glucagon in the liver (Parrilla et al, 1974 ; Vinicor et al, 1976 ; Hems, 1977). In fact, Neville and Jamieson (personal communication) noted that dibutyryl cAMP lowered β-hydroxybutyrate/acetoacetate ratio in rat hepatocytes in the presence of palmitate. Therefore, it is possible that cAMP may influence Ca^{2+} release from mitochondria by lowering the ratio of β-hydroxybutyrate/acetoacetate. The effect of cAMP on Ca^{2+} release from mitochondria was re-examined using

palmitoyl CoA or palmitoylcarnitine as substrates. The effect of cAMP was also examined using the substrates succinate and ATP.

Starvation is known to increase the levels of glucagon in blood (Gerich, 1976) which would be expected to increase the cAMP levels in liver (Garrison and Haynes, 1973). Therefore the effect of starvation on Ca^{2+} release from the mitochondria was also investigated.

7.2 METHODS AND MATERIALS.

The preparation of rat liver mitochondria was as described in chapter 1, section 1.1 . Continuous measurement of Ca^{2+} uptake, retention and release was monitored using the Ca-electrode (described in chapter 1, section 1.4). In order to calculate the amount of Ca^{2+} released in the presence of cAMP, the output of the amplifier connected to the Ca-electrode was connected to a second amplifier containing an antilog stage. Using this amplifier a linear output to the recorder could be obtained in the range 10^{-5}M to 10^{-6}M. The antilog recorder was calibrated with additions of 50 nmoles Ca^{2+}. The radio-isotope technique was also used to measure Ca^{2+} transport. The standard incubation mixture was used. Further additions are indicated in the legend to the respective figures. Substrates were adjusted to pH 7.3 - 7.4 with Tris base prior to use. It should be noted that 3-isobutyl-1-methylxanthin (IBM) is not readily soluble in distilled water. To prepare a 5 mM solution of IBM, 5.6 mg IBM was ground with 4 drops of 1 M Tris base and water was added slowly to a final volume of 5 ml (pH 9.0). The pH of the solution was then adjusted to pH 7.5 with 1 N HCl.

Endogenous Ca^{2+} in the mitochondria was estimated using Arsenazo III. 0.5 ml of the mitochondrial suspension was centrifuged for 3 min (9,750 x g) and the mitochondrial pellets washed with iso-osmotic sucrose medium, wet ashed with HNO_3 and $HClO_4$, evaporated to dryness, dried over NaOH in vacuo for 40 min and finally dissolved in 3 ml, 0.1 M Tris HCl pH 7.6 . Ca^{2+} was then estimated by reaction with Arsenazo III as described in chapter 1, section 1.5.5.2 .

Free fatty acids in mitochondrial suspension was determined by the method of Soloni and Sardina (1973) (chapter 2, section 2.2.4). Pyridine nucleotides in mitochondria prepared from fed and starved rats were estimated by enzymic and fluorimetric assays (chapter 1, section 1.8).

Cyclic AMP, dibutyryl cAMP and cGMP were obtained from Boehringer (Australia Pty. Ltd., Hardner Rd., Mt. Waverley, Vic.); S-palmitoyl-coenzyme A, palmitoyl-L-carnitine chloride and 3-isobutyl-1-methylxanthin were from SIGMA Chem. Co., St. Louis, Mo., U.S.A.

7.3 RESULTS

When studying the effect of cyclic AMP, dibutyryl cAMP or cGMP on Ca^{2+} release from mitochondria using the Ca^{2+} electrode, the test experiment was examined first, prior to the control experiment. The reason for this is with the Ca-electrode only 1 test situation can be examined at one time (approximately 30 min per test) thus examining the test first ensures that the release observed was not due to "aging" effect. It was observed that the control mitochondria released their accumulated Ca^{2+} slightly earlier when examined 1 hr after isolation compared with the control mitochondria which was examined immediately after isolation (i.e. after the final wash of the mitochondrial pellets).

7.3.1 The effect of 75 μM cAMP, dibutyryl cAMP and cGMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA, carnitine, ATP and rotenone studied by the Ca-electrode.

The energy substrates used for the investigations below were:- 20 μM palmitoyl CoA, 2 mM carnitine and 1 mM ATP. 10 μM rotenone was added to inhibit oxidation of NADH via the respiratory chain.

7.3.1.1 The effect of 75 μM cAMP.

As shown in fig 60, the addition of carnitine, palmitoyl CoA, rotenone and cAMP to the incubation medium containing Pi, Mg^{2+}, K^{+} and Ca^{2+} did not affect the sensitivity of the Ca-electrode. The addition of ATP resulted in an immediate chelation of approximately 275 nmoles Ca^{2+} out of a total of 400 nmoles present in the medium.

FIGURE 60

Effect of 75 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA, carnitine and rotenone.

Mitochondria (7.8 mg protein) were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 2 mM DL-carnitine HCl, 20 μM palmitoyl CoA (pal.CoA), 10 μM rotenone and 1 mM ATP. The addition of cAMP (final concentration = 75 μM) is as shown. Incubation temp. = 25°C. The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. The result shown is the superimposed tracings of 2 separate experiments.

(——) control experiment

(----) + 75 μM cAMP

mito. = mitochondria

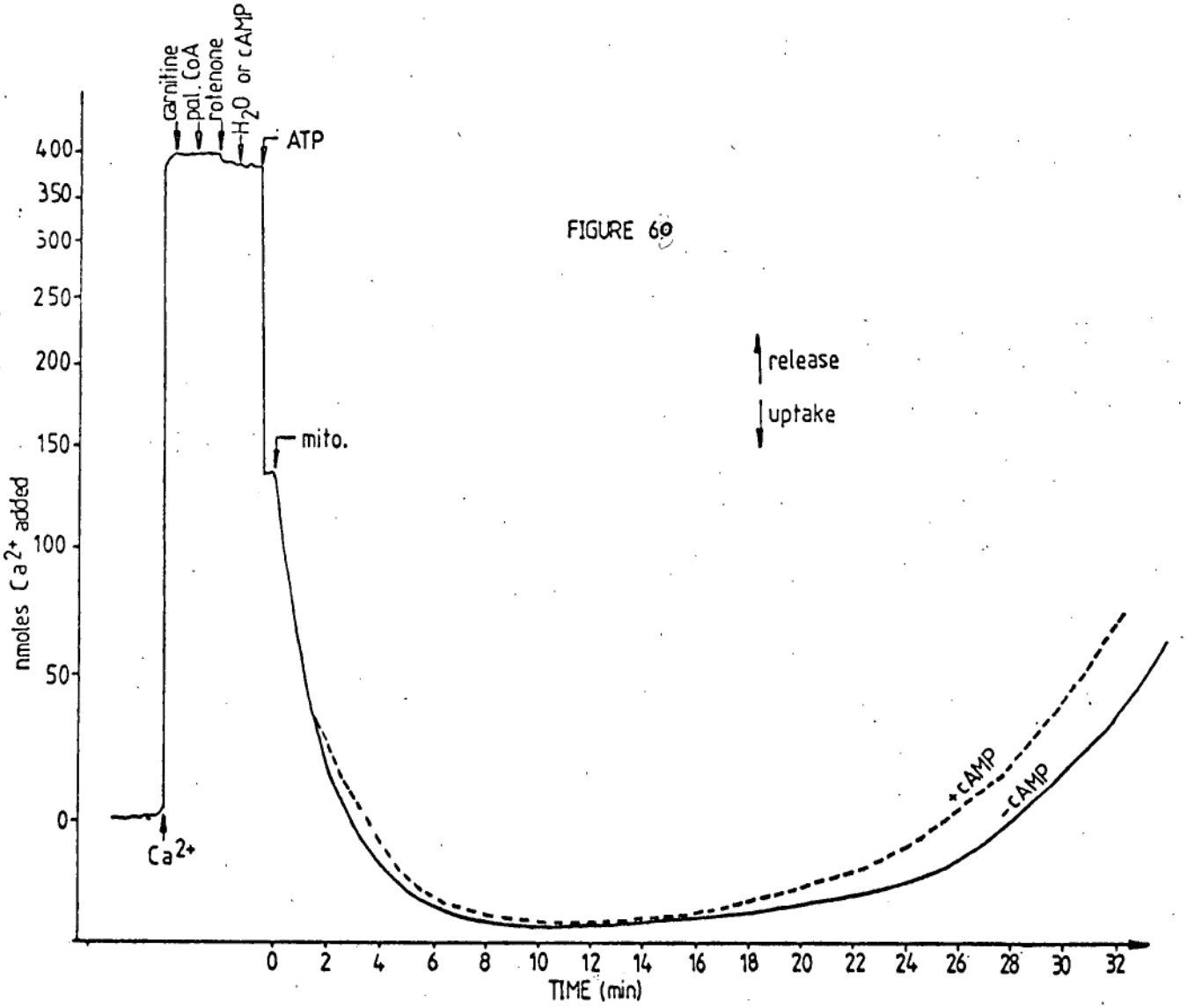

carnitine
pal. CoA
rotenone
H_2O or cAMP
ATP
mito.
release
uptake
nmoles Ca^{2+} added
400
350
300
250
200
150
100
50
0
Ca^{2+}
+cAMP
-cAMP
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
TIME (min)

FIGURE 6a

The test mitochondria, in the presence of cAMP, released their accumulated Ca^{2+} slightly earlier than the control mitochondria as shown in fig 60.

7.3.1.2 The effect of 75 μM dibutyryl cAMP.

The test mitochondria, in the presence of 75 μM dibutyryl cAMP, also released accumulated Ca^{2+} relatively earlier than the control mitochondria as shown in fig 61.

7.3.1.3 The effect of 75 μM cGMP.

The presence of 75 μM cGMP did not appear to cause Ca^{2+} release from the mitochondria as shown in fig 62.

7.3.2 The effect of 75 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA, carnitine and ATP (without rotenone), studied by the Ca-electrode.

The energy substrates used for this experiment was the same as that used previously (section 7.3.1) except that rotenone was omitted. Ca^{2+} release from the mitochondria was examined using the Ca-electrode which was connected to an antilog amplifier in order to give a linear response (section 7.2).

Fig 63 shows that the presence of 75 μM cAMP did not affect Ca^{2+} uptake; however, the mitochondria in the presence of cAMP started to release the accumulated Ca^{2+} at approx. 7 min, while the control mitochondria still retained their accumulated Ca^{2+} for the 24 min duration of the experiment. It was calculated that out of 66 nmole Ca^{2+}/mg protein accumulated by the test mitochondria, 8 - 10 nmoles Ca^{2+} were released in 20 min.

FIGURE 61

Effect of 75 μM dibutyryl cAMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA, carnitine and rotenone.

Mitochondria (6.6 mg protein), were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 2 mM DL-carnitine HCl, 20 μM palmitoyl CoA (pal.CoA), 10^{-5}M rotenone and 1 mM ATP. The addition of dibutyryl cAMP (db.cAMP) is as shown. Incubation temp. = 25°C. The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Tracings from 2 separate experiments were superimposed.

(——) control experiment

(----) + db.cAMP (final concentration = 75 μM)

mito. = mitochondria

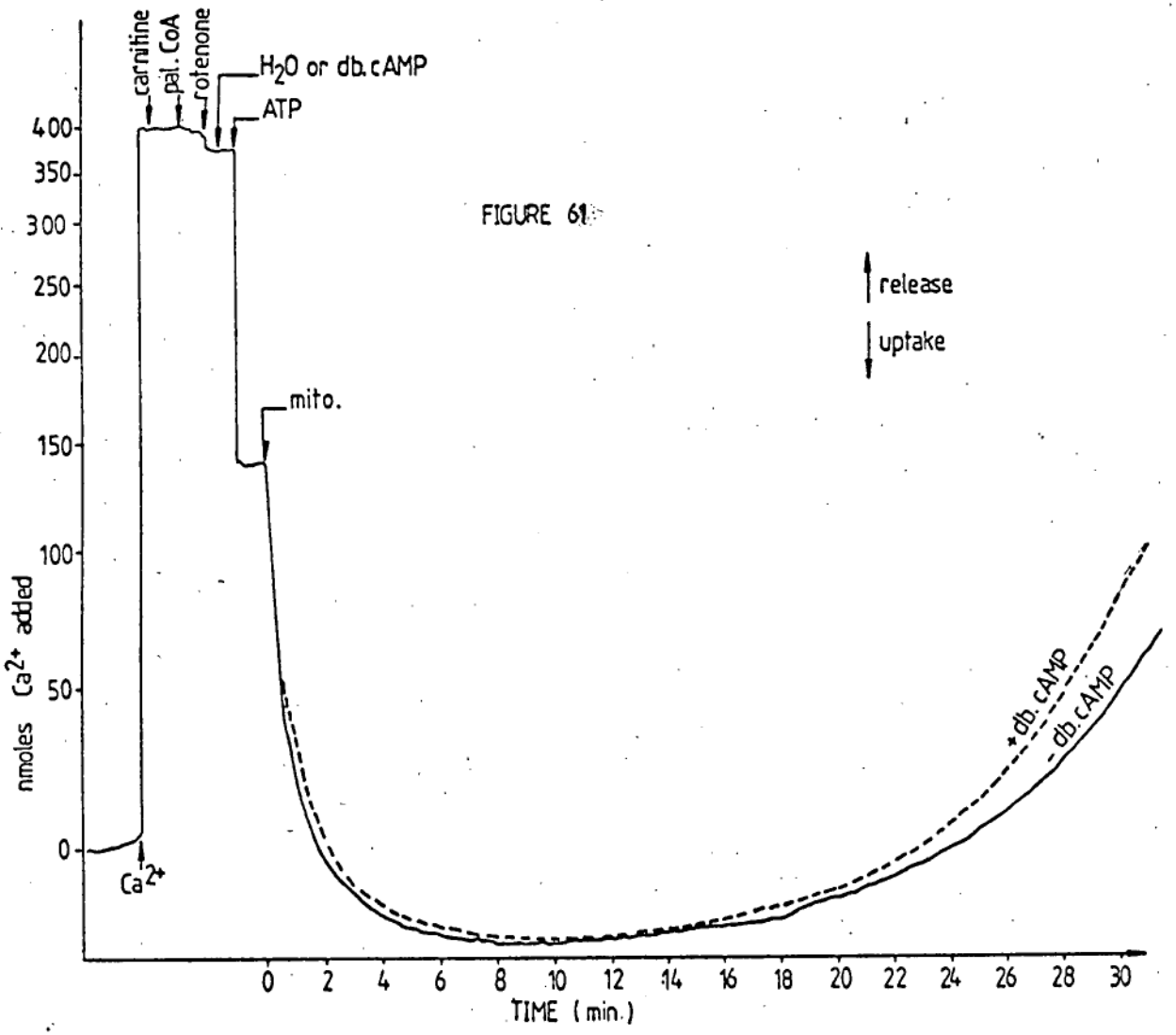

carnitine
pal. CoA
rotenone
H_2O or db. cAMP
ATP
mito.
Ca^{2+}
nmoles Ca^{2+} added
400
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (min.)
+db. cAMP
-db. cAMP
release
uptake

FIGURE 61

FIGURE 62

Effect of 75 μM cGMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA, carnitine and rotenone.

Mitochondria (7.1 mg protein), were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 2 mM DL-carnitine HCl, 20 μM palmitoyl CoA (pal.CoA), 10^{-5}M rotenone and 1 mM ATP. The addition of cGMP (final concentration = 75 μM) is as shown. Incubation temp. = 25°C. The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Tracings from 2 separate experiments were superimposed.

(——) control experiment

(----) + cGMP

mito. = mitochondria

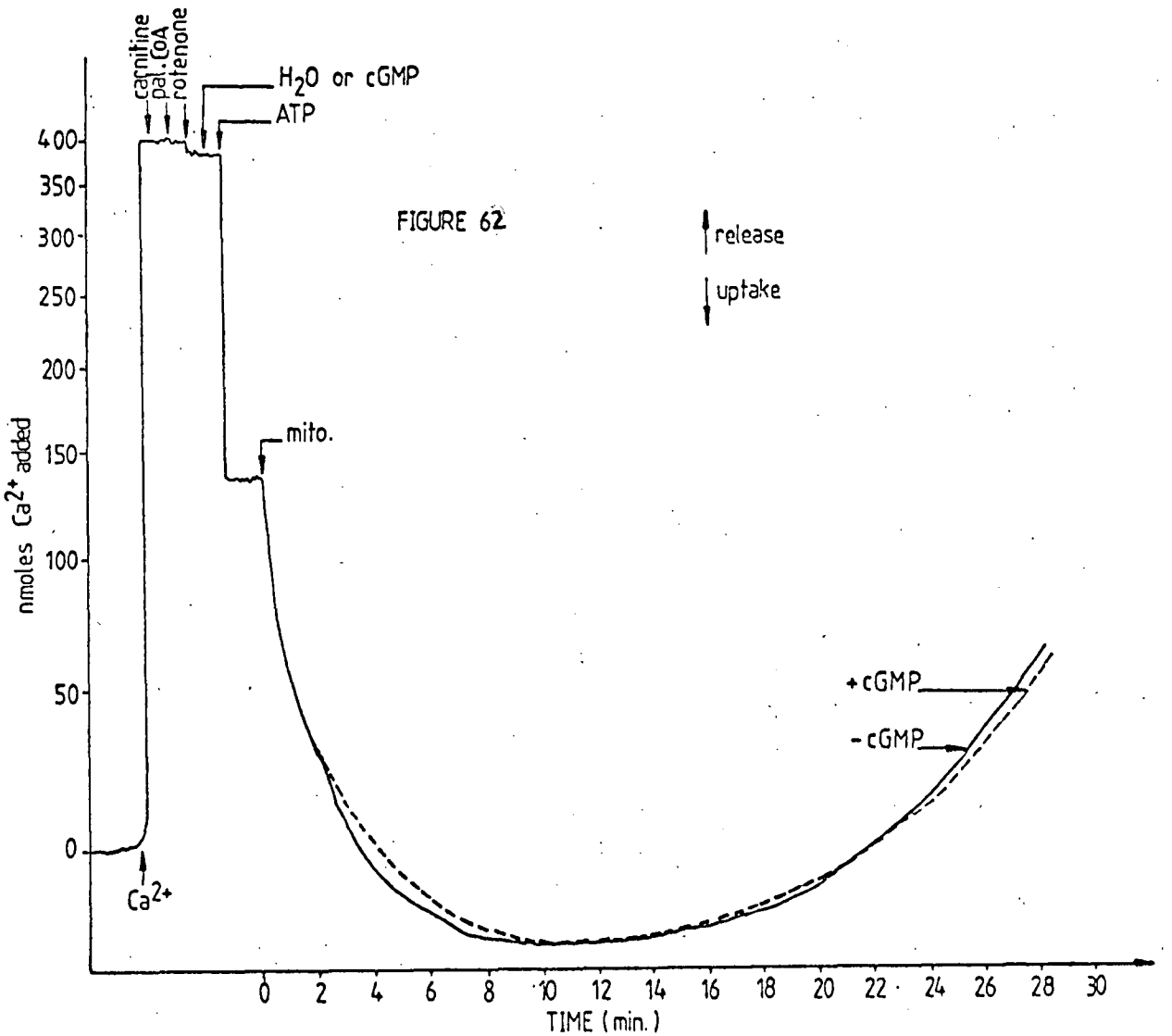

carnitine
pal.CoA
rotenone
H_2O or cGMP
ATP
mito.
Ca^{2+}
+cGMP
-cGMP
release
uptake
nmoles Ca^{2+} added
400
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (min.)

FIGURE 62

FIGURE 63

Effect of 75 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA and carnitine.

Mitochondria (6.0 mg protein), were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 2 mM DL-carnitine HCl, 20 μM palmitoyl CoA (pal.CoA) and 1 mM ATP. The addition of cAMP (final concentration = 75 μM) is as shown. Incubation temp. = 25°C. The Ca^{2+} added is shown on a log scale. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Tracings from 2 separate experiments were superimposed.

control experiment: - cAMP

test experiment: + cAMP

mito. = mitochondria

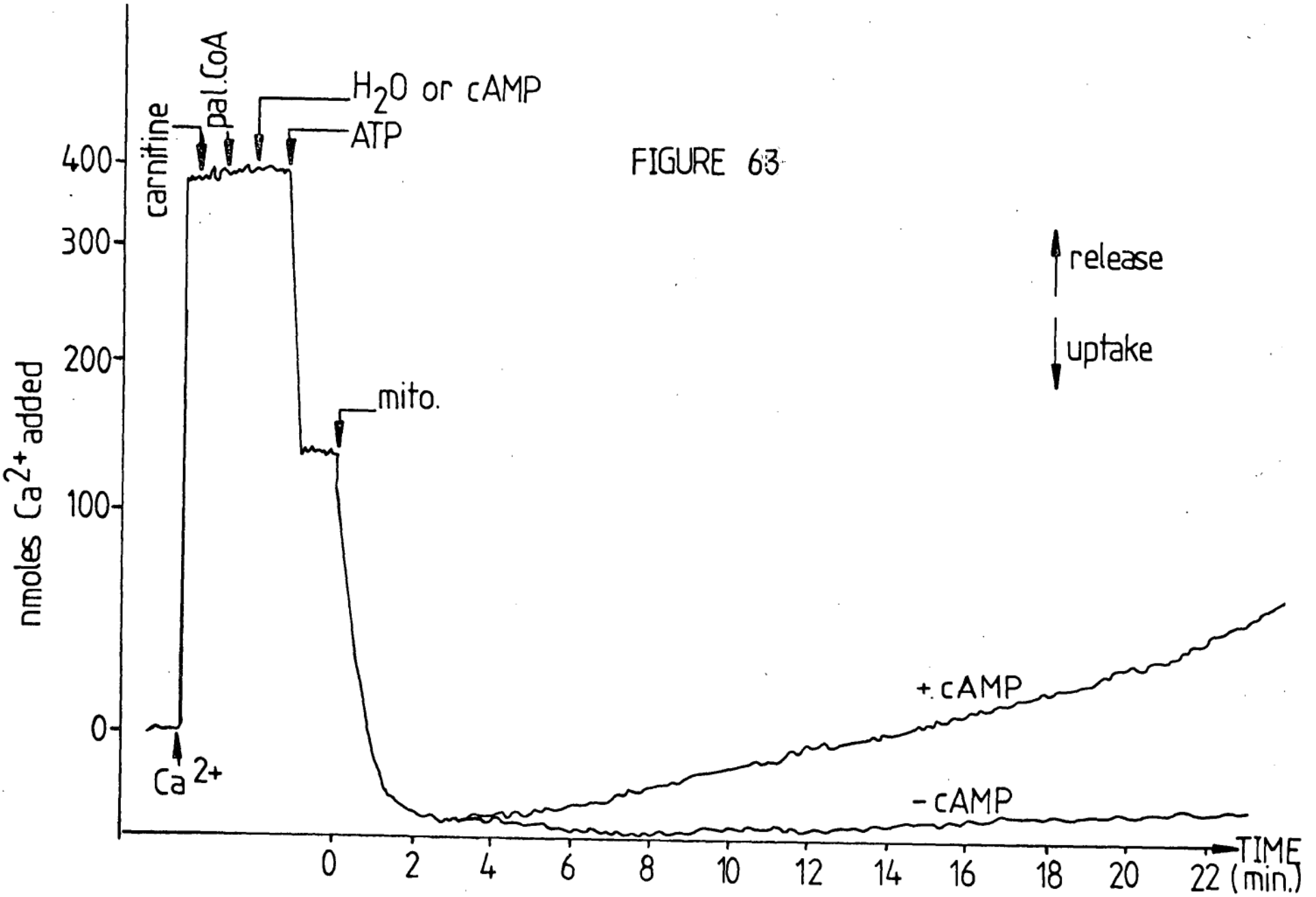

carnitine
pal.CoA
H_2O or cAMP
ATP
mito.
release
uptake
nmoles Ca^{2+} added
400
300
200
100
0
Ca^{2+}
+ cAMP
- cAMP
0
2
4
6
8
10
12
14
16
18
20
22 (min.)
TIME

FIGURE 63

7.3.2.1 The effect of 75 μM cAMP on Ca^{2+} release from preloaded mitochondria in the presence of 50 μM 3-isobutyl-1-methylxanthin (IBM)

Phosphodiesterase, which may be present in the mitochondrial preparation, could convert cAMP to AMP during the incubation. Therefore, the effect of cAMP on Ca^{2+} release from mitochondria was also examined in the presence of 3-isobutyl-1-methylxanthin (IBM), an inhibitor of phosphodiesterase.

The result indicated that after 20 min incubation, cAMP caused a release of approx. 7 - 9 nmoles Ca^{2+} out of the 51 nmoles Ca^{2+} accumulated/mg protein. The control mitochondria, in the absence of added cAMP, retained their accumulated Ca^{2+} during the 25 min duration of the experiment.

7.3.2.2 The effect of 75 μM cAMP on Ca^{2+} release from mitochondria not preloaded with Ca^{2+} in the presence of 50 μM IBM

For this particular investigation the mitochondria were prepared as described in chapter 1, section 1.1 but without EGTA in the homogenising medium. The mitochondria contained approx. 20 nmoles Ca^{2+}/mg protein, estimated by reaction with Arsenazo III dye described in chapter 1, section 1.5.5.2. Ca^{2+} release from the mitochondria was examined using the Ca-electrode which was connected to the antilog amplifier in order to give a linear response.

As shown in fig 64, the test mitochondria (with 75 μM cAMP), released relatively more of the accumulated Ca^{2+} after 12 min, compared with the control mitochondria. By calibration with additions of 50 nmoles Ca^{2+}, it was estimated that after 20 min incubation approx. 3 nmoles Ca^{2+}/mg mitochondrial protein were

FIGURE 64

Effect of 75 μM cAMP on Ca^{2+} release from mitochondria not preloaded with Ca^{2+}.

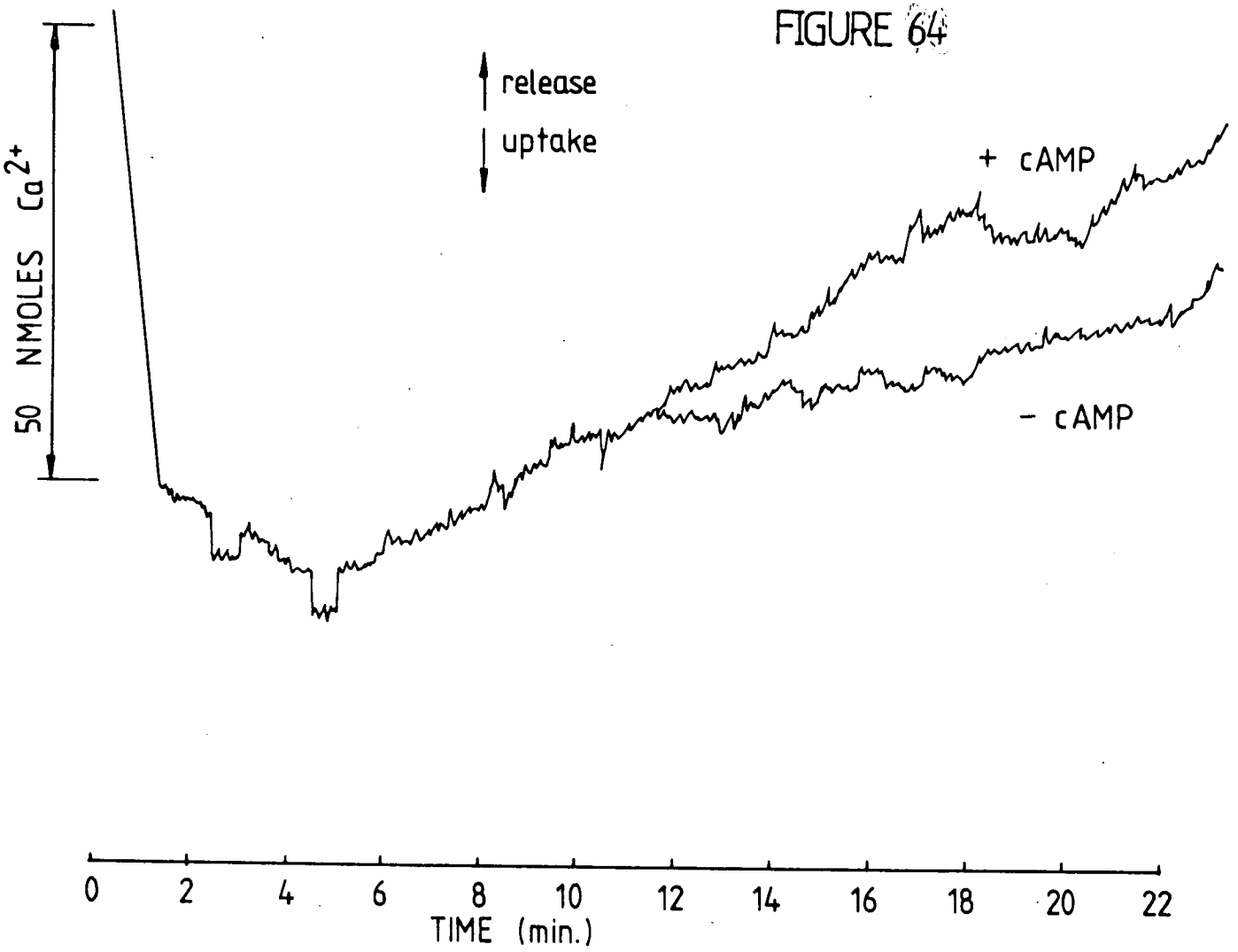
Mitochondrial Ca^{2+} movement was studied by means of the Ca-electrode which was connected to a second amplifier containing an antilog stage (to give a linear response).

Mitochondria (12 mg protein) were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 2 mM DL-carnitine HCl, 20 μM palmitoyl CoA, 50 μM IBM and 1 mM ATP. cAMP (final concentration = 75 μM) was added to the test experiment before the mitochondria. Incubation temp. = 25°C. The opposing arrows show the direction of Ca^{2+} uptake or release by the mitochondria. Tracings from 2 separate experiments were superimposed.

control experiment: - cAMP

test experiment: + cAMP

FIGURE 64


50 NMOLES Ca^{2+}
release
uptake
+ cAMP
– cAMP
0
2
4
6
8
10
12
14
16
18
20
22
TIME (min.)

~~protein were~~ released from the mitochondria in the presence of cAMP out of the 20 nmoles Ca^{2+}/mg protein initially present in the mitochondria.

7.3.3 The effect of cAMP on Ca^{2+} release from mitochondria in the presence of succinate, ATP and rotenone

An attempt was made to see whether cAMP affects Ca^{2+} release from mitochondria when 2 mM succinate (in the presence of 10^{-5} M rotenone) and 1 mM ATP were the energy sources for Ca^{2+} uptake. The Ca-electrode was used to monitor continuous movement of Ca^{2+} in and out of the mitochondria. The effect of various concentrations of cAMP, 3 μM, 10 μM and 75 μM were examined.

As shown in fig 65, the addition of ATP to the incubation medium resulted in an immediate chelation of approx. 275 nmoles Ca^{2+} out of the 400 nmoles Ca^{2+} in the medium. Inclusion of 3 μM cAMP in the incubation medium did not affect Ca^{2+} release or retention, compared with the control mitochondria, during the 30 min duration of the experiment. Similar result was obtained with 10 μM cAMP.

Fig 66 shows that 75 μM cAMP did not affect Ca^{2+} uptake by the mitochondria, neither did it cause Ca^{2+} release. In fact, the control mitochondria seemed to release their accumulated Ca^{2+} slightly earlier than the test mitochondria. (Since the control experiment was examined approx. 45 min after the test experiment, most likely the observed earlier Ca^{2+} release from the control mitochondria was due to "aging effect".)

FIGURE 65

Effect of 3 μM cAMP on Ca^{2+} release from mitochondria in the presence of succinate and rotenone.

Mitochondria (7.1 mg protein), were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 10^{-5}M rotenone, 2 mM Na succinate (succ.) and 1 mM ATP. The addition cAMP (final concentration = 3 μM) is as shown. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C. The opposing arrows show the direction of Ca^{2+} uptake and release by the mitochondria. Tracings from 2 separate experiments were superimposed.

(——) control experiment

(----) + cAMP

mito. = mitochondria

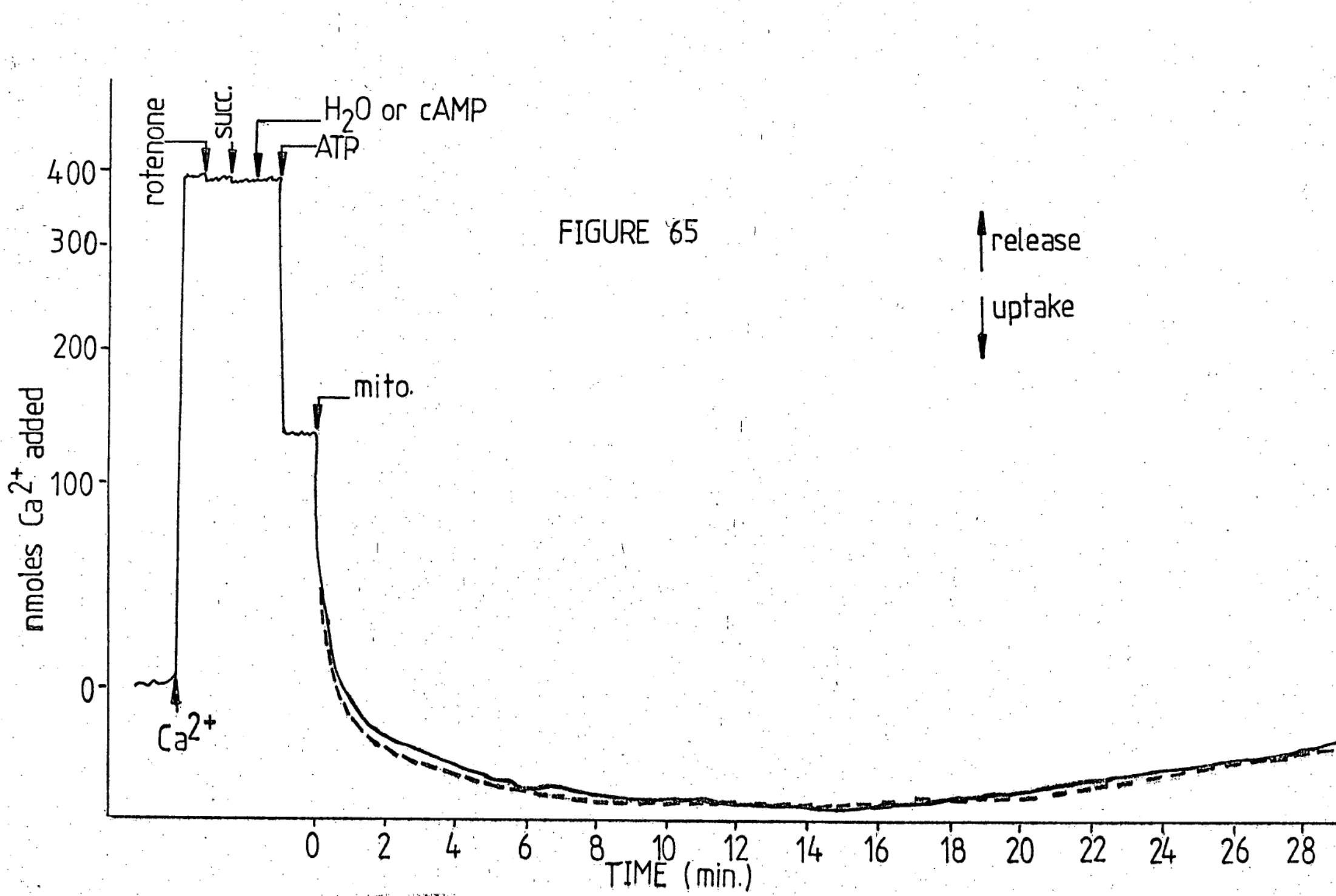

rotenone
succ.
H_2O or cAMP
ATP
mito.
Ca^{2+}
400
300
200
100
0
nmoles Ca^{2+} added
release
uptake
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
TIME (min.)

FIGURE 65

FIGURE 66

Effect of 75 μM cAMP on Ca^{2+} release from mitochondria in the presence of succinate and rotenone.

Mitochondria (7.8 mg protein) were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 10^{-5}M rotenone, 2 mM Na succinate (succ.) and 1 mM ATP. The addition of cAMP (final concentration = 75 μM) is as shown. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C. The opposing arrows show the direction of Ca^{2+} uptake and release by the mitochondria. Tracings from 2 experiments were superimposed.

(——) control experiment; - cAMP

(----) test experiment; + cAMP

mito. = mitochondria

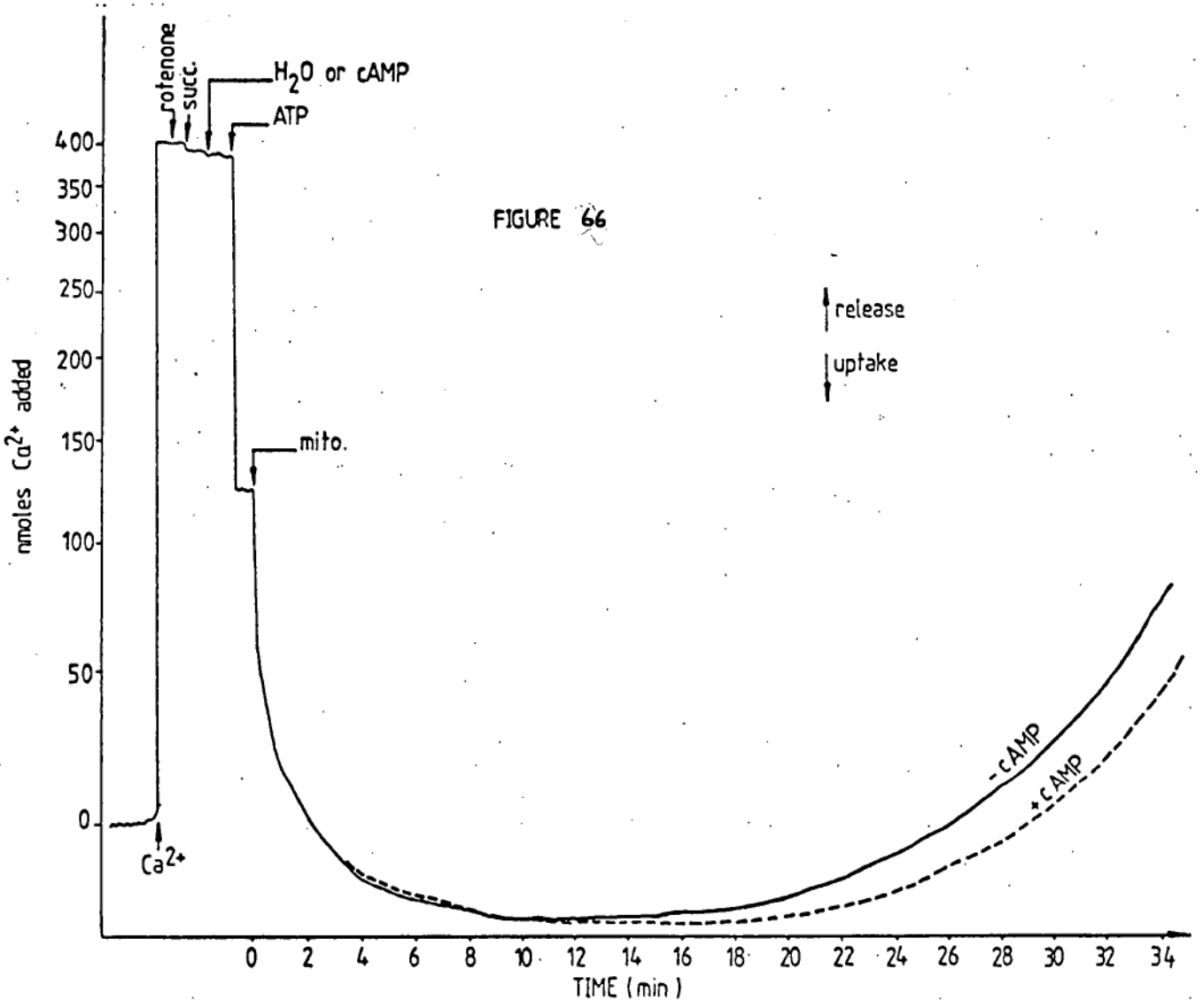

rotenone
succ.
H_2O or cAMP
ATP
mito.
400
350
300
250
200
150
100
50
0
nmoles Ca^{2+} added
Ca^{2+}
release
uptake
-cAMP
+cAMP
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
TIME (min)

FIGURE 66

7.3.4 The effect of cAMP on Ca^{2+} release from mitochondria in the presence of palmitoylcarnitine and ATP studied by means of the Ca-electrode.

It was observed in this chapter, and also in section 5.3.2, that prior to adding the mitochondria, the addition of ATP (final concentration 1 mM) resulted in an immediate chelation of approx. 275 nmoles Ca^{2+} out of the 400 nmoles, even in the presence of 1 mM Mg^{2+}. The Ca-electrode only measures free Ca^{2+} and cannot detect Ca^{2+} chelated to ATP or to the mitochondria. Therefore, it is possible that the actual amount of Ca^{2+} released from the mitochondria was underestimated due to combination of this Ca^{2+} with the ATP remaining in the incubation medium (note : 1 mM ATP was initially present in the incubation medium; however, after 30 min incubation in the presence of mitochondria, the concentration of ATP in the medium was approx. 0.5 mM (section 5.3.4.5). Therefore, instead of using the substrates 20 µM palmitoyl CoA, 2 mM carnitine and 1 mM ATP, the effect of cAMP was examined in the presence of 50 µM palmitoylcarnitine and a lower concentration of ATP, i.e. 0.1 mM. The effect of 75 µM, 1 µM and 0.1 µM cAMP on Ca^{2+} release from mitochondria using the latter substrates were examined.

As shown in fig 67, the addition of palmitoylcarnitine (final concentration 50 µM) to the incubation medium did not affect the sensitivity of the Ca-electrode. Addition of ATP (final concentration 0.1 mM) resulted in a slight fall in the ionic Ca^{2+} (less than 50 nmoles Ca^{2+}). Compare this with fig 60 where 1 mM ATP combines with 275 nmoles Ca^{2+}.

The control mitochondria accumulated all the added Ca^{2+} within 5 min after addition and started to release this Ca^{2+} at approx. 14 min (fig 67). The test mitochondria in the presence of 75 μM cAMP started to release their accumulated Ca^{2+} at approx. 7 min. A significant release of Ca^{2+} was observed from the test mitochondria compared with the contol mitochondria (also c.f. fig 63).

cAMP (final concentration 1 μM) was added at 4 min when the uptake of Ca^{2+} was almost completed (fig 68). The control mitochondria accumulated all the added Ca^{2+} after 6 min, i.e. at 45 nmoles Ca^{2+}/mg protein and released this Ca^{2+} rather rapidly at approx. 26 min. The test mitochondria in the presence of 1 μM cAMP started releasing their accumulated Ca^{2+} at approx. 21 min, i.e. 5 min earlier than the control mitochondria. (fig 68)

Inclusion of cAMP (final concentration 0.1 μM) at 4 min resulted in an earlier Ca^{2+} release from the mitochondria relative to the control mitochondria. As shown in fig 69, rapid release of Ca^{2+} from the test mitochondria occurred at 21 min while the control mitochondria released their accumulated Ca^{2+} 3 min later, i.e. at 24 min incubation.

7.3.5 The effect of cAMP on Ca^{2+} release from mitochondria studied by the radio-isotope technique

An alternative method of measuring the Ca^{2+} movement into and out of the mitochondria was the radio-isotope technique described in chapter 1, section 1.4. Unlike the Ca-electrode which measures free Ca^{2+} in the medium, the radio-isotope technique indicates the amount of Ca^{2+} actually present in the mitochondria.

FIGURE 67

Effect of 75 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoylcarnitine and lower concentration of ATP.

Mitochondria (7.5 mg protein) were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 50 μM palmitoylcarnitine, and 0.1 mM ATP. The addition of cAMP (final concentration = 75 μM) is as shown. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C. Tracings from 2 separate experiments were superimposed.

control experiment: - cAMP

test experiment: + cAMP

mito. = mitochondria

FIGURE 67

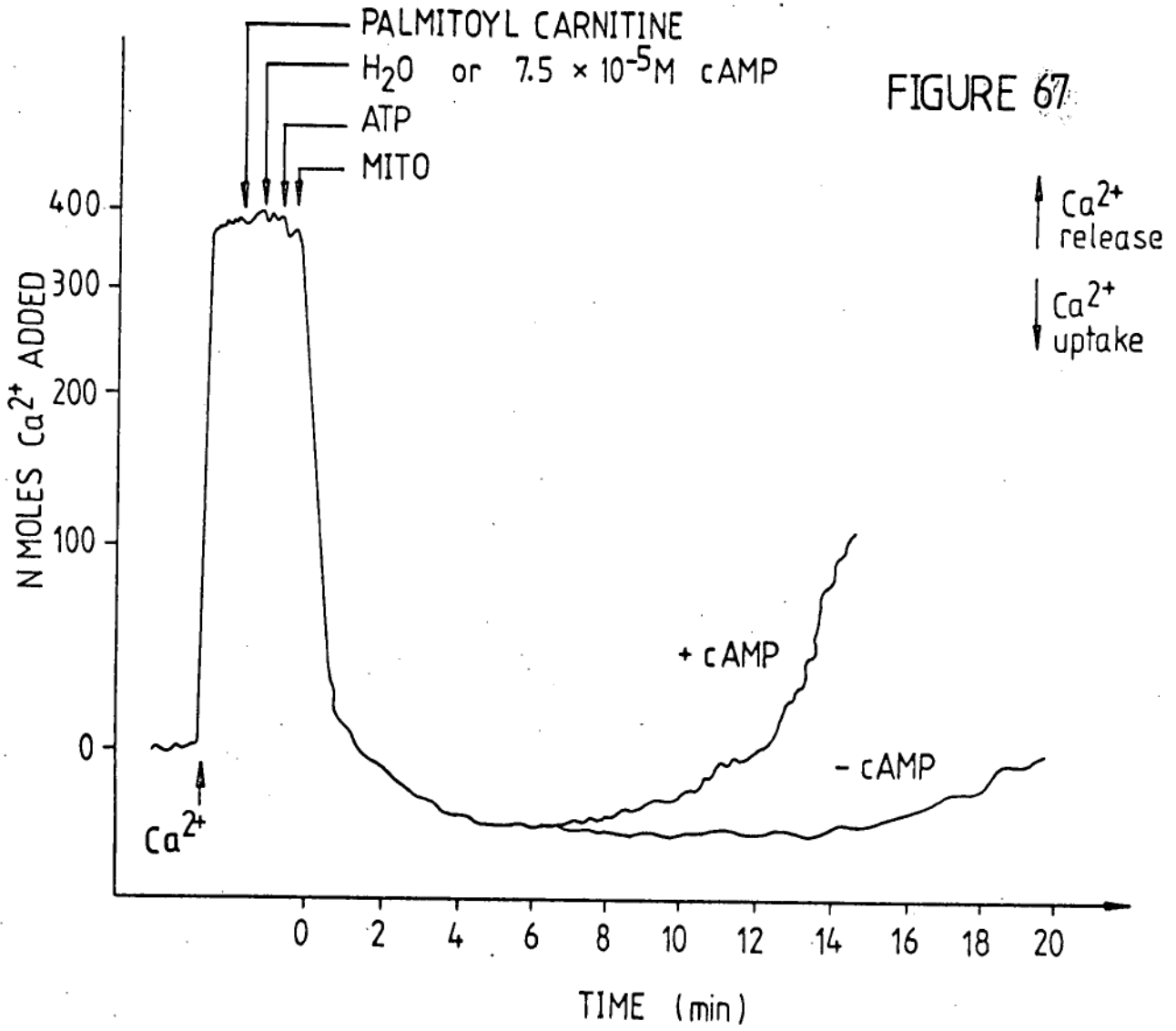

PALMITOYL CARNITINE
H_2O or 7.5×10^{-5}M cAMP
ATP
MITO
Ca^{2+} release
Ca^{2+} uptake
N MOLES Ca^{2+} ADDED
400
300
200
100
0
+ cAMP
- cAMP
Ca^{2+}
0
2
4
6
8
10
12
14
16
18
20
TIME (min)

FIGURE 68

Effect of 1 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoylcarnitine and ATP (0.1 mM)

Mitochondria (8.8 mg protein) were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 50 μM palmitoylcarnitine and 0.1 mM ATP. The addition of cAMP (final concentration = 1 μM) was made at 4 min after adding the mitochondria. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C. Tracings from 2 separate experiments were superimposed.

control experiment: - cAMP

test experiment: + cAMP

mito. = mitochondria

FIGURE 68

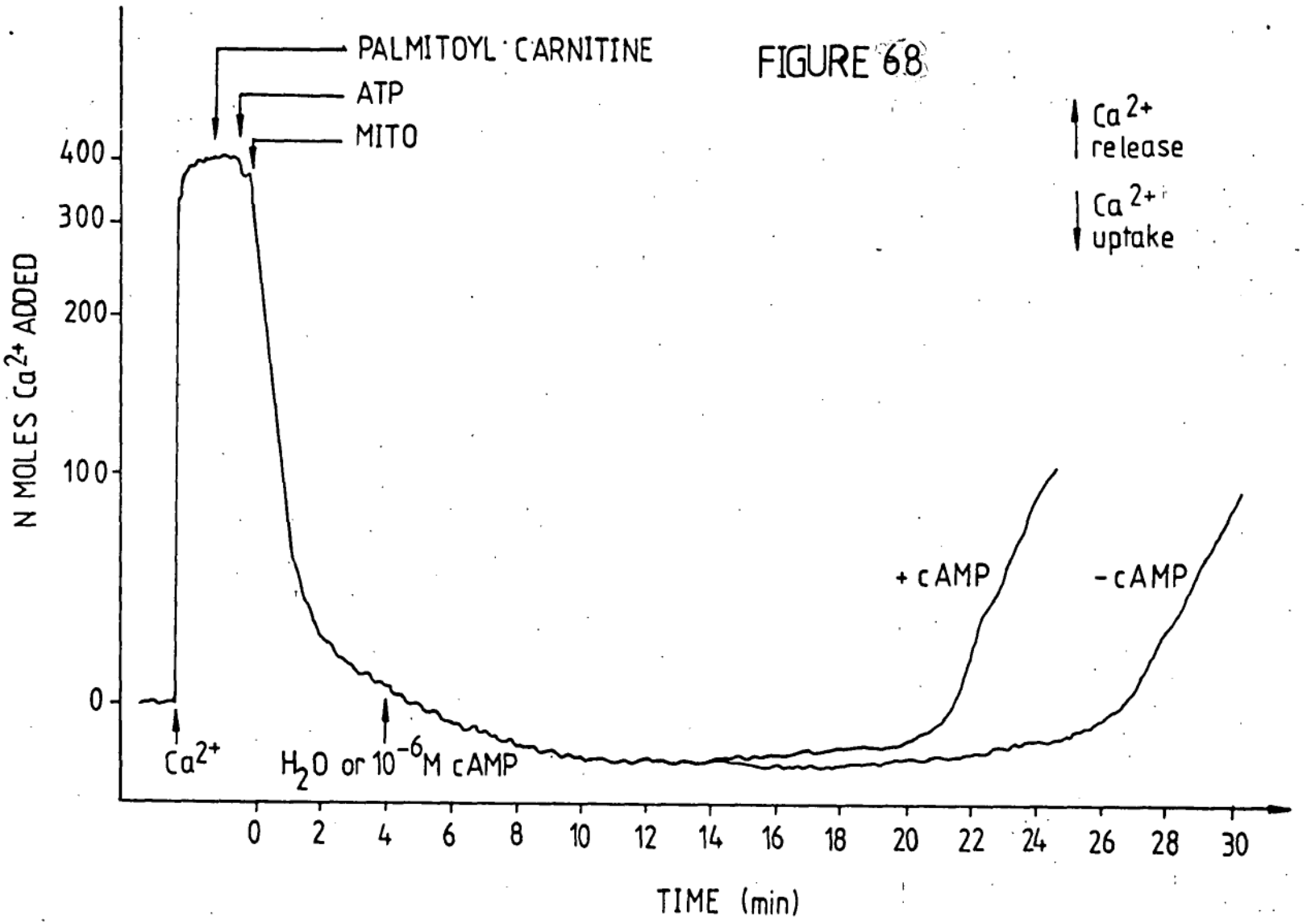

PALMITOYL CARNITINE
ATP
MITO
Ca2+ release
Ca2+ uptake
N MOLES Ca2+ ADDED
400
300
200
100
0
+ cAMP
- cAMP
Ca2+
H2O or 10-6M cAMP
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (min)

FIGURE 69

Effect of 0.1 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoylcarnitine and ATP (0.1 mM).

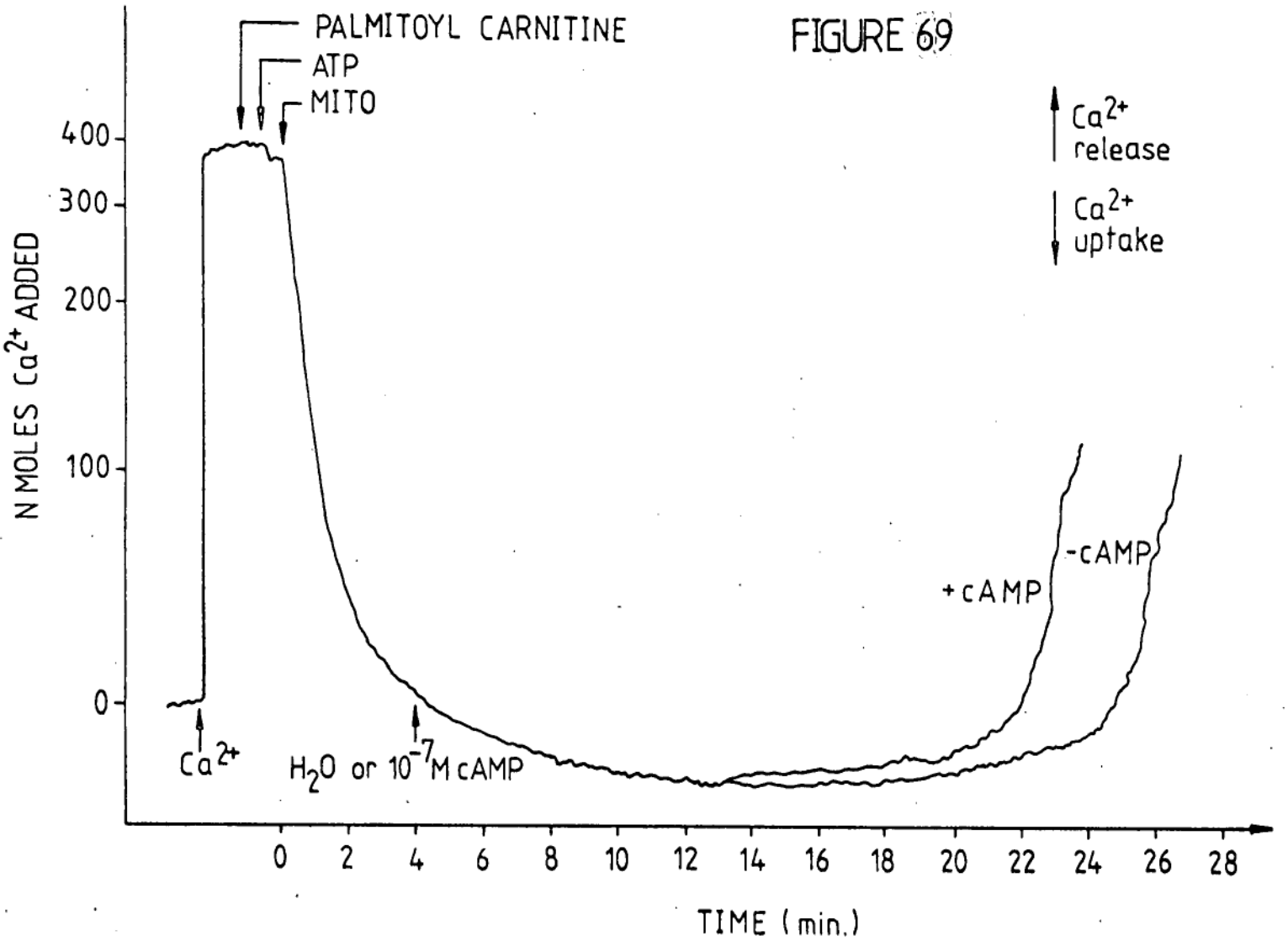
Mitochondria (8.5 mg protein) were added to the incubation medium (final vol. = 5 ml) containing the standard incubation mixture, 400 nmoles Ca^{2+}, 50 μM palmitoylcarnitine and 0.1 mM ATP. The addition of cAMP (final concentration = 0.1 μM) was made at 4 min after adding the mitochondria. The Ca^{2+} added is shown on a log scale. Incubation temp. = 25°C. Tracings from 2 separate experiments were superimposed.

control experiment: - cAMP

test experiment: + cAMP

mito. = mitochondria

FIGURE 69


PALMITOYL CARNITINE
ATP
MITO
Ca2+ release
Ca2+ uptake
N MOLES Ca2+ ADDED
400
300
200
100
0
+cAMP
-cAMP
Ca2+
H2O or 10-7M cAMP
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
TIME (min.)

The effect of 38 μM cAMP and 1 μM cAMP on Ca^{2+} release from mitochondria was examined in sections 7.3.5.1 and 7.3.5.2.

7.3.5.1 The effect of 38 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA, carnitine and ATP studied by the radio-isotope technique

Substrates were added to the standard incubation mixture. The substrates present were 20 μM palmitoyl CoA, 2 mM carnitine and 1 mM ATP. Fig. 70 indicates that at 10 min incubation, the test mitochondria (with cAMP) and the control mitochondria accumulated similar amounts of Ca^{2+}. At 20 min incubation, the control mitochondria contained 58 nmoles $^{45}Ca^{2+}$/mg protein; the test mitochondria, on the other hand, had 50 nmoles $^{45}Ca^{2+}$ per mg protein, suggesting that 38 μM cAMP had caused a release of 8 nmoles Ca^{2+} per mg protein.

FIGURE 70

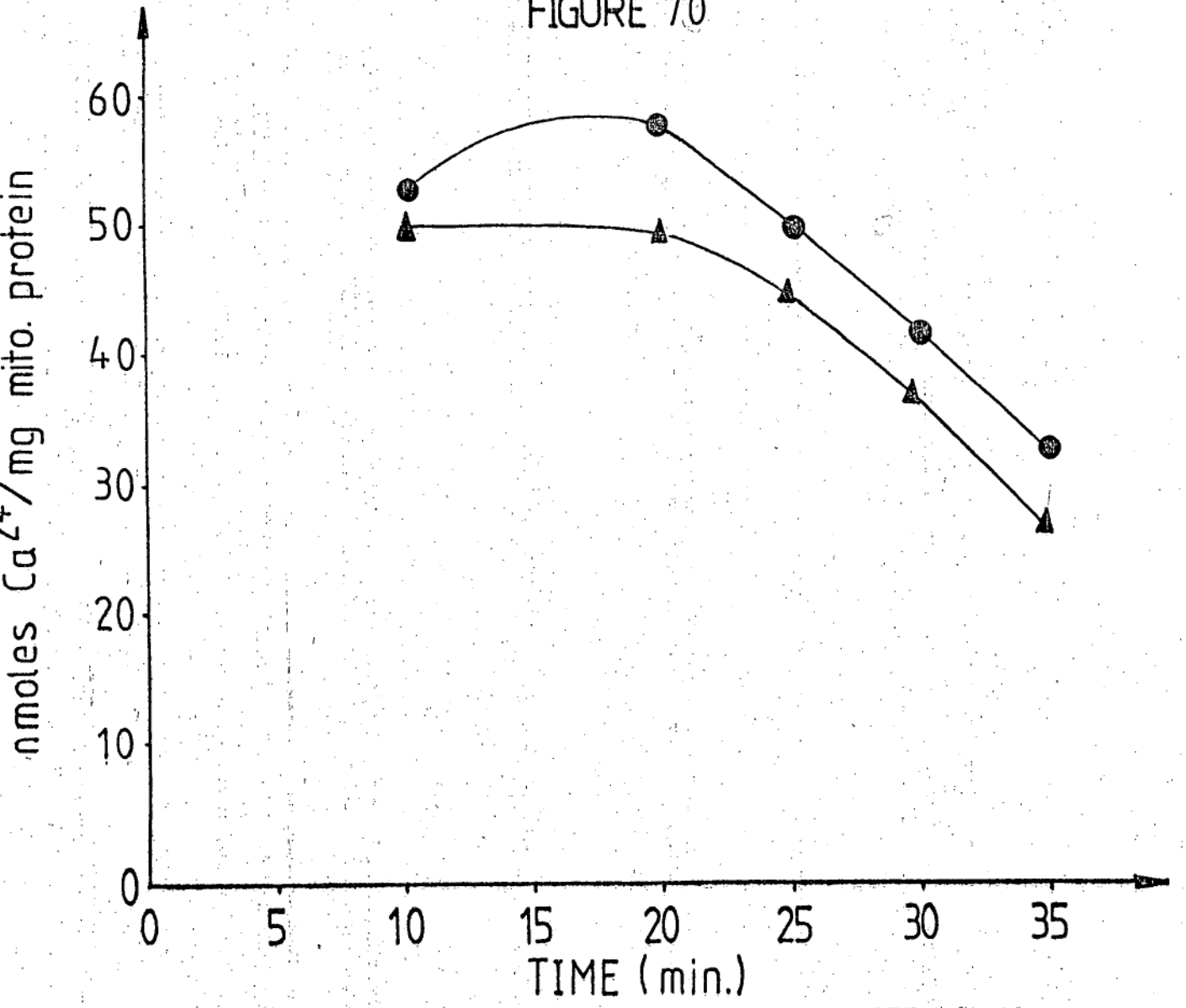
Effect of 38 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA and carnitine studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 20 μM palmitoyl CoA, 2 mM DL-carnitine HCl, 1 mM ATP and mitochondria (5.2 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. The addition of cAMP (final concentration = 38 μM) was made before adding the $^{45}Ca^{2+}$.

control

+ cAMP (38 μM)

FIGURE 70


nmoles Ca^{2+}/mg mito. protein
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
TIME (min.)

7.3.5.2 The effect of 1 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoylcarnitine and ATP studied by the radio-isotope technique.

The substrates present were 50 μM palmitoylcarnitine and 0.1 mM ATP. As shown in fig 71, the control mitochondria accumulated approx. 39 nmoles $^{45}Ca^{2+}$/mg protein at 4 min and started releasing this Ca^{2+} thereafter. The test mitochondria incubated with 1 μM cAMP accumulated 38 nmoles $^{45}Ca^{2+}$/mg protein, and released this Ca^{2+} 2 min earlier than the control mitochondria. At 12 min incubation, the test mitochondria contained 28 nmoles $^{45}Ca^{2+}$/mg protein while the control mitochondria had 32 nmoles/mg protein.

7.3.6 The effect of starvation on Ca^{2+} release from mitochondria studied by the radio-isotope technique.

For this particular investigation, mitochondria were prepared from normal stock fed rats and from their litter mates that were starved (with adequate water) for 40 hr in a cage which prevented coprophagy. Preparation of the mitochondria was described in chapter 1, section 1.1 . Mitochondria from fed and starved rats were prepared and used simultaneously. Since a number of test situations were examined simultaneously, the radio-isotope technique was used to monitor Ca^{2+} movement into and out of the mitochondria.

7.3.6.1 The effect of starvation on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA, carnitine and ATP.

Mitochondria from starved rats (7.6 mg protein) or from fed rats (8.1 mg protein) were added to the standard incubation

FIGURE 71

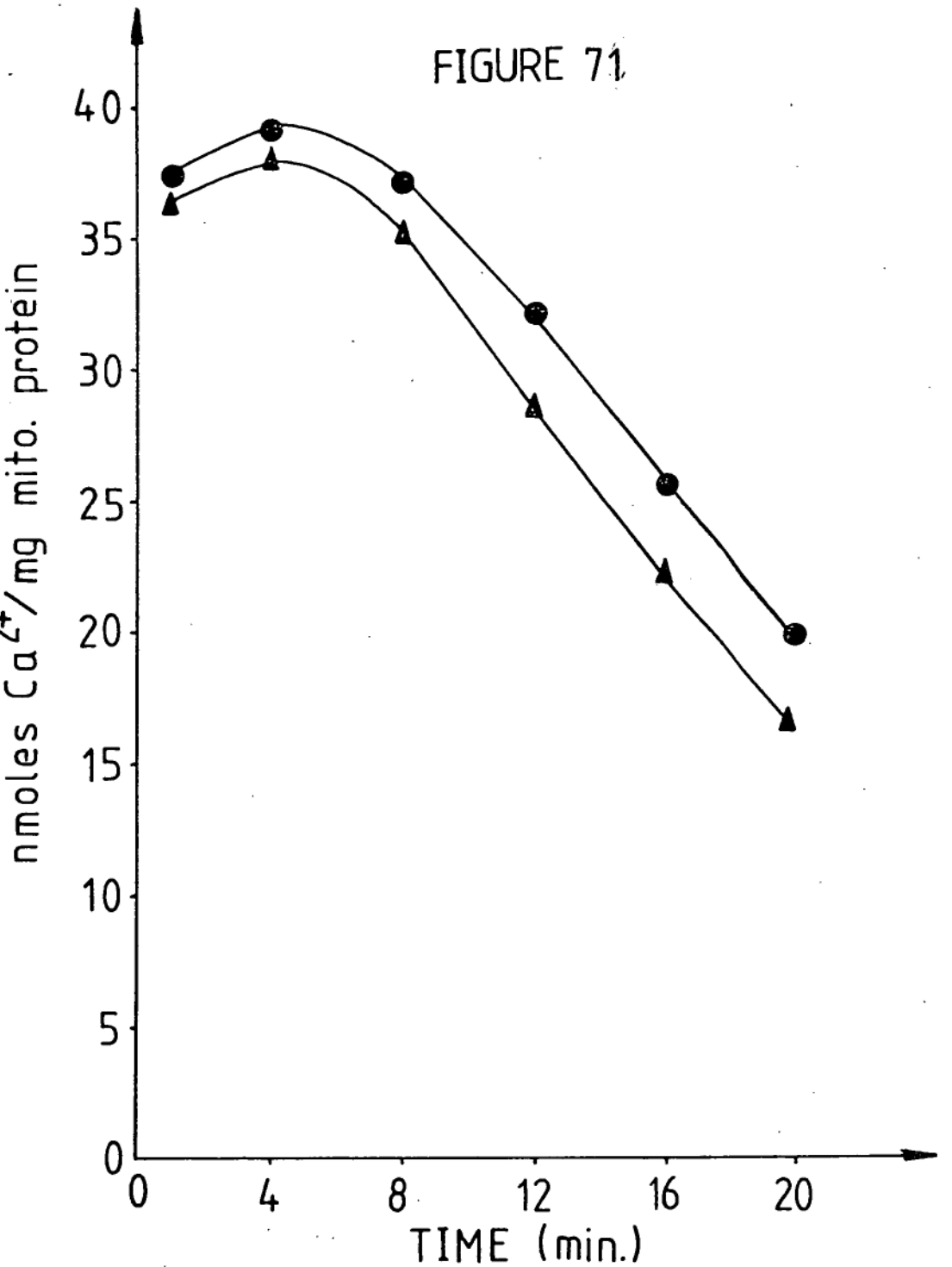
Effect of 1 μM cAMP on Ca^{2+} release from mitochondria in the presence of palmitoylcarnitine studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 50 μM palmitoylcarnitine, 0.1 mM ATP and mitochondria (9.3 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C. The addition of cAMP (final concentration = 1 μM) was made before adding the $^{45}Ca^{2+}$.

control

+ cAMP (1 μM)

FIGURE 71


nmoles Ca^{2+}/mg mito. protein
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
TIME (min.)

medium containing 400 nmoles Ca^{2+}, 20 μM palmitoyl CoA, 2 mM carnitine and 1 mM ATP. Since the amounts of mitochondria added were different, the results were expressed as percentage of maximum possible Ca^{2+} in the mitochondria, rather than nmole Ca^{2+}/mg protein.

As shown in fig 72, the mitochondria from starved rats started releasing their accumulated $^{45}Ca^{2+}$ at 20 min, but the control mitochondria retained their $^{45}Ca^{2+}$ at that time. In fact at 40 min, the control mitochondria (i.e. from fed rats) still contained 85 % of the maximum possible $^{45}Ca^{2+}$, while the test mitochondria only had 59 % (fig 72). Fig 72 also shows that the uptake of $^{45}Ca^{2+}$ by the mitochondria from starved rats was relatively slower than the mitochondria from fed rats.

7.3.6.2 The effect of starvation on Ca^{2+} release from mitochondria in the presence of succinate and ATP.

In the presence of 2 mM succinate and 1 mM ATP, the mitochondria from starved rats started to release the accumulated $^{45}Ca^{2+}$ at 10 min. The mitochondria from fed rats still retained ~~retained~~ their $^{45}Ca^{2+}$ during the 40 min duration of the experiment (fig 73).

Since this experiment was performed at the same time and using the same mitochondria as the experiment in section 7.3.6.1, the results can be compared. The effect of starvation on Ca^{2+} release from mitochondria was greater when palmitoyl CoA, carnitine and ATP were substrates (fig 72), than when succinate and ATP were the substrates (fig 73).

FIGURE 72

Effect of starvation on Ca^{2+} release from mitochondria in the presence of palmitoyl CoA and carnitine studied by the radioassay technique.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 20 μM palmitoyl CoA, 2 mM DL-carnitine HCl, 1 mM ATP and mitochondria from starved rats (7.6 mg protein) or from fed rats (8.1 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 μCi). Incubation temp. = 25°C.

—○— mitochondria from fed rats

---●--- mitochondria from starved rats

FIGURE 72

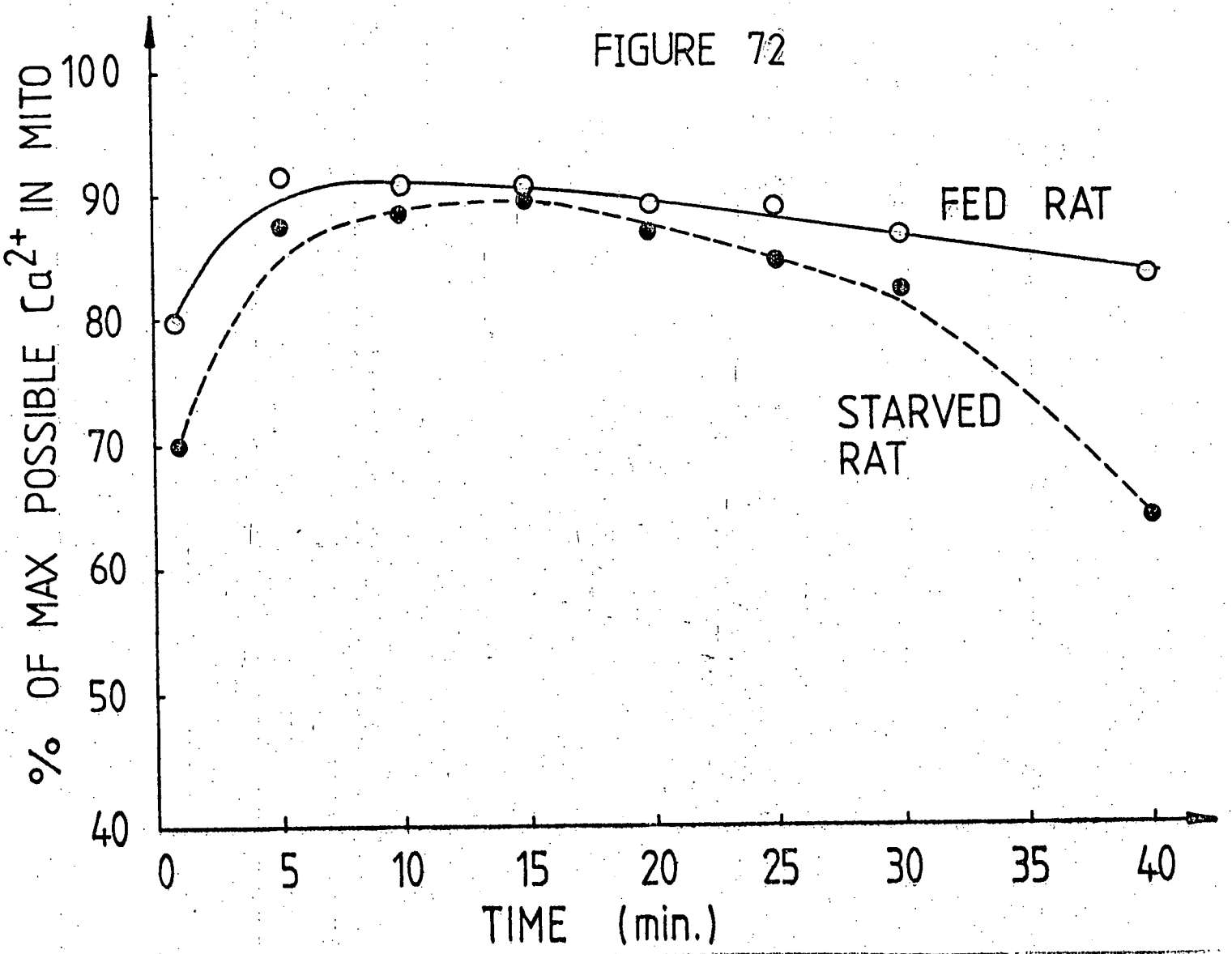

% OF MAX POSSIBLE Ca^{2+} IN MITO
100
90
80
70
60
50
40
0
5
10
15
20
25
30
35
40
TIME (min.)
FED RAT
STARVED RAT

FIGURE 73

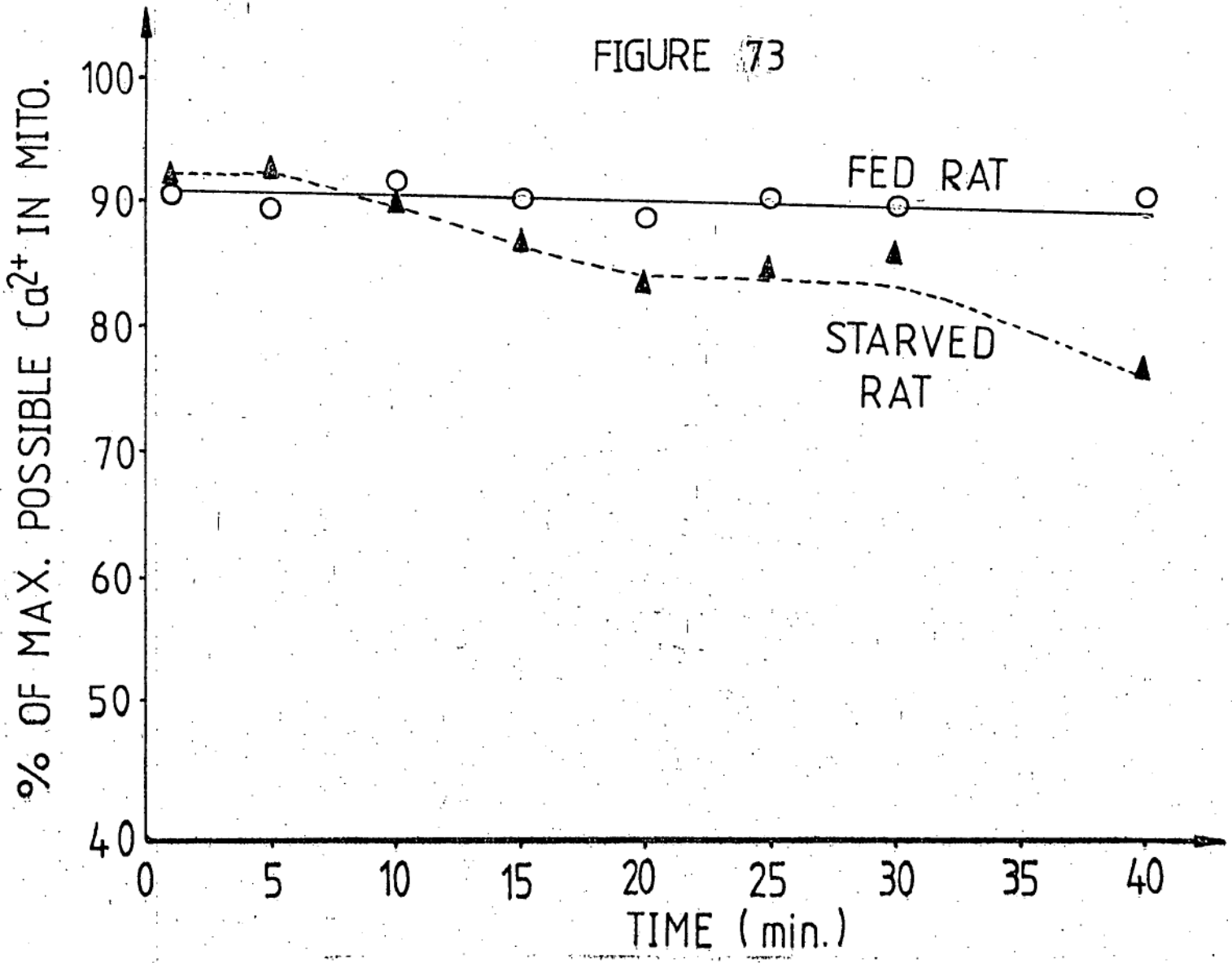
Effect of starvation on Ca^{2+} release from mitochondria in the presence of succinate.

The incubation system (final vol. = 5 ml) contained the standard incubation mixture, 2 mM succinate, 1 mM ATP and mitochondria from starved rats (7.6 mg protein) or from fed rats (8.1 mg protein). The reaction was started by the addition of 400 nmoles $^{45}Ca^{2+}$ (1 µCi). Incubation temp. = 25°C.

—○— mitochondria from fed rats

—▲-- mitochondria from starved rats

FIGURE 73


% OF MAX. POSSIBLE Ca^{2+} IN MITO.
100
90
80
70
60
50
40
0
5
10
15
20
25
30
35
40
TIME (min.)
FED RAT
STARVED RAT

7.3.6.3 The concentrations of Ca^{2+}, pyridine nucleotides and free fatty acids in mitochondria from starved and fed rats.

The free fatty acid content of the liver mitochondria was slightly higher in the starved rats (43 - 49 nmoles/mg mitochondrial protein) than the fed rats (39 - 47 nmoles/mg protein).

Mitochondria (10 mg protein) from starved and fed rats were added to 5 ml of incubation medium containing Pi, Mg^{2+}, K^{+}, palmitoyl CoA, carnitine, ATP and 400 nmoles Ca^{2+}. Calcium and pyridine nucleotides were estimated at 1 min after adding the mitochondria to the incubation medium. The results shown in table 16 suggest that at 1 min incubation, the Ca^{2+} content in mitochondria from starved rats (24 nmoles/mg) was less than mitochondria from fed rats(30 nmoles/mg) and the $NADH/NAD^{+}$ ratio was lower (starved = 0.06, fed = 0.15). It was also observed that NADP concentration in mitochondria from starved rats were higher than that obtained from fed rats.

TABLE 16

Concentrations of calcium and pyridine nucleotides in starved and fed rat liver mitochondria.

Calcium uptake by rat liver mitochondria was by the radioassay technique: 10 mg of mitochondrial protein from starved (40 hrs starvation) or fed rats were added to 5 ml of 0.25M sucrose, 2.5 mM HEPES pH 7.4, 2 mM P_i pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 20 μM palmitoyl CoA, 2 mM carnitine, 1 mM ATP and 400 nmoles Ca^{2+}. Calcium and pyridine nucleotides in mitochondria were estimated at 1 min. after adding mitochondria in the incubation medium.

	nmoles/mg mitochondrial protein	
	FED	STARVED
Calcium	30	24
NAD^+	2.6	3.3
NADH	0.4	0.2
$NADP^+$	2.6	4.5
NADPH	not detectable	not detectable
$NADH/NAD^+$ ratio	0.15	0.06

7.4 DISCUSSION

There is considerable evidence to indicate that efflux of Ca^{2+} from mitochondria differs from the influx mechanism (Peng et al, 1977; Nicholls, 1978(b); Crompton et al, 1978; Caroni et al, 1978). While there have been numerous reports on the mechanism of Ca^{2+} uptake by mitochondria, the mechanism of Ca^{2+} release from mitochondria is still uncertain.

Na^{+} has been found to cause Ca^{2+} release from mitochondria isolated from rat heart and brain (Carafoli et al, 1974; Crompton et al, 1976), but not mitochondria from rat liver and kidney.

In the chick small intestine, Hamilton and Holdsworth (1975) reported that Ca-binding protein from chick duodena caused Ca^{2+} release from intestinal mitochondria.

Palmitoyl CoA induced Ca^{2+} release from rabbit heart mitochondria (Asimakis and Sordahl, 1977) and rat liver mitochondria (section 3.3.5); however, this effect was inhibited in the presence of carnitine and ATP. Peng et al (1974) reported that phosphoenolpyruvate (PEP) caused Ca^{2+} release from rat mitochondria; however, it was shown in the present study (section 5.3.3) that inclusion of pyruvate kinase together with PEP in the incubation medium helped Ca^{2+} retention, due to regeneration of ATP in the system. Since ATP, carnitine and pyruvate kinase are present in the rat liver and heart cytosol, it is therefore unlikely that PEP or palmitoyl CoA regulates Ca^{2+} release from mitochondria under physiological conditions.

For some years, cAMP has been favoured as a "messenger"

in the control of cell Ca^{2+} (Rasmussen, 1970). In fact, Borle (1974) reported that between 0.1 to 3 μM cAMP caused a rapid and massive release of Ca^{2+} from mitochondria of rat liver, heart and kidney. He used succinate and ATP as the energy sources for Ca^{2+} uptake by mitochondria. The cAMP releasing effect was also obtained by Matlib and O'Brien (1974). However, the effect was not observed by other workers such as Scarpa et al (1976). Borle in 1976 reinvestigated the cAMP effect and out of the 442 experiments performed only 6 % showed a significant release from liver or kidney mitochondria, and so this finding of the cAMP effect was retracted by Borle (1976). It should be noted that Scarpa et al (1976) and Borle (1976) reexamined the cAMP effect in the presence of ATP and/or respiratory substrates. However there are reasons to suspect that cAMP could cause Ca^{2+} release. Christiansen (1977) reported that glucagon lowered the ratio of β-hydroxybutyrate/acetoacetate of rat hepatocytes (i.e. reflecting a lowered ratio of mitochondrial $NADH/NAD^+$) when palmitate was substrate. Glucagon is known to increase the level of cAMP in hepatocytes (Garrison and Haynes, 1973) and Neville and Jamieson of this department in fact noted that dibutyryl cAMP lowered β-hydroxybutyrate/acetoacetate ratio in rat hepatocytes in the presence of palmitate. A lowering of the steady state ratio of $NADH/NAD^+$ would favour Ca^{2+} release (Lehninger et al, 1978(b)). Hence it is possible that cAMP could cause Ca^{2+} to be released via its effect on mitochondrial $NADH/NAD^+$ ratio. This chapter of the thesis re-examined the effect of cAMP on Ca^{2+} release from mitochondria using palmitoyl CoA or palmitoylcarnitine as

substrate.

Effect of cAMP on Ca^{2+} release in the presence of palmitoyl CoA, carnitine and ATP.

The results obtained in the present study revealed that cAMP caused an earlier release of Ca^{2+} from test mitochondria than control mitochondria. In the presence of 20 μM palmitoyl CoA, 2 mM carnitine, 1 mM ATP and 10^{-5}M rotenone, the cAMP effect was small (fig 60). Dibutyryl cAMP also stimulated the mitochondria to release their accumulated Ca^{2+} relatively earlier than the control mitochondria (fig 61). The effect was however not observed with a similar concentration of cGMP (fig 62).

When rotenone was excluded from the incubation medium, a more significant Ca^{2+} release from the mitochondria by cAMP was observed as shown in fig 63 (c.f. fig 60). Thus it appears that rotenone partially inhibits the effect of cAMP.

The presence of 3-isobutyl-1-methylxanthin (IBM), an inhibitor of phosphodiesterase, did not increase the effect of cAMP any further, since similar amounts of Ca^{2+} were released from the mitochondria by cAMP in the presence or absence of IBM (fig 63, ~~69~~). The results suggest that either (1) phosphodiesterase contamination in the mitochondrial preparation was insignificant, or (2) the amount of cAMP added was excessive so that even in the presence of contaminating phosphodiesterase there was sufficient cAMP remaining in the medium to affect Ca^{2+} release from the mitochondria.

Effect of cAMP on Ca^{2+} release from mitochondria not preloaded with Ca^{2+}.

Cyclic AMP also caused an earlier release of Ca^{2+} from the mitochondria which were not preloaded with Ca^{2+}, but contained 20 nmoles Ca^{2+}/mg protein (fig 64). A release of approx. 3 nmoles Ca^{2+}/mg protein from the mitochondria by cAMP was observed after 20 min incubation. The substrates used for this particular investigation were 20 μM palmitoyl CoA, 2 mM carnitine and 1 mM ATP. In order to measure the release of small amounts of Ca^{2+} from the mitochondria, the output of the amplifier of the Ca-electrode was connected to a second amplifier containing an antilog stage with a linear output in the range of 10^{-5}M - 10^{-6}M. Due to the high concentration of ATP added to the medium, the true release of Ca^{2+} was probably greater than appears in fig 64 since some of the released Ca^{2+} combines with the ATP present in the medium. It was reported earlier (in chapter 5 and in this chapter) that even though 1 mM Mg^{2+} was present in the incubation medium, ATP still combines with a large proportion of the ionic Ca^{2+} in the medium.

The Ca^{2+} estimated in the mitochondria was most likely the endogenous Ca^{2+}. The value obtained agrees with Heaton and Nicholls (1976) (i.e. 18 nmoles endogenous Ca^{2+}/mg mitochondrial protein) and Vinogradov et al (1972) (10 - 15 nmoles Ca^{2+}/mg protein).

Effect of cAMP on Ca^{2+} release in the presence of palmitoylcarnitine and ATP.

The Ca-electrode cannot detect Ca^{2+} chelated to ATP or that transported into the mitochondria. When the Ca-electrode was

used to monitor Ca^{2+} movement into and out of the mitochondria, the presence of a high concentration of ATP in the medium appeared to mask the full extent of Ca^{2+} release from the mitochondria by cAMP.

In order to detect a significant effect of cAMP on Ca^{2+} release, a lower concentration of ATP was necessary. By using 50 μM palmitoylcarnitine as substrate, a low concentration of ATP, i.e. 0.1 mM could be used. Indeed, under the stated conditions, cAMP at the concentration range examined, i.e. between 0.1 μM to 75 μM, caused significant release of Ca^{2+} from the mitochondria (figs 67, 68 and 69). Comparing the result in fig 67 with fig 63, the release of Ca^{2+} from mitochondria by cAMP appeared to be small in the presence of palmitoyl CoA, carnitine and 1 mM ATP but was more obvious when 0.1 mM ATP was present.

It was also observed that the effect of cAMP on Ca^{2+} release from the mitochondria increased with increasing concentration of cAMP within the concentration range examined. For example in the presence of 75 μM cAMP, the test mitochondria started to release the accumulated Ca^{2+} approx. 8 min earlier than control mitochondria (fig 67); however, with 0.1 μM cAMP the releasing time was only 3 min earlier than the control (fig 69). It should be noted that the time at which release of Ca^{2+} from the control mitochondria was first detected differed slightly from one experiment to the other, even though the incubation conditions were similar (refer to section 2.4).

In 18 experiments, significant release of Ca^{2+} from the mitochondria was obtained with cAMP. No effects were obtained

in experiments in which the mitochondria were kept in ice for longer than 1 hr, although these mitochondria were still capable of Ca^{2+} uptake and retention. For this reason only 1 test situation (with the control) was examined per mitochondrial preparation, when examining the effect of cAMP using the Ca-electrode. The radioassay technique on the other hand was not sensitive enough to detect the cAMP releasing effects, although slight release of Ca^{2+} was noted (figs 70,71).

Effect of cAMP on Ca^{2+} release in the presence of succinate, rotenone and ATP

In the present study, the release of Ca^{2+} from mitochondria was not observed in the presence of the substrates succinate, rotenone and ATP. This result is in agreement with the observations by Scarpa et al (1976). (The latter workers also reported negative results when other respiratory substrates were used, such as glutamate + malate or pyruvate + malate). Palmitoyl CoA (in the presence of carnitine and ATP) or palmitoylcarnitine with ATP seemed to be the essential substrates for the release of Ca^{2+} by cAMP, suggesting that the β-oxidation of fatty acid may be involved. Earlier, Otto and Ontko (1978) reported that Ca^{2+} accumulation by mitochondria increased the oxidation of added palmitate in the mitochondrial matrix, resulting in an increased production of ketone bodies (acetoacetate plus β-hydroxybutyrate) from acetyl CoA via the hydroxymethylglutaryl-CoA (HMG-CoA) pathway (also known as the Lynen Cycle). The latter workers also noted an increase in β-hydroxybutyrate/acetoacetate ratio (i.e. reflecting an increased mitochondrial $NADH/NAD^+$ ratio).

In this study, the observed release of Ca^{2+} from the mitochondria by cAMP in the presence of palmitoyl CoA or palmitoylcarnitine

as substrates is possibly due to cAMP altering the activity of certain enzyme/s involved in ketogenesis, so that the β-hydroxybutyrate/acetoacetate ratio is lowered. It is known that cAMP increases lipolysis whereby the non-activated lipase is converted to the activated form (Sutherland Jr et al, 1968). It is therefore possible that cAMP activated the lipase, which in turn may alter the ratio of β-hydroxybutyrate/acetoacetate. A relatively more oxidised steady state of mitochondrial pyridine nucleotides would therefore favour Ca^{2+} release from the mitochondria. Unfortunately, it was rather difficult to examine simultaneously Ca^{2+} release from the mitochondria by cAMP and the concentrations of mitochondrial pyridine nucleotides. In future, attempts should be made to estimate mitochondrial NADH/NAD^+ ratio during Ca^{2+} release by cAMP. One of the problems one might face when examining the effect of cAMP on Ca^{2+} release from mitochondria is that Ca^{2+} accumulation itself increased the oxidation of added palmitate resulting in an increase in mitochondrial NADH/NAD^+ ratio (Otto and Ontko, 1978). Carnitine also stimulates palmitate oxidation making the mitochondrial pyridine nucleotides more reduced (Christiansen, 1977). Assuming that cAMP lowers the mitochondrial NADH/NAD^+ ratio, a favourable condition must be achieved so that a more oxidised steady state mitochondrial pyridine nucleotides is obtained. A possible means to overcome the above problem is to use a lower concentration of Ca^{2+} and carnitine in the incubation medium.

Effect of starvation on Ca^{2+} release.

Starvation is known to increase the levels of glucagon in blood (Gerich, 1976) which would be expected to increase the cAMP levels in liver (Garrison and Haynes, 1973). Therefore, in the present study, the effect of starvation on Ca^{2+} release from the mitochondria was examined. The results obtained indicated that the mitochondria from the starved rats released their accumulated Ca^{2+} earlier than the mitochondria from fed rats. Interestingly, the above effect was also obtained when using the substrates succinate plus ATP although the effect was not as marked as when palmitoyl CoA, carnitine and ATP were the substrates (figs 72, 73). A probable explanation is as follows:- during starvation, the levels of glucagon (Gerich, 1976) and free fatty acids (Bohmer, 1967) in blood, and of carnitine in liver (McGarry et al, 1975) increased. As the concentration of free fatty acids in blood increased, the concentration of acylcarnitine in liver increased, resulting in an increase in the rate of fatty acid oxidation (Bohmer, 1967). Apparently, glucagon may have a "push-pull" effect on fatty acid oxidation by making more acyl-CoA and acylcarnitine available for β-oxidation and at the same time facilitating the oxidation with a lowered redox potential, i.e. a decrease in β-hydroxybutyrate/acetoacetate ratio (Bremer et al, 1978). Presumably the pyridine nucleotides in mitochondria isolated from starved rats are in a relatively more oxidised steady state which would explain why the mitochondria from starved rat released their accumulated Ca^{2+} earlier than the mitochondria from fed rat, whether the substrates used were palmitoyl CoA (plus carnitine and ATP) or succinate (plus ATP).

Indeed it was observed in the present study, that the mitochondria isolated from starved rat had a $NADH/NAD^+$ ratio = 0.06 compared with the $NADH/NAD^+$ ratio of 0.15 in the mitochondria from fed rat. (Pyridine nucleotides were estimated at 1 min during Ca^{2+} accumulation by the mitochondria). The free fatty acid content of the mitochondrial suspensions was only slightly higher in starved rat 43 - 49 nmoles/mg protein than the fed rat 39 - 47 nmoles/mg protein. It is unlikely that the slightly higher free fatty acid in mitochondrial suspension from starved rat would have caused the earlier release of Ca^{2+} from the mitochondria.

Other workers have reported that mitochondria isolated from glucagon-treated fed rats accumulated added Ca^{2+} in the incubation medium and retained this Ca^{2+} for a longer period compared with control mitochondria (Hughes and Barritt, 1978; Prpic et al, 1978). Glucagon (25 µg/100 g body weight) or normal saline was administered to fed rats by intraperitoneal injection or by perfusion of livers from fed rats. Although glucagon administration to fed rats should have a similar effect to starvation, the results above contradict the observation in the present study that the mitochondria isolated from starved rats released their accumulated Ca^{2+} earlier than the mitochondria from fed rats. It should be noted that Hughes and Barritt, 1978; Prpic et al, 1978; administered glucagon to the rats and waited 1 hr before isolating the mitochondria, whereas glucagon effects on mitochondria were noted within minutes of treatment (Haynes, 1976; Garrison and Haynes, 1975; Yamazaki, 1975). A suggestion for future experiment is to determine the $NADH/NAD^+$ ratio in mitochondria isolated from starved rats and glucagon-treated fed rats.

The observations in the present study suggested that cAMP caused Ca^{2+} release from the mitochondria when examined *in vitro*. Cyclic AMP may well be the physiological modulator for Ca^{2+} release from mitochondria, thus regulating the levels of ionic Ca^{2+} in the cytosol in μM concentration range.

7.5 SUMMARY

1. cAMP (75 μM) did not affect Ca^{2+} uptake by the mitochondria.

2. cAMP (0.1 - 75 μM) caused Ca^{2+} release from the mitochondria provided that:-
   (a) the mitochondria prepared from fed rats were used within 1 hr after isolation
   (b) palmitoyl CoA + carnitine + ATP or palmitoylcarnitine + ATP were used as the substrates for oxidation by the mitochondria.

   Cyclic AMP did not cause Ca^{2+} release from the mitochondria when using the substrates succinate + ATP (in the presence of rotenone).

3. Using palmitoyl CoA + carnitine + ATP (in the presence of rotenone) it was found that:-
   (a) cAMP (75 μM) caused an earlier release of Ca^{2+} from the mitochondria
   (b) Dibutyryl cAMP (75 μM) also stimulated an earlier Ca^{2+} release
   (c) cGMP (75 μM) had no effect on Ca^{2+} release.

4. Using palmitoyl CoA + carnitine + ATP (without rotenone) it was found that:-
   (a) the effect of cAMP (75 μM) on Ca^{2+} release was more apparent than that in the presence of rotenone
   (b) 3-isobutyl-1-methylxanthin, an inhibitor of phosphodiesterase did not appear to increase the effect of 75 μM cAMP on Ca^{2+} release
   (c) cAMP (75 μM) caused release of Ca^{2+} from the

mitochondria which were not preloaded with Ca^{2+} but contained 20 nmoles Ca^{2+}/mg protein.

5. Using palmitoylcarnitine and a low concentration of ATP (0.1 mM), it was found that cAMP (0.1 - 75 μM) caused significant release of Ca^{2+} from the mitochondria.

6. Mitochondria isolated from starved rats had a lower $NADH/NAD^{+}$ ratio and released their Ca^{2+} earlier than the mitochondria from fed rats (substrates were (a) palmitoyl CoA + carnitine + ATP and (b) succinate + ATP). The free fatty acid content in the mitochondrial suspensions isolated from starved rats was only slightly higher than the fed rats.

7. cAMP may be the physiological modulator of Ca^{2+} release from mitochondria.

CHAPTER 8

FINAL DISCUSSION

In the previous chapters, the effect of the rat cytosol and various components of the cytosol on Ca^{2+} movement into and out of the isolated mitochondria was investigated. The effect of the redox state of mitochondrial pyridine nucleotides on Ca^{2+} release and retention by the mitochondria was also examined. The aim of this particular chapter is to integrate the findings reported in the previous chapters. A possible mechanism of Ca^{2+} uptake and release by rat liver mitochondria is discussed in this chapter, based on the results obtained in the present study and on the published reports by other workers.

Techniques used for *in vitro* studies of mitochondrial Ca^{2+} transport

The two techniques employed to monitor Ca^{2+} movement into and out of the mitochondria are a) radioassay technique whereby radioactive calcium, $^{45}Ca^{2+}$, was used. b) Ca-electrode was used to monitor changes in ionic Ca^{2+} concentration in the medium. A comparison between the two techniques used is summarised in the table below.

Ca-electrode	Radioassay technique
1. Continuous monitoring of Ca^{2+} movement into and out of the mitochondria.	1. Incubation mixtures were sampled at various time intervals. When a maximum of 8 test situations was examined at one time, a time interval of less than 3 min was not feasible.
2. Only one test situation could be examined at one	2. A number of test situations could be examined simultaneously under

Ca-electrode	Radioassay technique
time. This method was not used when examining a number of test situations which require long incubations periods since "aging" of the mitochondria may give misleading results.	similar incubation conditions. This technique was particularly useful for comparing various test situations which require long incubation periods, e.g. factors that help Ca^{2+} retention in the mitochondria.
3. Ca-electrode only measures free ionic Ca^{2+} in the medium and therefore cannot detect Ca^{2+} transported into the mitochondria or chelated to other substances in the medium, e.g. ATP, the outer membrane of the mitochondria.	3. The EGTA-ruthenium red quench technique (Reed and Bygrave, 1974(a)) ensured discrimination of Ca^{2+} transported into the mitochondria from that bound externally to the inner mitochondrial membrane.
4. The Ca-electrode was connected to a special high performance amplifier. The output could be obtained in the range 10^{-4}M to 10^{-6}M. The output of this amplifier in some experiments was also connected to a second amplifier containing an antilog stage. Using this amplifier, a linear output to the recorder could be obtained in the range 10^{-5}M to 0.5×10^{-6}M. The lower concentration range measureable with the electrode depends on	4. For detection of small quantities of Ca^{2+}, a high specific activity of $^{45}Ca^{2+}$ is required. The smallest concentration of $^{45}Ca^{2+}$ detectable by the radioassay technique was approx. 2×10^{-6}M.

Ca-electrode	Radioassay technique
the ion-exchange materials used in the electrode. The Radiometer electrode (the most sensitive available, No. F2112) was only able to measure down to 10^{-6}M.	

Although there are some advantages and disadvantages in the two techniques used, nevertheless they were complementary. For example the Ca-electrode which allows continuous monitoring of Ca^{2+} transport by the mitochondria was useful when studying Ca^{2+} cycling in the mitochondria on alternately adding oxaloacetate and β-hydroxybutyrate, or when observing the effect of added adenine nucleotide on the initial Ca^{2+} uptake by the mitochondria after exhaustion of the endogenous substrates. Since the electrode was connected to a stable and sensitive amplifier, it was possible to detect the effect of cAMP on the release of small amounts of Ca^{2+} from the mitochondria which were not preloaded with Ca^{2+}. The radioassay technique on the other hand was useful when examining a number of test situations simultaneously; for example, when studying the effect of palmitoyl CoA, palmitoyl-carnitine or palmitate on Ca^{2+} transport by the mitochondria. A few of the experiments in this thesis were done by both the radioassay technique and the Ca-electrode and the two methods gave consistent results.

Since the EGTA-ruthenium red quench medium was used to inhibit Ca^{2+} uptake and removed Ca^{2+} bound to the outer surface of the inner mitochondrial membrane, the $^{45}Ca^{2+}$ estimated in the mitochondria would be the transported $^{45}Ca^{2+}$. The supernatant contained the soluble $^{45}Ca^{2+}$ which would include free ionic $^{45}Ca^{2+}$, $^{45}Ca^{2+}$ combined

to ATP in the medium and $^{45}Ca^{2+}$ removed from the low affinity binding sites (on the outer side of the inner mitochondrial membrane) by the EGTA-ruthenium red quench medium.

The Ca-electrode on the other hand, only measures free ionic Ca^{2+} in the medium. The mitochondria appeared to take up more than the total amount of added Ca^{2+} when studied by the Ca-electrode, possibly due to minute traces of Ca^{2+} remaining in some of the reagents despite attempts to remove contaminants by filtration through columns of Chelex-100. It should be noted that the Ca^{2+} contamination which would be in μM concentration range was exaggerated by the log scale. The concentration of free Ca^{2+} in the medium, after Ca^{2+} uptake by the mitochondria in the presence of energy substrate/s, was approximately 1 μM as calibrated with the Ca-nitrilotriacetic acid buffers (Reed and Bygrave, 1975(a)).

When using the Ca-electrode it was noted that prior to adding the mitochondria, 1 mM ATP combines with 275 nmoles Ca^{2+} out of the 400 nmoles added. Although the electrode cannot detect Ca^{2+} chelated to ATP or that transported into the mitochondria, obviously, the mitochondria have a higher affinity for the Ca^{2+} in the medium than has ATP, since examination by means of the EGTA-ruthenium red quench technique indicated that 85 - 90 % of the externally added Ca^{2+} was transported into the mitochondria in the presence of 1 mM ATP.

Conditions under which Ca^{2+} movement was studied

The iso-osmotic sucrose medium (pH 7.3 - 7.4) used to study Ca^{2+} transport by the mitochondria contained 1 mM Mg^{2+}, 2 mM Pi pH 7.4 and 72 mM K^{+} (unless otherwise stated), i.e. at concentration almost similar to those found in the rat cytosol. The energy sources for Ca^{2+} uptake were 1 mM ATP and 2 mM respiratory substrate such as β-hydroxybutyrate, succinate.

The P:O ratio for each batch of the mitochondrial preparation was determined to ensure that well coupled mitochondria were used for the study.

The amount of mitochondria or Ca^{2+} added to the incubation medium was such that no greater than 100 nmoles Ca^{2+} was accumulated per mg mitochondrial protein in order to avoid massive Ca^{2+} loading which would otherwise result in swelling, membrane damage and organelle lysis (Lehninger et al, 1967). Generally, 400 nmoles Ca^{2+} (80 μM final conc.) and 4 - 8 mg mitochondrial protein were present in the 5 ml incubation medium. It should ~~also~~ be pointed out that the actual amount of soluble Ca in the cytosol is probably 0.1mM (Foden and Randle, 1978) but in the presence of 4 - 6 mM ATP, 2mM Mg^{2+} and mitochondria, the concentration of free Ca^{2+} can be about 1 μM. Some other workers have exposed the mitochondria to much higher concentrations of Ca^{2+} (2 - 4 mM) when alteration in mitochondrial structure or function was observed (Vasington and Murphy, 1962; DeLuca and Engstrom, 1961; Brierley et al, 1963(b); Rossi and Lehninger, 1963; Carafoli et al, 1964; Borle, 1974).

It should be noted that even though the incubation conditions for each experiment were kept constant, the time at which release of Ca^{2+} from the control mitochondria was first detected varied with different mitochondrial preparations used, possibly due to varying concentrations of contaminating free fatty acids, endogenous substrates or even the physiological state of the rat prior to decapitation. For these reasons, the results obtained were compared with the control of the respective experiment. Since it was not possible to group experiments, statistics on the results was not carried out. However, each experiment presented in this thesis was repeated at least 3 times, using different mitochondrial preparations, to obtain consistent results.

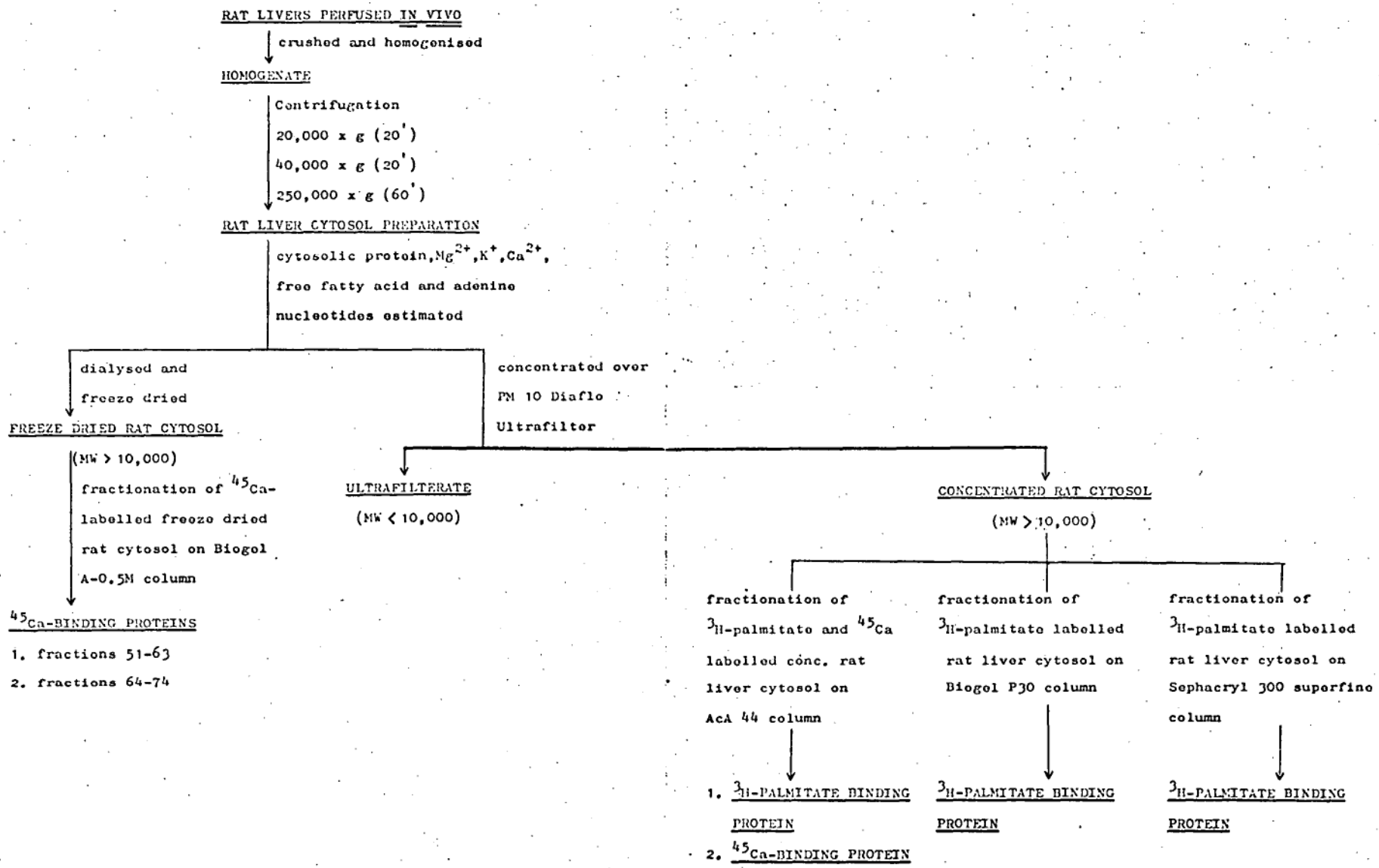
Ca^{2+} movement in the presence of rat cytosol and some of the components present in the cytosol

The preparation of the rat liver cytosol and the subsequent fractionations of the concentrated rat cytosol (MW $>$ 10,000) and the freeze dried rat cytosol (MW $>$ 10,000) is outlined in diagram 11. The rat cytosol preparation (i.e. the 250,000 x g supernatant) contained approximately half the reported concentrations of cytosolic protein, K^+ and Ca^{2+} suggesting that only 50 % of the cells were broken by the method of preparation.

The presence of the concentrated rat liver cytosol or the freeze dried rat cytosol in the incubation medium inhibited Ca^{2+} uptake and caused an immediate release of Ca^{2+} from the mitochondria respiring on added respiratory substrates, with or without ATP as the additional energy source for Ca^{2+} uptake (figs 5, 21). The inhibitory factor was most likely fatty acids bound to protein, since inclusion of carnitine and ATP prevented release (fig 8). In support of such a conclusion, partial removal of the fatty acids present in the concentrated rat cytosol by florisil treatment helped to diminish some of the inhibitory effect (fig 8). It was also observed that the addition of BSA caused reuptake of the Ca^{2+} released by the mitochondria in the presence of the concentrated rat cytosol (fig 6), suggesting that the release observed was due to uncoupling of oxidative phosphorylation by fatty acids and that BSA helped to reactivate the uncoupled oxidative phosphorylation (Helinski and Cooper, 1960).

The ultrafiltrate obtained after concentrating the rat cytosol preparation contained substances less than 10,000 MW at only 1/7 the concentration present in the original liver cells. Inclusion of the ultrafiltrate in the incubation medium did not affect Ca^{2+} uptake by the mitochondria (fig 4).

DIAGRAM 11


RAT LIVERS PERFUSED IN VIVO
crushed and homogenised
HOMOGENATE
Centrifugation
20,000 x g (20')
40,000 x g (20')
250,000 x g (60')
RAT LIVER CYTOSOL PREPARATION
cytosolic protein, Mg^{2+}, K^{+}, Ca^{2+}, free fatty acid and adenine nucleotides estimated
dialysed and freeze dried
concentrated over PM 10 Diaflo Ultrafilter
FREEZE DRIED RAT CYTOSOL
(MW > 10,000)
fractionation of ^{45}Ca-labelled freeze dried rat cytosol on Biogol A-0.5M column
^{45}Ca-BINDING PROTEINS
1. fractions 51-63
2. fractions 64-74
ULTRAFILTERATE
(MW < 10,000)
CONCENTRATED RAT CYTOSOL
(MW > 10,000)
fractionation of ^{3}H-palmitate and ^{45}Ca labelled conc. rat liver cytosol on AcA 44 column
fractionation of ^{3}H-palmitate labelled rat liver cytosol on Biogel P30 column
fractionation of ^{3}H-palmitate labelled rat liver cytosol on Sephacryl 300 superfine column
1. ^{3}H-PALMITATE BINDING PROTEIN
2. ^{45}Ca-BINDING PROTEIN
^{3}H-PALMITATE BINDING PROTEIN
^{3}H-PALMITATE BINDING PROTEIN

The effects of some of the cytosolic components present in the concentrated cytosol and the ultrafiltrate which would regulate Ca^{2+} transport by the mitochondria are summarised below :

Key : + = Ca^{2+} retention in mitochondria
- = release of Ca^{2+} from preloaded mitochondria
0 = no effect on Ca^{2+} uptake or release.

	Cytosolic components	Effect on mitochondrial Ca^{2+} transport	Ref.
1.	^{3}H-palmitate-binding protein (when saturated with ^{3}H-palmitate) (> 10,000 MW)	-	fig. 20
2.	Defatted rat albumin (30 µM) (> 10,000 MW)	+	fig. 10
3.	Ca-binding proteins (i.e fractions BA (51 - 63) and fractions BA (64 - 74) (> 10,000 MW)	+	fig. 21
4.	Palmitoyl CoA (5 - 20 µM)	-	fig. 28
5.	Palmitoylcarnitine (10 - 20 µM)	+	fig. 28
6.	K^{+} (2 - 146 mM)	0	fig. 39
7.	Na^{+} (0 - 10 mM)	0	fig. 38

	Cytosolic components	Effect on mitochondrial Ca^{2+} transport	Ref.
8.	NAD^+ (0.5 mM)	+	fig. 59
9.	NADH (0.5 mM)	+	fig. 59
10.	$NADP^+$ (0.125 ; 0.5 mM)	0	fig. 59
11.	NADPH (0.5 mM)	-	fig. 59
12.	Adenine nucleotides	ATP and ADP but not AMP support ~~the initial~~ Ca^{2+} uptake. The rate of ADP-supported Ca^{2+} uptake was relatively slow compared with ATP. Inclusion of antimycin A did not affect the ATP-supported transport of Ca^{2+} by the mitochondria ; however rotenone promoted longer retention of Ca^{2+} by the mitochondria. Approx. 1-3 % of the added ATP or ADP was transported into the mitochondria (Chapter 5).	
13.	Pi	Omitting external Pi and in the presence of oligomycin and NEM only 15% of the added Ca^{2+} was transported into the mitochondria (Chapter 5).	
14.	Respiratory substrates	The mitochondria retained Ca^{2+} for a longer period in the presence of	

Cytosolic components	Effects on mitochondrial Ca^{2+} transport	Ref
	succinate compared with the other NAD^+-linked substrates examined (fig 29,30).	
15. Mg^{2+} (0-3 mM)	Inclusion of Mg^{2+} did not inhibit or increase Ca^{2+} uptake. 4-9 % of the externally added Mg^{2+} was accumulated by the mitochondria and the additional Mg^{2+} accumulated prolonged Ca^{2+} retention in the mitochondria. During release of Ca^{2+} from the mitochondria there was a corresponding release of mitochondrial Mg^{2+} (Chapter 4).	

It is necessary to review some of the findings in the present study before discussing their implications.

High molecular weight components of the cytosol

The ^{3}H-palmitate-binding protein (MW 30,000 - 50,000) was eluted from the 3 different columns used, i.e. AcA 44, Biogel P30 and Sephacryl 300 superfine column. The double antibody precipitation and the immunoelectrophoresis techniques were used to show that the ^{3}H-palmitate-binding protein was not rat albumin, which is also known to bind fatty acids. The exact physiological function of the fatty acid-binding protein in the rat cytoplasm is not known, although it might be an intracellular transport protein for fatty acids. In a recent publication, Barbour and Chan (1979) reported that the defatted liver fatty acid-binding protein reversed the inhibitory effect of palmitoyl CoA on adenine nucleotide transport in rat liver mitochondria.

Defatted rat albumin helped to retain Ca^{2+} in the mitochondria (fig 10), possibly by protecting the mitochondria from the uncoupling effects of endogenous fatty acids produced by phospholipase A. The Ca-binding activity of the rat albumin (30 μM) in the presence of 400 nmoles Ca^{2+} was negligible as determined by using the Ca-electrode (fig 11). Therefore it is unlikely that in vivo, the trace amounts of rat albumin present in the liver cytosol would lower the concentration of the free Ca^{2+}.

Other higher molecular weight components of the rat liver cytosol are the Ca-binding proteins. These proteins were eluted as one small peak from AcA 44 column corresponding to molecular weight between 86,000 - 364,000 (fig 12). Fractionation of the freeze dried cytosol on Biogel A - 0.5 M column revealed two main Ca-binding protein namely fractions BA(51 - 63) (MW ~ 150,000), and fractions BA(64 - 74) (MW 150,000 - 364,000) (fig 19). The physiological function of the Ca-binding proteins is not known. Presumably these proteins combine with some of the free Ca^{2+} in the cytosol reducing the concentrations of the ionised Ca^{2+}.

Effects of inorganic cations.

It should be noted that although the effect of K^+, Na^+ and Mg^{2+} on Ca^{2+} transport by mitochondria have been reported by other workers, the effects of these ions have often been studied in the absence of an important substrate such as Pi or ATP (Drahota and Lehninger, 1965; Sordahl, 1974; Crompton et al, 1976; Akerman et al, 1977) or omitting Mg^{2+} when studying the effects of Na^+ and K^+ (Drahota and Lehninger, 1965; Crompton et al, 1976). In the present study, the incubation medium

contained all the factors that are known to be required for Ca^{2+} uptake and retention, and under the stated conditions, which were intended to simulate the in vivo cytosolic situation, the control mitochondria were able to retain their Ca^{2+} for the 30 - 45 min duration of the experiment. Previous work on Ca^{2+} transport by mitochondria shows variable times for Ca^{2+} release from control mitochondria, depending on the incubation mixtures, and in some reports Ca^{2+} is lost in 5 - 10 min (Dorman et al, 1975; Asimakis and Sordahl, 1977; Hughes and Barritt, 1978). The latter workers for example, reported a release of 50 % of the accumulated Ca^{2+} after 4 min. Their incubation medium did not contain Mg^{2+} and ATP, which are required for Ca^{2+} retention in mitochondria.

Effect of palmitoyl CoA.

Palmitoyl CoA caused Ca^{2+} release from rabbit heart mitochondria respiring on succinate with or without ATP and in the absence of Mg^{2+} (Asimakis and Sordahl, 1977). In the present study, it was found that palmitoyl CoA caused Ca^{2+} release from rat liver mitochondria in the presence of Mg^{2+}, ATP and β-hydroxybutyrate as the respiratory substrates.

Effect of adenine nucleotides.

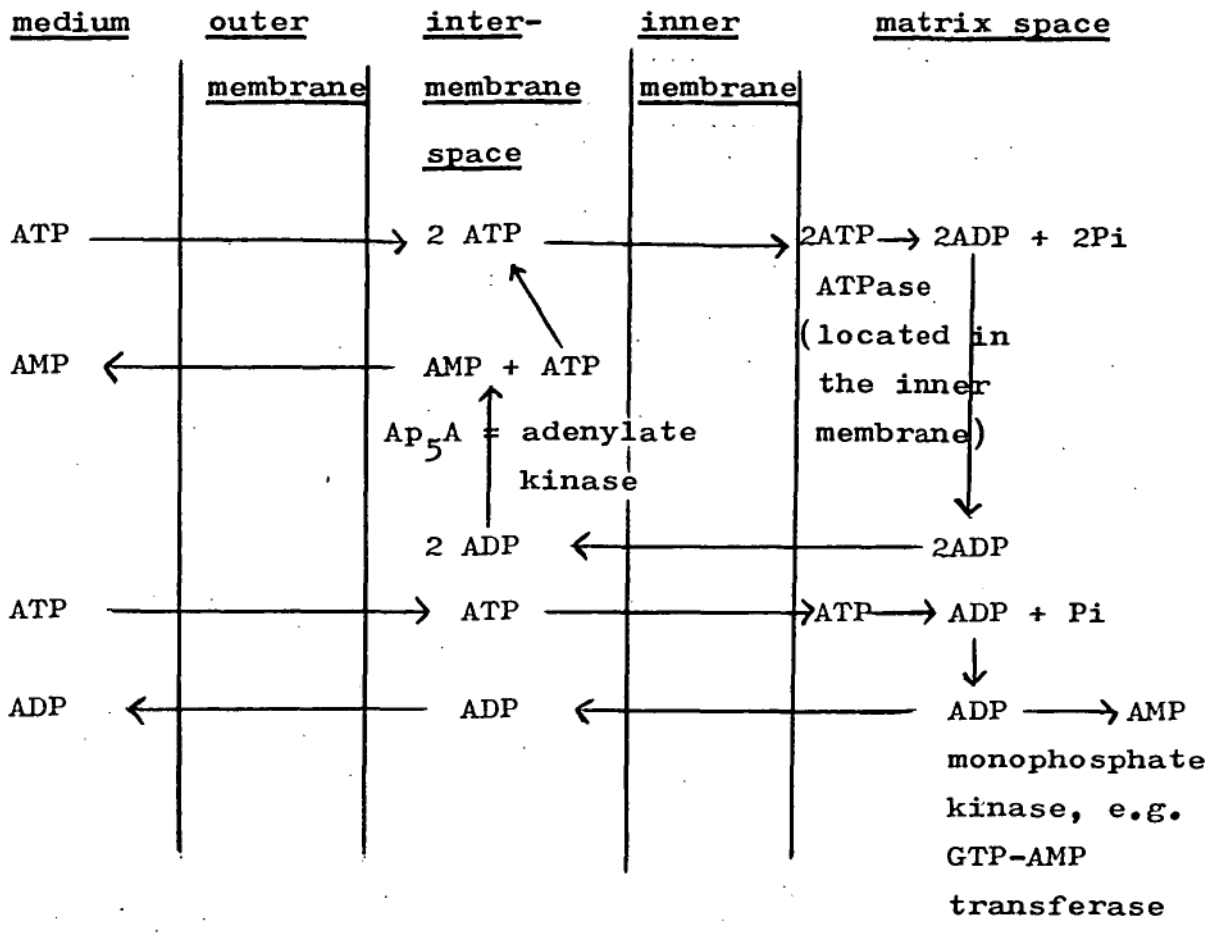
Previous workers have used inhibitors of respiratory chain such as antimycin A, CN^-, rotenone in the absence or presence of oligomycin in attempts to show that ATP supported Ca^{2+} uptake by mitochondria (Brierley et al, 1964; Spencer and Bygr ave, 1973). In the present study, a new approach was employed to show that ATP and ADP, but not AMP, support the initial Ca^{2+} uptake by the mitochondria. The adenine

nucleotide was added just as the mitochondria began to release their Ca^{2+}, i.e. at the point where endogenous energy sources became depleted.

The ATP analogues Bγ-methylene ATP and αB-methylene ATP were used to demonstrate that ATP had to be metabolised in order to support Ca^{2+} uptake.

The concentration of ATP, ADP and AMP present in the system during Ca^{2+} retention and release by the mitochondria was also investigated in the present study. The initial concentration of the ATP in the incubation medium was 1 mM ATP. In the in vitro system, it was estimated that the mitochondria contained only 5 - 10 % of the total adenine nucleotides. Various substances were used to cause an earlier Ca^{2+} release or to prolong Ca^{2+} retention in the mitochondria. (The substances used to prolong Ca^{2+} retention were, palmitoyl-carnitine plus ATP, PEP plus pyruvate kinase, defatted BSA and EHDP. The substances used to induce Ca^{2+} release were PEP, glucose plus hexokinase, palmitoyl CoA and fatty acid bound to rat albumin). During prolonged retention of Ca^{2+} by the mitochondria, the total ATP (i.e. that in the medium plus in the mitochondria) remained high, ADP remained steady and AMP was low. During Ca^{2+} release the total ATP decreased, ADP remained steady and the AMP concentration increased. The earlier release of Ca^{2+} from the mitochondria in the presence of the "Ca^{2+}-releasing substances" is possibly due to depletion of ATP rather than an increase in AMP in the medium, since the release was not affected when the formation of AMP was reduced significantly by Ap_5A, an inhibitor of adenylate kinase. The depletion of external ATP would result in an

increase in AMP in the medium, possibly by the reaction shown below.



In addition, it was observed that Ca^{2+} retention in the mitochondria was favoured by an ATP regenerating system (PEP plus pyruvate kinase) while an ATP trapping system (glucose plus hexokinase) caused an earlier Ca^{2+} release.

Effect of the redox state of mitochondrial pyridine nucleotides.

Lehninger et al (1978) postulated that whenever the cytosolic phosphate potential, i.e. $[ATP]/[ADP][Pi]$ ratio, decreases, a more oxidised mitochondrial NAD^+ is favoured via reversibility of site 1 phosphorylation in the respiratory chain:-

$NADH + H^+ + Pi + ADP + fumarate \rightleftharpoons NAD^+ + ATP + succinate + H_2O$.

The cytosolic phosphate potential could also influence the respiratory control of the electron transported via the following reaction (Williamson, 1976) :-

$$NADH + 2 \text{ cyt } a_3^{3+} + 3 ADP + 3 Pi \rightleftharpoons NAD^+ + 2 \text{ cyt } a_3^{2+} + 3 ATP.$$

The observed marked decrease in ATP and a fairly steady ADP in the medium during Ca^{2+} release from the mitochondria agrees with this theory, i.e. a decrease in cytosolic phosphate potential would favour a more oxidised mitochondrial NAD^+ which in turn would favour Ca^{2+} release.

The following evidence suggest that the redox state of the mitochondrial pyridine nucleotides is important in controlling release and retention of Ca^{2+} by the mitochondria.

(1) The substrates oxaloacetate and acetoacetate (that lower the mitochondrial $NADH/NAD^+$ ratio) caused release of Ca^{2+}, without altering the concentrations of adenine nucleotides (chapter 6).

(2) The effect of palmitoyl CoA on heart mitochondria had previously been shown not to be due to the uncoupling of mitochondrial respiration but was consistent with the idea that palmitoyl CoA was a potent inhibitor of adenine nucleotide translocase (Asimakis and Sordahl, 1977) Inhibition by palmitoyl CoA is competitive with ATP and ADP and the Ki for palmitoyl CoA is less than 0.5 μM. (Shrago et al, 1974). Since the experimental work for this thesis was completed, recent evidence put forward by Wolkowics and Wood (1979) showed that addition of palmitoyl CoA after Ca^{2+} accumulation caused Ca^{2+} release and also resulted in a rapid oxidation of mitochondrial pyridine nucleotides. Separate addition of either palmitoyl CoA or Ca^{2+} did not result in the observed effects. Thus possibly palmitoyl CoA inhibits the mitochondrial adenine nucleotide transloc-

case which in turn may result in rapid oxidation of the mitochondrial pyridine nucleotides. The exact mechanism by which palmitoyl CoA influences the redox state of mitochondrial pyridine nucleotides is not known. It would be interesting to see whether the other "Ca^{2+} releasing" substances (such as PEP or Na^{+}) have any influence on the redox state of mitochondrial pyridine nucleotides.

(3) A possible explaination for the observed Ca^{2+} retention in the presence of palmitoylcarnitine is the elevation of $NADH/NAD^{+}$ ratio as a result of activation of mitochondrial palmitate oxidation by Ca accumulation into the mitochondria (Otto and Ontko, 1978). The latter workers observed that the initial rapid uptake of Ca^{2+} in the presence of palmitate, CoA, carnitine, Pi, Mg^{2+} and ATP caused a decrease in β-hydroxybutyrate/acetoacetate ratio, but the ratio subsequently increased; they therefore concluded that the Ca^{2+} accumulation in the mitochondria, and not the Ca^{2+} transport per se, was the prerequisite for the development of an enhanced fatty acid oxidation and an elevated $NADH/NAD^{+}$ ratio. They also calculated that the additional NADH produced via increase in fatty acid oxidation was more than required for Ca^{2+} transport and for the increase in the reduction of acetoacetate. However the mechanism by which Ca^{2+} stimulates fatty acid oxidation is not known.

From direct measurement of NAD^{+} and NADH in rat mitochondria incubated with palmitate, ATP, carnitine and CoA, Otto and Ontko (1978) showed that when the mitochondria accumulated 80 nmoles Ca^{2+}/mg protein, the mitochondrial

$NADH/NAD^+$ ratio increased from 0.14 to 0.41 (see table below).

Ca^{2+} nmole/mg mitochondrial protein	NADH nmole/mg mitochondrial protein	NAD^+	$NADH/NAD^+$ ratio
0	0.26	1.9	0.14
80	0.57	1.3	0.41

(4) During prolonged Ca^{2+} retention in the mitochondrial in the presence of BSA and EHDP, the ATP concentration remained high suggesting a high cytosolic phosphate potential which in turn would favour a high mitochondrial $NADH/NAD^+$ ratio. Infact the present study also show that BSA or EHDP helped reuptake of Ca^{2+} released by an oxidant of mitochondrial NADH such as oxaloacetate.

It was also observed in the present study that quinidine sulphate caused an immediate release of Ca^{2+} from the mitochondria however it did not result in marked depletion of ATP in the medium (chapter 5, section 5.3.4.9). It was

suggested that another mechanism of Ca^{2+} release is involved : possibly quinidine affects the redox state of the mitochondrial pyridine nucleotide directly.

It is also of interest to note that the concentrations of mitochondrial adenine nucleotide remained fairly steady although Ca^{2+} was gained or lost during Ca^{2+} cycling on alternately adding oxaloacetate and β-hydroxybutyrate.

Based on the observation in the present study that Ca^{2+} alters the retention time (observed by HPLC, in the non-polar μ-Bondapak C_{18} column) of $NADP^+$ and NADPH but not NAD^+ or NADH, presumably, Ca-$NADP^+$ and Ca-NADPH complexes formed in the less polar mitochondrial membrane could be direct modulators of Ca^{2+} release and retention in the mitochondria.

The effect of externally added pyridine nucleotide on Ca^{2+} movement into and out of the mitochondria was also examined in the present study. The results obtained suggest that NAD^+ and NADH prolonged Ca^{2+} retention, $NADP^+$ did not affect Ca^{2+} uptake or release while NADPH caused an earlier Ca^{2+} release (chapter 6, section 6.3.4). The mitochondrial membrane is considered to be impermeable to pyridine nucleotides. The NADH/NAD^+ ratio in the cytoplasm is maintained in a (contd. overleaf)

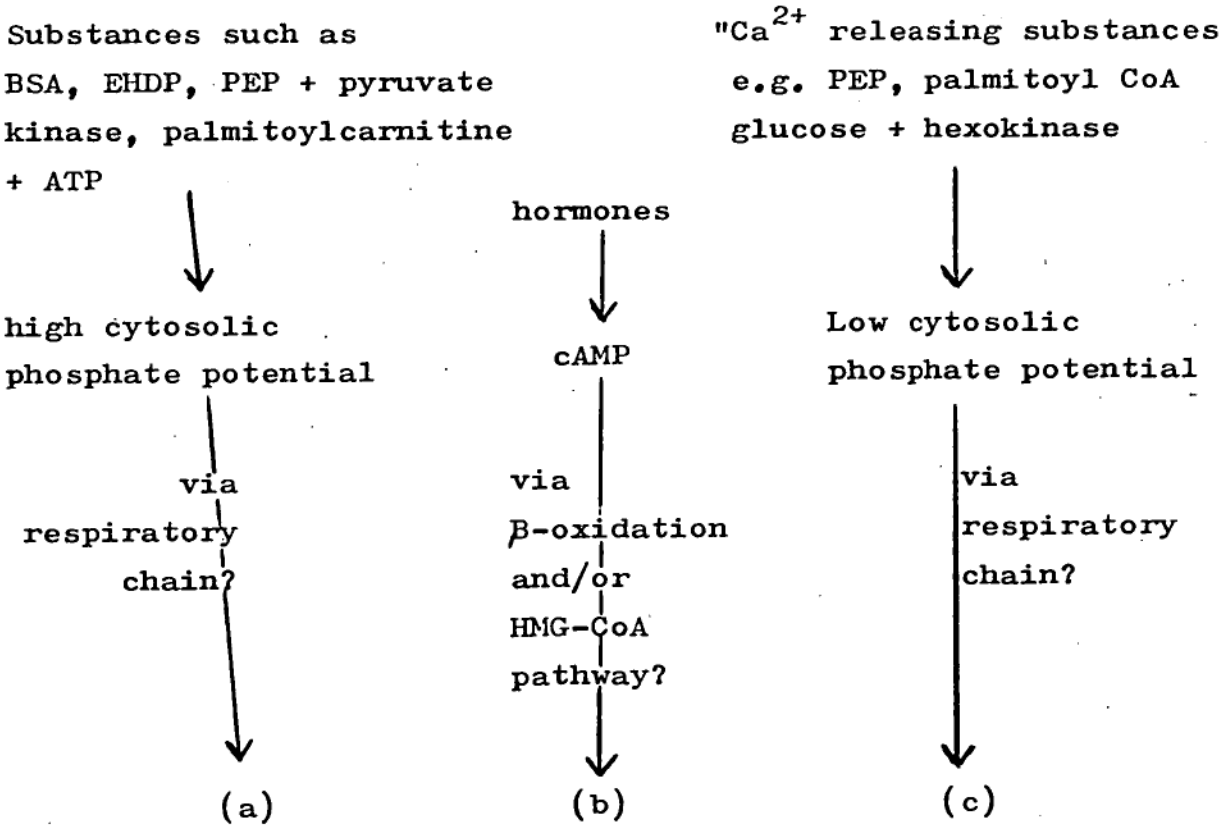
relatively highly oxidised state in order to ensure that the equilibrium of the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase lies in favour of glycolysis. Since the externally added NAD^+ prolonged Ca^{2+} retention in the mitochondria, it is possible that in vivo the highly oxidised state of cytosolic NAD^+ favours Ca^{2+} retention in the mitochondria. However, it is not known why the externally added NADPH caused an earlier release of Ca^{2+} from the mitochondria. The mitochondrial NAD^+ on the other hand is maintained in a much more reduced state in vivo in order to provide a sufficient driving force for the electron transport chain and oxidative phosphorylation. However a slight decrease in the mitochondrial $NADH/NAD^+$ ratio induced by a physiological modulator would cause a release of Ca^{2+} from the mitochondria. A rise in free ionic Ca^{2+} in the cytosol will in turn alter the activity of the Ca-dependent enzymes such as pyruvate kinase, phosphofructokinase and fructose 1-6 biphosphatase.

Based on the findings in the present study, it was suggested that indirectly cAMP could be the physiological modulator of Ca^{2+} release from the mitochondria. The effect of cAMP (0.1 μM - 75 μM) on Ca^{2+} release was observed in the presence of the substrates palmitoyl CoA or palmitoylcarnitine but not the NAD^+-linked substrates such as glutamate + malate or pyruvate + malate (Scarpa et al, 1976) or succinate as substrate (section 7.3; Scarpa et al, 1976), suggesting that β-oxidation of fatty acid in the mitochondrial matrix may be involved. Presumably cAMP alters the activity of certain enzyme/s involved in ketone production from acetyl CoA (via the HMG-CoA pathway) which would result in a decrease in

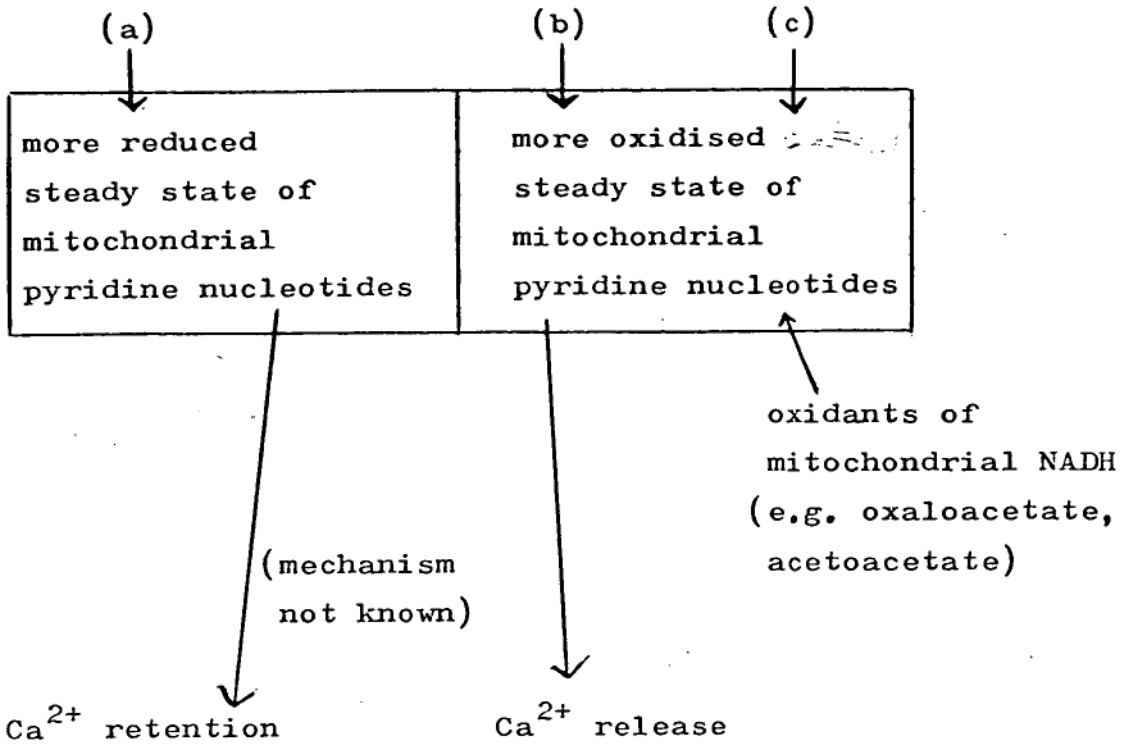
β-hydroxybutyrate/acetoacetate ratio (i.e. reflecting a decrease in mitochondrial NADH/NAD^+ ratio).

An alternative way to investigate the effect of cAMP on Ca^{2+} release from the mitochondria was to starve the rats, which would be expected to increase the cAMP levels in liver (Garrison and Haynes, 1973). Interestingly, it was observed that the mitochondria isolated from starved rats had a lower NADH/NAD^+ ratio and released their Ca^{2+} earlier than the mitochondria from fed rats.

Based on the findings in the present study and that reported by Lehninger et al (1978), the importance of the redox state of the mitochondrial pyridine nucleotides in controlling mitochondrial Ca^{2+} release and retention <u>in vitro</u> is summarised below:-



(continued overleaf)



Factors involved in Ca^{2+} uptake and release in vitro.

Before discussing the possible mechanism of Ca^{2+} transport in rat liver mitochondria, the factors required for an in vitro Ca^{2+} uptake and factors affecting release and retention by the mitochondria are summarised below:-

Factors required for Ca^{2+} uptake

1. Energy source from oxidation of respiratory substrate or from ATP hydrolysis.
2. A permeant anion that can donate proton to the mitochondrial matrix, e.g. Pi, acetate.

Factors favouring Ca^{2+} retention

1. Both ATP and respiratory substrate especially succinate present in the incubation medium.
2. Mitochondrial NAD^+ in a relatively reduced steady state.
3. BSA, rat albumin (defatted).
4. EHDP.

5. An ATP regenerating system (PEP + pyruvate kinase)
6. Ca-binding proteins isolated from rat liver cytosol.
7. Palmitoylcarnitine plus ATP.
8. Additional Mg^{2+} accumulated by the mitochondria from the medium.
9. Externally added NAD^+ or NADH.

Factors causing Ca^{2+} release

1. Palmitate binding protein isolated from rat liver cytosol containing 'bound' fatty acids.
2. Palmitoyl CoA, palmitate.
3. Quinidine sulphate.
4. PEP.
5. ATP trapping system (glucose + hexokinase)
6. Externally added NADPH.
7. Mitochondrial NAD^+ in a more oxidised steady state.
8. cAMP, dibutyryl cAMP.
9. Starvation.

A possible mechanism of Ca^{2+} uptake and release by rat liver mitochondria.

Since a number of the cellular reactions are sensitive to Ca^{2+} in the μM concentration range, it is essential that cell Ca^{2+} is controlled precisely. Mitochondria can accumulate Ca^{2+} and the properties of mitochondrial Ca^{2+} transport observed *in vitro* suggest that mitochondria may act as an effective buffer for cell Ca^{2+}, i.e. rapidly accumulating excess Ca^{2+} from the cytosol and releasing their store when the Ca^{2+} level in cytosol drops.

The total content of Ca^{2+} in the cytosol is in the range

of 100 - 400 μM (Hamilton and Holdsworth, 1975; Foden and Randle, 1978) of which only 0.1 - 10 μM is the free ion (Rasmussen et al, 1975). Therefore, a considerable amount of the soluble Ca^{2+} in the cytosol exists as bound Ca^{2+}. In fact, the cytosol contains numerous Ca^{2+} chelators such as ATP, ADP, citrate, Ca-binding proteins which may help to lower the concentration of free ionic Ca^{2+} in the cytosol. It is of interest to note that in the present study it was found that in the incubation medium containing 1 mM ATP and 80 μM Ca^{2+} even with 1 mM Mg^{2+}, approximately 50 μM Ca^{2+} was chelated to the ATP. In vivo, there is 6 mM ATP in the cytosol (Soboll et al, 1976) ; so even if Mg^{2+} has more affinity for ATP than Ca^{2+} has, there is enough ATP at 1 mM Mg^{2+} (the physiological concentration of Mg^{2+}, Veloso (1973)) to combine with the Ca^{2+} reducing the concentration of free Ca^{2+} . This seems to be a point overlooked by most workers.

Claret-Berthon et al (1977) reported distinct pools of intracellular Ca^{2+} (see p.32). The distribution of intracellular Ca^{2+} in liver cell is shown in diagram 12.

A possible mechanism of Ca^{2+} uptake and release by rat liver mitochondria is discussed below and the proposed model is as shown in diagrams 13 and 14.

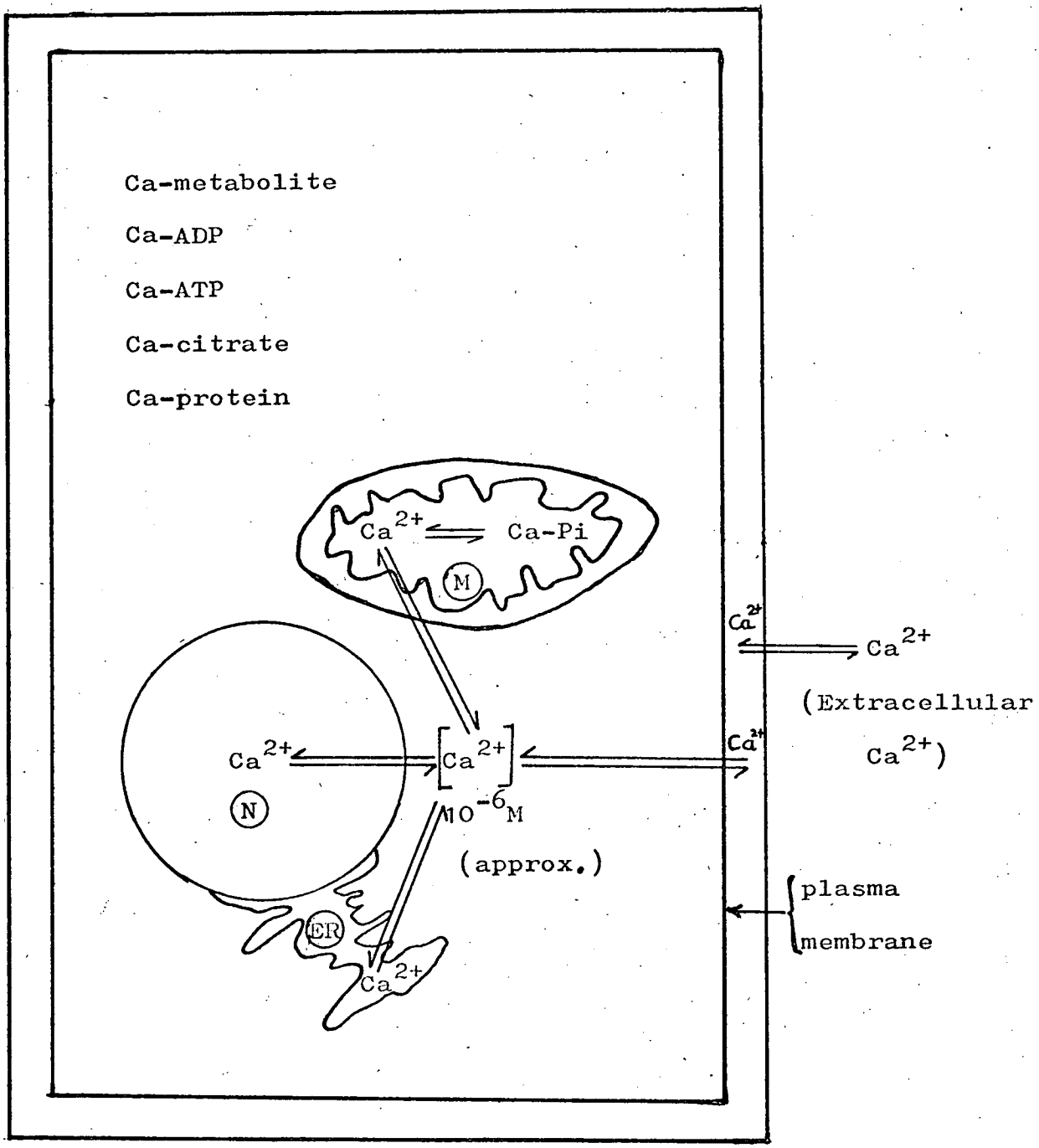
(1) Ca^{2+} uptake

The first step in the uptake of Ca^{2+} by the mitochondria is the energy independent binding of Ca^{2+} to the inner membrane (note : the outer mitochondrial membrane is permeable to Ca^{2+}). Apparently there are two binding sites, namely, a) the low — affinity binding sites which are non-specific for Ca^{2+}, and possibly associated with the functional groups of the lipids and/

DIAGRAM 12

Distribution of intracellular Ca^{2+} in liver cell

(based on reports by Claret-Berthon et al, 1977)



(N) = nucleus

(M) = mitochondria

(ER) = endoplasmic reticulum

or protein constituents of the inner membrane b) the high affinity-binding site, presumably the active site of the Ca^{2+} -specific carrier (Reynafarje and Lehninger, 1969).

The next step is the transport of Ca^{2+} across the inner mitochondrial membrane via a Ca^{2+} specific glycoprotein carrier (Sottocasa et al, 1971, 1972; Prestipino et al, 1974; Panfili et al, 1976). Ca^{2+} uptake by the mitochondria is an electrophoretic process, (with a net charge transfer of 2 per Ca^{2+} transported) via the carrier in response to a membrane potential, negative inside, that is generated across the inner membrane as a result of ATP hydrolysis or oxidation of respiratory substrates. It is not certain whether the carrier is mobile or just a superficial specific Ca^{2+} receptor. Permeant anion such as Pi or acetate that can donate protons to the mitochondrial matrix are required for Ca^{2+} uptake and to stimulate the capacity for Ca^{2+} transport.

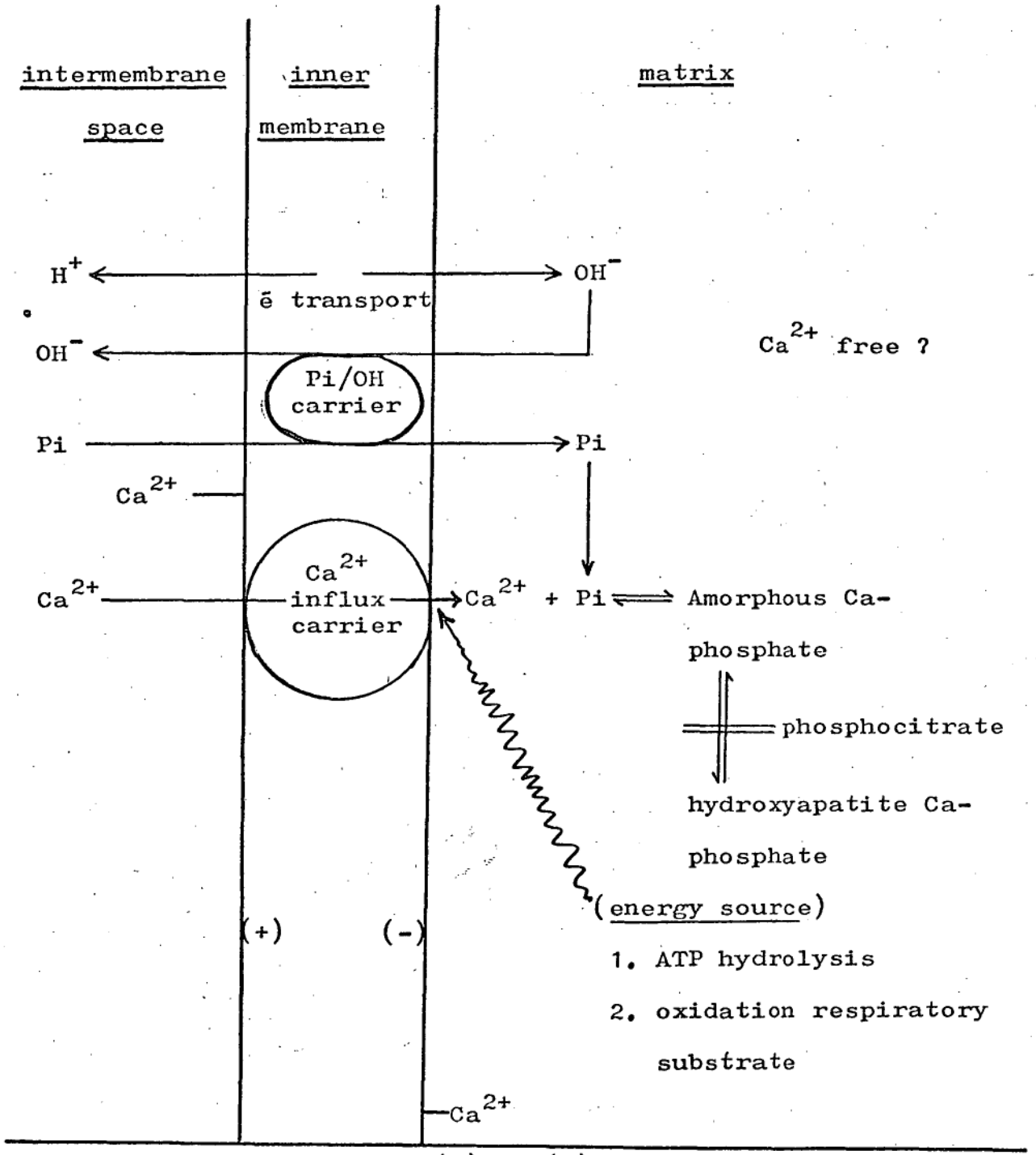
At present it is uncertain whether the Ca^{2+} accumulated in the mitochondria exist as a free form in the matrix space, bound to the inner side of the inner mitochondrial membrane or precipitated in the matrix space in the form of Ca-phosphate. There is evidence from electron microscopy and electron microprobe analysis that amorphous calcium granules may be present in the mitochondrial matrix (Greenwalt et al, 1964). Lehninger et al (1978(a)) suggested that phosphocitrate may act as an inhibitor of hydroxyapatite formation in the matrix and therefore stabilise the amorphous $Ca_3(PO_4)_2$ in the matrix.

(2) Ca^{2+} release

The mechanism of Ca^{2+} release from the mitochondria is still poorly understood. There is evidence to indicate that the efflux mechanism of Ca^{2+} from the mitochondria differs from the influx

DIAGRAM 13

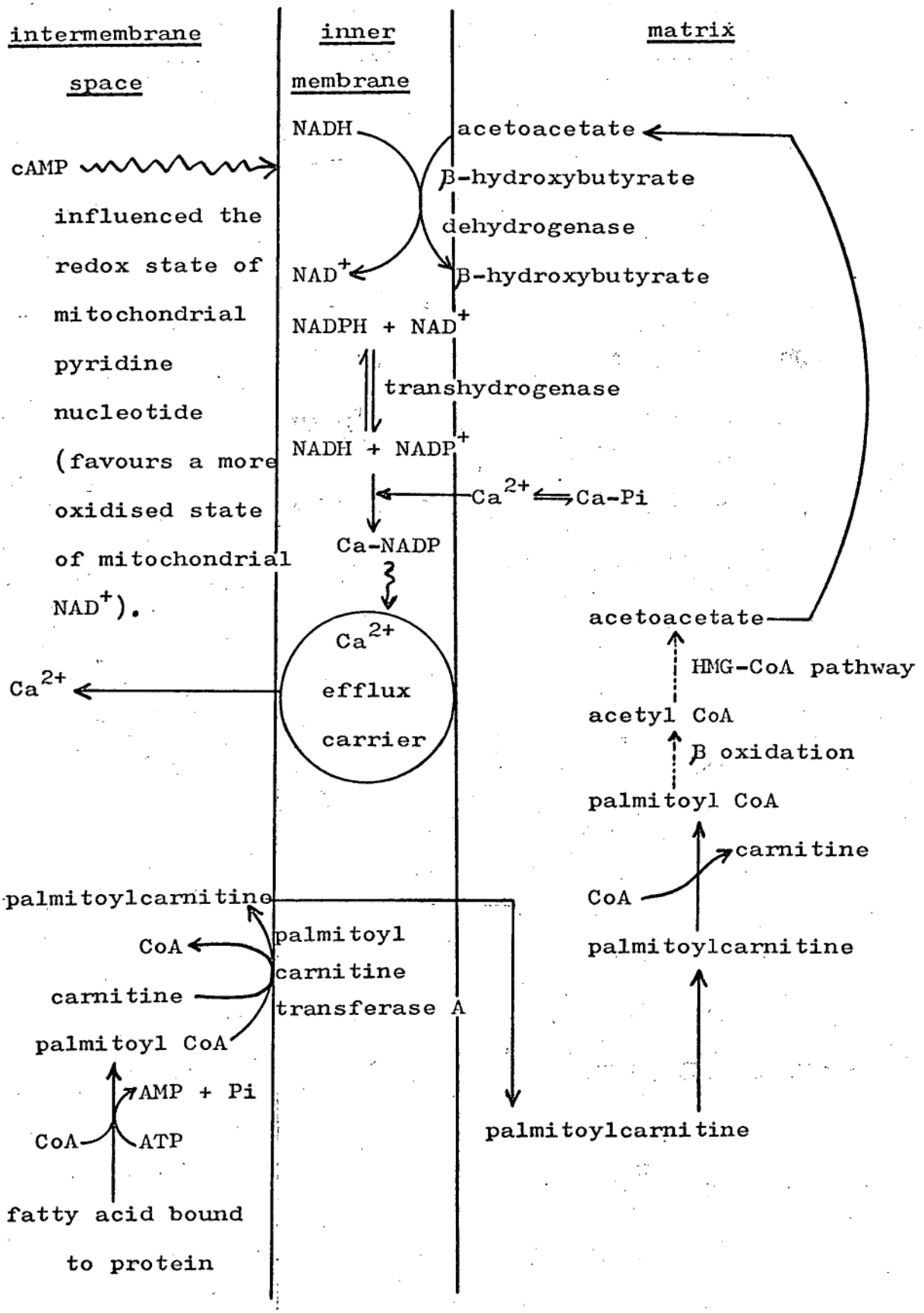
Possible mechanism of Ca^{2+} uptake by rat liver mitochondria.



Note: membrane potential (+) or (-)

DIAGRAM 14

Possible mechanism of Ca^{2+} release from rat liver mitochondria.



mechanism (Peng et al, 1977; Nicholls, 1978(b); Crompton et al, 1978; Caroni et al, 1978). Apparently the release of Ca^{2+} from the mitochondria is via a glycoprotein carrier (Sandri et al, 1976); however, it is not known whether it is via the influx glycoprotein carrier but at a different active site, or via a different carrier altogether. It is also uncertain whether the release of Ca^{2+} from the mitochondria is an energy independent process, i.e. by mere diffusion down a concentration gradient or an energy dependent process.

Although some components of the rat liver cytosol such as palmitate (bound to palmitate-binding protein), palmitoyl CoA and PEP caused Ca^{2+} release from preloaded mitochondria when examined in vitro, it is unlikely that these substances are the physiological modulators for the release of Ca^{2+}. Formation of palmitoylcarnitine in cytosol from palmitate or palmitoyl CoA would prevent the releasing effect of these factors and in fact helped Ca^{2+} retention in the mitochondria (chapter 3). The presence of pyruvate kinase in the cytosol ensures Ca^{2+} retention in the presence of PEP (chapter 5).

The present study suggests cAMP as the possible physiological modulator for Ca^{2+} release. Presumably cAMP directly/indirectly alters the activity of the enzyme/s involved in the HMG-CoA pathway which in turn would result in a decrease in β-hydroxybutyrate/acetoacetate ratio, reflecting a decrease in mitochondrial $NADH/NAD^+$ ratio. Lehninger et al (1978) have reported that when the mitochondrial NAD^+ is in a relatively more oxidised steady state, Ca^{2+} release is favoured. This observation was confirmed in this thesis (chapter 6). The present study also supports the theory of the formation of a Ca-NADP or Ca-NADPH complex in the less polar inner mitochondrial membrane (chapter 6). A lowered mitochondrial $NADH/NAD^+$ ratio would also result in a decrease in $NADPH/NADP^+$ ratio via the action of the energy linked pyridine dinucleotide transhydrogenase which is located in the inner mitochondrial membrane. Possibly Ca-NADP formed in the inner mitochon-

drial membrane could stimulate the Ca-efflux mechanism while Ca-NADPH could be the inhibitory modulator. The exact mechanism by which cAMP influences the redox state of the mitochondrial pyridine nucleotide is however not known.

(3) Equilibrium between Ca^{2+} uptake and release and its physiological consequences

It is likely that a dynamic situation exists in vivo where Ca influx and efflux by mitochondria reach a steady state. There have been few reports suggesting that Ca^{2+} cycling does occur in mitochondria (Stucki and Ineichen, 1974; Grist and Baum, 1975). Cyclic AMP possibly regulates the rate of Ca^{2+} efflux from the mitochondria via the redox state of the mitochondrial pyridine nucleotides. Since the concentration of free ionised Ca^{2+} in the cytosol is approx. 1 μM, (Rasmussen et al, 1975) a release of a small amount of Ca^{2+} from the mitochondria would cause a relatively large increase in the free Ca^{2+} concentration in the cytosol.

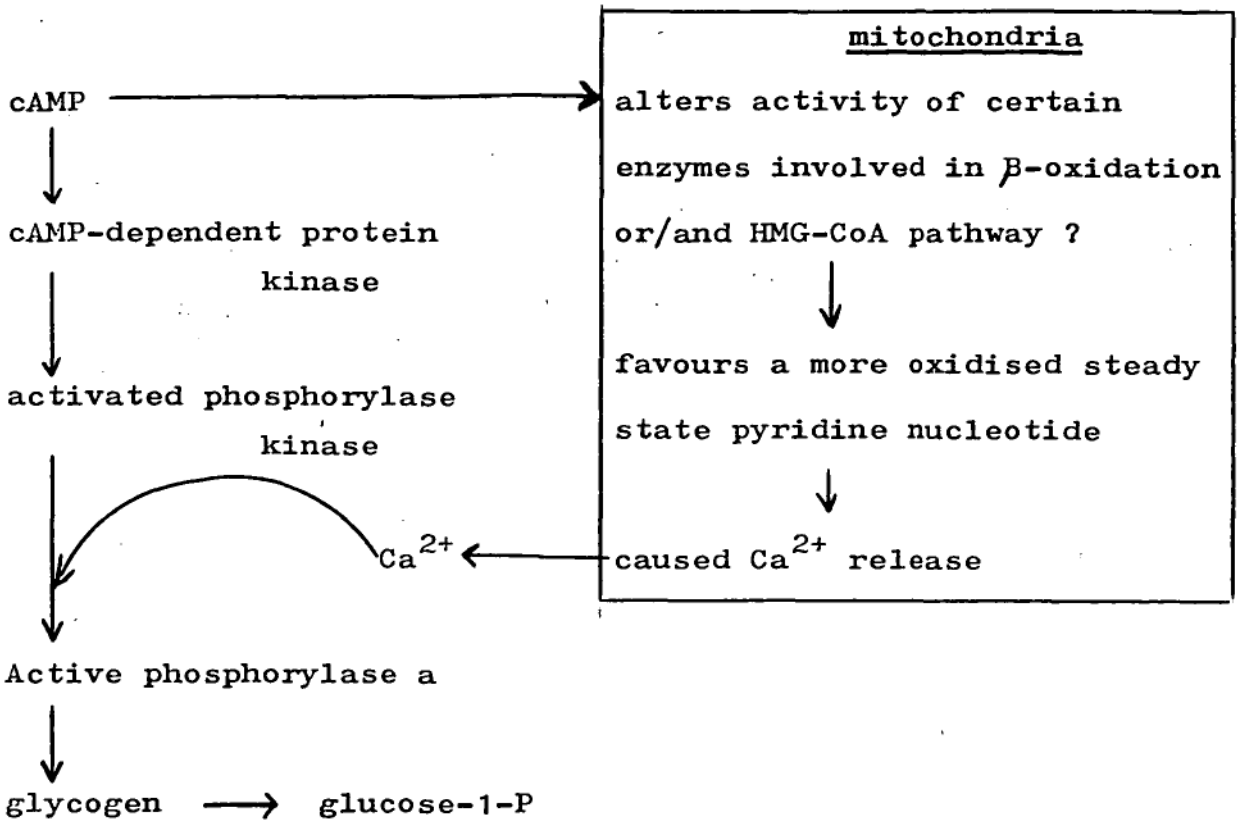
A rise in the ionised Ca^{2+} in the cytosol may alter the activity of some cytosolic enzymes listed below. Apparently Ca^{2+} in the mitochondria can also regulate the activity of certain mitochondrial enzymes (see table below).

Ca^{2+}-sensitive enzymes

Mitochondria	Cytosol
pyruvate dehydrogenase phosphatase	cyclic nucleotide phosphodiesterase
pyruvate dehydrogenase kinase	phosphorylase kinase
isocitrate dehydrogenase	glycerol phosphate dehydrogenase

It is also possible that an increase in the level of cAMP in the cytosol and the subsequent increase in the rate of Ca^{2+} efflux from mitochondria may reinforce one another in

regulating the activity of certain enzymes, for example, phosphorylase kinase in the regulation of glycogen breakdown as shown below.



According to Cohen (1978) the phosphorylase kinase from mammalian skeletal muscle possesses the structure $(\alpha\beta\gamma\delta)_4$ where the α- and β- subunits are the components phosphorylated by cAMP-dependent protein kinase and the δ-subunit is identical to the Ca-binding protein, calmodulin (Cohen et al, 1978).

On the other hand, pyruvate kinase in the liver cytosol is inactivated by phosphorylation via the cAMP-dependent protein kinase (Engstrom, 1978) and ionised Ca^{2+} in the cytosol has been shown to inhibit the activity of pyruvate kinase (Meli and Bygrave, 1972).

The examples given above suggest that the effect of cAMP

and that of Ca^{2+} are inter-related. Most of the effects of cAMP are mediated through cAMP-dependent protein kinases while that of Ca^{2+} is mediated via calcium-binding proteins such as calmodulin (Cheung, 1980). Other examples on the interactions of Ca^{2+} and cAMP are:-

1) effects of Ca^{2+}, via calmodulin, on the synthesis of cAMP by brain adenylate cyclase (Brostrom et al, 1975; Cheung et al, 1975).
2) effects of Ca^{2+}, via calmodulin, on the degradation of cAMP by cAMP phosphodiesterase (Cheung, 1970).
3) the role of cAMP-mediated phosphorylations in the regulation of Ca^{2+}-activated contractile systems.

The important role of cAMP in controlling Ca^{2+} release from the rat liver mitochondria has been suggested in the present study. However, many problems remain to be solved. For example:-

1) the mechanism by which cAMP influences the redox state of the mitochondrial pyridine nucleotides.
2) the mechanism by which the redox state of the mitochondrial pyridine nucleotides influences Ca^{2+} release and retention by the mitochondria.
3) whether the Ca^{2+} released from the mitochondria is derived from a specific Ca^{2+} pool in the matrix.
4) whether cAMP affects Ca^{2+} release from mitochondria isolated from other tissues such as heart, kidney, intestine and brain.

To summarise, therefore, cAMP possibly regulates the rate of Ca^{2+} efflux from the mitochondria via the redox state of mitochondrial pyridine nucleotides. It is also possible that

the function and metabolism of cAMP and Ca^{2+} are related.

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