THE SYNTHESIS OF TWO PHOSPHOCITRATE ANALOGUES AND THEIR EFFECTIVENESS AS CALCIFICATION INHIBITORS

by

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ABSTRACT

The synthesis of two structural analogues of the naturally occurring compound phosphocitrate (PC) was investigated, with a view to producing compounds more enzymatically stable than PC, yet with similar anti-calcifying properties. It was envisaged that such compounds might prove to be suitable agents for preventing the deposition of insoluble calcium salts associated with pathological calcification, especially kidney stones.

The analogues sought were the sulphamate and phosphoramidate derivatives of PC; namely N-phospho-2-amino tricarballylate (PAT) and N-sulpho-2-amino tricarballylate (SAT). The preparation of PAT, which was ultimately achieved after investigating a number of unsuccessful synthetic routes, involved two distinct stages: a) synthesis of a new precursor in trimethyl 2-amino tricarballylate, and b) coupling of the latter compound with 2-cyanoethyl phosphate, followed by alkaline hydrolysis. The preparation of SAT was effected by the coupling of 2-amino tricarballylate with pyridine-sulphur trioxide. The same synthetic route was also utilized to yield [^{35}S]-labelled SAT.

Systems which were developed to aid in the ultimate purification and characterization of synthetic products included ion-exchange and thin layer chromatography, electrophoresis, isotachophoresis and chemical assays. Additional structural proof of the new compounds was obtained through [^{1}H]-NMR and infra-red spectroscopy.

The ability of the PC analogues to inhibit calcification *in vitro* was assessed. PAT was as potent as PC in preventing hydroxyapatite formation, while SAT was less potent but still a strong inhibitor. Both compounds also inhibited calcium oxalate crystallization, the order of potency being PC > SAT >> PAT.

The chemical and biological stabilities of the molecules were studied. *In vitro*, PAT was found to be readily hydrolyzed at acid pH, unstable at neutral pH and susceptible to the action of alkaline phosphatase. SAT was shown to be completely stable at neutral and alkaline pH, relatively stable in acid and totally resistant to the actions of the hydrolytic enzymes sulphamatase and sulphatase. The stability of SAT was confirmed *in vivo* from metabolic studies utilizing [^{35}S]-SAT. When given orally, SAT was well absorbed across the gut and rapidly cleared unchanged to the urine from blood and all tissues.

The effectiveness of the compounds in arresting renal calcification *in vivo* was also studied using well established test systems. Comparisons were made with other inhibitors including PC. SAT was proven capable of inhibiting calcium oxalate crystallization, whereas PC and PAT were ineffective. With hydroxyapatite formation, different trends were observed; PAT and SAT had no effect whereas PC produced pronounced inhibition. Rationalization of these findings have been given.

Results presented suggest that, in terms of future possible therapeutic value, PAT would be unsuitable, whereas PC and SAT might prove useful under certain situations. The studies help define further the structure-activity relationships of PC and its new analogues in terms of anti-calcifying potential and stability. On this basis, avenues for future research are discussed for the development of further inhibitor molecules that might have greater activity and hence ultimately prove more useful agents for stone prevention.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Urolithiasis, or the formation of stones within the kidney and urinary tract, is one of the oldest and most widespread diseases known to man. Characteristically, stones can occur in a variety of shapes, colours, textures and sizes, the latter ranging from the size of a pinhead to that of a coconut. Their presence causes acute pain and discomfort to the sufferer and may result in stasis and renal failure. Although at present urolithiasis has a variety of age, sex and geographic correlations, nevertheless the aetiology, pathogenesis and successful treatment of this disease has still to be resolved.

Stones (also called calculi) are formed by the precipitation of relatively insoluble material, the concentration of which has reached supersaturation point within the urinary tract. Due to the complex nature of urine and its many component ions, stones that do form are never pure and may contain other precipitating substances that have also exceeded saturation point, or components that have been bound and subsequently become incorporated into the stone such as protein. The majority of stones that form in the urinary tract contain calcium in the form of either calcium oxalate, calcium oxalate mixed with calcium phosphate in the form of hydroxyapatite or as brushite. Other stone constituents include magnesium ammonium phosphate, uric acid and cystine. The majority of work described in this thesis has been directed at stones containing calcium, and in particular, those formed within renal tissue.

1.2 PHYSICOCHEMICAL ASPECTS OF UROLITHIASIS

The intricate processes associated with stone initiation and development are perhaps better understood after considering the process from a physicochemical level. Crystallization, i.e. the formation of an inorganic solid from an ionic solution, is the fundamental mechanism contributing to stone formation. The initial event of this transformation is termed nucleation, i.e. the formation of infinitesimal fragments of the solid. Nucleation may be of two types; a) homogenous, where nucleation occurs in the absence of impurities, and b) heterogenous, which occurs in

the presence of impurities. A necessary requirement for nucleation to occur is the supersaturation of the respective salts in the solution. Varying degrees of supersaturation may exist and, for example, a metastable range is the range in which nucleation will only occur when induced by another agent, i.e. a nucleating agent. The unstable region is a range of saturation where rapid spontaneous nucleation occurs. The critical level separating these two regions is defined as the formation product, whereas the dividing line between supersaturation and undersaturation below which precipitation will not occur, is termed the solubility product.

Nucleation is followed by a stage of crystal growth, whereby through further growth the initial crystals develop in size. Again, a necessity for this stage is the supersaturation of the component ions in solution, irrespective of whether the degree of supersaturation lies within or above the metastable region. The stages of crystal nucleation and growth are also collectively termed crystal formation (Fleisch, 1978). The final stage associated with crystallization occurs through the aggregation of crystals to form small or large crystal aggregates.

Since these stages, i.e. crystal nucleation, growth and aggregation are essential processes associated with crystallization, and therefore stone development, factors that may regulate these stages have a major influence on the formation of stones. In this regard, three main mechanisms are currently recognized as being important: 1) the degree of saturation or supersaturation of urinary components, 2) the role of molecules that may act by promoting the stages associated with crystal formation and aggregation, and 3) the effect of molecules that may inhibit crystal formation and aggregation.

1.2.1 URINARY SATURATION

Whilst the state of saturation of a particular salt is recognized as a potential factor that may influence the risk of stone formation, nevertheless exact measurements of saturation levels occurring in urine is complicated, due to the interfering interactions of the many other salts. Robertson (1969) was perhaps the first investigator to establish a successful method for calculating the saturation levels of calcium salts in urine, expressing the values as their activity product. This product was found to be dependant on urine pH and the concentration of ions including calcium, phosphate, oxalate, as well as other divalent and monovalent ions in magnesium, sulphate, sodium, and potassium. The method has since been used to measure the state of saturation in the urine of normal individuals

and in patients suffering from recurrent stone disease (Roberton, 1976; Crassweller et al., 1978; Oreopoulos et al., 1976). Whilst reports are conflicting as to whether the urines of stone-formers are significantly more saturated with respect to calcium salts than normal individuals, nevertheless two important features have emerged. It has been found in the majority of cases that the urine of both stone-formers and non-stone formers is supersaturated with respect to calcium oxalate. In addition, whilst some individuals from both groups have urine saturation above the formation product, most individuals are found to have saturation in the metastable zone.

Similar studies relating the degree of saturation of brushite and octocalcium phosphate (both insoluble forms of calcium phosphate) between stone-formers and non stone-formers have shown that the level of saturation of these salts may be slightly elevated in the case of stone-formers. Nevertheless in respect to these salts a significant proportion of individuals from both groups also appear to have saturation levels within the metastable range (Pak and Chu, 1973; Oreopoulos et al., 1976).

1.2.2 PROMOTERS AND THEORIES OF STONE FORMATION AND DEVELOPMENT

These preceding observations have raised a topic of some controversy in urolithiasis research. That is, given that a large proportion of stone suffering patients have urinary salt saturation in the metastable region, what factors are responsible for inducing the initial nucleation of crystals in these patients? Two theories, outlined below, have been presented to account for this, in 1) the matrix theory, and 2) the precipitation-crystallization theory. Agents that may induce nucleation by the mechanisms described in these theories are known as promoters. Whilst these theories have been implicated as a mechanism for crystal nucleation, a further mechanism known as epitaxis, has also been proposed as a factor that may promote nucleation and further stages of crystallization. The role of epitaxis in stone development is also discussed.

1.2.2.1 Matrix Theory

The presence of protein in stone matrix has led to speculation as to whether such substances might play an active role in stone development, i.e. act as nucleating agents. The basis for the matrix theory then arose from initial observations that urinary proteins can induce calcification in vitro (Boyce et al., 1955), and qualitative differences are found to exist in protein from the urine of stone-formers and non stone-formers (Boyce et

al., 1962). These findings have since received support from a number of investigators (Resnick et al., 1981; Joost et al., 1981; Hallson et al., 1981). The site of stone origin in renal tissue has been speculated to be extraluminal; i.e. within the renal tubular cells, where matrix substances containing protein capable of binding calcium participate actively in crystal nucleation (Pak, 1978). It would be likely that the calcium binding capacity is conferred upon matrix substances by the presence in them of γ-carboxyglutamic acid, an amino acid previously identified in calcium containing renal stones (Lian et al., 1977). This theory of stone formation clearly obviates the problem of anchorage of the initially precipitated small nuclei (or nidus). Nevertheless its major drawback is the physical separation of the matrix involved in initiating stone formation, and the urine from which mineral constituents must be derived.

A further modification to this hypothesis which might overcome the above problem has been presented by Pak (1978), who suggests that matrix substances may eventually appear intraluminally and become anchored. By what mechanism this would occur has not been proposed, and as yet, whilst this remains an attractive idea, it remains to be confirmed by investigation. Further, despite the presence of organic matrix in urinary calculi, there is still no indisputable evidence that such urinary proteins play a significant role in stone formation. Other explanations have been presented to explain the presence of proteins and may be just as valid. Deposition may occur secondary to the irritation from the stone and to infection (Fleisch, 1978). Co-precipitation of protein with the mineral or secondary binding onto the crystals (Vermeulen et al., 1964; Leal and Finlayson, 1977) could also be postulated.

1.2.2.2 Precipitation-Crystallization Theory

An alternate hypothesis for renal stone development is presented in the precipitation-crystallization theory. Precipitation or crystallization of stone forming salts from urine supersaturated with respect to such salts could occur intraluminally, and would not require an aetiological role of organic matrix for nidus formation and stone growth. Presumably the initial formation of a nidus may occur spontaneously when the supersaturation of salts transiently exceeds the formation product. Thereafter the presence of the nidus promotes further nucleation and crystal growth which leads ultimately to the development of the stone through crystal aggregation. However, with this theory, the rate at which crystals attain a critical size for retention within the tubules must exceed the transit time of urine

through the tubule. Unfortunately, at present, experimental verification for this is lacking (Finlayson, 1978), so the validity of this model is also questionable.

1.2.2.3 Epitaxis as an Influence in Stone Development

Regardless of the mechanism by which the crystal nidus is formed and anchored within renal tissue, once established, it is then possible for stone formation to occur through further nucleation, crystal growth and aggregation. In stone sufferers, it is possible that a mechanism known as epitactic induction may also play a contributing role in stone development. This phenomenon occurs where the crystallization of a salt is induced by crystals of a different nature. Lattice similarities exist between crystals of uric acid, calcium oxalate and calcium phosphate and epitactic induction does occur amongst them *in vitro* (Lonsdale, 1968). Epitaxis could explain why many stones are a mixture of different salts. Pak *et al.* (1971) have presented data to suggest that brushite functions as a nucleator for calcium oxalate stones, but arguments just as cogent have been raised against this theory (Williams, 1974). Nevertheless support for epitaxy as a contributing factor continues to build up. Coe (1978) found that allopurinol, a drug that decreases the excretion of urate, was successful in reducing stone formation in calcium stone patients who frequently are hyperuricosuric (Coe and Kavalach, 1974). This observation, in association with the well documented epitaxis of the abovementioned salts *in vitro*, suggests that epitaxis between components may be an important factor *in vivo*. At the present time however, although epitactic induction remains an attractive theory and might account in part to the promotion of stone development, there is no direct evidence that epitaxis is the major mechanism attributing to this process.

1.2.3 INHIBITORS AND STONE FORMATION

1.2.3.1 Inhibition of the Stages of Crystallization

As previously stated, normal urine is frequently supersaturated with respect to stone forming components (especially calcium oxalate) and furthermore, may contain crystals of these salts. Considering this, as well as the possibility of the promotion of stone development by factors mentioned above, the next question to be answered is why is stone formation not a more prevalent disorder? While one possible explanation is that non stone-formers may have less promoters in their urine, it is now apparent

that the presence of substances known as inhibitors could play a vital role in safeguarding against stone formation in normal urine. Such compounds could act by preventing the formation of crystals and inhibiting crystal aggregation. Pyrophosphate (PP_i) was one of the first of such inhibitors to be recognized and successfully isolated from urine (Fleisch and Bisaz, 1962). Since then more extensive investigations have led to the identification of a variety of other natural urinary inhibitors. The majority of such compounds appear capable of inhibiting both calcium oxalate and calcium phosphate crystal formation and aggregation, although some may preferentially inhibit one salt or the other. Similarly, although all stages of crystallization (e.g. nucleation, growth and aggregation) may be inhibited, some inhibitors act preferentially at one or more of these stages.

As to which area of crystallization is the most important in the formation of stones, and hence a focus for inhibition studies, is still a matter of some controversy. In the past, major attention has been directed toward the inhibition of crystal growth. Meyer and Smith (1975a) have suggested that the crystal growth of stone material is a major factor in stone formation, because if the growth is inhibited, the initially precipitated crystals are cleared harmlessly to the urine. The importance of crystal aggregation has also been highlighted in recent years, and Ryall et al. (1981) have suggested that urine has a greater ability to inhibit calcium oxalate crystal aggregation than crystal growth. Fleisch (1978) has also noted the importance of crystal aggregation and has suggested that aggregation could be the mechanism which distinguishes the crystalluria that is found in normal urine from that of stone formers. This belief is supported by the observation that usually only individual calcium oxalate crystals are excreted in normal urine, whereas large aggregates of the salt are more often found in the urine of stone-formers (Robertson et al., 1969; Robertson and Peacock, 1972).

1.2.3.2 Nature of Urinary Inhibitors

The inhibitors that that have so far been identified as present in urine may be placed into two broad categories; those of low molecular weight such as intermediary metabolites and inorganic ions, and those of high molecular weight, or organic macromolecules. In general, the macromolecules seem more potent in the inhibition of crystal aggregation, whereas the smaller molecular weight inhibitors affect all stages of crystallization. Thus the major urinary inhibitors of crystal nucleation

and growth are PP_i (Fleisch and Bisaz, 1962), magnesium (Oreopouos et al., 1975), zinc (Bird and Thomas, 1963), fluoride (Bachra and Fischer, 1969), citrate (Bisaz et al., 1978) and isocitrate (Sutor et al., 1978). The most prominent of the low molecular weight urinary metabolites with inhibitory activity towards crystal aggregation are PP_i and citrate (Fleisch and Monod, 1973; Hansen et al., 1976). The most potent macromolecular inhibitors of crystal aggregation appear to be the urinary heteropolysaccharides, of which almost all probably consist of glycosaminoglycans (GAGS) (Kitamura et al., 1982). GAGS which appear responsible for inhibitory activity in urine are chondroitin sulphate, heparin, hyaluronic acid, and dermatan sulphate (Fleisch and Monod, 1973; Hansen et al., 1976; Robertson et al., 1973). Glycoproteins and ribonucleic acids are other macromolecules offering potent inhibition (Nakagawa et al., 1978; Schrier et al., 1981).

In the past the presence of GAGS and other macromolecules in urine suggested that these compounds play a significant role in the prevention of nephrolithiasis under normal conditions. Recent work by Edyvane et al. (1984) has however challenged this interpretation. Their data suggest that the bladder is the main source of macromolecules in urine. Additional support for these findings has been presented by Martin et al. (1984). The inference of these new observations is that while GAGS and other macromolecules might be important factors in preventing bladder stone formation, they may play little part in protecting against renal stones.

1.2.3.3 Mechanism of Action of Inhibitors

Although the precise relationship between structure and inhibitory activity of the different types of inhibitors is not well understood, the basic mechanism of action of crystal inhibitors appears to be related to their binding onto the crystal surface whereby they inhibit further nucleation, growth and aggregation (Fleisch, 1978). Whilst a relationship does exist between the inhibitor concentration on the crystal surface and the extent of inhibition (Bliznakov, 1965), it is not essential for the entire surface to be covered to obtain potent inhibition. Specific binding of inhibitors to crystal growth sites is sufficient, hence inhibition may occur when less than one percent of the surface is covered. Several types of binding sites however are recognized to exist. The affinity for only one particular class of sites appears to be related to an inhibition of crystal formation, which suggests that in this situation, binding is occurring to sites responsible for crystal growth (Jung et al., 1973). The fact that the

inhibition of the formation rate of calcium oxalate (Meyer and Smith, 1975b) and brushite (Marshall and Nancollas, 1969) is well described by the Langmuir adsorption isotherm adds further support to this postulated mechanism. The inhibition of crystal aggregation however, is more likely to occur through changes in zeta potential that result in repulsion between crystals. For example, the polyanionic nature of GAGS possibly is responsible for the potency of this class of compounds in the inhibition of crystal aggregation.

Despite recognition that the binding of anionic inhibitors to crystal sites is mediated probably through the calcium ion, the nature of the binding is not directly related to the chelating ability of the inhibitor for calcium. For example EGTA, a powerful in vitro chelator of calcium, is a poor inhibitor of calcification. Nevertheless various structural factors have been implicated as essential for an inhibitor of calcification to be potent. In studies on the inhibition of hydroxyapatite formation in vitro, Williams and Sallis (1979, 1982) have established structural and stereochemical criteria required for a molecule to be a strong inhibitor. Their data has indicated that an inhibitor should possess at least one phosphate group and at some other proximal position another acidic group, be it phosphate or carboxylate. When this requirement is fulfilled, further chelating groups can confer increased inhibitory activity, dependant upon other factors. These are a) the proximity of the active groups, b) the nature of the additional groups, c) stereochemical arrangements, d) steric factors, e) stability of the molecule, and f) lipophilicity. Recently, the hydrophilicity of an active group has also been suggested as a critical factor (Moreno et al., 1984). This may determine the ability of the inhibitor to adsorb to the crystal by the displacement of water molecules adsorbed to this site.

1.2.3.4 Synergistic Interactions between Inhibitors

Whilst the mechanism of inhibitory action of a single ionic species may be readily explicable in an in vitro calcification system, the interactions that occur in a solution containing many different ions make the mechanism of action of inhibitors in such a system more difficult to determine. For example, it is a commonly observed phenomenon whereby the inhibition produced by two compounds together, is equal to or greater than the sum of their individual effects. The precise interactions responsible for this synergistic behaviour between ions are often difficult to discern, as several mechanisms seem feasible. It could be rationalized in terms of

the binding of different inhibitors to different active growth sites on the crystal and when this occurs, a more efficient coverage of the crystal surface results such that growth and nucleation are more effectively hindered. A second factor, possibly attributing to the synergism observed between Mg^{2+} and certain organic phosphates (Williams, 1981) is the chelation between these species, thus increasing the stability of the organic phosphate on the crystal surface, where hydrolysis reactions are known to occur (Krane and Glimcher, 1962; Meyer, 1984). In an *in vivo* situation where urine, for example, is multicomponent in nature, synergistic interactions are obviously important. Hence because of this, one must be wary in trying to calculate the relative contributions of various inhibitors in urine. As synergism is difficult to accurately assess, it is often a more meaningful parameter to consider the inhibitory activity of the urine as a whole.

1.2.4 RELATIONSHIP BETWEEN URINARY LEVELS OF INHIBITORS, PROMOTERS AND SALT SATURATION IN STONE FORMING INDIVIDUALS

Whilst the presence in urine of inhibitors of crystallization may safeguard against stone formation, especially when the concentration of stone forming components lies within the metastable range, a variety of abnormal states can exist where the supersaturation of stone forming components is well above normal values. Under these conditions, compensation by normal levels of inhibitors in urine may not be possible. The most common of these disorders are hypercalciuria, hyperoxaluria and to a lesser extent, hyperuricosuria. While stone formation generally ensues, this is not always true as it depends on the degree and cause of the condition.

It is quite common nevertheless, for stone formation to occur when the levels of stone forming components, although elevated, lie still within the normal range for urinary constituents. Under such conditions, speculation arises as to whether stone—formers have a lower level of inhibitors in their urine as compared to normal urine. Some workers have found for example, that the levels of citrate and GAGS in urine are significantly lower in stone-formers than in normal urine (Robertson, 1976; Schwille *et al.*, 1982; Kitamura *et al.*, 1982). Other studies have indicated no differences in levels of inhibitors (Oreopoulos *et al.*, 1976) and in some instances inhibitory activity of stone patients has been recorded as elevated compared to normal controls (Randolph *et al.*, 1981). The possible reasons for these discrepancies are not clear. Robertson *et al.*, (1976)

have suggested that the inhibitor level should be compared to the salt saturation level to give a value termed the saturation/inhibition index. Using this parameter, separation of stone-formers from normal controls becomes possible, whereas if saturation and inhibition indices are studied separately, a significant overlap can occur.

Recognizing some of the shortcomings, such as the difficulty and the time involved in determining these parameters; the group have subsequently attempted a different approach to the problem. Following the observation that stone-formers display a higher urinary pH, excrete more calcium, oxalate and uric acid, and less GAGS, Robertson et al. (1978) have calculated the frequency distributions of the single variables. Using this data, it is possible to quantitate the risk of stone formation and so obtain an overall probability of forming stones for each risk factor. However, one pitfall with this updated model is that all variables considered must be stochastically independant, and since Robertson and Peacock (1980) have reported a correlation between calcium and oxalate in a stone forming population, this approach also has limitations. Other workers have reported that the ratio oxalate/citrate x GAGS may be useful, and is capable of separating more than 80% of calcium oxalate stone-formers from controls (Baggio et al., 1982).

At present then, although uncertainty still remains as to what specific urinary constituents contribute most to the risk of forming a stone, the most widely accepted belief is that stone formation results from a concentration imbalance in urine between stone promoting factors, such as lithogenic salts, or perhaps even specific macromolecules that may induce crystallization, and factors that normally prevent crystal formation and aggregation, i.e. inhibitors.

1.3 METHODS OF TREATMENT OF STONE DISEASE

1.3.1 GENERAL APPROACH

Successful treatment of stone disease for the most part, still remains a hope more than an accomplishment. Significant advances have been made in recent years, and it is now feasible to remove stones without major kidney surgery. New techniques include disintegration of the stone by ultrasonic methods or by bombardment with high energy shock waves. In either case, the stone is shattered into small fragments which are then removed or passed in the urine. Whilst stone removal is often essential to relieve stasis, nevertheless the patient may still have the disposition to

form further stones due to the recurrent nature of the disease, particularly if any stone fragments are not recovered.

Irrespective of stone removal, it is therefore essential that stone-formers undergo a treatment regimen that will prevent the development of new stones, or arrest the growth of existing stones. Many treatment programs in the past were used too broadly without regard to the specific cause of the stone formation. Nowadays a more selective approach has been advocated, whereby treatment is chosen specifically for particular causes of stone disease (Pak, 1982; Danielson et al., 1983). The elements of this specific approach are that treatment 1) corrects physicochemical abnormality in urine, 2) overcomes physical derangements in stone-formers, 3) inhibits the formation of stone, 4) has minimal side-effects and 5) prevents extrarenal manifestation of the disease process.

1.3.2 SELECTIVE TREATMENT METHODS

A number of carefully chosen methods have been tested, and in selected instances some have proven to be useful in the treatment of stone disease resulting from specific disorders. Most agents that have proven so far to prevent stones may do so by correcting the promoter/inhibitor imbalance that has been suggested as prevalent in stone-formers. Oral administration of such agents may correct this imbalance by decreasing the urinary concentration of lithogenic salts, raising the level of inhibitors in urine, or both.

A high fluid intake alone may be of limited value as treatment. Although urine dilution may cause some dissociation of the various soluble complexes of calcium (although not significantly in the case of calcium oxalate), it has the disadvantage of lowering the concentrations of the urinary inhibitors (Danielson et al., 1983). Nevertheless, in association with other forms of treatment it may prove effective. For example, a high fluid intake in association with a low calcium diet has been proven to correct the physicochemical abnormalities in the urine of stone-formers with absorptive hypercalciuria type II, and correspondingly leads to fewer stone attacks.

Cellulose phosphate, in conjunction with a low calcium diet, is one form of treatment that has in some cases proven successful in calcium stone patients with absorptive hypercalciuria type I, by virtue of its complexing of the calcium in the gut (Pak et al., 1974). More recent work however, has indicated that undesirable secondary effects occur with this agent such as a reduction in magnesium and an increase in oxalate in the urine (Pak,

1981). This, in association with the fact that the agent is expensive, bulky and must be taken in quantities of 15-30g/day makes this mode of therapy unpopular.

Allopurinol has been suggested as an effective agent in the treatment of calcium stone-formers that have hyperuricosuria. This drug is thought to act by reducing the excretion of sodium urate crystals, which act as effective nucleators of calcium oxalate crystallization (Coe, 1978). Nevertheless, not all investigators agree with this school of thought and many challenge the efficacy of this agent (Baggio et al., 1983; Danielson et al., 1983).

Thiazide diuretics perhaps represent one of the most effective drugs of those tested to date in relation to calcium stone prevention, with one particular study showing that stone progression ceased in 90% of patients undergoing chronic hydrochlorothiazide treatment (Yendt and Cohanim, 1978). Although the precise mechanism of action of these drugs is not clear, they do produce an increase in the excretion of the urinary inhibitor zinc (Bird and Thomas, 1963), decrease urinary calcium (Yendt and Cohanim, 1978) and may lower the incidence of crystalluria (Hallson and Rose, 1976). Other data is directly conflicting with any claims that thiazides may be useful. Scholz et al. (1981) have reported a reduction in the relative supersaturation of calcium oxalate in stone-formers following thiazide treatment, but they found also a reduction of the same magnitude when other stone-formers were given a placebo dose. Ljunghall et al. (1981) have found that chronic administration leads to a decline in serum magnesium which they suggest ultimately might lead to magnesium deficiency. Other undesirable effects reported are a reduction in urinary citrate and a reduction in body potassium stores (Klein and Griffith, 1981) and adverse changes to bone (Pak, 1982). Considering these findings, present indications are that thiazide treatment could be useful if the patient is carefully selected. In specific cases magnesium and/or potassium supplements may be used to overcome some of the difficulties.

Magnesium may be used by itself as a therapeutic agent either in the form of an hydroxide or an oxide. However, during administration, urinary pH rises significantly, and in some patients an increase in urine calcium may occur. Treatment does lead to an increase in the urinary magnesium concentration, but at present no objective evidence for the beneficial effects of magnesium alone in calcium nephrolithiasis can be found.

Methods of raising the urinary excretion of the inhibitor citrate,

is one approach that has recently been investigated. While citrate administration itself has proven effective, a variety of other natural organic acids also do so by increasing the renal tubular secretion of citrate (Leskovar et al., 1981). This treatment has in specific cases proven capable of reducing the recurrence rate of stone formation. In particular, it has proven successful in patients who form stones as a result of renal tubular acidosis; where the urine is characteristically low in levels of citrate (Pak, 1982). Treatment of these patients with bicarbonate has led to an increased urinary citrate excretion, and correspondingly fewer stone attacks (Backman et al., 1981).

1.3.3 PHOSPHORYLATED COMPOUNDS AS INHIBITORS

Whilst many of the aforementioned agents, when used according to the selective treatment approach have proven effective in specific cases, they are nevertheless still not without their limitations. Specifically, the underlying cause of stone formation may not be easily recognized in a number of situations, the true efficacy of these agents are in many cases questionable and their administration may in some instances lead to adverse side-effects. The fact that not only PP_i but a number of other phosphorylated derivatives have proven extremely potent as anti-calcifying agents, has raised the possibility that methods that lead to an increase in the urinary excretion of these agents might prove a superior treatment than many in vogue and have a more widespread application in the prevention of stone disease.

The oral administration of orthophosphates not only results in a significant increase in the urinary concentration of PP_i (Smith, 1976) and citrate (Danielson et al., 1983), but has the added advantage of binding calcium in the gut, thus reducing the intestinal absorption of this ion and hence its urinary excretion. This form of treatment has had some degree of success in reducing the stone recurrence rate in patients, but nevertheless it has not been totally effective. In a proportion of patients, stone formation actually has been seen to be increased (Peacock et al., 1981).

Since administration of PP_i itself is limited, as the compound undergoes rapid inactivation by enzymic hydrolysis with pyrophosphatases, extensive research has been undertaken in recent years to discover compounds of similar structure but more resistant to enzymic attack. Such inhibitor compounds could prove effective therapeutic agents if excreted unchanged in the urine following oral administration. Research has been directed in two areas a) the synthesis of compounds exogenous to normal

urine, and b) an examination of some of the other naturally occurring organic phosphates that may be more stable than PP_i.

1.3.3.1 Phosphonates and Bisphosphonates

The phosphonates, which are characterized by C-P bonds, and more specifically the bisphosphonates (formerly called diphosphonates), which contain the P-C-P linkage, are synthetic compounds that have proven to be extremely potent inhibitors of calcification *in vitro* (Ohata and Pak, 1973; Meyer *et al.*, 1977). In contrast to PP_i and other natural phosphate esters, they are believed to be totally resistant to breakdown in the body (Fleisch, 1981). For these reasons their use in the therapy of stone disease would initially appear attractive.

Whilst bisphosphonates have been shown to very efficient inhibitors for experimentally induced renal calcification (Fleisch *et al.*, 1970; Potokar and Schmidt-Dunker, 1978), other research has shown however, that their usefulness as therapeutic agents is severely restricted for a number of reasons. Pharmacokinetic studies, most of which have been done using 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) [previously called ethane-1-hydroxy 1,1-diphosphonate (EHDP)], have shown that these compounds are poorly absorbed (1-10%) following oral administration (Recker and Saville, 1973). While approximately half of any HEBP absorbed does find its way to urine, the majority of the remainder enters bone (Michael *et al.*, 1972). The half time for its release from this latter site is generally slow because of both its strong affinity for hydroxyapatite and its resistance to enzymes such as phosphohydrolases that might ordinarily hydrolyze phosphate groupings and lead to its subsequent removal. Various investigators have shown that upon reaching bone, bisphosphonates disrupt bone metabolism by inhibiting resorption; a process essential for normal bone development (Schenk *et al.*, 1973). The effect of bisphosphonates on bone resorption has been suggested as being mediated through mechanisms other than purely physicochemical effects on the hydroxyapatite at bone (Shinoda *et al.*, 1983). This is supported by the observation that bisphosphonates alter the morphology of osteoclasts (cells associated with bone resorption), both in culture (Rowe and Hausmann, 1976) and when administered *in vivo* (Schenk *et al.*, 1973).

Such studies have stimulated active research into the biochemical effects of bisphosphonates. A variety of responses have now been described, and are extensively reviewed in a recent article by Fleisch (1983). Nevertheless, despite the shortcomings of the currently available

bisphosphonates, research is progressing into the development of alternate phosphonates and bisphosphonates by varying the substitution on the basic skeleton. Hopefully such alterations might lead to compounds with fewer side-effects, therefore enabling the compounds to become more valuable in the treatment of urolithiasis.

1.3.3.2 Phosphocitrate

One compound that has recently emerged as a prospective urinary inhibitor is phosphocitrate (PC). This compound was first reported by Howard (1976) who found that the acidification of a solution containing phosphate, citrate and calcium followed by neutralization led to a marked increase in the inhibitor potential of the mixture. Purification and partial characterization of an impure material derived from this reaction disclosed that it contained both phosphate and citrate moieties, and by implication was tentatively assigned the structure of PC. This material was found to have similar properties to a material isolated from urine by the same workers. Nevertheless, as suggested by Williams (1981), its existence in urine was not confirmed by this report, as the ion-exchange system used to isolate it from urine may also have caused artefactual formation.

The availability of high-purity synthetic PC (Williams and Sallis, 1980; Tew et al., 1980) has since allowed a more reliable evaluation of the presence of this compound in biological material. PC has now shown unambiguously to occur in rat and rabbit, both in whole tissue homogenates (liver, kidney and intestine), and mitochondria isolated from these tissues (Williams, 1981). The presence of PC in urine has also received some support from the work of Williams (1981), who detected the presence of a compound containing hydrolyzable phosphate and citrate eluting at the same volume as synthetic PC following ion-exchange chromotagraphy of deproteinized human urine. However, due to the interference of other ions co-eluting with PC, verification by further analysis was not possible.

In vitro, PC has been shown to interfere at very low concentrations, with the conversion of amorphous calcium phosphate to hydroxyapatite (Williams and Sallis, 1979). It is more potent than any other inhibitor tested to date, which includes PP_i as well as the synthetic compounds HEBP and imidodiphosphate. In relation to calcium oxalate crystallization, PC, although weaker than GAGS, has still proven to be a potent inhibitor, being more potent than PP_i (Williams and Sallis, 1981).

The implicated presence of PC in urine, and its potent anti-calcifying activity in vitro has raised many important questions. At

present it seems that the known inhibitors can account for only part of the total inhibitory power of urine. Possibly then, PC may be a major inhibitor of stone formation and account for the remainder of the inhibitory activity in normal urine. Since it is likely that the metabolism of PC and citrate is closely linked, another possibility is that the low levels of citrate that have been reported in the urine of a proportion of stone formers is also parallelled by a reduction in PC excretion. If PC was in urine, this possible reduction in citrate excretion by stone-formers takes on additional significance. It has recently been shown that citrate and PC are strongly synergistic in vitro (Williams and Sallis, 1981; Tew et al., 1980). Hence small changes in urinary citrate could result in a large change in the inhibitory activity in urine by virtue of its synergism with PC, even if the level of PC in urine was the same in stone formers and non stone formers. If the urinary level of PC also was lower in stone formers, this would further amplify the differences in inhibitory activity between the two groups. Whilst these questions are yet to be resolved, clearly they are areas that deserve investigation in the future.

The preceding arguments raises the possibility that PC may play a major, but as yet unrecognized, role in preventing kidney stone disease. PC therefore might appeal as an ideal therapeutic agent. It is one of the most powerful anti-calcifying agents in vitro presently known. In addition, because of its natural occurrence it would therefore be likely to be free of the biochemical side-effects and adverse reactions to bone metabolism that are evident following the long term therapy with, for example, the bisphosphonates. The nature of the PC molecule however does not preclude enzyme degradation. In fact, Williams and Sallis (1981) have shown that following the intravenous administration of [^{32}P]-PC, the compound is rapidly destroyed in the kidney, as evidenced by their failure to detect any of the radiolabelled PC passing through to urine but only its [^{32}P]-phosphate cleavage product. This finding suggests that exogenous PC may also have limited value in the treatment of urolithiasis. Despite this, successful treatment might be achieved by measures that stimulate the endogenous production of the compound, leading to its ultimate passage into urine. As yet this is not possible, since the biosynthetic pathways leading to this metabolite as well as its physiological role have yet to be elucidated.

1.3.3.3 Phosphocitrate Analogues as Inhibitors of Stone Formation

In view of the possible shortcomings of PC, structural analogues of PC might ultimately prove more useful in the treatment of calcium lithiasis

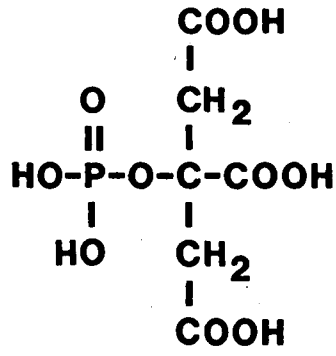
than the parent molecule. The requirements for such molecules are that they possess a) a similar structure to PC and hence retain potent anti-calcifying activity, b) more stability to enzymatic hydrolysis than PC, c) no harmful side-effects, eg. disruption of bone metabolism, and d) absorptive properties which make them suitable for oral administration, and yet ultimately allow them to be cleared to the urine.

The aims of this thesis were therefore to develop analogues of PC that would satisfy structural criteria, and then to evaluate the usefulness of these compounds by determining whether they would satisfy other criteria mentioned above. Since the site of enzymatic attack on PC occurs at the P-O-C bond (see PC structure; Fig. 1.1), analogues that differed in substitution at this position were investigated. A number of possibilities were apparent. Substitution of the O atom could be made by N, C or S to form the phosphoramidate, phosphonate or thiophosphate derivatives respectively. Replacement of the P for S would yield a sulphate derivative. A further possibility was replacement of P-O for S-N giving the sulphamate derivative.

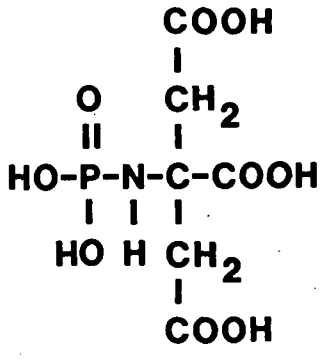
To decide which of these potential analogues warranted investigation, the suitability of the compounds were evaluated on a) their ease of synthesis, and b) the predicted stability of the formed product. Using these guidelines, a number of analogues were then eliminated. The phosphonate derivative was not favoured because of the toxic effects reported to be associated with compounds containing this linkage, and further, it was apparent that a complicated synthetic route might be necessary. This latter factor was also apparent with the thiophosphate derivative, and the fact that such linkages are readily lyzed by alkaline phosphatase (Fernley, 1971) also made this derivative unsuitable. The synthesis of the sulphate derivative was not feasible, since sulphate does not form a stable ester when attached to a tertiary carbon atom, as would be the case with this PC derivative (Kharasch, 1961).

The synthesis of the phosphoramidate and sulphamate analogues did appear readily achievable, and the structures of these analogues are compared to PC in Fig. 1.1. The sulphamate bond in particular has been proven to be very stable *in vivo* (Spillane and Benson, 1978; Spillane *et al.*, 1979), and although sulphamatases are found in the body, these are highly specific for natural substrates (Friedman and Arsenis, 1974). Despite their extreme stability however, no evidence exists as to whether such compounds might exert toxic effects at the bone site (c.f. bisphosphonates). Both the sulphamate and sulphate moieties occur as major

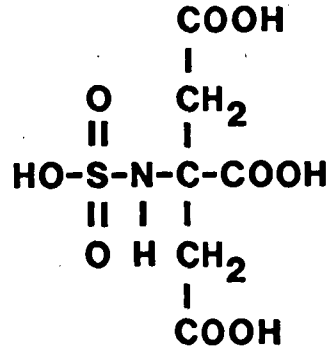
Fig. 1.1 The structures of phosphocitrate (PC) and potential synthetic analogues.



Phosphocitrate (PC)



N-phospho-2-amino tricarballylate (PAT)



N-sulpho-2-amino tricarballylate (SAT)

functional groups of GAGS, and it is thought that in these macromolecules, such groups contribute inhibitory activity toward crystal aggregation by ionization to give the polyanionic nature of GAGS. At present the ability of the sulphamate linkage to contribute inhibitor potency to a low molecular weight molecule has not been explored.

In relation to the phosphoramidate linkage, whilst the P-N bond is not cleaved by bacterial alkaline phosphatase (Reid and Wilson, 1971) enzymes such as alkaline phosphatase (Winnick, 1947) and phosphoamidases (Waldschmidt-Leitz and Köhler, 1933) derived from mammalian sources have been shown capable of splitting this bond in a variety of natural and synthetic substrates in vitro. Nevertheless, the rate at which these enzymes might lyze a sterically hindered phosphoramidate linkage such as that of N-phospho-2-amino tricarballylate (PAT) has not been reported. In some respects, as inferred from the preceding discussion, it would be a desirable attribute for PAT to have some degree of lability, provided it was more stable to bio-degradation than PC. With respect to the inhibitory capabilities of the phosphoramidate group, the majority of work has been done on imidodiphosphate, the phosphoramidate analogue of PP_i. This compound has actually proven to be stronger inhibitor than PP_i in specific test systems (Robertson and Fleisch, 1970; Williams and Sallis, 1979).

Due to the nature of the thesis topic, the work described herein is divided into two sections. In Part A of the thesis, it was proposed that, after the exploration of various synthetic strategies, suitable methods would be found and undertaken to prepare the desired compounds. In addition, it was foreseen that the development of a variety of physical and chemical characterization techniques would be required to establish the purity and identity of the new products. In Part B, the anti-calcifying and metabolic properties of the compounds would be explored. It was envisaged that these analogues would be compared with PC as to their effectiveness to inhibit calcification in vitro. Further, it was anticipated that the stability and metabolism of these compounds could be assessed both in vitro and following administration in vivo, possibly by the use of radiolabelled material. Upon establishing these parameters, it was finally proposed to test the effectiveness of these compounds on the inhibition of biological calcification by the utilization of appropriate test systems. From the collected data, it was hoped that some predictions could be made on the future clinical applications of the new compounds in relation to kidney stone disease. Furthermore, additional insight obtained into the structure-activity relationship of these types of molecules could prove useful in any future development of other anti-calcifying agents.

PART A

METHODS FOR THE SYNTHESIS AND CHARACTERIZATION OF N-PHOSPHO-2-AMINO TRICARBALLYLATE AND N-SULPHO-2-AMINO TRICARBALLYLATE

CHAPTER 2

PREPARATION OF 2-AMINO TRICARBALLYLATE AND ITS TRIMETHYL ESTER; PRECURSORS OF THE PHOSPHOCITRATE ANALOGUES

2.1 INTRODUCTION

The synthetic strategies that appeared most suitable for the preparation of the phosphoramidate and sulphamate analogues of phosphocitrate involved the phosphorylation or sulphonation of either 2-amino tricarballylic acid (AT) or its tri-ester, depending on the nature of the reactants. Since in the developmental stages of this project it was not possible to predict what routes the syntheses would take, there was a necessity to have both agents (i.e. AT and its tri-ester) available. The precursors themselves could not be obtained commercially.

Literature research established two reports on the preparation of AT (Dornow and Rombusch, 1955; Connors et al., 1962), but the synthesis of a tri-ester of this compound was not reported. Whilst the esterification of AT was one possibility, alternate routes leading to the production of trimethyl 2-amino tricarballylate were also apparent. For example, the synthesis of the trimethyl ester of 2-nitro tricarballylate, a similar compound, has been demonstrated (Kaji and Zen, 1973; Zen and Kaji, 1970). Much of the work described in this chapter then involves the reduction of this nitro compound and the purification of reaction products to yield the corresponding amine, trimethyl 2-amino tricarballylate.

2.2 MATERIALS

Diethyl acetonedicarboxylate and methyl bromoacetate were both products of the Fluka Chemical Co., Buchs, Switzerland, and aluminium oxide (neutral, activity II) was obtained from Ajax Chemicals, Sydney, Australia. Methyl nitroacetate was prepared from nitromethane according to the method of Zen et al. (1976). Chelex-100 resin (Na^+ form; 200-400 mesh) was a product of the Bio-Rad Laboratories, Richmond, California, and the resin was converted to the H^+ form before use. All other chemicals were reagent grade and obtained commercially.

2.3 METHODS

2.3.1 SYNTHESIS OF 2-AMINO TRICARBALLYLATE

Diethyl acetonedicarboxylate (b.p. 93-95°C/1.0mm Hg) was initially prepared by reaction of chlorosulphonic acid with citric acid as described by Gerner (1964). However, it subsequently proved more convenient to purchase the compound directly from the Fluka Chemical Company (Buchs, Switzerland). This product was converted to AT by a two step procedure as outlined by Dornow and Rombusch (1955) (Fig. 2.1).

In the first stage, 0.1mol of diethyl acetonedicarboxylate, 0.35mol ammonium carbonate and 0.11mol potassium cyanide were reacted in 170ml of 55% ethanol at 60°C for 6h. Following this, the solution was concentrated by rotary evaporation to 30ml and the pH adjusted to 7.0. Upon cooling, 5,5-di(ethyloxycarbonyl methyl) hydantoin crystallized. Recrystallization from 50% ethanol gave a pure product of m.p. 142-144°C with an overall yield of 50%.

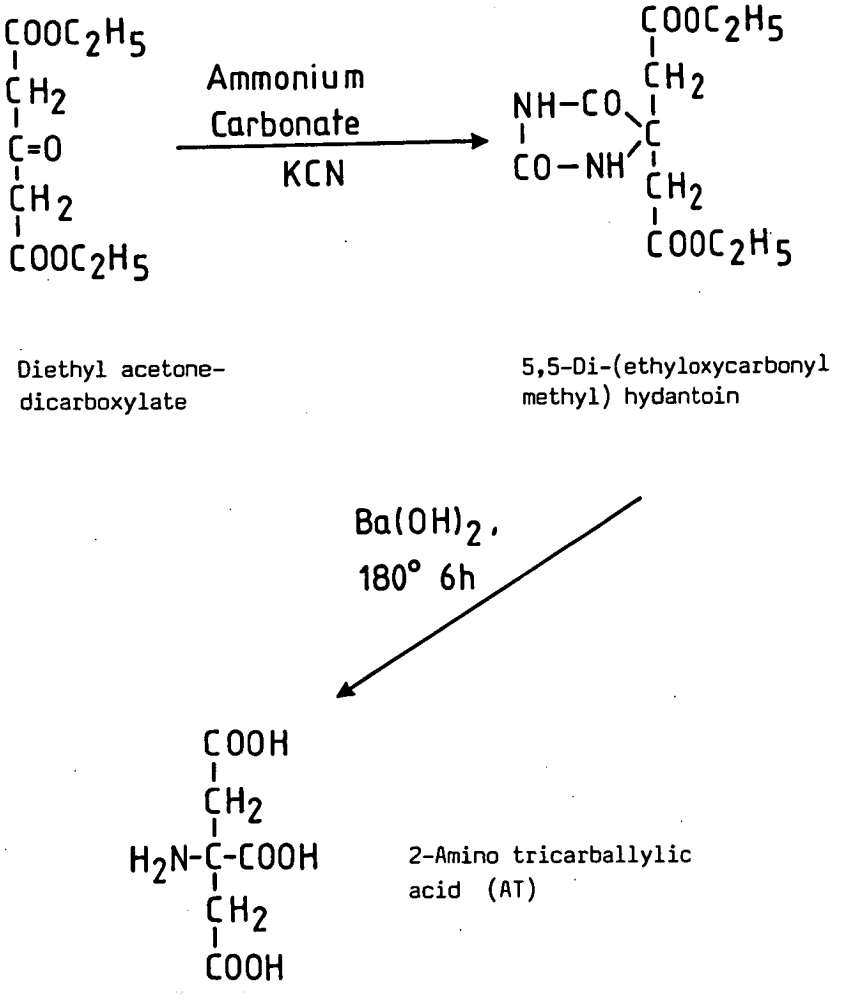
Subsequently, the hydantoin was converted to AT by base hydrolysis. Hence, 2.7g of hydantoin and 11.0g of $Ba(OH)_2.8H_2O$ were added to 50ml of water and heated at 180°C for 6h using a pressure bomb. After cooling, the resultant mixture was adjusted to pH 3.5 with 5M H_2SO_4, filtered and the filtrate passed through a column of Chelex-100 (H^+; 2 x 5cm). The column was washed with a further 50ml of water and the combined eluants were lyophilized. This crude fraction was recrystallized from 80% ethanol to give 1.4g (75%) of 2-amino tricarballylic acid (m.p. 264-266°C).

2.3.2 PREPARATION OF TRIMETHYL 2-AMINO TRICARBALLYLATE

In their investigation of the alkylation of methyl nitroacetate by methyl bromoacetate, Kaji and Zen (1973) employed two different approaches. With one method 1M sodium methylate was added to equivalent proportions of methyl nitroacetate and methyl bromoacetate to yield the mono-alkylated product, dimethyl nitrosuccinate in a 66% yield. In the second method the sodium salt of methyl nitroacetate was reacted with an equivalent amount of methyl bromoacetate to yield dimethyl nitrosuccinate in a 23% yield, as well as the dialkylation product, trimethyl 2-nitro tricarballylate, in a 16.4% yield.

Using these guidelines, it was hoped that by suitable alteration of the reaction conditions, the yield of trimethyl 2-nitro tricarballylate could be maximized. Once this product was obtained, it was envisaged that catalytic reduction would yield the ultimate desired product, trimethyl

Fig. 2.1 Synthetic route for the preparation of 2-amino tricarballylic acid (AT).



2-amino tricarballylate. The overall scheme for this synthesis is presented in Fig. 2.2.

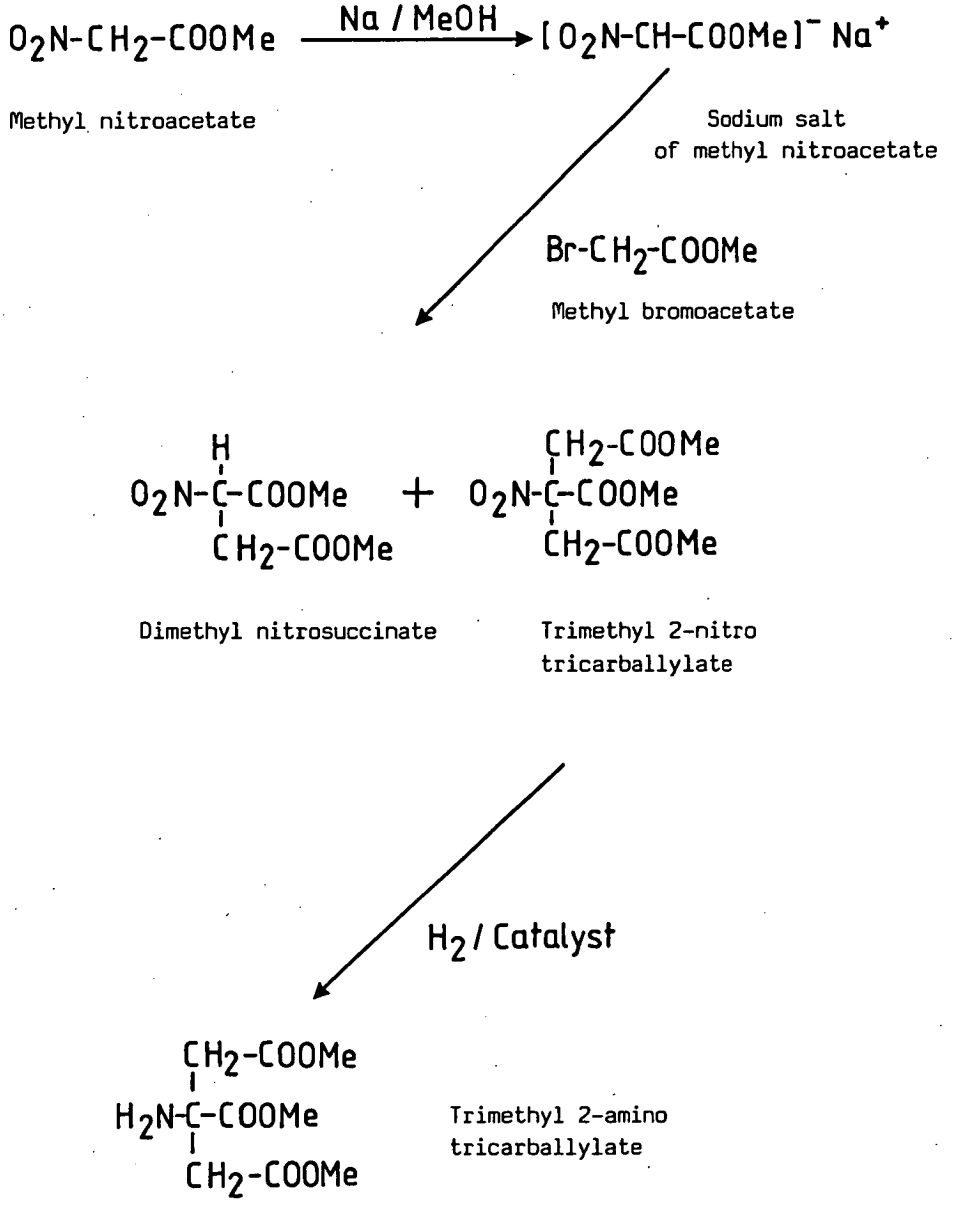
2.3.2.1 Synthesis of Trimethyl 2-Nitro Tricarballylate

In brief, the essential elements of the synthesis of trimethyl 2-nitro tricarballylate, as described by Kaji and Zen (1973), were as follows. The sodium salt of methyl nitroacetate was prepared by adding methyl nitroacetate in ether to an equivalent amount of sodium methoxide in methanol. The precipitated salt was isolated by centrifugation, dried and resuspended in a flask containing dimethylsulphoxide (DMSO). The reaction mixture was placed under an atmosphere of nitrogen, then methyl bromoacetate was added in an equivalent amount dropwise with vigorous stirring at 25°C. After total addition, the mixture was left to react under these conditions overnight. Ice-cold water was then added to the reaction mixture and the products extracted with benzene. After drying (Na_2SO_4), the combined benzene extracts were concentrated to give a golden oil.

Isolation and separation of the products, dimethyl nitrosuccinate and trimethyl 2-nitro tricarballylate, from the oil was achieved by high vacuum distillation. Dimethyl nitrosuccinate distilled at 76-78°C/0.06mm Hg (21-25% yield). Trimethyl 2-nitro tricarballylate remained as a gummy residue in the distillation flask and was crystallized after cooling by the addition of a minimum volume of ether followed by overnight refrigeration. This impure product could be further purified by recrystallization from an acetone/ether/diisopropyl ether mixture to yield trimethyl 2-nitro tricarballylate (13-15%) as colourless prismatic crystals, m.p. 88-89°C.

Whilst attempts to vary the above standard conditions were unsuccessful in increasing the yield of trimethyl 2-nitro tricarballylate, it was found that the overall efficiency of the process could be increased by utilizing the isolated dimethyl nitrosuccinate. This compound could be converted to trimethyl 2-nitro tricarballylate by reaction with methyl bromoacetate. Hence dimethyl nitrosuccinate was added to an equivalent amount of sodium methylate and the solution concentrated to give a sticky solid. This was then dissolved in DMSO, and, in an identical fashion to that described above, the reaction was initiated and allowed to proceed before product isolation. Distillation of products yielded 50% unreacted dimethyl nitrosuccinate, as well as the compound of interest, trimethyl 2-nitro tricarballylate in a 20% yield.

Fig. 2.2 Synthetic route for the preparation of trimethyl 2-amino tricarballylate.



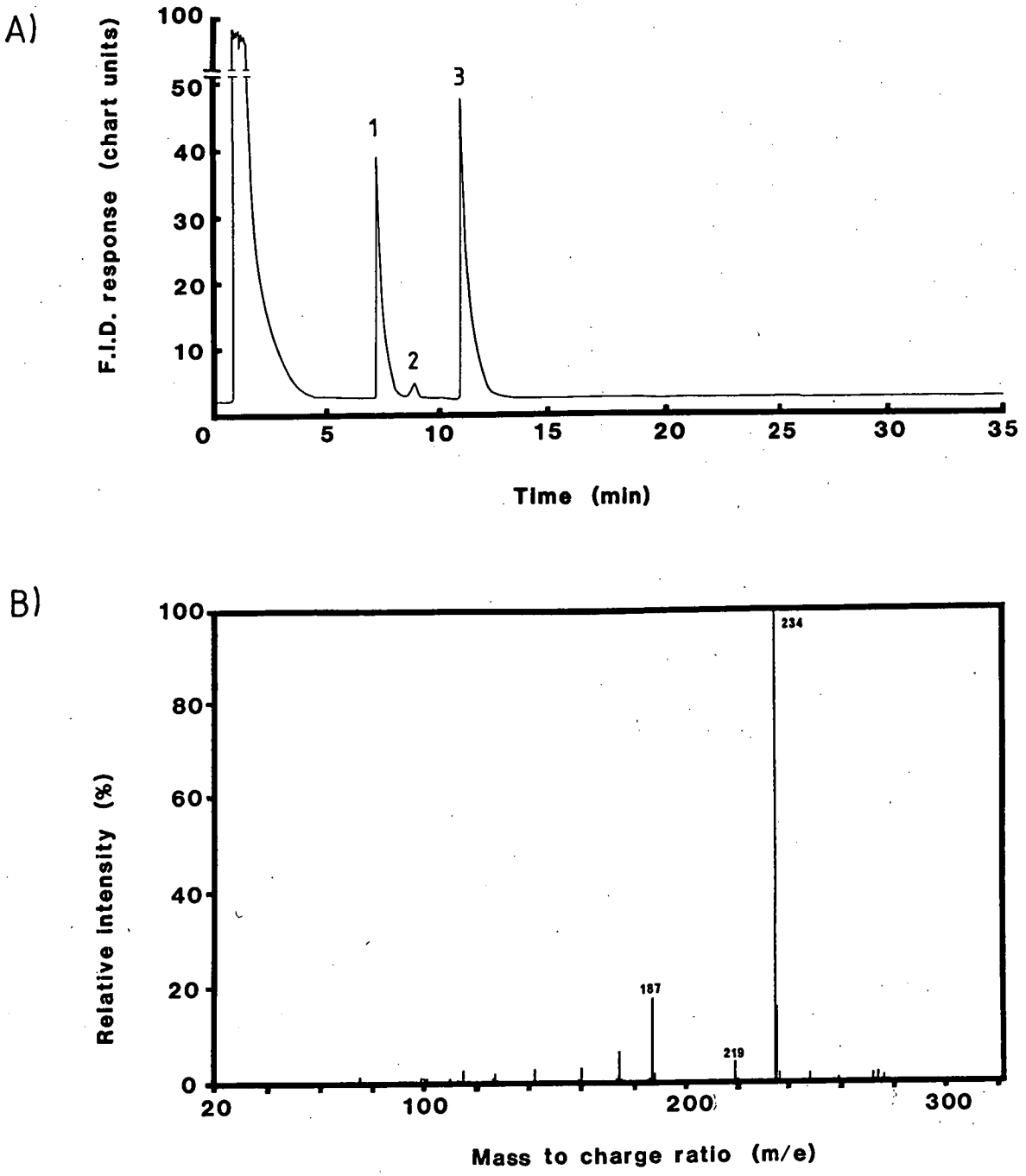
2.3.2.2 Reduction of Trimethyl 2-Nitro Tricarballylate

The catalyst used for the hydrogenation of trimethyl 2-nitro tricarballylate to yield trimethyl 2-amino tricarballylate was Raney Nickel T-1, which was prepared by the method of Dominguez *et al.* (1961). This catalyst has been used for the conversion of similar nitro aliphatic compounds (including dimethyl nitrosuccinate) to their corresponding amines in yields ranging from 90-100% (Kaji and Zen, 1973). For the reduction of trimethyl 2-nitro tricarballylate, 16g of the compound was suspended in 100ml of methanol and hydrogenated with 2ml of the Raney Nickel T-1 catalyst (500mg/ml suspension) under 40-60 p.s.i. of hydrogen in a Parr low-pressure hydrogenator. The course of the hydrogenation was followed by removing aliquots at various time intervals, fitering, and analyzing by gas chromatography (GC). The end point was indicated by the complete disappearance of trimethyl 2-nitro tricarballylate from the reaction mixture, which generally took 2-4 days.

From the GC trace of the fully hydrogenated sample, it was apparent that although no trimethyl 2-nitro tricarballylate remained, its conversion to trimethyl 2-amino tricarballylate was not quantitative, since three peaks were evident (Fig.2.3A). To gain insight into the identity of these components, and therefore assist in the development of methods for the recovery of trimethyl 2-amino tricarballylate, the filtrate from the hydrogenation mixture was concentrated by rotary evaporation at 30°C to give a green oil (14g) and subjected to further analysis prior to separation. Hence, the mixture was analyzed by mass spectroscopy (2.3B), and also by mass spectroscopy after resolution of components by GC (i.e. GC-MS analysis).

When the structures of the major components 1 and 3 (as defined in Fig. 2.3A) derived from the above reduction were ultimately recognized as being the trimethyl esters of tricarballylate and 2-amino tricarballylate respectively, it was then possible to review the conditions of the hydrogenation in order to achieve optimum conversion to trimethyl 2-amino tricarballylate. Thus it was found that either increasing the amount of catalyst used or elevating the reaction temperature much above 25°C, would only increase the rate of by-product formation. Nevertheless, if the temperature reduced much below room temperature or if less catalyst was used, it was found that reduction occurred at a significantly slower rate without any appreciable change in product ratios. Similar changes were noted under lower pressure. Substitution of the catalyst was also investigated; however the use of platinum oxide gave a minimum yield of trimethyl

Fig. 2.3 Analysis of the reaction mixture produced from the catalytic hydrogenation of trimethyl 2-nitro tricarballylate by A) gas chromatography (the retention time of trimethyl 2-nitro tricarballylate was 18-19min), and B) mass spectroscopy with chemical ionization.



2-amino tricarballylate. In view of these findings, the conditions of the hydrogenation that were adopted initially (1.0g Raney Nickel; 40-60 p.s.i. at room temperature for 4-6 days) were routinely used in subsequent preparations.

2.3.2.3 Separation of Trimethyl 2-Amino Tricarballylate from the Hydrogenated Mixture

In initial work, high vacuum fractional distillation was used to attempt the resolution of trimethyl 2-amino tricarballylate from other reaction components present in the hydrogenated mixture. The majority of the mixture distilled at 90-110°C/0.05mm Hg and because of the wide boiling range, approximately ten fractions of equal volume were collected. However, GC analysis indicated that each fraction contained both major components in ratios ranging from 60:40 in the first fraction to 40:60 in the last. This suggested that distillation was incapable of resolving these components and other separative methods were investigated.

It was subsequently proven that the components could be successfully purified from the hydrogenated mixture by column chromatography using aluminium oxide (neutral, activity II). Full details for this procedure are given in Fig. 2.4. Fractions found to be pure, as determined by GC, were pooled and concentrated by rotary evaporation. The yields of the products, which were later characterized as being trimethyl tricarballylate and trimethyl 2-amino tricarballylate were 3.8g and 8.0g respectively.

Whilst this method proved satisfactory, another method involving solvent extraction ultimately proved to be superior, since it was simpler, less expensive and more rapid. This method is summarized in Fig. 2.5 and is detailed as follows. The hydrogenated mixture (14g) was taken up in toluene (300ml), transferred to a separating funnel and then extracted with 2 x 150ml of ice-cold 0.2M acetic acid. Both the aqueous and organic phases were then retained and treated separately. The toluene phase was washed with water (100ml), separated, dried over anhydrous Na_2SO_4 (5g), then concentrated to yield trimethyl tricarballylate as a clear oil (3.6g). The combined acid extracts were washed with further toluene (200ml), the pH of the acid phase was adjusted to 7.0 with 2M NaOH and the solution lyophilized. Anhydrous sodium sulphate (40g) was then added and components extracted by washing with ethyl acetate (150ml) at 60°C for 10min and the organic phase recovered by filtration. This process was repeated with a further 150ml of ethyl acetate. The combined extracts were cooled to room

Fig. 2.4 Resolution of components derived from the hydrogenation of trimethyl 2-nitro tricarballylate by column chromatography (Al_2O_3). The hydrogenated product (14g) was taken up in 50ml of toluene and applied to a 40 x 4cm column previously equilibrated with toluene. Elution was then carried out as shown. Fractions (100ml) were collected and aliquots (5µl) subjected to GC analysis for the identification and quantitation of components. As shown, the major components were subsequently identified as trimethyl tricarballylate and trimethyl 2-amino tricarballylate.

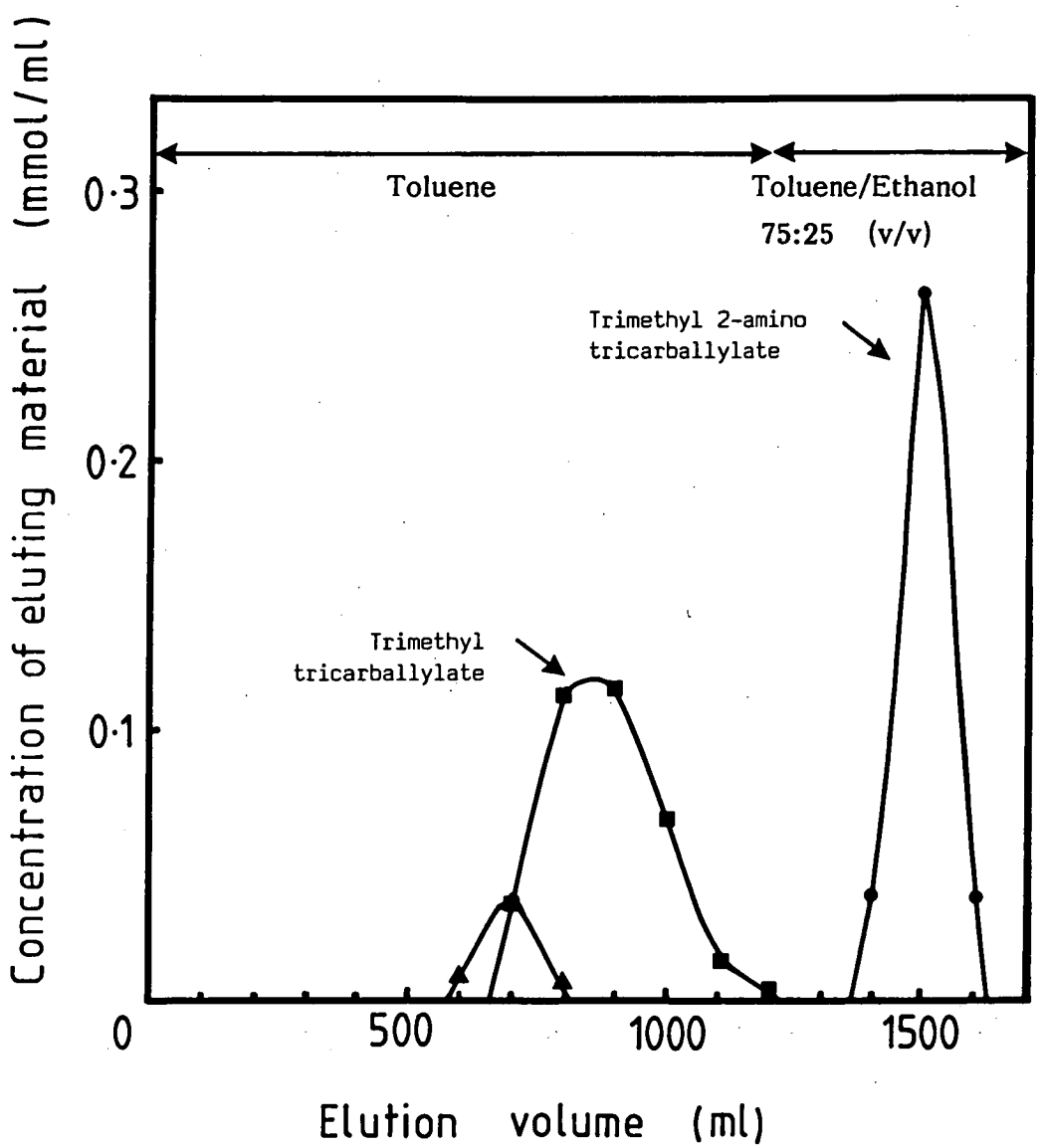
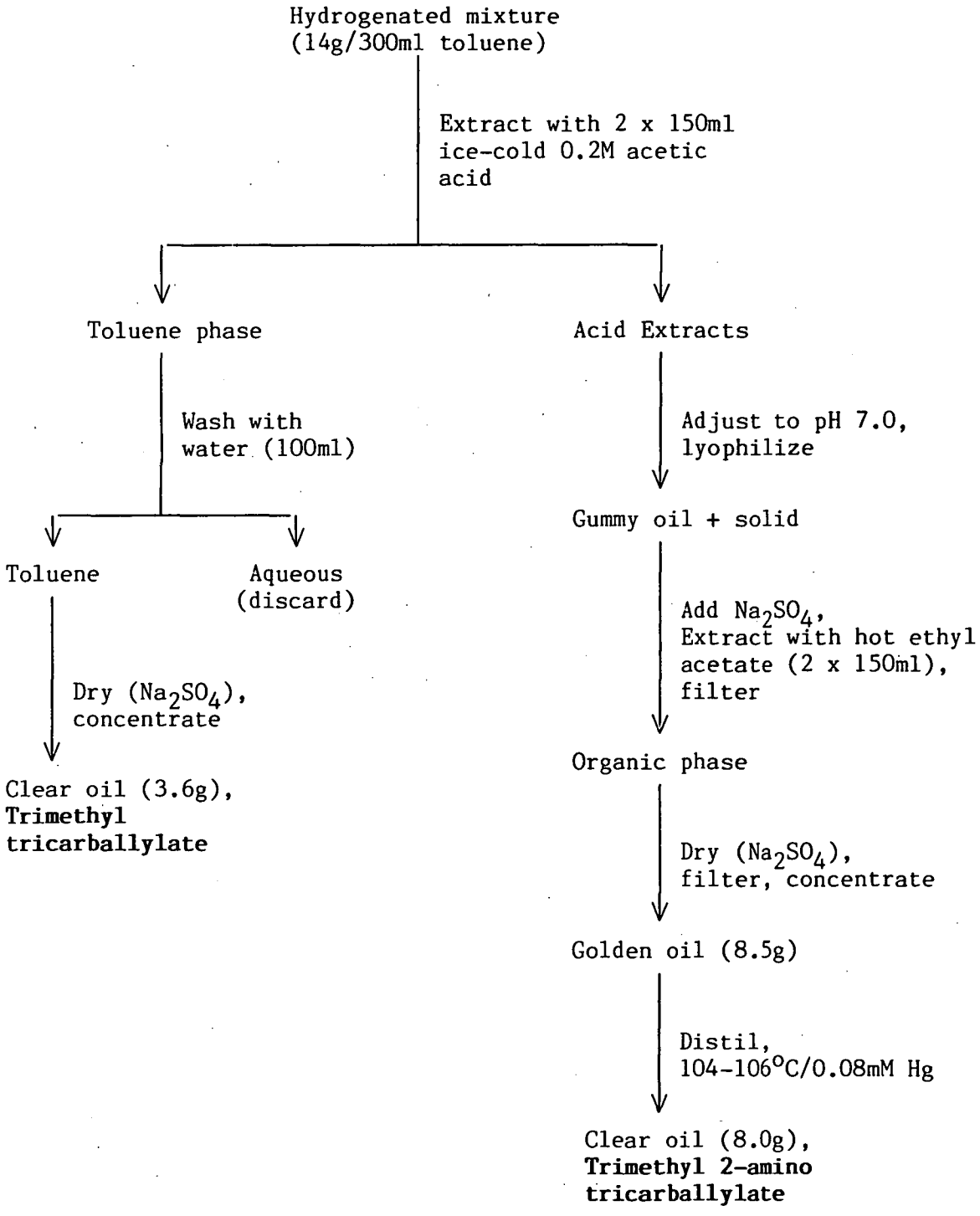


Fig. 2.5 The separation and purificaton of trimethyl 2-amino tricarballylate following solvent extraction of the impure mixture obtained by the catalytic reduction of trimethyl 2-nitro tricarballylate.



temperature, dried again over anhydrous Na_2SO_4 (10g), filtered, and concentrated by rotary evaporation at 30°C to give 8.5g of a golden oil. This product could then purified further by vacuum distillation to yield 8.0g of trimethyl 2-amino tricarballylate (b.p. 104-106°C/0.08mm Hg).

2.3.3 ANALYSIS OF PRODUCTS

2.3.3.1 Spectroscopic analysis

[^{1}H]-NMR were determined with a 4H Jeol Continuous Wave Instrument at an operating frequency of 100MHz using D_2O (at pH 7.0) or $CDCl_3$ as solvent. Mass spectroscopic analysis was performed using a VG 7070F spectrometer, with samples being ionized by either electron bombardment or chemically with ammonia.

2.3.3.2 Gas Chromatography

Gas chromatography (GC) of samples was performed using a Varian Aerograph 1520 machine fitted with a Scott OV-17 low-polarity silicone column (silonised and deactivated) and a flame ionization detector (F.I.D.). The injector temperature was 150°C, column temperature 150°C and the detector temperature 215°C. The gas flow rate was set at 10ml/min. Routinely, 2-5µl aliquots of sample (10-50mg/ml solvent) were analyzed.

When GC was combined with mass spectroscopy (GC-MS analysis), samples where resolved by GC using similar running conditions to those described, and the resolved fractions were analyzed by mass spectroscopy following electron ionization.

2.3.3.3 Nitrogen Determination

The nitrogen content was determined as ammonium following digestion with concentrated sulphuric acid, as outlined by Johnson (1941).

2.4 RESULTS

2.4.1 CHARACTERIZATION OF AT

AT was not rigorously characterized here, since the authors who originally prepared this compound established structure confirmation through elemental analysis (Dornow and Rombusch, 1955). However the product purity was confirmed through nitrogen determination [theoretical 7.33% (w/w), experimental 7.29%] and furthermore the m.p. obtained was in good agreement to that obtained by Dornow and Rombusch (1955). One unique form

of analysis undertaken here which further supported these findings was [^{1}H]-NMR spectroscopy in D_2O at pH 7.0. Under these conditions the methylene protons of the compound produced a typical AB splitting pattern with chemical shift (δ) values of 2.92, 3.02, 3.12 and 3.29.

2.4.2 CHARACTERIZATION OF INTERMEDIATES AND PRODUCTS INVOLVED IN THE SYNTHESIS OF TRIMETHYL 2-AMINO TRICARBALLYLATE

2.4.2.1 Trimethyl 2-Nitro Tricarballylate and Dimethyl Nitrosuccinate

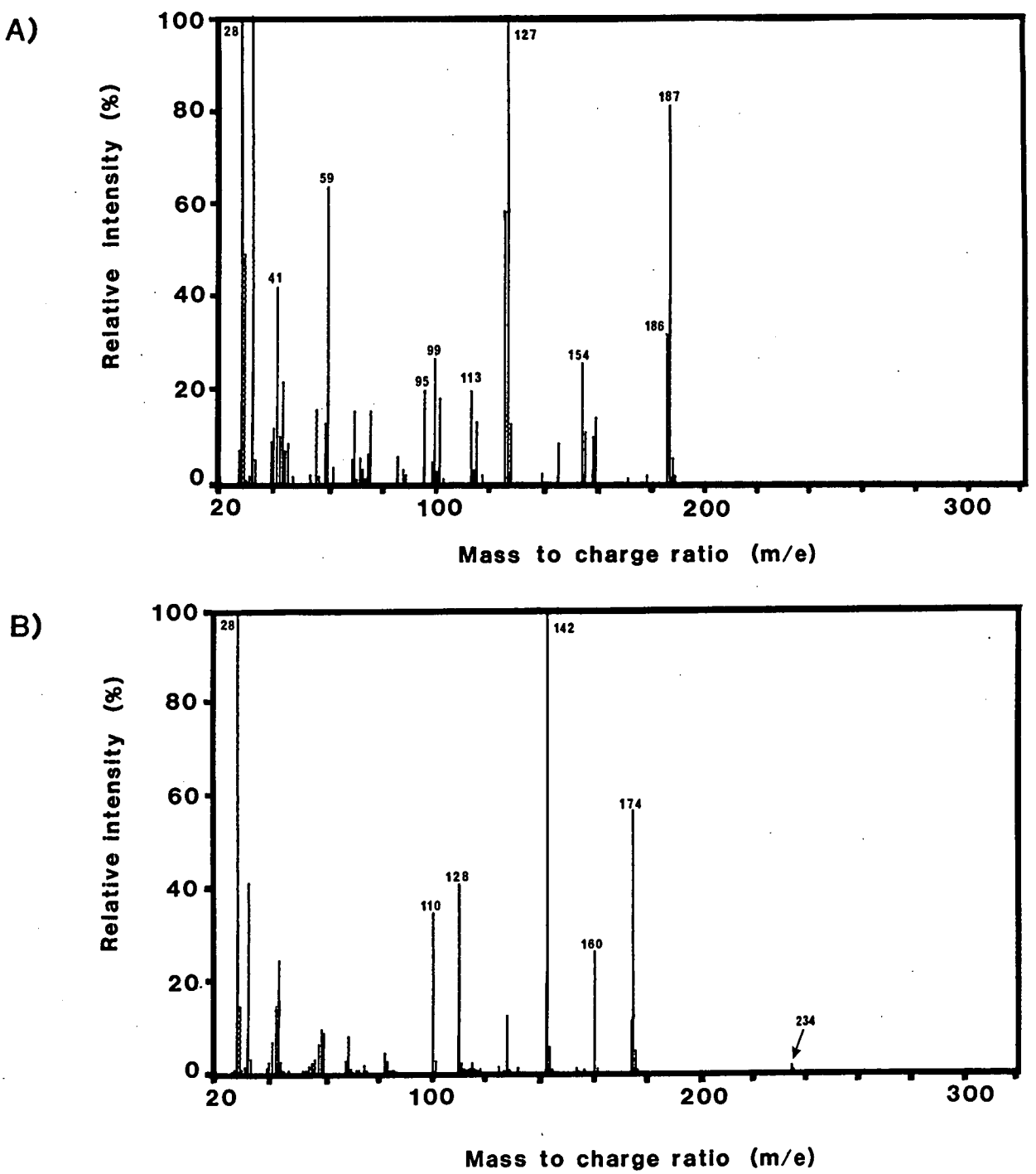
The original authors who prepared trimethyl 2-nitro tricarballylate and dimethyl nitrosuccinate successfully identified these products (Kaji and Zen, 1973), so again rigorous characterization of the products was not pursued. However, the m.p. and b.p. were determined for the respective products and found to be in agreement to those published. Further characterization employed here included [^{1}H]-NMR, which supported their structure (not shown), and GC analysis. With the latter form of analysis, the retention times of trimethyl 2-nitro tricarballylate and dimethyl nitrosuccinate were found to be 18-19min and 8-9min respectively.

2.4.2.2 Impure Mixture Derived From the Catalytic Hydrogenation of Trimethyl 2-Nitro Tricarballylate

As previously presented, the initial form of analysis of the reaction mixture derived from the catalytic hydrogenation of trimethyl 2-nitro tricarballylate was GC, which clearly showed the mixture to be impure and consisting of three products (Fig. 2.3A). Prior to purification, this mixture was subjected to two further forms of analysis, namely mass spectroscopy (chemical ionization; Fig. 2.3A) and GC-MS analysis (electron ionization; Fig. 2.6). One advantage of ionizing by chemical means is that basic nitrogen compounds (eg. amines) protonate very readily and often give abundant $M^+ + 1$ ions (quasi-molecular ions) in their spectra, whereas M^+ ions may be of negligable abundance or absent in electron bombardment spectra (Williams and Fleming, 1973). This was evident in the spectrum (see Fig. 2.3B) where a high intensity signal occurred at the mass to charge ratio (m/e) of 234, of which a likely assignment was the $M^+ + 1$ of trimethyl 2-amino tricarballylate (M.Wt. = 233). Another significant band in this spectrum was one of smaller intensity occurring at 219. It was recognized that this could be attributed to the $M^+ + 1$ of trimethyl tricarballylate, a possible by-product derived by deamination.

To gain insight into which of the peaks (see Fig. 2.3A)

Fig. 2.6 GC-MS analysis of the mixture derived from the hydrogenation of trimethyl 2-nitro tricarballylate. Components 1 and 3 (as defined in Fig. 2.3A) were resolved by GC and the separated fractions analyzed by mass spectroscopy (electron ionization). The respective components were identified as A) trimethyl tricarballylate, and B) trimethyl 2-amino tricarballylate.



corresponded to trimethyl 2-amino tricarballylate, the gas chromatograph was coupled to the mass spectrometer, thus enabling mass spectrum analysis on individual components following resolution by gas chromatography. The mass spectra derived from this technique of the major components are displayed in Fig. 2.6. Unfortunately chemical ionization was not available for this method and so ionization had to be accomplished with the more harsh treatment of electron bombardment. Although the molecular ion (M^+) of trimethyl 2-amino tricarballylate was not observed with any of the spectra, nevertheless the spectrum of one fraction did reveal a signal corresponding to $M^+ + 1$ of the product (Fig. 2.6B). This could be due to "auto-ionization" of trimethyl 2-amino tricarballylate resulting from ionization by one of its own ion fragments. Neither spectra however, produced M^+ or $M^+ + 1$ equivalent to trimethyl tricarballylate.

2.4.2.3 Identification of Trimethyl 2-Amino Tricarballylate From the Purified Fractions

The fractions that were separated by solvent extraction from the hydrogenation mixture were analyzed by a variety of techniques to aid in purity determination and structure identification. All forms of analysis supported the structure of the toluene soluble component as being trimethyl tricarballylate, and the acid soluble component being trimethyl 2-amino tricarballylate.

GC was the initial form of analysis, and was utilized to check both the recoveries of components during various phases of separation and the purity of the ultimate products. This showed that the recovery of the individual components in final fractions from the original mixture was quantitative, and the final fractions were of a high purity state (Fig. 2.7).

Nitrogen analysis of the purified fractions provided initial information on their elemental composition. Hence the acid soluble fraction was found to contain 5.9% N by weight (theoretical amount for trimethyl 2-amino tricarballylate = 6.0%) whereas the toluene soluble fraction had less than 0.1%.

The [^{1}H]-NMR spectrum gave structural proof to the identity of the acid soluble fraction as trimethyl 2-amino tricarballylate (Fig. 2.8A). With the spectrum, two singlets at δ = 3.70 and 3.65 with the integral ratio 6:3 were recognized as due to the three methyl ester groups. Since two ester groups are equivalent, these would give rise to the signal at δ = 3.70. The AB splitting pattern at 3.6-3.9 δ with integration value 4

Fig. 2.7 GC analysis of purified fractions containing A) trimethyl tricarballylate, and B) trimethyl 2-amino tricarballylate.

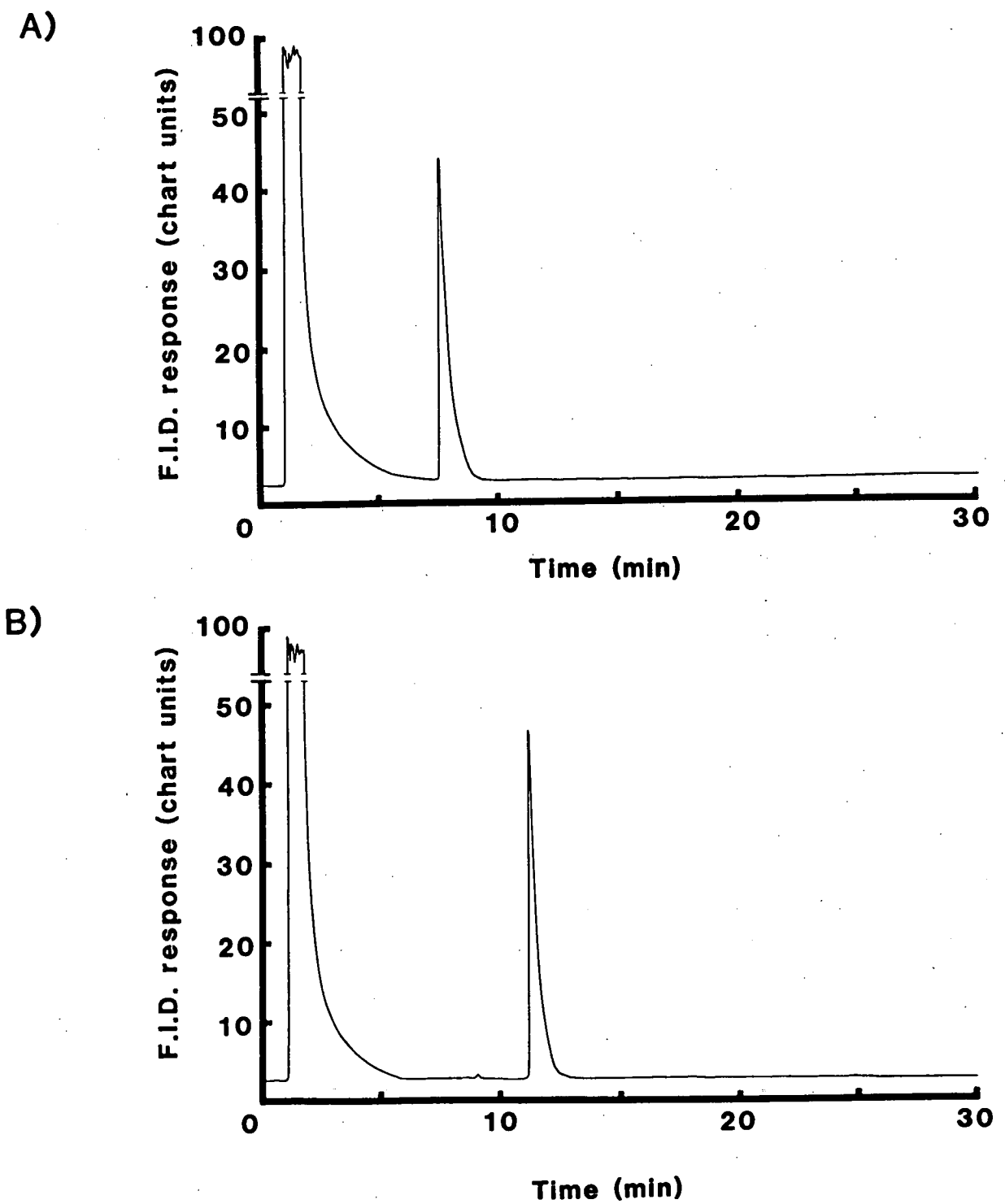
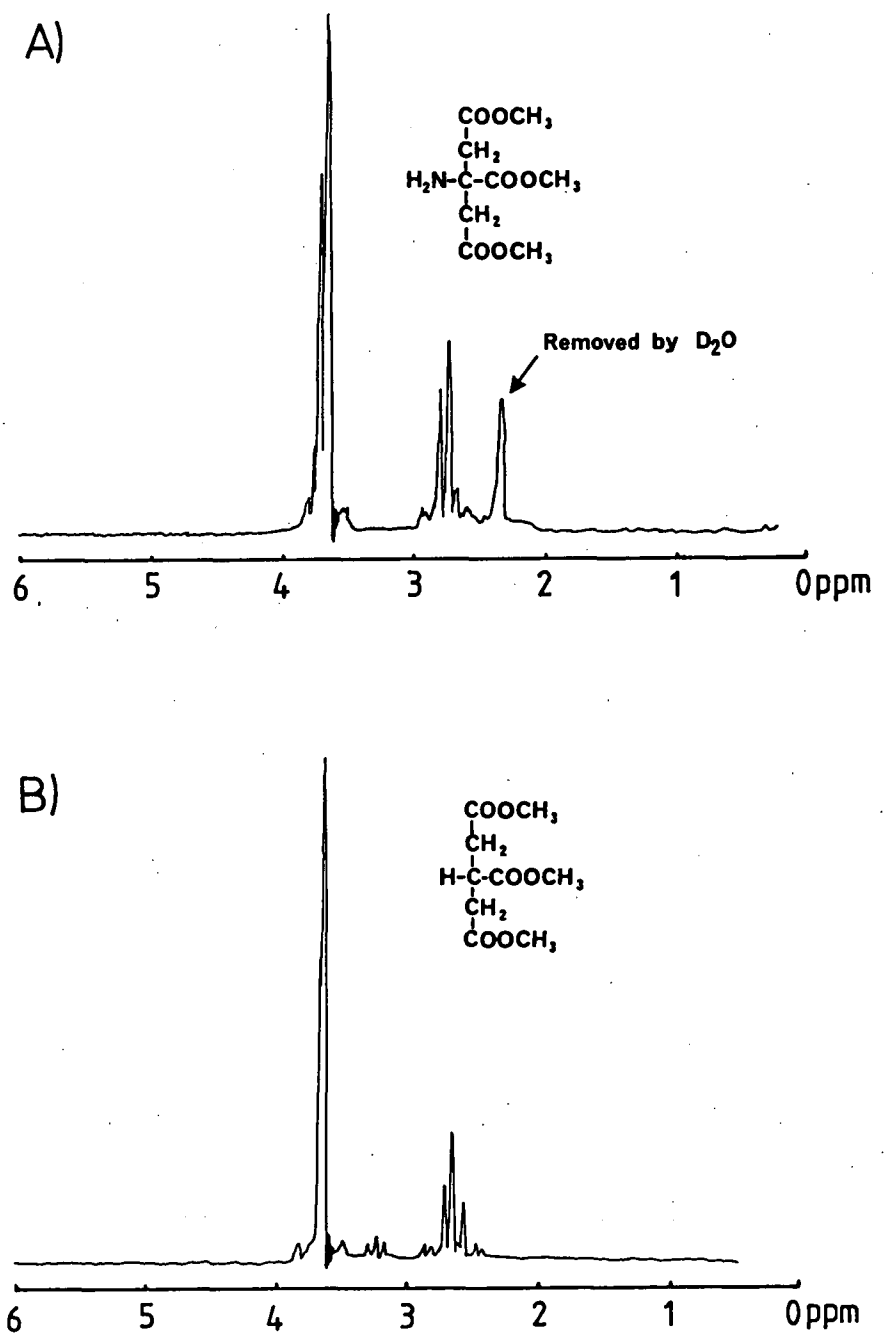


Fig. 2.8 [^{1}H]-NMR spectra of the fractions identified as A) trimethyl 2-amino tricarballylate, and B) trimethyl tricarballylate. Spectra were determined in $CDCl_3$.



was assigned to the two methylene groups of trimethyl 2-amino tricarballylate. The signal at $\delta = 2.35$ with integration value 2 was removed by D_2O, which indicated exchangeable protons and supported the presence of an amino group. The spectrum of the other purified fraction (Fig. 2.8B) supported its identity as trimethyl tricarballylate. Again the signals at 3.7 were diagnostic of methyl ester groupings and had an integration ratio of 9 (made up of 3 + 6). Two multiplets were visible at $\delta = 2.2$-2.3 and 2.5-2.9 with integration ratios of 1 and 4 and hence were attributed to the methylidine and methylene protons of trimethyl tricarballylate respectively. With both spectra, the values for δ were in close agreement to those estimated from the proposed structures by theoretical considerations (Williams and Fleming, 1973).

Given these interpretations from the purified fractions, it was then possible to further examine the spectra from the GC-MS analysis of the unpurified mixture. As previously observed, the mass spectrum of the component now assigned as trimethyl 2-amino tricarballylate, produced a signal of low intensity at 234 which would correspond to $M^+ + 1$ for trimethyl 2-amino tricarballylate (Fig. 2.6B). The identity of this component was also supported by intense signals at m/e of 174 and 160, which would correspond to the loss of $-COOCH_3$ and $-CH_2COOCH_3$ respectively from the parent molecule. The signal of highest intensity (142) would be attributable to losses of both $-COOCH_3$ and CH_3OH from the compound, and the loss of a further CH_3OH could account for m/e at 110. The loss of CH_3OH is characteristic of compounds containing methyl ester groupings. Looking at the mass spectrum of the other major component, no M^+ or $M^+ + 1$ ion was evident corresponding to trimethyl tricarballylate, nevertheless its identity as such was inferred from the spectrum. The signal of highest intensity was at 127, which could be explained by the loss of both $-COOCH_3$ and CH_3OH from trimethyl tricarballylate (analogous to the m/e of highest intensity for trimethyl 2-amino tricarballylate). Loss of $-OCH_3$ and CH_3OH from this molecule could attribute to m/e occurring at 187 and 186 respectively. Whilst the identity of the minor component in the unpurified mixture (component 2 in Fig. 2.3A) was not investigated, it appeared from its mass spectrum to be similar to the major components that were successfully purified and identified (not shown). Intense signals were found at 100, 110, 128, 142, 156, 174 and 188.

Similar characterization carried out on the purified fractions derived from the less favoured purification method of column chromatography produced similar data to those just presented.

2.5 DISCUSSION

The preparation of AT and its trimethyl ester, compounds required as precursors for the synthesis of PAT and SAT, was successfully achieved by the methods described in this chapter. Whilst AT was obtained by already documented methods (Dornow and Rombusch, 1955), a synthetic procedure still had to be devised for its trimethyl ester. Since the trimethyl ester of a closely related compound, viz, 2-nitro tricarballylate had been developed by other workers (Kaji and Zen, 1973), it appeared that the trimethyl ester of 2-amino tricarballylate could be produced simply by the reduction of the former compound. A literature survey suggested that catalytic hydrogenation with Raney Nickel was the superior method for this conversion.

Following the hydrogenation of trimethyl 2-nitro tricarballylate, it was evident that three products were formed. The two major products were subsequently proven by analysis to be trimethyl 2-amino tricarballylate, which was produced in a 60% yield, and its deaminated derivative, trimethyl tricarballylate. Subsequent literature research in fact revealed that partial or total deamination may occur from the aliphatic nitro group reduction by catalytic hydrogenation in cases where the group is attached to a tertiary carbon atom (Freifelder, 1971), as is the case with trimethyl 2-nitro tricarballylate. An attempt was made to limit the deamination and thus increase the yield of the amine by altering the conditions of hydrogenation, but this proved unsuccessful. The possibility of reducing the nitro function by chemical means was considered, but quickly discarded when it became obvious that the methods a) were not necessarily quantitative, and b) would involve the subsequent removal of reducing agents or derived products, which might have proven difficult. Therefore it was decided to continue with the current method, i.e. catalytic hydrogenation with Raney Nickel, and accept the 60% yield for the conversion.

The separation of the reaction components from the hydrogenated mixture was attempted by three methods; namely a) vacuum distillation, b) chromatography and c) solvent extraction. Distillation alone proved unsuccessful for resolving the products directly, but it was used as a method of final purification following major separation of the products by solvent extraction. The system adopted made use of the fact that trimethyl 2-amino tricarballylate, being a charged molecule at low pH was more soluble in a weakly acidic aqueous solution than in toluene. With trimethyl tricarballylate, the reverse was true. Hence extraction with these solvents gave a clear and definitive separation of these products in a minimum of

time and thus enabled further purification of the fraction enriched in trimethyl 2-amino tricarballylate by distillation. Whilst column chromatography was found an equally capable method of resolving products, it was one of second choice, because it generally took longer and utilized a larger amount of solvent and aluminium oxide. Characterization of the products derived from either of these purification techniques by mass and [^{1}H]-NMR spectroscopy, nitrogen determination and GC analysis confirmed both the purity and the identification of the separated products.

The synthesis of trimethyl 2-amino tricarballylate by the methods presented could be described as time-consuming, expensive and producing a low yield. Nevertheless, it does have distinct advantages over other methods leading to a tri-ester. Firstly, the trimethyl ester is prepared, and since methyl ester groupings are generally the most labile, this could be advantageous for the ultimate synthesis of the appropriate analogues of phosphocitrate, where hydrolysis of these groupings could be required. In considering alternate methods, it was clear that the tri-esterification of AT would be involved. Whilst various agents are capable, the use of such agents generally means prior protection of the amino group by acetylation. The yields with these reagents, as determined by a literature survey, were generally less than quantitative (especially where the amino group is in a sterically hindered environment; eg. AT), and nevertheless their use would also require final product purification by methods similar to those described herein. In addition, AT which would be a precursor for such methods, was not itself a commonly available reagent, and its own preparation was time consuming and expensive. In summary then, despite the shortcomings of the synthesis, no superior methods were clearly evident. The synthesis did produce trimethyl 2-amino tricarballylate of high purity, and further, in an amount sufficient to support the synthetic strategies that were proposed for the preparation of PAT and/or SAT.

2.6 SUMMARY

1. The synthesis of 2-amino tricarballylate (AT) was achieved by following documented methods.

2. Methods were investigated for the synthesis of trimethyl 2-amino tricarballylate; this new synthesis was ultimately achieved following reduction of trimethyl 2-nitro tricarballylate and subsequent product purification.

3. Trimethyl 2-amino tricarballylate could be separated from other materials derived from the reduction by both chromatography and solvent

extraction followed by distillation. The latter technique proved more practical.

4. The yield of trimethyl 2-amino tricarballylate although low, was nevertheless sufficient to furnish subsequent needs for coupling with phosphorylating and/or sulphating agents to prepare PAT and SAT.

CHAPTER 3

THE SYNTHESIS OF N-PHOSPHO-2-AMINO TRICARBALLYLATE

3.1 INTRODUCTION

The first phosphocitrate analogue considered for synthesis was N-phospho-2-amino tricarballylate (PAT), a compound differing only in the replacement of the P-O-C bond present in PC by a P-N-C bond. The choice of this particular analogue was stimulated by findings from other workers who compared the inhibitory potential of pyrophosphate (PP_i; P-O-P bond) and its derivative imidodiphosphate (P-N-P). Imidodiphosphate showed a greater inhibitory activity towards calcium phosphate crystallization (Williams and Sallis, 1982; Robertson and Fleisch, 1970) and the difference in activity was suggested as being due to the greater stability of imidodiphosphate, especially on calcium phosphate surfaces, which are known to catalyze trans-phosphorylation reactions. If then, such properties were shared by PAT, the PC analogue might offer good inhibitory potential, while at the same time displaying more stability towards degradation than the parent PC.

Molecules such as PAT which contain the P-N bond, are termed phosphoramidates, and several examples of such compounds have been shown to exist in nature. These include phosphocreatine, phosphotaurocyamine and phosphoarginine. Biological studies have also shown that other compounds containing this linkage, namely proteins phosphorylated at the imidazole ring of the histidine residue, are often involved as intermediates in biological phosphorylation. These natural phosphoramidates, as well as other synthetic phosphoramidates have, in the past, been shown to be hydrolyzed by various biological extracts, which some workers have ascribed to the activity of distinct enzymes termed phosphoamidases (Waldschmidt-Leitz and Köhler, 1933). Conflicting reports exist however, and Winnick (1947) found that the hydrolysis of phosphocreatine by biological extracts was attributable totally to alkaline phosphatase activity. While such accounts suggest that PAT could be hydrolyzed *in vivo* (whether it be by phosphoamidase or phosphatase), because the phosphoramidate linkage of PAT would exist in a sterically hindered environment, it becomes difficult to speculate on its degree of susceptibility to those enzymes. Total resistance to enzyme degradation is perhaps undesirable, as evident from studies

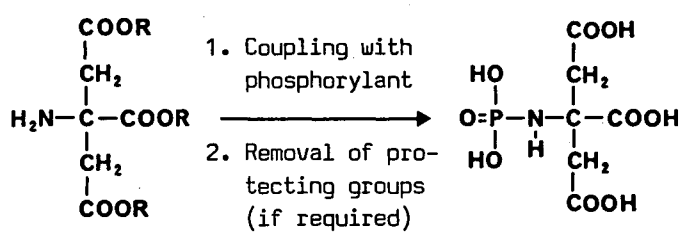
reported with HEBP (Schenk et al., 1973). If PAT were to display some degree of lability to enzymes, it might prove beneficial, provided it still had a relatively greater resistance to enzyme attack than PC.

The synthetic approaches considered for the production of PAT involved either (a) selective phosphorylation of the amino group of 2-amino tricarballylate (AT), or (b) the phosphorylation of trimethyl 2-amino tricarballylate by non-selective agents and subsequent treatment to yield PAT. A number of phosphorylating agents are available for use with either of these two approaches (Fig. 3.1). In reference to a) above, several procedures have been reported for the phosphorylation of polyfunctional amines, in particular amino acids. Jampel et al., (1968) have described the use of either N-phosphoryl N'-methyl imidazole (PMI) and N-phosphoryl-γ-picoline (P-γ-P) to prepare a variety of phosphoramidates from compounds including glycine, serine and ethanolamine in relatively high yields (25-95%). In an earlier communication, Winnick and Scott (1947) demonstrated the synthesis of three phosphoramidates by the action of phosphorous oxychloride on glycine, alanine and glutamate in aqueous magnesia solution. With this method, the yields were however lower than those achieved with PMI and ranged from 10-50%.

While these methods appeared attractive because the phosphoramidate would be synthesized in a minimum number of steps, the more powerful non-specific phosphorylating agents were also considered. Although their use would require further treatment to eliminate protecting groups, a greater coupling of phosphorylant to amine could ensure a more superior overall yield of phosphoramidate. Of the more powerful agents available, o-phenylene chlorophosphate is considered to be one of the best (Slotin, 1977), and has been used to synthesize PC in a 60% yield after coupling with the sterically hindered hydroxy group of triethyl citrate (Tew et al., 1980). Under similar conditions o-phenylene chlorophosphate has been shown to couple with amines (Dulog and Dewacle, 1974). Such an agent therefore might be more favoured for a synthesis of PAT, since the amino group of trimethyl 2-amino tricarballylate exists in a similar steric environment to the hydroxy group of triethyl citrate.

Other reagents capable of coupling with trimethyl 2-amino tricarballylate would include phosphorous oxychloride, dibenzyl chlorophosphate and 2-cyanoethyl phosphate (CEP), although these reagents are generally less effective when coupling with sterically hindered molecules. The agent CEP for example, has been coupled successfully with the hydroxy group of triethyl citrate to synthesize PC, albeit in a poor yield (7%).

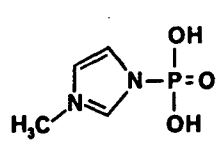
Fig. 3.1 The synthetic approach for the preparation of N-phospho-2-amino tricarballylate (PAT), and the phosphorylating agents that were considered.



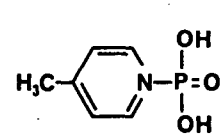
2-Amino tricarballylate or trimethyl ester

N-Phospho-2-amino tricarballylate (PAT)

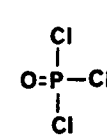
PHOSPHORYLATING AGENTS:



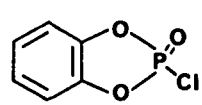
N-Phosphoryl N'-methyl imidazole (PMI)



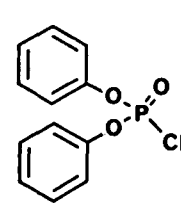
N-Phosphoryl -γ-picoline (P-γ-P)



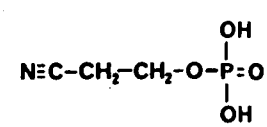
Phosphorous oxychloride



o-Phenylene chlorophosphate



Dibenzyl chlorophosphate



2-Cyanoethyl phosphate (CEP)

Nevertheless, at least this synthetic route did afford a final product of high purity (Williams and Sallis, 1980). Sciarini and Fruton (1949) used the dibenzyl derivative of chlorophosphate in an attempt to synthesize N-phosphorylated amino acids from diethyl glutamate and ethyl glycine. However, after coupling and hydrogenolysis to remove the phenyl groups, these workers encountered difficulties in hydrolyzing the ethyl esters by base to yield the appropriate phosphoramidates. One possible explanation related to the lack of any calcium in the medium. Base hydrolysis of triethyl phosphocitrate under similar conditions is known to yield only the monoethyl ester as the main product (Williams and Sallis, 1980). When calcium is included the hydrolysis is driven to completion by virtue of the insolubility of the calcium salt of PC. Presumably then, if the trimethyl ester of PAT was formed, similar conditions would ensure complete hydrolysis and the formation of PAT as a calcium salt.

Several synthetic approaches were ultimately attempted to prepare PAT, utilizing all the aforementioned phosphorylating agents. This chapter summarizes the unsuccessful routes, and then reports in detail on the successful approach. In addition, methods that were developed for the purification of the final product are detailed, and the full characterization of synthetic PAT is also presented.

3.2 MATERIALS

Dibenzyl chlorophosphate was a product of the Aldrich Chemical Co., Milwaukee, Wisconsin and o-phenylene chlorophosphate was purchased from the Fluka Chemical Co., Buchs, Switzerland. Platinum oxide was obtained through BDH Chemicals Australia, Kew, Victoria. N-phosphoryl N'-methyl imidazole (PMI) and N-phosphoryl-γ-picoline (P-γ-P) were synthesized as described by Jampel et al. (1968). The method of Tener (1961) was employed to prepare 2-cyanoethyl phosphate (CEP). Trimethyl 2-amino tricarballylate and AT were prepared by methods described in the previous chapter.

Benzene and pyridine were dehydrated by overnight storage over molecular sieve 4A. The ion-exchange resins AG 1-X8 (100-200 mesh; Cl^- form) and AG 50W-X8 (200-400 mesh; H^+ form) were products of the Bio-Rad Laboratories, Richmond, California. Before use, the resins were cycled and the AG 1-X8 resin converted to the bicarbonate form.

3.3 METHODS

3.3.1 SUMMARY OF THE GENERAL METHODS USED FOR THE SYNTHESIS OF PAT

The preparation of PAT involved the investigation of a variety of synthetic strategies; this section therefore summarizes basic procedures adopted with the coupling and subsequent treatment for both the unsuccessful and successful routes. A more detailed description of the successful synthesis of PAT (which was ultimately achieved via coupling with CEP), and the procedures associated with product purification, are given in the subsequent section.

In initial experimentation, the synthesis of PAT was attempted by the direct action of selective phosphorylants on the amine group of AT. The first phosphorylants investigated were PMI and P-γ-P. The general procedure for coupling with these agents, as described by Jampel et al. (1968), was as follows: To an aqueous solution of phosphorylant at pH 11.0 and 25°C was added a 10% excess of amine. The solution was maintained at the same pH and temperature for 24h after which time the crude product was precipitated by the addition of barium chloride. Another selective method tried was phosphorous oxychloride ($POCl_3$) in the presence of an excess of magnesium oxide (Winnick and Scott, 1947). Solid MgO was added to an aqueous solution of amine before the dropwise addition of $POCl_3$ in carbon tetrachloride. Addition was regulated such that the temperature could be maintained at 0-5°C, and the pH remained in the vicinity of 8.0. At the end of the addition, the impure product was precipitated as a magnesium salt by the addition of ethanol.

The less selective phosphorylation methods utilized such agents as o-phenylene chlorophosphate, dibenzyl chlorophosphate, $POCl_3$ and CEP to couple with trimethyl 2-amino tricarballylate. With the former three agents, the method used for coupling was essentially that employed by Tew et al. (1980) for the synthesis of PC. Hence, to a solution of phosphorylant dissolved in benzene was added dropwise with stirring an equivalent amount of both amine and pyridine, also in benzene. The solution was filtered, solvent removed and the resulting oil was treated then in one of two manners: a) The solution was hydrogenated to remove aromatic protecting groups if present (Tew et al., 1980), followed by mild base hydrolysis (Williams and Sallis, 1980) to remove the methyl ester functions and to precipitate the impure product as a calcium salt. b) The solution was subjected to base hydrolysis under more rigorous conditions to cleave both the aromatic and methyl protecting groups simultaneously,

precipitating the product finally as a barium salt (Zervas and Katsoyannis, 1955). These methods are depicted in Fig. 3.2 in the case of o-phenylene chlorophosphate. The final and ultimately successful phosphorylating agent investigated was 2-cyanoethyl phosphate, and the method used for coupling with this agent was similar to that described by Williams and Sallis (1980), although with notable modifications. The method, as described in greater detail in a subsequent section, involved reacting phosphorylant with an equivalent amount of amine in pyridine as the solvent with an excess of dehydrating reagent (dicyclohexylcarbodiimide) for 6 days. After this time, the solvent and excess dehydratant were removed, the resulting oil was subjected to base hydrolysis and the products precipitated as calcium salts.

In all instances, the final method of purification involved conversion of the insoluble salts (i.e. magnesium, calcium or barium) to a soluble form (sodium) which was then fractionated by ion-exchange chromatography.

3.3.2 SYNTHESIS OF PAT BY COUPLING TRIMETHYL 2-AMINO TRICARBALLYLATE WITH 2-CYANOETHYL PHOSPHATE

The synthesis of PC in a 7% yield has been achieved by the coupling of CEP with triethyl citrate in the presence of dicyclohexylcarbodiimide; a powerful dehydratant. By analogous methods it was proposed that PAT could be prepared after coupling CEP with trimethyl 2-amino tricarballylate (Fig. 3.3).

3.3.2.1 Coupling of Trimethyl 2-Amino Tricarballylate with CEP

The barium salt of 2-cyanoethyl phosphate (12g), dissolved in 200ml of water, was converted to the pyridinium salt by passage through AG 50W-X8 (H^+; 100ml resin volume), and after filtration, the filtrate adjusted to pH 6.0 with pyridine before lyophilization. The resulting oil was dissolved in 200ml anhydrous pyridine, and added to a flask containing 8.0g of trimethyl 2-amino tricarballylate. The mixture was concentrated to an oil by rotary evaporation at 30°C, further pyridine (300ml) was added and the solution again concentrated. This process was repeated twice before addition of 300ml of pyridine and dicyclohexylcarbodiimide (50g). The reaction flask was stoppered and allowed to stand for 6 days at room temperature with occasional shaking. Pyridine was removed by rotary evaporation, 300ml of ice-cold water added, and the contents stirred vigorously at 4°C for 2h to destroy excess dehydratant. The suspension was

Fig. 3.2 The synthetic routes available for the synthesis of PAT following the coupling of o-phenylene chlorophosphate with trimethyl 2-amino tricarballylate.

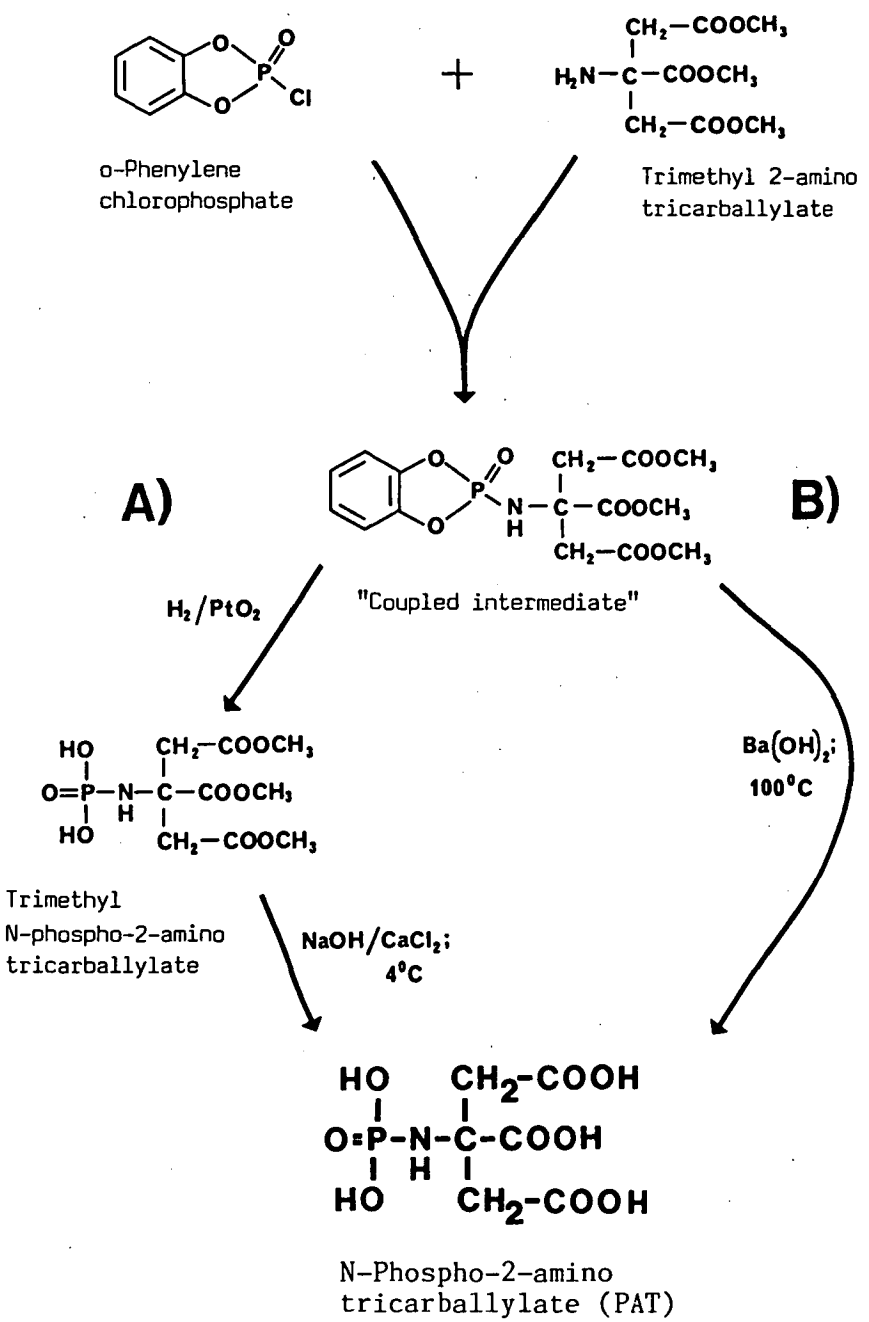
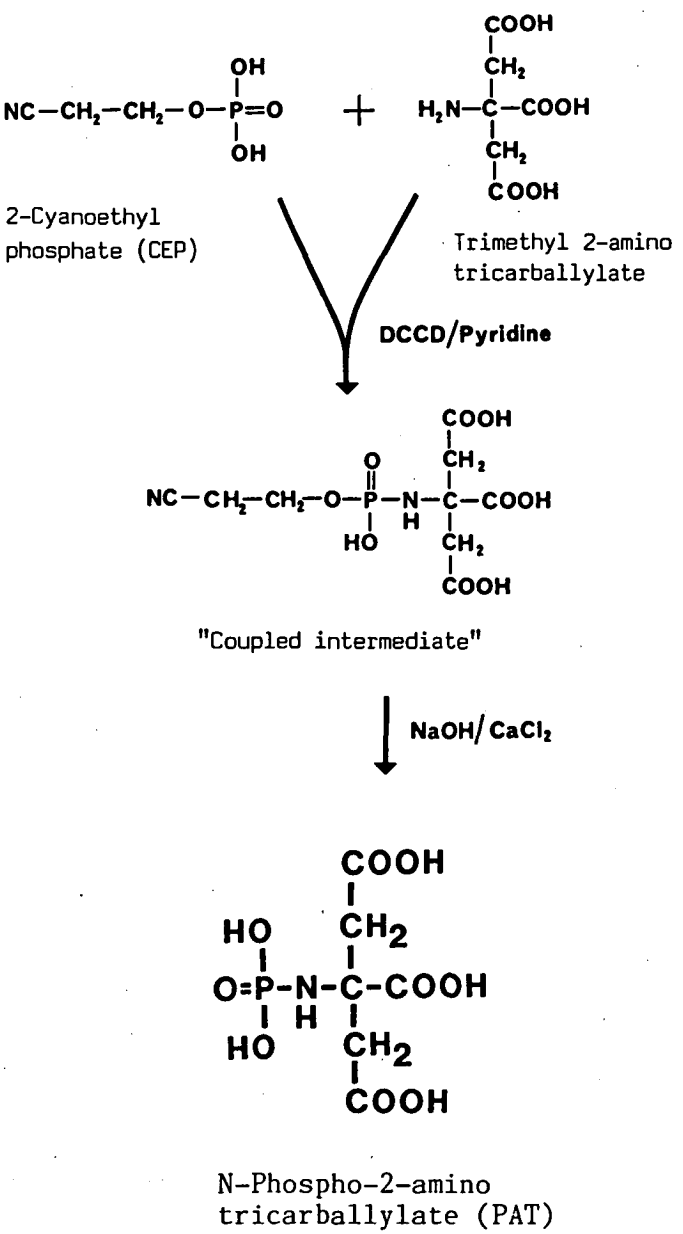


Fig. 3.3 The synthetic route for the preparation of PAT following the coupling of trimethyl 2-amino tricarballylate with CEP.



filtered and the precipitate washed with a further 50ml of water to ensure complete recovery of the coupled product. The combined filtrates were adjusted to pH 7.0 with 1M NaOH and lyophilized to give 10g of a brown oil.

3.3.2.2 Hydrolysis of Coupled Product

The oil was taken up in 100ml of water, 500ml of 0.3M NaOH was added and the solution heated at 50°C for 10min, before the addition of a calcium chloride solution (12g/50ml). The solution was allowed to stir at 4°C overnight, and then adjusted to pH 8.5 before lyophilization. The resulting solid was resuspended in 150ml of water and left at 4°C for 3h before the precipitate was collected by centrifugation. After successive washes with alcohol, acetone and finally diethyl ether the solid was allowed to air-dry. The yield of calcium salt was 6.5g.

3.3.2.3 Final Purification of PAT by Ion-Exchange Chromatography

The calcium salt was converted to the soluble sodium form by stirring the solid in 50ml water with an excess of AG 50W-X8 (H^+; 50ml resin volume) at 0°C for 5min. The suspension was filtered and the filtrate adjusted to pH 8.0 with 2M NaOH prior to loading onto a column of AG 1-X8 (HCO_3^-; 3.5 x 30cm) for the final purification of PAT. The column, which was maintained at 4°C by a water jacket, was washed initially with 1.5l of 0.25M $NaHCO_3$. Pilot studies indicated that inorganic phosphate (P_i) and AT were successfully eluted under these conditions. The stronger binding material was then removed by applying a linear gradient (0.25-0.50M $NaHCO_3$; 800ml each compartment). Eluants were collected in 10ml fractions, a 3ml aliquot was removed from every fifth fraction for phosphate analysis, and a 4ml aliquot for the analysis of the AT moiety. A fraction eluting at 0.33M $NaHCO_3$ was shown to contain high concentrations of both hydrolyzable phosphate and AT. Tubes contributing to this fraction were collected and their contents pooled and decarbonated by stirring with an excess of AG 50W-X8 (H^+) at 0°C for 3-5min. The suspension was filtered, the filtrate adjusted to pH 7.0 with 2M NaOH, and then lyophilized. This process was repeated to ensure total removal of bicarbonate. The yield of product, shown to be PAT by subsequent characterization, was 150mg (1%).

Whilst excellent resolution was achieved using AG 1-X8 resin of PAT and other reaction components derived by coupling with CEP, nevertheless problems were encountered in initial preparations in the final recovery of the compound from the bicarbonate eluant. Decarbonation using AG 50W-X8

(H^+) resulted in a reduction of the pH to 3-5, and it was found that when carried out at 4-8°C for 10min, significant hydrolysis (ca. 20%) of the product occurred.

Attempts to overcome this problem were examined by several methods. Recrystallization of the product was attempted by the selective precipitation of PAT as a lithium salt. This method had been utilized to purify 3-phospho histidine (a phosphoramidate), removing P_i and histidine (Hultquist et al., 1966). The rationale was that the initial removal of P_i was possible as an insoluble lithium salt by dissolving the product in 0.5M lithium chloride solution and adding ethanol up to a concentration of 50% (v/v). The precipitation of pure PAT might then be accomplished by increasing the ethanol concentration, leaving behind AT as a soluble salt. In practice however, after removal of P_i, PAT could not be precipitated even at a concentration of 95% ethanol.

An effort was made to eliminate the use of AG 50W-X8 by eluting with a triethylamine bicarbonate buffer, a salt that is completely removed upon lyophilization. However, with this buffer it was evident that a much higher bicarbonate concentration was required to elute PAT, and the resolution with this system was poor. Switching to ammonium bicarbonate, did improve the situation as the elution pattern for PAT was found to be identical to that seen with sodium bicarbonate. This system was adopted and decarbonation attempted by precipitation as barium carbonate following the addition of an equivalent amount of a saturated barium hydroxide solution. In theory, this might have yielded the ammonium salt of PAT upon lyophilization of the filtrate. Whilst PAT isolated by this method was free of hydrolytic products, nevertheless phosphate analysis revealed that up to 90% of PAT was lost by co-precipitation as a barium salt. Finally, it was found that hydrolysis of the product could be minimized by conducting the decarbonation strictly at 0°C for 3-5min and these were the conditions ultimately used. Although hydrolysis was still evident under these conditions (final sample contained 2% P_i), nevertheless it was minimized to an extent to yield a product of an acceptable purity (as determined by characterization) for testing the anti-calcifying properties of PAT both in vitro and in vivo.

3.3.3 ANALYSIS OF PRODUCTS

3.3.3.1 Total and Free Phosphate

Total phosphate was determined by the method of Fiske and Subbarow (1925) after acid hydrolysis of the samples at 100°C for 1h in 1M H_2SO_4 to

release organically bound phosphate. Whilst this method was most useful for the quantitation of total phosphate in the final sample and the routine analysis of fractions eluting from ion-exchange chromatography, a second method was used to quantitate P_i. This method, as described by Lowry and Lopez (1946), utilized milder assaying conditions and is therefore a more applicable assay for P_i in the presence of labile phosphate esters.

3.3.3.2 Nitrogen

The nitrogen content of the sample was determined according to the method of Johnson (1941).

3.3.3.3 Detection of AT from Column Fractions

Whilst phosphate assays provided general detection of phosphate containing compounds from column fractions, it became evident that in association a more specific assay for PAT was also required. Detection with ninhydrin was not feasible, since preliminary observations had shown that AT gave a poor reaction with this agent. A specific assay was developed by first converting the AT moiety (or compounds containing the AT moiety, i.e. PAT) to citrate. This conversion was achieved by incubation with nitrous acid, an agent capable of both cleaving the phosphoramidate linkage (Winnick and Scott, 1947) and converting amines to a mixture of alcohols (including the parent alcohol) and alkenes (Morrison and Boyd, 1976). The mixture was then assayed specifically for citrate.

The general procedure was as follows: Following ion-exchange chromatography, 4ml aliquots from the collected fractions were taken from every fifth tube, and decarbonated by the addition of a small amount of AG 50W-X8 (H^+). After removal of the resin by centrifugation, supernatants were transferred to digestion tubes and evaporated to dryness at 120°C. The tubes were cooled to room temperature before the addition of 1.5% $NaNO_2$ (1.0ml) and acetic acid (100µl). The tube contents were mixed, and incubated at room temperature for 30min with occasional shaking. The fractions were then assayed for citrate by the spectrophotometric method of Taylor (1953).

When standard AT was assayed by this method, the absorption value was 40% of that observed for an equivalent amount of standard citrate treated in identical fashion. This reflects the incomplete conversion of AT to citrate by nitrous acid treatment. Nevertheless, this procedure provided a sensitive and qualitative means by which compounds containing the AT moiety could be easily detected from column fractions.

With the above method established as satisfactory for the purpose, some modifications were ultimately tested. In this respect, the substitution of $NaHCO_3$ with NH_4HCO_3 as the eluting buffer for ion-exchange chromatography simplified the assay. PAT was found to elute at identical volumes with either buffer, but the use of NH_4HCO_3 eliminated the necessity of the AG 50W-X8 resin step in the assay, since the salt was totally eliminated as CO_2 and NH_3 upon boiling in the first stage. Whilst this system was therefore superior for AT analysis of column fractions, it was not suitable when determining the nitrogen content of the final product. Obviously, any contamination of NH_4HCO_3 would lead to an erroneous result, so with this in mind, $NaHCO_3$ was preferred for ion-exchange chromatography.

3.3.3.4 High-Voltage Paper Electrophoresis

Samples (5µl; 10mg/ml) were applied to Whatman 3MM paper and electrophoresed at 2000V for 1h using 0.05M triethanolamine bicarbonate (pH 7.5) as the buffer system. The paper was oven-dried (60°C) prior to staining procedures.

3.3.3.5 Thin Layer Chromatography

Samples (1µl; 10mg/ml) were applied to cellulose plates (0.25mm thickness; MN 300HR cellulose from Machery-Nagel, Germany) and developed in a solvent mixture of isobutanol : tetrahydrofuran : water : acetone [80:60:50:10 (v/v)]. Plates were dried at 60°C and then sprayed with a stain to detect hydrolyzable phosphates.

3.3.3.6 Staining Procedures

3.3.3.6.1 Compounds Absorbing Ultra-Violet Light

Prior to staining, the paper or plate was examined under ultra-violet light (254nm) to detect any U.V. absorbing components.

3.3.3.6.2 Phosphate and Organic Phosphates

The paper or plate was sprayed with a mixture comprising of 60% perchloric acid : 1M HCl : 4% ammonium molybdate : water [5:10:25:60 (v/v)], dried (85°C for 1min) and exposed to U.V. light. Under these conditions phosphate esters are revealed as blue spots with P_i yellow-green (Dawson et al., 1969).

3.3.3.6.3 Organic Phosphates and Acids

Electrophoretograms were dipped through solutions of 1.1% ammonium thiocyanate in acetone and, after air-drying, $FeCl_3$ in acetone (0.03%). P_i, organic phosphates and organic acids appeared as bleached white zones and amino acids as creamy-yellow zones against a pink background (Firmin and Gray, 1974).

3.3.3.7 Isotachophoresis

Isotachophoretic analysis was performed using isotachophoretic equipment constructed in our laboratories. A conductimetric detector was used. The buffer system comprised of 10mM HCl/arginine pH 10.6 (which included 0.2% hydroxypropyl methylcellulose) as the leading electrolyte and 10mM glycine/$Ba(OH)_2$ (0.02% hydroxypropyl methylcellulose) as the terminator.

3.3.3.8 [^{1}H]-NMR Spectroscopy

[^{1}H]-NMR spectra were determined with a 4H Jeol Continuous Wave Instrument at an operating frequency of 100MHz using D_2O as solvent. Samples were buffered at pH 7.0.

3.4 RESULTS

3.4.1 SUMMARY OF THE ATTEMPTS FOR THE SYNTHESIS OF PAT

Of the number of phosphorylating agents and synthetic routes that were investigated, all, with the exception of coupling with CEP, proved unsatisfactory (Table 3.1). Although there were instances when specific difficulties seem to arise with each agent, more generally, the problems could be categorized as due to a) poor coupling of phosphorylant to amine, or b) difficulties in the removal of protecting groups, which led to an impure and inferior recovery of product. Poor coupling was generally evidenced by the so-called selective agents; whilst such compounds have been shown by others to be capable of phosphorylating the amine group, results here suggest their use is limited in the case of groups existing in a sterically hindered environment.

o-Phenylene chlorophosphate was initially the most promising of the phosphorylants tested, with the results indicating an initial coupling of $> 60\%$. The difficulty with this reagent was the elimination of the aromatic protecting groups. Hydrogenation with PtO_2 was an attractive method, however even under extreme conditions the reaction mixture could not be

Table 3.1 The summary of the various synthetic routes investigated for the synthesis of PAT.

PHOSPHORYL-ATING AGENT	AMINE[#]	STAGES PRIOR TO PURIFICATION	FRACTIONS OBTAINED[*] BY CHROMATOGRAPHY	COMMENTS
PMI	AT	Direct coupling	Two fractions eluting at 0.29 & 0.33M	0.29M = PMI; 0.33M = PAT, low yield (< 0.5%), contaminated with PMI
P-γ-P	AT	Direct coupling	One fraction; 0.38-0.42M	Fraction contained PP_i, no PAT detected
$POCl_3$ + MgO	AT	Direct coupling	Two fractions; 0.33 & 0.38M	0.33M = PAT; 0.38M = PP_i, yield of PAT < 0.5%
o-Phenylene chloro-phosphate	TM-AT	Coupling, then a) hydrogenation b) base hydrolysis	One fraction; 0.30-0.40M	Partial hydrogenation, 0.30-0.40M mainly hydroxy phenyl phosphate, PAT present but impure
"	"	Coupling, then base hydrolysis	"	0.30-0.40M hydroxy phenyl phosphate, little PAT present
Dibenzyl chloro-phosphate	TM-AT	Coupling, hydrogenation, base hydrolysis	n.d.	Partial hydrogenation, did not pursue further
$POCl_3$	TM-AT	Coupling, base hydrolysis	n.d.	Poor coupling; did not pursue further
CEP	TM-AT	Coupling, base hydrolysis	One fraction; 0.33M	0.33M proved to be PAT, high purity; yield = 1%

[#] TM-AT = Trimethyl 2-amino tricarballylate.

[*] Analyzed for hydrolyzable phosphate.

n.d. : Not determined

fully hydrogenated (as determined by [^{1}H]-NMR). The poor yield and low purity state of PAT derived from the hydrolysis of this partially hydrogenated fraction suggested that the complete removal of the aromatic moiety was a prior necessity. Direct base hydrolysis of coupled product under harsher conditions was also unsuccessful and confirmed this prediction.

Whilst the coupling of CEP with trimethyl 2-amino tricarballylate still gave a low yield of PAT, the yield was significantly higher than that obtained by any other method, and further, the resolution of PAT from impurities was complete. This route then, was the one used for the preparation of PAT as required for characterization and testing.

3.4.2 CHARACTERIZATION OF THE PRODUCTS DERIVED FROM THE COUPLING WITH 2-CYANOETHYL PHOSPHATE

3.4.2.1 Coupled Product

The reaction mixture obtained following the coupling of CEP with trimethyl 2-amino tricarballylate was analyzed both by electrophoresis and thin layer chromatography (TLC) to detect coupled product (Fig. 3.4). Analysis showed this fraction to contain three phosphorylated compounds, two showing similar migration to P_i and CEP, with the third spot, presumably being attributable to a coupled product.

3.4.2.2 Synthetic PAT

A range of techniques were employed to establish the identity and the purity of the material eluting at 0.33M $NaHCO_3$, derived from the hydrolysis of the mixture containing coupled product. The product, which was tentatively assigned as N-phospho-2-amino tricarballylate (PAT) by inference from its elution profile (Fig. 3.5), was subjected to total phosphate and P_i analysis, nitrogen analysis, [^{1}H]-NMR, electrophoresis, TLC and isotachophoresis.

Phosphate assays showed that synthetic PAT contained a total of 2.20µmol of phosphate/mg of which 2% was present as P_i. Nitrogen analysis gave a value of 2.13µmol N/mg; the P:N ratio of 1.04 therefore was in good agreement to the theoretical value for PAT of 1.00. Electrophoresis and TLC (Fig. 3.4 and Table 3.2) confirmed the purity of the product, as no contaminants were detected by these analyses. Isotachophoretic analysis (Fig. 3.6) further supported this product, although a small trace of P_i was detected ($\simeq$ 2%), which confirmed the results from chemical assays. In

Fig. 3.4 The analysis by A) thin layer chromatography, and B) high-voltage electrophoresis of fractions obtained after the coupling of CEP with trimethyl 2-amino tricarballylate, and following hydrolysis and final purification by ion-exchange chromatography.

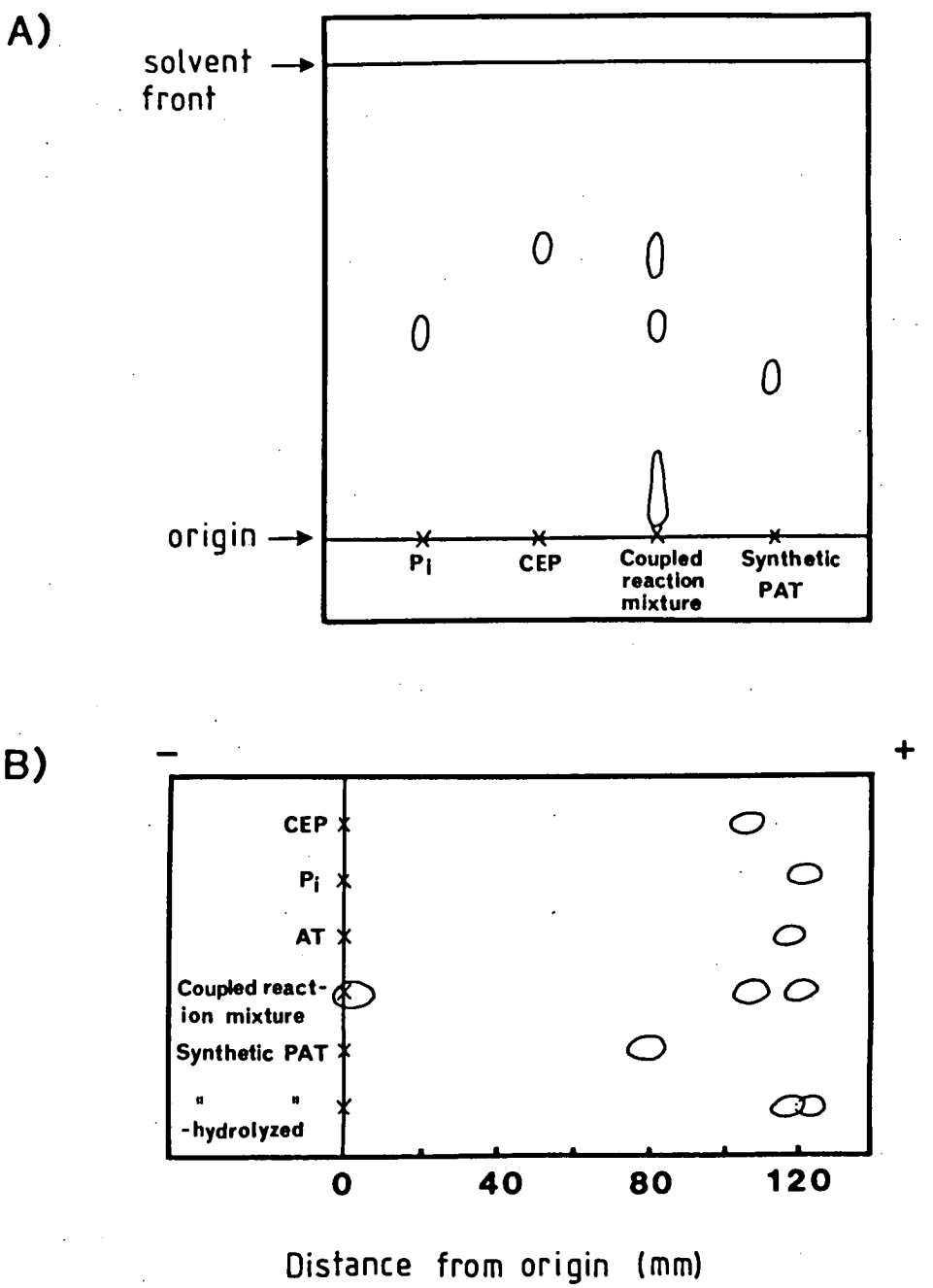


Fig. 3.5 The purification of PAT by ion-exchange chromatography of the reaction components derived after the coupling of trimethyl 2-amino tricarballylate with CEP and subsequent hydrolysis.

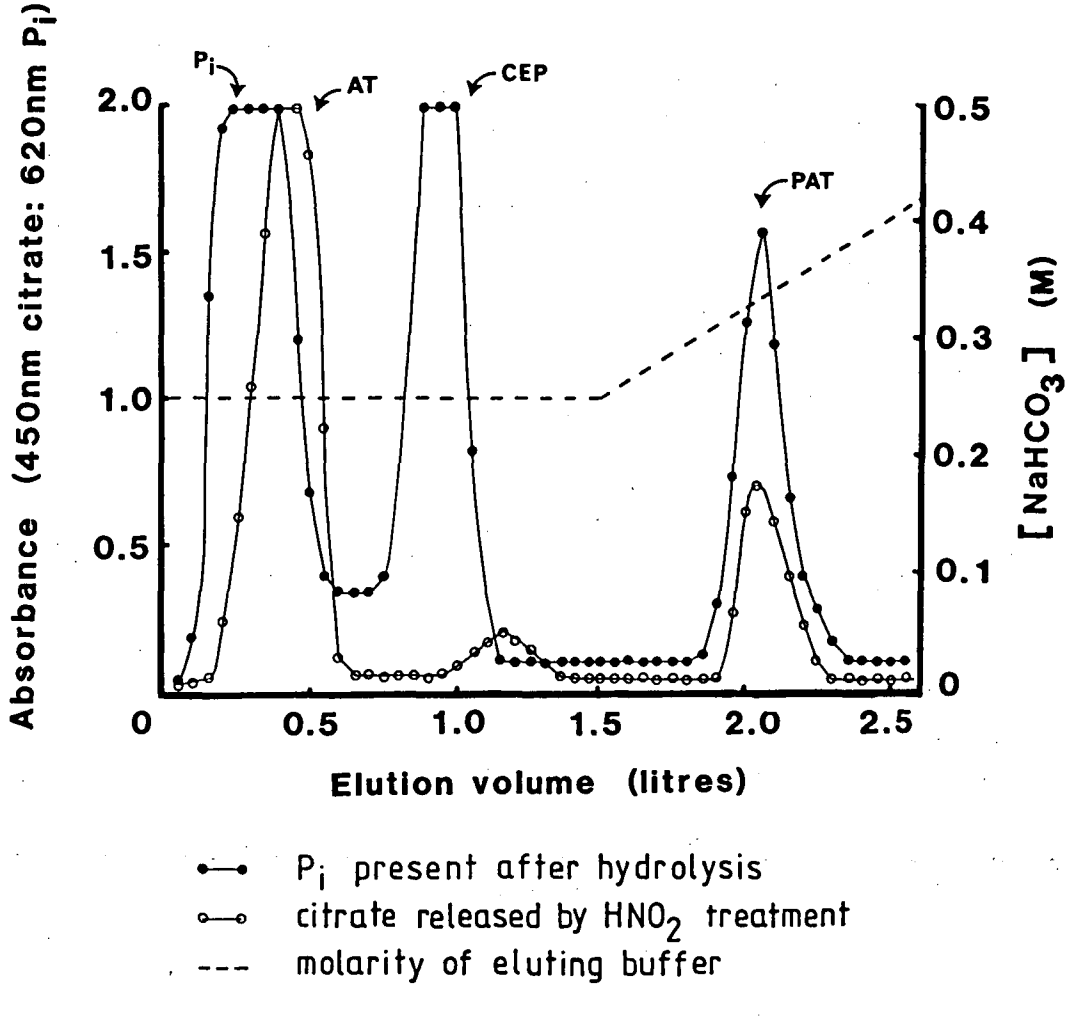
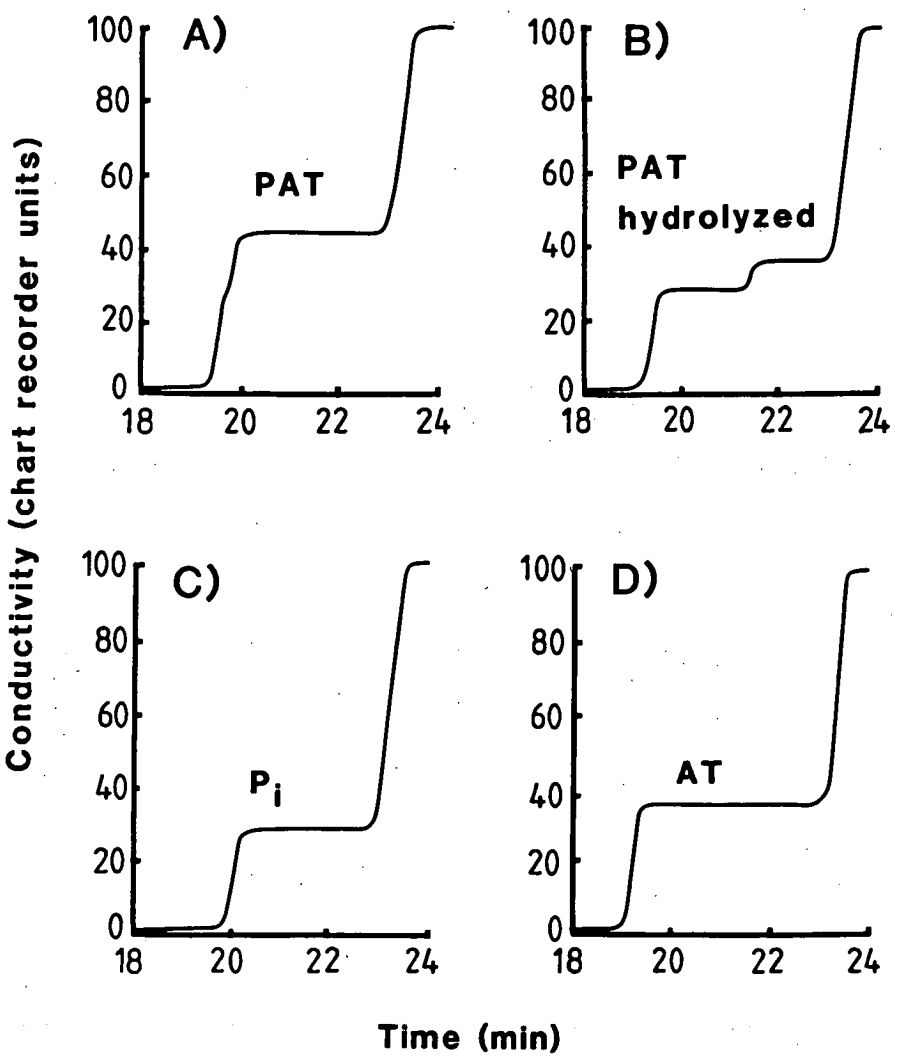


Table 3.2 The resolution of synthetic PAT, P_i and various other phosphate esters by high-voltage electrophoresis (HVE) and thin layer chromatography (TLC).

COMPOUND	TLC-R_f	HVE-Migration*
P_i	0.44	1.00
CEP	0.62	0.93
PMI	0.55	0.48
PP_i	0.18	0.50
2-hydroxy phenyl phosphate	0.63	0.84
PAT	0.32	0.68
PC	0.20	0.72

* Migration relative to P_i.

Fig. 3.6 Isotachophoretic analysis at pH 10.6 of A) synthetic PAT, B) synthetic PAT after hydrolysis, C) standard P_i, and D) standard AT.



addition, after hydrolysis of the sample in aqueous solution at neutral pH (1mg/100µl; 1h at 100°C), samples analyzed by isotachophoresis and electrophoresis showed the formed products to have similar mobilities to P_i and AT.

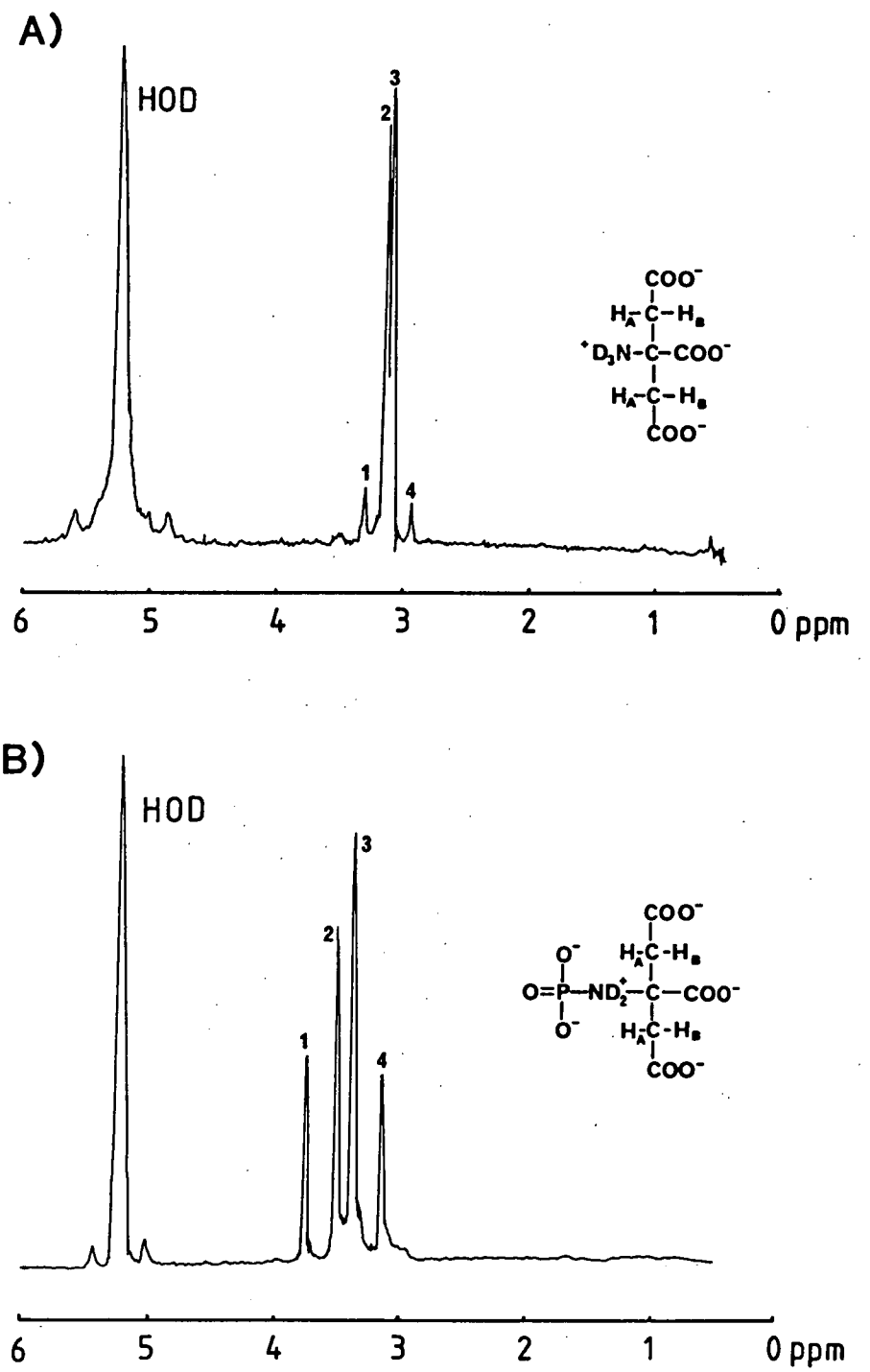
The [^{1}H]-NMR of both AT and synthetic PAT were established at pH 7.0 in D_2O for direct comparison, when only the methylene protons ($-CH_2-$) of the compounds produce signals (Fig. 3.7). Both spectra displayed a typical AB splitting pattern, with the signals for PAT being shifted downfield relative to those of AT. Similar splitting trends have been reported for citrate and PC (Williams and Sallis, 1979). As well as providing structural information for the identification of a compound, NMR has the added versatility of yielding information on the state of purity. From the [^{1}H]-NMR of synthetic PAT, no AT was detectable, nor were any other compounds present having protons producing signals in the region. Hence all forms of analysis indicated the identity of the isolated product as PAT, and confirmed the purity of the preparation.

3.5 DISCUSSION

A variety of synthetic routes involving a range of phosphorylating agents were investigated for the synthesis of N-phospho-2-amino tricarballylate (PAT). The phosphorylant that ultimately proved to be the most effective was 2-cyanoethyl phosphate (CEP). PAT was prepared then from the coupling of this agent with trimethyl 2-amino tricarballylate, followed by base hydrolysis, and final purification by ion-exchange chromatography. All forms of analysis were consistent with the identity and the high-purity state of the final product.

Characterization of PAT utilized a number of available techniques, including TLC, high-voltage electrophoresis, isotachophoresis, phosphate and nitrogen analysis, and [^{1}H]-NMR spectroscopy. P_i analysis showed the final product to have $\simeq 2\%$ P_i present, whereas the combination of total phosphate and nitrogen analysis, suggested a P:N ratio of 1.04 which was consistent with the stoichiometric ratio expected for PAT. The TLC and high-voltage electrophoresis systems used for the analysis of PAT, were developed so as to give good resolution between PAT and any other possible product contaminants. This enabled clear interpretation of the state of purity of the final product and identification of any impurities. For example, AT, P_i, PP_i (derived from the coupling of CEP with itself) and CEP were all recognized as potential contaminants in the final product and so were included as reference standards in the analysis of PAT. With both

Fig. 3.7 [^{1}H]-NMR spectra of A) AT, and B) synthetic PAT. Samples were analyzed at pH 7.0 in D_2O.



these analytical systems, synthetic PAT was shown to be free of such impurities, and to have a distinct mobility from all of these products. Isotachophoretic analysis reinforced these findings, and in addition, being a more sensitive technique it was able to detect the presence of a trace of P_i. Analysis of synthetic PAT by these analytical systems following exposure to hydrolytic conditions further supported its identity. Upon heating an aqueous solution of PAT, an analysis of the resultant reaction mixture by high-voltage electrophoresis and isotachophoresis showed the products to have identical migration properties to P_i and AT, i.e. the hydrolytic products of PAT.

Comparison of the elution of PAT from AG 1-X8 and its migration under high-voltage electrophoresis with those documented for PC (Williams, 1981), provided further proof of the new product. The basis of separation of both of these techniques is largely dependant upon charge density and the nature of the ionizable groups within the molecule. Because of the structural and functional group similarities between the two molecules, their state of ionization at a given pH would be very similar. Hence their migration or elution properties should closely correspond with these systems. This indeed proved to be the situation, as the mobilities (relative to P_i) for PAT and PC were 0.68 and 0.72 with high-voltage electrophoresis (see Table 3.2), and elution from AG 1-X8 occurred at 0.33 and 0.34M $NaHCO_3$ buffer respectively. The slight differences suggests that PC might be slightly more negatively charged at the operating pH values of these systems (7.4-8.0), perhaps reflecting partial ionization (i.e. protonation) of the secondary amino group of PAT, since the pKa of such groups lie generally in the range of 6.0-7.0.

The ion-exchange chromatography system of AG 1-X8 (HCO_3^-) that was adopted for the final purification of PAT was chosen because it appeared superior to others for the following reasons. As mentioned above, the elution properties of PC had been established with this system (Williams, 1981) and this gave an indication where PAT would elute. The system was also clearly capable of resolving PC from predicted contaminants, and further, ultimate removal of sodium bicarbonate using the resin AG 50W-X8 (H^+) permitted a salt-free sample to be easily prepared.

The detection of PAT eluting from AG 1-X8 resin was facilitated by the ultimate development of a specific assay capable of detecting the presence of the AT moiety. The basis of this assay was the treatment of fractions with nitrous acid, which from theoretical considerations would first hydrolyze PAT to its constituents AT and P_i, and then convert AT to

citrate (plus other products). Assay of citrate by established specific methods was then possible. The development of this assay greatly aided in detecting the presence of PAT, since its elution was indicated by the presence of fractions containing both the AT moiety and hydrolyzable phosphate. Synthetic PAT was obtained following the collection of the 0.33M eluting fraction from the AG 1-X8 resin, which clearly, from the elution profile (see Fig. 3.5), was a fraction with intense overlapping peaks of both hydrolyzable phosphate and AT. This, therefore was further evidence for the identity of this fraction containing PAT.

The [^{1}H]-NMR spectrum of PAT provided unambiguous structural evidence for the identity of PAT, as well as reinforcing conclusions from other characterization data. The spectrum was determined at pH 7.0 in D_2O, under which conditions the methylene protons gave rise to a typical AB splitting pattern. An AB system is one consisting of two mutually coupled protons, A and B, which are not coupled to any other protons. In the case of a methylene group with no protons on adjacent carbon atoms, if the group is part of an aliphatic chain the two protons may be chemically equivalent. If however, as is the situation with both PAT and AT, the group is part of a chain in which rotation is restricted, the possibility of the non-equivalence of the protons arises. Under such circumstances, the resonances of the two protons will be split due to the mutual coupling of the A and B protons. Thus the AB splitting patterns observed for AT and PAT allows various characterizing data to be calculated [as defined by Williams and Fleming (1973)], including the chemical shifts (δ) for hypothetical non-interacting H_A and H_B, differences in chemical shifts between H_A and H_B (δ_{AB}), and coupling constants for H_A and H_B (J_{AB}). These parameters will be presented in greater detail in a subsequent chapter, when comparing AT, PAT and the other PC analogue, N-sulpho-2-amino tricarballylate (SAT). Nevertheless it is useful to make some comment on the salient features of the two spectra now; clearly the position of the spectrum of PAT is shifted downfield relative to that of AT, and it is most likely that deshielding produced by the electron withdrawing phosphoryl group would be the major force accounting for this trend.

To review the synthetic strategies, initial attempts to prepare PAT were directed at the use of selective agents such as PMI, P-γ-P and $POCl_3$ + MgO to phosphorylate the amine group of AT. As evidenced by the poor coupling with these agents, their use gave a significantly lower yield than that obtained with CEP. In addition, PMI was difficult to resolve from PAT by ion-exchange chromatography, and hence the final sample obtained with

this agent contained PMI as an impurity. In considering the more powerful non-selective agents, o-phenylene chlorophosphate initially appeared the most promising. Although the initial coupling of reagents seemed almost quantitative, the general difficulty associated with the use of this agent was that the cleavage of the phenyl group by catalytic reduction was not complete. Various factors were investigated to overcome this, such as addition of fresh catalyst, greater amounts of catalyst, higher operating pressures, longer reaction times, activation of catalyst and prior purification of starting products. Nevertheless, all failed to result in total hydrogenation. When the partially hydrogenated sample was subjected to base hydrolysis and ion-exchange purification, the product was contaminated with 2-hydroxy phenyl phosphate, and low in yield of PAT. Direct hydrolysis of the coupled product, under more vigorous base conditions again did not circumvent this problem, and such treatment followed by ion-exchange purification, revealed 2-hydroxy phenyl phosphate as the only detectable compound.

Dibenzyl chlorophosphate, a less powerful phosphorylant than the latter was investigated briefly; nevertheless it was clear that hydrogenolysis of the aromatic groups of the coupled product derived from this reagent was also incomplete. The direct action of $POCl_3$ on trimethyl 2-amino tricarballylate was also explored, but this synthetic route was abandoned when no indication of coupling was evident. Ultimately, pure PAT was prepared from the coupling of trimethyl 2-amino tricarballylate with CEP. Whilst the yield was low (1%), it was significantly greater than that possible by the other described synthetic methods, and did compare to a yield of PC derived from coupling triethyl citrate with this agent of 7% (Williams and Sallis, 1980). In both cases the major factor accounting for the low degree of coupling would be the steric hindrance surrounding the phosphorylation site. Nevertheless, despite the poor yield, the product obtained was of high purity and the amount generated was sufficient for full characterization of the product by methods described in subsequent chapters.

3.6 SUMMARY

1. A variety of methods were investigated for the preparation of PAT involving either a) selective phosphorylation of AT, or b) phosphorylation of trimethyl 2-amino tricarballylate by non-specific agents followed by removal of protective groups.

2. Selective agents were found incapable of phosphorylating the

sterically hindered amino group of AT to any extent.

3. Of the more powerful non-selective agents, o-phenylene chlorophosphate appeared the most promising, with a high yield for the initial coupling with trimethyl 2-amino tricarballylate. However, difficulties were encountered with the subsequent removal of protecting groups.

4. CEP ultimately proved the most capable agent for preparing PAT. The yield obtained with this agent was superior to other phosphorylants. Further, the resolution by ion-exchange chromatography of PAT and impurities derived from this synthesis was excellent.

5. Synthetic PAT was analyzed by a variety of characterization techniques. All confirmed both the purity and identity of the product.

6. The amount obtained was of sufficient quantity for the further needs of the project.

CHAPTER 4

THE SYNTHESIS OF UNLABELLED AND [^{35}S]-LABELLED N-SULPHO-2-AMINO TRICARBALLYLATE

4.1 INTRODUCTION

The second PC analogue that was investigated was N-sulpho-2-amino tricarballylate (SAT). As was evident with PAT, this compound also has structural similarity to PC, where the $-O-PO_3H_2$ group of PC is substituted for a $-NH-SO_3H$ (sulphamate) group to give SAT. Whilst structural similarity was paramount in order to confer upon the molecule a similar anti-calcifying potency *in vitro* to that seen with PC, the other major criteria looked for was a greater resistance to breakdown in the body than that which occurs with PC. The sulphamate bond of SAT would represent the most likely site of attack if enzymic degradation of SAT were to occur. Examples of this type of linkage do exist in nature, and are commonly associated with glucosamine or galactosamine residues. These particular sulphamates are cleaved however, by specific sulphamatases *in vivo* (Friedman and Arsenis, 1974). Nevertheless, the metabolic studies of Spillane and Benson (1978) on the non-nutritive sweeteners cyclopentylmethylsulphamate and cyclopentylsulphamate showed that following administration, these sulphamates are virtually unmetabolized *in vivo*. Such observations support the specificity of the natural sulphamatases, and suggest that SAT might also be biologically stable.

The two synthetic approaches that were apparent for the preparation of SAT were analogous to those initially envisaged for the synthesis of PAT (Chapter 3). These were: a) the sulphonation of trimethyl 2-amino tricarballylate using non-specific reagents followed by the hydrolysis of the methyl groups, or b) the direct sulphonation of the amino group of 2-amino tricarballylate (AT) by a selective agent. Agents that have been employed for amine sulphonation include concentrated sulphuric acid (Reitz *et al.*, 1944), a sulphur dioxide/sulphur trioxide mixture (Coleman *et al.*, 1953 - cited by Warner and Coleman, 1958), chlorosulphonic acid, sodium chlorosulphonate (Audrieth and Sveda, 1944), a pyridine/chlorosulphonic acid mixture (Reitz *et al.*, 1946) and various addition products between strong electron donors such as dioxane, dimethyaniline and pyridine with sulphur trioxide (Sisler and Audrieth, 1946). Sodium sulphamate itself has also been utilized to sulphonate various amino alkanes, but this reaction has

limitations in that it only proceeds at high temperatures (Bowen et al., 1966).

While the majority of the sulphonating agents are non-specific and/or give variable yields, Warner and Coleman (1958) have demonstrated that the pyridine-sulphur trioxide complex is capable of selectively sulphonating polyfunctional amines in an aqueous solution manner when the pH is maintained between 9 and 10. These workers demonstrated the N-sulphonation of serine, ethanolamine and 3-aminopropanol to yield the corresponding sulphamates with yields ranging from 25-57%. The agent appeared suitable for the synthesis of SAT and superior to the use of other agents for the following reasons: a) AT could be sulphonated directly to yield SAT, b) AT was more readily available than its trimethyl ester (Chapter 2) and c) literature research indicated that pyridine-sulphur trioxide was an equal if not more powerful sulphonating agent than many of the non-specific agents.

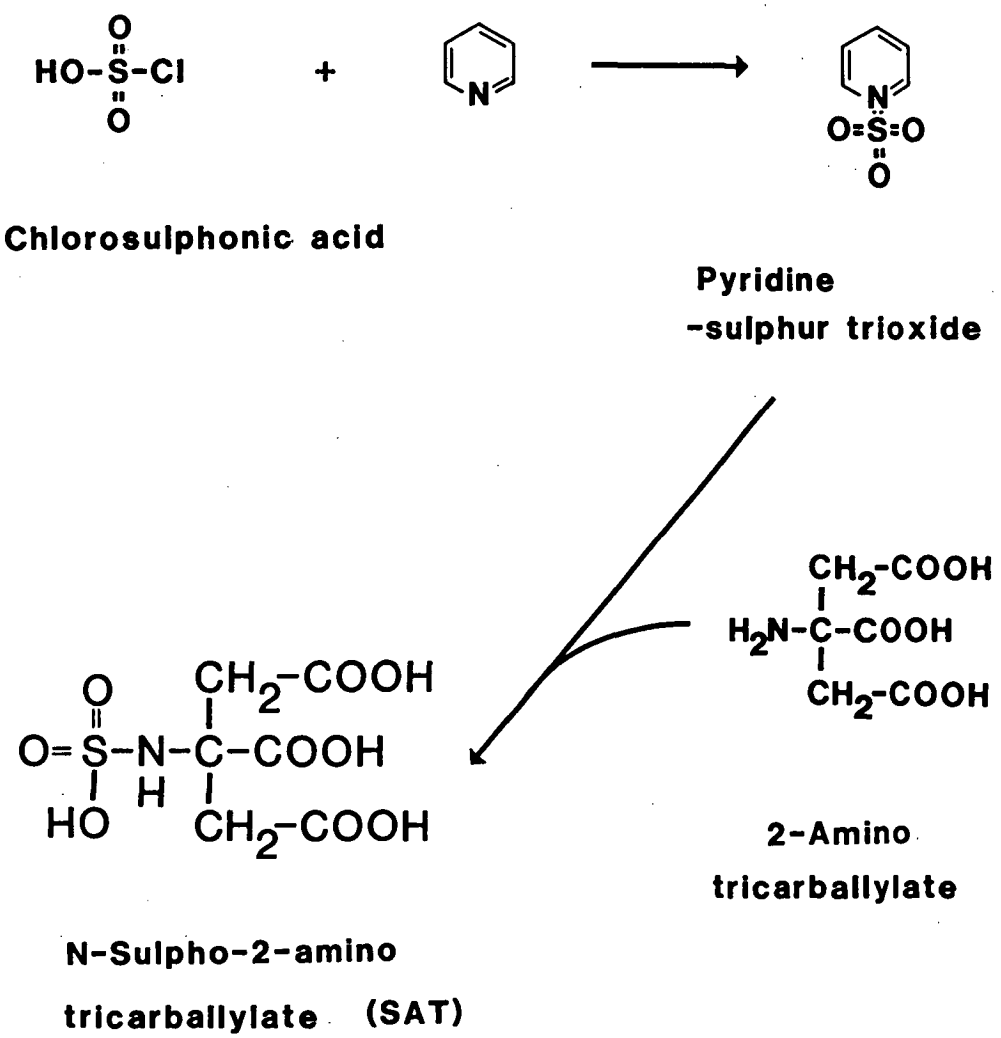
An additional requirement for any synthetic method for SAT was that it could be utilized also to prepare radiolabelled SAT. This was of prime consideration, since isotopically labelled SAT would be of potential value for subsequent studies to determine the metabolic fate and stability of SAT in biological systems. Clearly, the proposed synthetic route would allow the preparation of [^{35}S]-SAT since pyridine-[^{35}S]-sulphur trioxide could be prepared from chloro-[^{35}S]-sulphonic acid by adaptation of the existing method for non-radiolabelled pyridine-sulphur trioxide synthesis (Sisler and Audrieth, 1946).

This chapter therefore describes the synthesis of both unlabelled and [^{35}S]-labelled SAT by a two-step procedure as summarized in Fig. 4.1. Methods developed for the purification and characterization of these products are also detailed.

4.2 MATERIALS

The ion-exchange resins AG 2-X8 (Cl^-; 100-200 mesh) and AG 50W-X8 (H^+; 200-400 mesh) were products of Bio-Rad Laboratories, Richmond, California. The resins were cycled before use and the AG 2-X8 resin was converted to the bicarbonate form. AT was prepared by the method of Dornow and Rombusch (1955), summarized in Chapter 2. Chloro-[^{35}S]-sulphonic acid (2mCi/5µl) and [^{35}S]-Na_2SO_4 were supplied by the Radiochemical Centre, Buckinghamshire, England. Scintillation fluid comprised of 18ml of a mixture of 0.6% 2,5 diphenyloxazole in toluene, and 2-ethoxy ethanol (10:6 v/v). Pyridine and chloroform were dried before use by storage over molecular sieve 4A.

Fig. 4.1 Synthetic route for the preparation of unlabelled and [^{35}S]-radiolabelled N-sulpho-2-amino tricarballylate (SAT).



4.3 METHODS

4.3.1 SYNTHESIS OF UNLABELLED SAT

4.3.1.1 Preparation of the Pyridine-Sulphur Trioxide Complex

Although the compound is now commercially available (Aldrich Chemical Company, Milwaukee, U.S.A.), it was found more convenient to prepare this compound directly by the method of Sisler and Audrieth (1946). Chlorosulphonic acid was added dropwise to anhydrous pyridine in chloroform whilst maintaining the temperature at 0°C. At the end of the addition the product was isolated by filtration, washed with ice cold chloroform and stored at -15°C.

4.3.1.2 Synthesis of SAT

A solution of 2.2g of AT in 50ml of water was prepared; the temperature lowered to 10°C and the pH adjusted to 9.6 with 2M NaOH. Pyridine-sulphur trioxide (5.0g) was added in small portions with continuous stirring over a 90min period. During this time, the pH was maintained at 9.6 with 2M NaOH using a pH-stat (Radiometer, Copenhagen, Denmark) whilst the temperature was held constant. After total addition, a large amount of pyridine-sulphur trioxide remained unreacted. The mixture was allowed to stir for a further 2h with the pH still maintained at 9.6 while allowing the temperature to rise slowly to 20°C. At the end of this time, the resulting clear yellow solution was passed through a 2.5 x 1.5cm column of AG 50W-X8 (H^+) at 4°C to remove pyridine and unreacted AT. After washing the column with 100ml of water, the eluants were pooled. Following dilution of this fraction approximately 4-fold with water at room temperature, 100ml of a 6.6% $BaCl_2$ solution was added with stirring and the pH adjusted to 3.0 with 2M NaOH. The mixture was left to stir for a further 5min, the solution filtered, and the filtrate adjusted to pH 9.0 before lyophilization.

The solid was resuspended in 150ml of water, 2.6g of $BaCl_2$ added, and the mixture stirred at 4°C for 1h. The precipitate was collected by filtration, washed with a minimum volume of ice-cold water, followed by ethanol, acetone and ether respectively before air drying. A yield of 2.7g of the partially pure product was obtained. Before final purification by ion-exchange chromatography, the barium was removed. This was done at 4°C by stirring the solid suspended in 50ml of water with AG 50W-X8 (H^+; 50ml resin volume) for 10min before pouring as a slurry onto a column of AG 50W-X8 (H^+; 3.5 x 4.0cm). Following a column wash with 50ml of water, the

combined eluants were adjusted to pH 9.0, before loading onto a 3.5 x 6.0cm column of AG 2-X8 (HCO_3^-). A preliminary wash with 500ml of 0.35M $NaHCO_3$ was initiated to remove residual sulphate, then SAT was recovered by elution with 0.60M $NaHCO_3$, collected in 100 fractions of 10ml volume. The presence of SAT was detected by sulphate analysis before and after hydrolysis. Fractions found to contain SAT were pooled and an excess of AG 50W-X8 (H^+) was added with stirring at 4°C for 10min to remove $NaHCO_3$. The suspension was filtered and the filtrate adjusted to pH 7.0 before lyophilization. This process was repeated with the freeze-dried product to ensure total decarbonation. The subsequent yield was 500mg (10%).

4.3.2 CHARACTERIZATION OF UNLABELLED SAT

4.3.2.1 Sulphate

Two methods were employed for the analysis of free sulphate and N-sulphate. For column chromatography, free sulphate was detected in 0.5ml aliquots from fractions by first decarbonating with 100µl of acetic acid and then assaying using the sodium rhodizonate method outlined by Terho and Hartiala (1971). To detect N-sulphate using this procedure, 0.5ml aliquots were incubated with 100µl of 15% $NaNO_2$ and 100µl acetic acid for 30min at room temperature before assaying. Under these conditions, sulphate is quantitatively released from N-sulphate linkages (Inoue and Nagasawa, 1976). While this method was found to be the most sensitive for the detection of inorganic and organic sulphate, a somewhat less sensitive turbidimetric assay was found to be more useful for the quantitative assay of sulphate in the final product. This was a modification of that described by Inoue and Nagasawa (1976) and was as follows. For N-sulphate, 0.5ml of a sample or standard (0.5-2.5µmol) was added to 0.5ml 5% $NaNO_2$ and 0.5ml of 33% (v/v) acetic acid, shaken, and left standing for 30min at room temperature. Trichloroacetic acid (2.0ml; 8% w/v) was added and the tubes shaken, followed by the addition of 1.0ml of a barium chloride-gelatin reagent (1g $BaCl_2$ and 0.5g gelatin/100ml) with immediate shaking. After standing for 20min, the turbidity of the solution was read at 500nm. This method was made applicable to free sulphate determinations by the substitution of 0.5ml of 5% $NaNO_2$ and 0.5ml of 33% acetic acid with 1.0ml of water.

4.3.2.2 Nitrogen

The nitrogen content of samples was determined as outlined by Johnson (1941).

4.3.2.3 Thin Layer Chromatography

Samples (1µl; 10mg/ml) of SAT, Na_2SO_4 or sodium sulphamate were applied to plates coated with cellulose (0.25mm; MN 300HR cellulose from Machery-Nagel, Germany) and developed in a solvent mixture of isobutyric acid : methanol : 7.5% (w/w) ammonia : 10% (w/v) trichloroacetic acid [40 : 100 : 10 : 15 (v/v)] for 3h. Plates were air-dried, and trichloroacetic acid removed by washing carefully in diethyl ether. Following ether evaporation, the plates were sprayed with a mixture containing equal volumes of 5% $NaNO_2$ and 33% acetic acid and placed in a chromatography tank equilibrated with the same mixture in such a manner that the plates did not come in contact with the liquid. This procedure effected the hydrolysis of the N-sulphate linkage in SAT. After 30min the plates were removed and dried at 60°C. Identification was made by spraying with a solution of equal volumes of Reagent A (20ml 10M acetic acid, 4ml 0.025M $BaCl_2$, 16ml 0.1M $NaHCO_3$ made up to 100ml with ethanol) and Reagent B (10mg sodium rhodizonate, 100mg ascorbic acid in 20ml of water made up to 100ml with ethanol). Sulphate and N-sulphate appear as white spots on a pink background.

4.3.2.4 High-Voltage Paper Electrophoresis

Samples (6µl; 10mg/ml) of SAT and AT, or SAT, Na_2SO_4 and sodium sulphamate were applied to Whatmann No. 3MM paper and electrophoresed at 2000V for 20min in an acid buffer [2.5% (v/v) acetic acid and 3% (v/v) formic acid]. The papers were dried at 100°C before further treatment. The paper with the applied sulphate compounds was treated with 5% $NaNO_2$ and 33% acetic acid followed by Reagent A and B in analogous fashion to that described in Section 4.3.2.3 for the analysis by TLC. (the paper was however dipped in the latter reagent rather than sprayed). Papers with only SAT and AT applied were stained by the method of Firmin and Gray (1974) (Section 3.3.3.6.3).

4.3.2.5 Isotachophoresis

Aliquots (2-10µl; 1mg/ml) of SAT or AT were subjected to isotachophoretic analysis using isotachophoretic equipment constructed in our laboratories. A conductimetric detector was used and the minimum amount of SAT detectable was 2µg. The buffer system comprised of 5mM HCl/aniline pH 4.5 in 0.2% hydroxypropyl methylcellulose as the leading electrolyte and 5mM glutamic acid (0.02% hydroxypropyl methylcellulose) as the terminator.

4.3.2.6 Spectroscopy

[^{1}H]-NMR spectra were determined at an operating frequency of 100MHz using a 4H-100 Jeol Continuous Wave Instrument. Samples were analyzed at pH 7.0 using D_2O as the solvent. Infra-red spectrophotometry was performed using a Beckman Acculab 1 Infra-red Spectrophotometer. A mull, prepared from the compound and hexachlorobutadiene was scanned for absorption between the range 4000 to 600cm^{-1}.

4.3.3 SYNTHESIS OF [^{35}S]-SAT

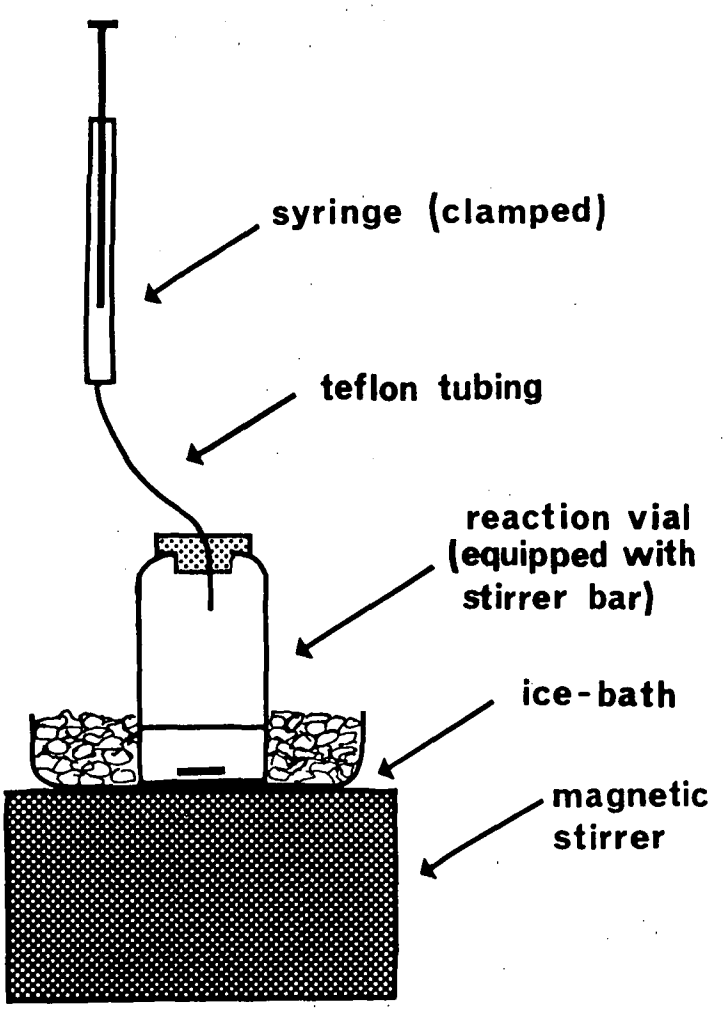
The synthesis of [^{35}S]-SAT was achieved following the same synthetic route as that for non-radiolabelled SAT, although the following important points are to be noted: a) the scale of the synthesis was greatly reduced; for this reason the preparation of pyridine-[^{35}S]-sulphur trioxide, although essentially the same as that described by Sisler and Audrieth (1946) for non-radiolabelled agent, utilized special equipment and techniques which are detailed below, and b) in contrast to the synthesis of unlabelled SAT, AT was in large excess for the second synthetic stage to allow a maximum incorporation of the radiolabel.

4.3.3.1 Preparation of Pyridine-[^{35}S]-Sulphur Trioxide

To a standard scintillation vial equipped with a magnetic stirrer bar was added 3.0ml of anhydrous chloroform and 160µl anhydrous pyridine. The vial was sealed with a rubber cap and cooled to 0°C by means of an ice-bath. Chloro-[^{35}S]-sulphonic acid (2mCi/5µl) was then diluted to 100µl with non-radiolabelled chlorosulphonic acid, and drawn up into an airtight syringe fitted with teflon tubing. The rubber cap of the reaction vial was pierced, the tip of the teflon tubing introduced, and the syringe was clamped in an upright position above the level of the vial as depicted in Fig. 4.2.

Chloro-[^{35}S]-sulphonic acid was then added dropwise from the syringe over a 10min period with stirring while the temperature was maintained at 0°C. At the end of this addition, the ampoule originally containing the chloro-[^{35}S]-sulphonic acid was rinsed with 100µl of dry chloroform. This was again drawn up into the syringe, and the contents added to the reaction vial as before. The reaction mixture was allowed to stir for a further 5min at 0°C before microfiltration under suction. The collected pyridine-[^{35}S]-sulphur trioxide was then washed with ice-cold anhydrous chloroform (2.0ml), which had been used to rinse the reaction vial. The solid was dried thoroughly *in vacuo* over concentrated sulphuric

Fig. 4.2 An apparatus for the preparation of pyridine-[^{35}S]-sulphur trioxide.



acid in a desiccator for 4-6h. The scintillation vial was retained for use in the next stage of the synthesis, and also dried in this fashion. The radiochemical yield of pyridine-[^{35}S]-sulphur trioxide was 27% (65mg of specific activity 8.3µCi/mg).

4.3.3.2 Preparation of [^{35}S]-SAT

To the scintillation vial used for the described preparation under 4.3.3.1 was added 500mg of AT in 10ml of water; the temperature was lowered to 10°C and the pH adjusted to 9.6 with 2M NaOH. The pyridine-[^{35}S]-sulphur trioxide (65mg) was added in 5 portions with continuous stirring over a 10min period. The pH was maintained at 9.6 with 2M NaOH using a pH-stat and the temperature held constant during this time. The mixture was then allowed to stir for a further 30min with the pH still maintained at 9.6 while allowing the temperature to rise to 20°C. The resulting clear yellow solution was passed through a 1.5 x 5.5cm column of AG 50W-X8 (H^+) at 4°C to remove pyridine and excess AT. After washing the column with a further 10ml of water, the combined eluants were adjusted to pH 9.0 and loaded onto a 2.2 x 1.5cm column of AG 2-X8 (HCO_3^-). After a preliminary wash with 250ml of 0.35M $NaHCO_3$ to remove [^{35}S]-sulphate, the stronger binding [^{35}S]-SAT was recovered by elution with 250ml of 0.60M $NaHCO_3$ collected in 5ml fractions. The presence of [^{35}S]-sulphate and [^{35}S]-SAT was determined by measuring the radioactivity of aliquots (100µl eluant/10mls scintillant) taken from the 5ml fractions collected. Relevant tube contents containing [^{35}S]-SAT were pooled and decarbonated by stirring at 4°C for 10min with an excess of AG 50W-X8 (H^+). The suspension was filtered, the filtrate adjusted to pH 7.0 with NaOH and then lyophilized to give a white powder (25mg; specific activity 3µCi/mg). The yield of radiolabel for this step was 15%, with the overall yield for both steps being 4%.

4.3.4 CHARACTERIZATION OF [^{35}S]-SAT

Inorganic and N-hydrolyzable sulphate analyses, nitrogen determination and high-voltage paper electrophoresis were utilized to confirm the purity of [^{35}S]-SAT. Electrophoresis was performed by applying samples (1µl; 1mg/ml) of [^{35}S]-SAT and [^{35}S]-sulphate standard diluted to the same specific activity to Whatman No.3 MM paper and running under the same conditions as non-radiolabelled SAT, although with a longer running time (1h). The paper was dried and marked in a grid pattern before cutting into sections (3cm wide and 1cm long) along the migration path of the compounds. These sections of paper were extracted with 2.0ml of 1M HCl, and the

extracts were added to vials with 18.0ml of scintillation fluid for counting.

4.4 RESULTS

Following partial purification by chromatographic and selective precipitation techniques, unlabelled N-sulpho-2-amino tricarballylate (SAT) was separated from the other reaction components by anion-exchange chromatography using an AG 2-X8 (HCO_3^-) resin (Fig. 4.3). After removal of free sulphate and other contaminating ions by a batch elution of 0.35M $NaHCO_3$ the stronger binding SAT was recovered by elution with 0.60M $NaHCO_3$. Considering the possibility that impurities not detected from ion-exchange chromatography were co-eluting with SAT, varieties of systems were employed for the characterization of the final product to confirm both its purity and identity. Sulphamate was recognized as a potential impurity in the final product, derived possibly through ammonium contamination in the reaction mixture or from the breakdown of SAT. For this reason, sodium sulphamate was included as a reference in the characterization techniques. High-voltage electrophoresis gave definitive separation of SAT, sulphate, sulphamate and AT, as did TLC. With both of these systems, synthetic SAT was shown to be free of these major components as well as any other detectable impurities (Fig. 4.4). This was also confirmed by isotachophoretic analysis, where only one band was evident (Fig. 4.5A).

Further support for both the identification and high-purity state of SAT was evident in the infra-red spectrum (Fig. 4.5B) and the [^{1}H]-NMR spectrum (Fig. 4.6). Characterization parameters arising from the latter spectrum are presented also in Table 4.1, where comparison is made to similar data derived for AT and PAT from previous chapters. The interpretation of both the [^{1}H]-NMR and infra-red spectra is discussed in detail in Section 4.5.

Sulphate assays indicated that < 0.01% of free sulphate was present in the final sample of SAT. Following hydrolysis, 2.36μmoles of sulphate were produced per milligram of SAT. Since nitrogen digestion revealed 2.41μmoles of nitrogen present per milligram of SAT, a sulphate to nitrogen ratio of 0.98 was obtained. This was in good agreement to the theoretical value of 1.00.

Radiolabelled [^{35}S]-SAT was also successfully prepared by this synthetic route, and its elution from AG 2-X8 (HCO_3^-) resin (see Fig. 4.7) was identical to non-radiolabelled SAT which had been fully characterized by the abovementioned physical and chemical techniques. The final product

Fig. 4.3 Purification of N-sulpho-2-amino tricarballylate (SAT) on an AG 2-X8 (HCO_3^-) resin column using sodium bicarbonate buffers. Aliquots were assayed for sulphate.

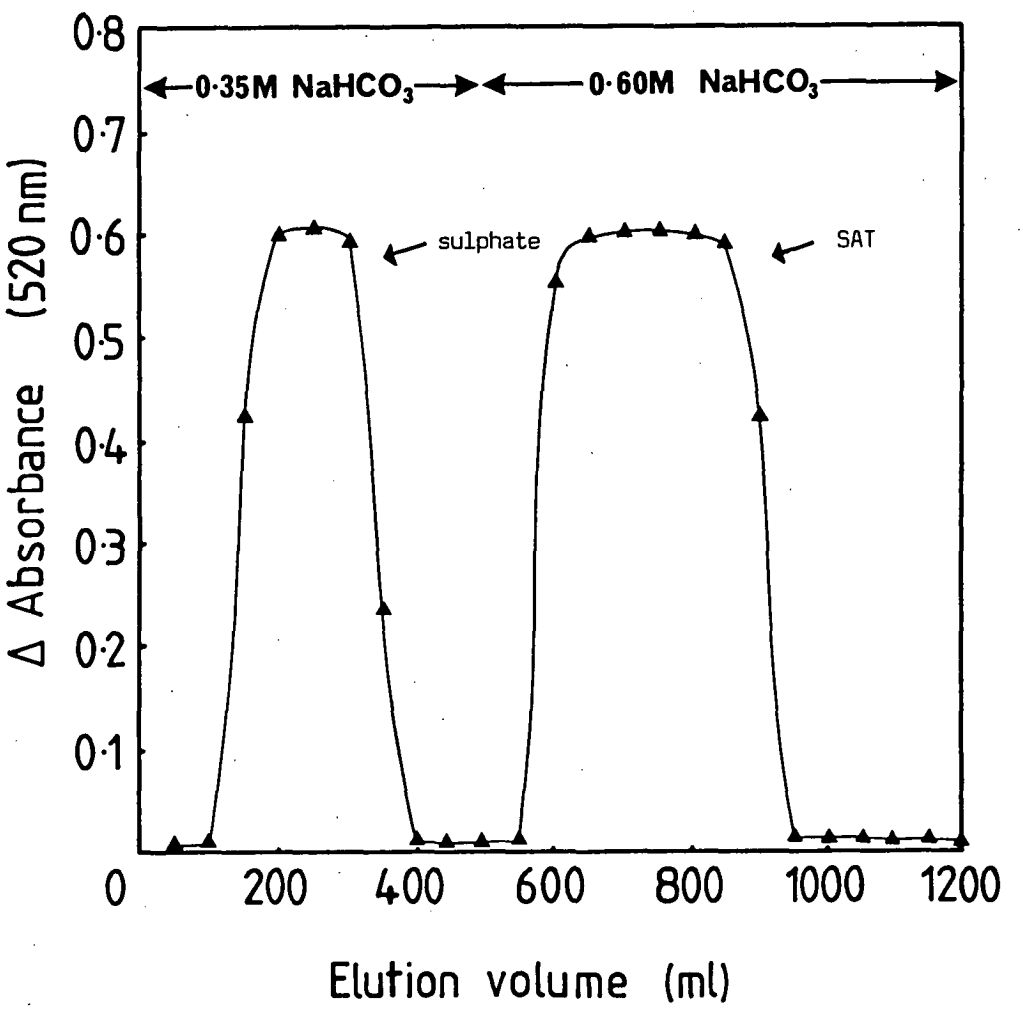


Fig. 4.4 The analysis of synthetic SAT by A) thin layer chromatography, and B) high-voltage electrophoresis. Possible contaminants were included in the systems as reference standards.

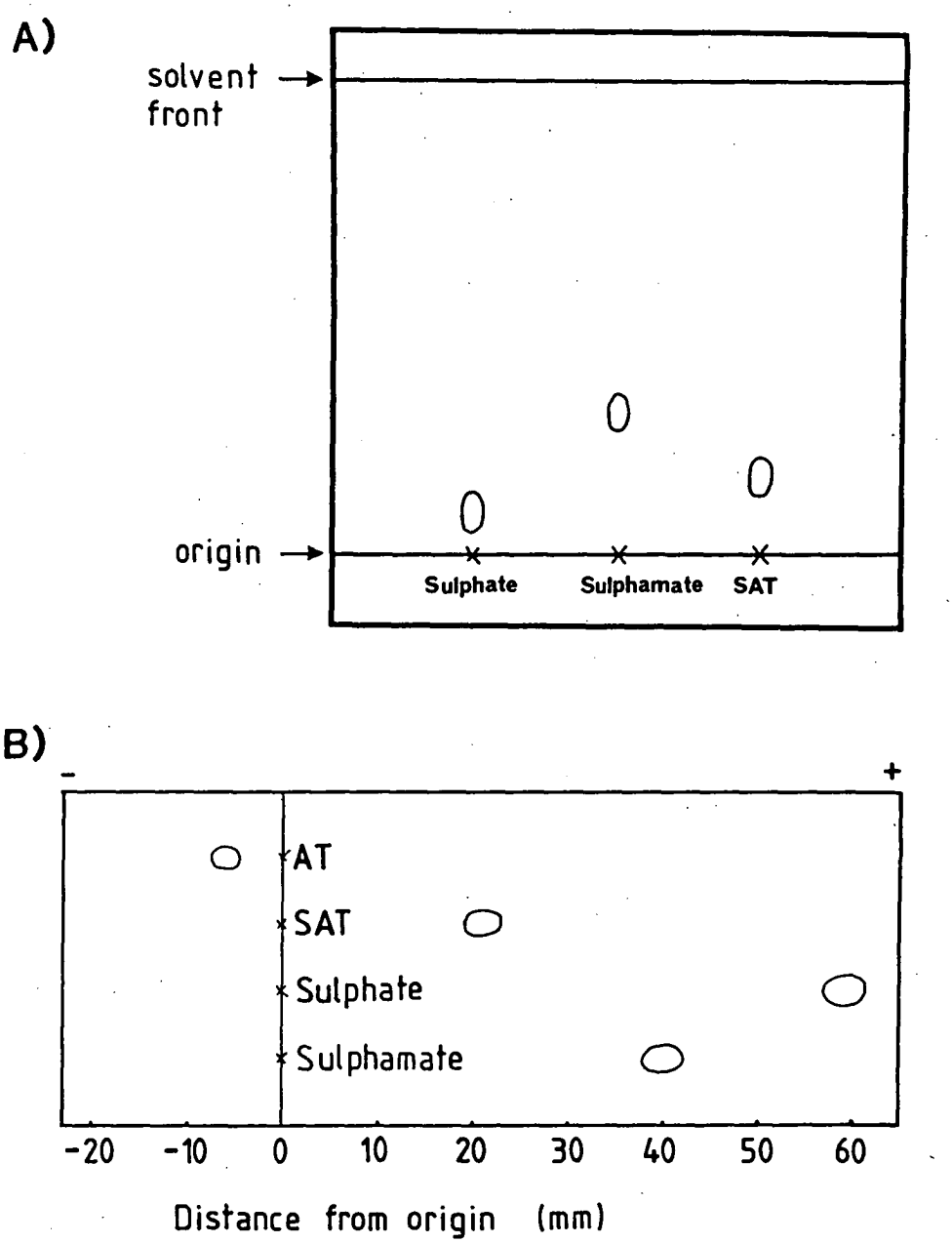


Fig. 4.5 A) Isotachophoretic analysis at pH 4.5 of synthetic SAT and comparison to AT.

B) Infra-red spectrum of SAT. A mull was prepared between SAT and hexachlorobutadiene (HCB) and absorption scanned between 4000-600cm^{-1}.

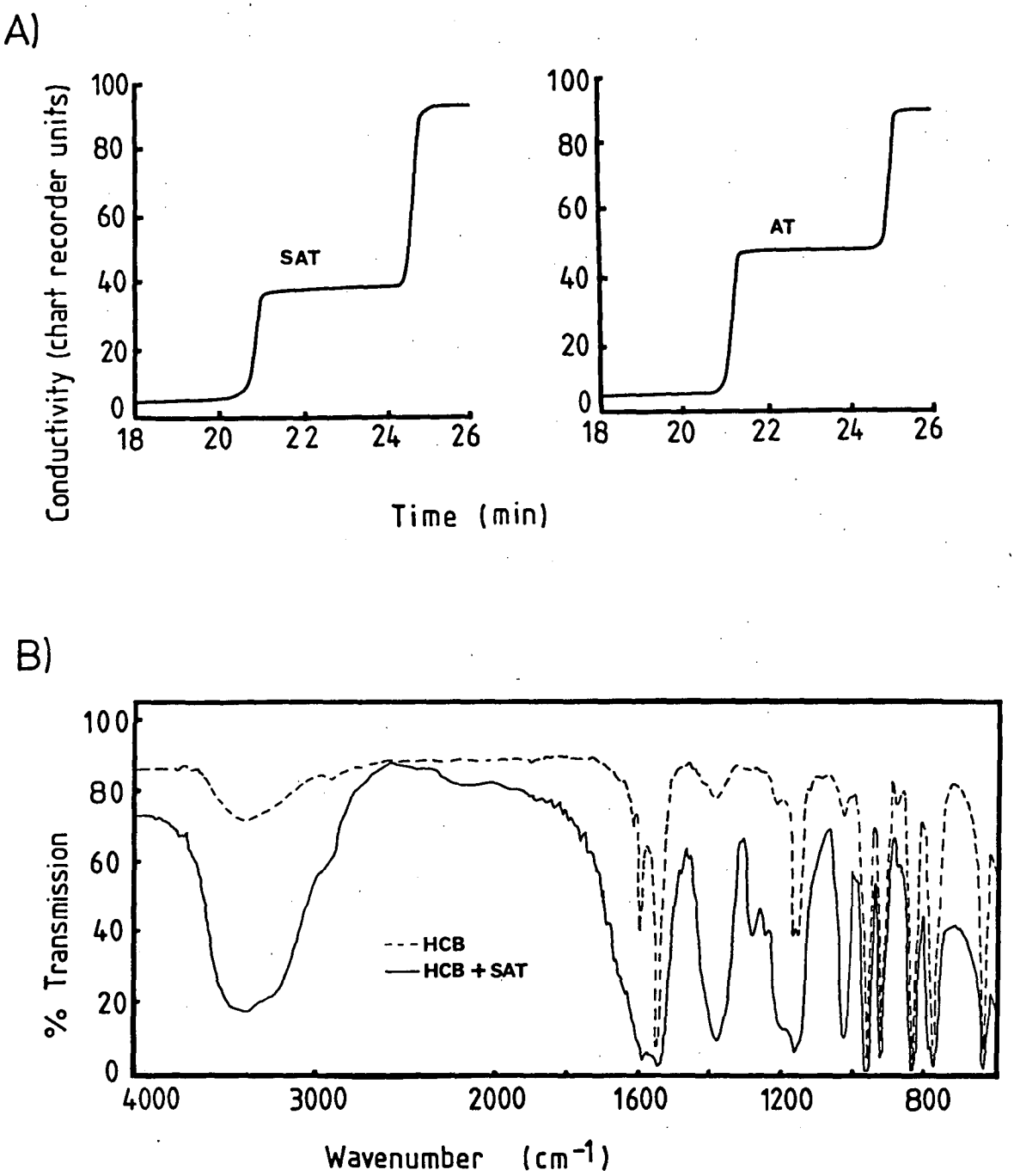


Fig. 4.6 [^{1}H]-NMR spectrum of synthetic SAT. The spectrum was determined at pH 7.0 using D_2O as the solvent.

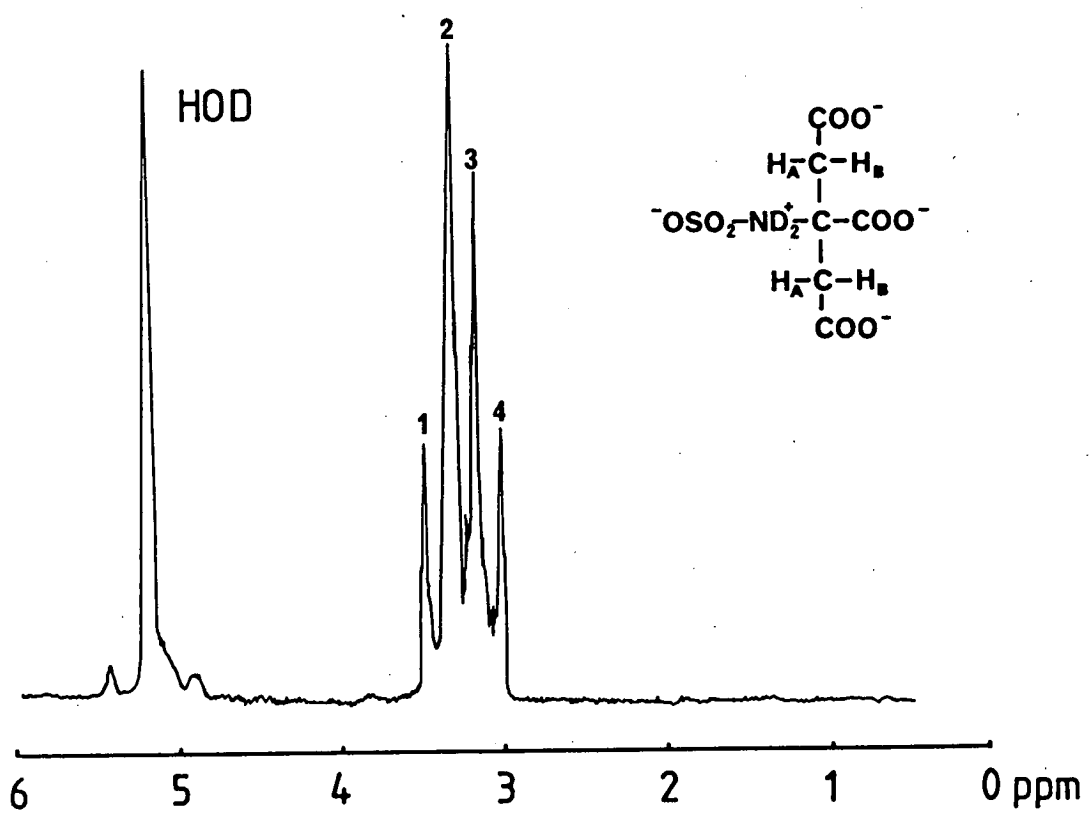


Table 4.1 [^{1}H]-NMR chemical shift (δ) data, coupling constants (J_{AB}) and differences in chemical shifts (δ_{AB}) between H_A and H_B for AT, PAT and SAT. [a]

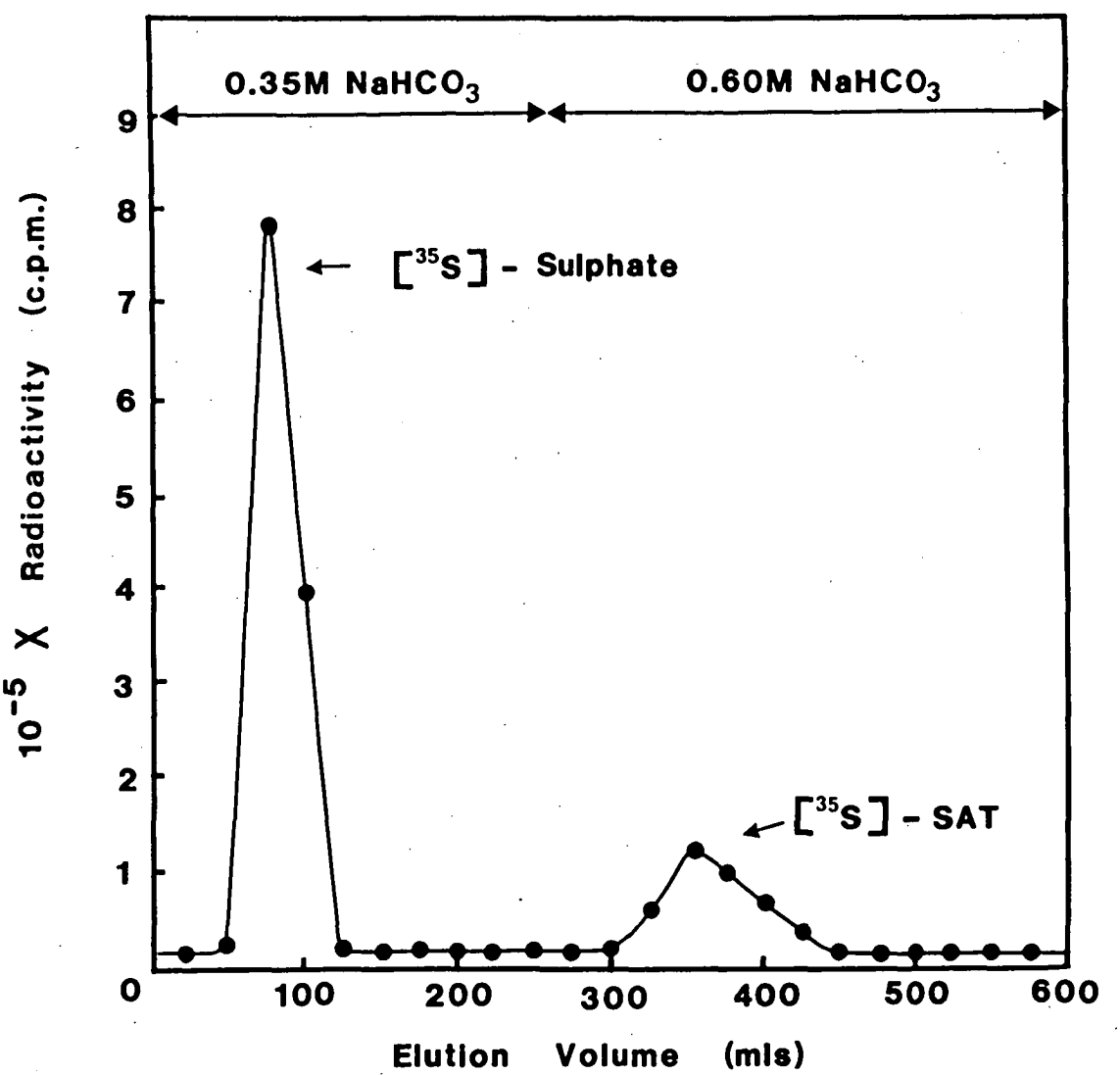
SAMPLE	PEAK [b]	δ (ppm)	PEAKS [c]	δ (ppm)	J_{AB} (Hz)	δ_{AB} (Hz)
AT	1	3.29	3.16		17	10.5
	2	3.12	3.16			
	3	3.09	3.05			
	4	2.92	3.05			
PAT	1	3.69	3.56		18	25.5
	2	3.50	3.56			
	3	3.37	3.31			
	4	3.19	3.31			
SAT	1	3.50	3.38		18	25.0
	2	3.32	3.38			
	3	3.19	3.15			
	4	3.01	3.15			

[a] Determined at pH 7.0 in D_2O, operating frequency = 100MHz, ambient temperature.

[b] See Figs. 3.7 and 4.6.

[c] Value calculated for uninteracting H_A and H_B.

Fig. 4.7 Chromatographic profile during the purification of [^{35}S]-SAT on an AG 2-X8 (HCO_3^-) resin column using sodium bicarbonate buffers. Suitable aliquots were taken from fractions, and the radioactivity determined.



was also shown to be of high purity containing < 0.1% free sulphate and had a hydrolyzable sulphate to nitrogen ratio of 0.98, as determined by chemical analysis. Electrophoretic analysis (Fig. 4.8) supported these findings, showing the final product to be both free of inorganic sulphate and any other detectable radiolabelled contaminants, and to have an identical migration to non-radiolabelled SAT.

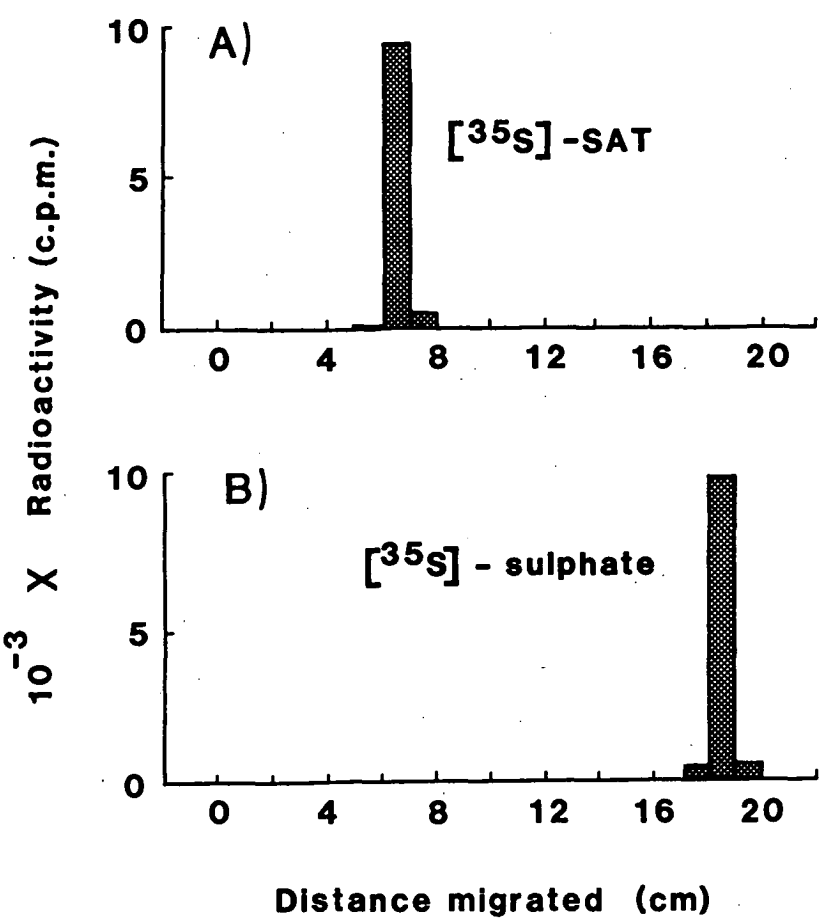
4.5 DISCUSSION

N-sulpho-2-amino tricarballylate was successfully prepared by the sulphonation of AT with pyridine-sulphur trioxide. All forms of analysis, confirmed both the identity and purity of the new compound. The achievement of this synthesis was possible by modifications of the methods originally described by Warner and Coleman (1958) for the sulphonation of amines by pyridine-sulphur trioxide. The purification methods described by these authors, which included a large number of crystallizations and extractions were replaced by steps that were more selective in removal of sulphate and unreacted amine and therefore minimized the loss of the sulphamate (in this case SAT) during purification. With this method, the final purification by anion-exchange chromatography clearly resolved SAT from any residual impurities to yield a pure product. Further, since the removal of excess sulphate was no problem, a large excess of pyridine-sulphur trioxide could be included in the reaction mixture to increase the yield of unlabelled SAT from AT. Whilst the final yield was low compared to the yields obtained by Warner and Coleman (1958) for the preparation of other sulphamates, this possibly reflects the steric hindrance associated with the amino group of AT, a factor discussed in the previous chapter and attributing to the low yield of PAT.

The infra-red spectrum of SAT showed five major absorption bands and of particular interest are those bands associated with the sulphamate portion of the molecule. The absorption bands at 1020-1040cm^{-1} and 1150-1200cm^{-1} are due to the symmetric and asymmetric stretching, respectively, of the S=O bond (Conley, 1966). The band at 1350-1400cm^{-1} is characteristic of sulphonic acid derivatives and its likely assignment is due to the further absorption of the sulphonyl grouping (Schreiber, 1949). The other major absorption bands in the spectrum occurring at 3000-3500cm^{-1} and 1580-1620cm^{-1} would be attributable to O-H and/or N-H stretching, and C=O stretching respectively.

While the infra-red spectrum did not clearly show the presence of the N-S bond in the molecule, the quantitative hydrolysis of SAT by nitrous

Fig. 4.8 A comparison of the electrophoretograms of A) [^{35}S]-SAT, and B) [^{35}S]-sulphate.



acid shown by the sulphate analysis as well as electrophoresis and TLC supported the presence of such a bond. This method of hydrolysis is specific for the cleavage of N-sulphate whereas it does not significantly hydrolyze an O-sulphate linkage (Inoue and Nagasawa, 1976).

The [^{1}H]-NMR of SAT was established at pH 7.0 in D_2O for comparison with the spectra obtained for PAT and AT. As was observed with AT and PAT, a typical AB splitting pattern pattern was produced, due to the mutual coupling of the methylene protons within the compound. The coupling constants (J_{AB}) between the methylene protons were of the same magnitude (17-18Hz) for all three compounds. These values were within the expected range of coupling of 12-18Hz displayed by such H_A-H_B systems (Williams and Fleming, 1973). The position of the signals (δ) within the spectrum of SAT were found to be intermediate between those for PAT and AT; i.e. downfield relative to PAT and upfield of AT. Deshielding produced by the electron withdrawing groups could explain these trends and suggest that in order of strength H_2PO_3-NH- > HO_3S-NH- > H_2N-. However, differences in intramolecular hydrogen bonding and steric factors arising from the introduction of the respective groups may also influence this effect. In particular, these last factors could markedly change the orientation of and increase the restriction of rotation about the methylene bonds of SAT and PAT compared to AT, therefore possibly enhancing the nonequivalence of these protons. This could explain the larger values of δ_{AB} found with PAT and SAT compared to AT. Similar splitting patterns have been observed, and similar relationships were found to exist in characterization parameters, between citrate and PC (Williams and Sallis, 1980). It is likely that factors mentioned above also contribute to these trends.

The method described for the preparation of [^{35}S]-SAT from chloro-[^{35}S]-sulphonic acid also proved to be successful. Since AT could easily be removed with AG 50W-X8 (H^+), this procedure enabled the use of a large excess of the amine for coupling at the second stage of the synthesis to allow a greater degree of incorporation of the radiolabel. While the overall radiochemical yield was low for reasons already discussed, nevertheless the product was of sufficient activity to support several pharmacokinetic experiments involving 20-30 laboratory rats.

4.6 SUMMARY

1. Unlabelled and [^{35}S]-labelled N-sulpho-2-amino tricarballylate were prepared by the new and modified synthetic routes described.

2. Methods for the purification of the products were developed so

as to allow the use of a large excess of pyridine-sulphur trioxide in the synthesis of unlabelled SAT, or AT in the case of [^{35}S]-SAT. The rationale for this was to maximize the yields with respect to the limiting reagent.

3. The analytical systems utilized and developed gave proof as to the identity and purity of the products.

4. Despite low yields, the amount of unlabelled and [^{35}S]-labelled SAT generated was sufficient to accommodate the requirements assessed for <u>in vitro</u> and <u>in vivo</u> studies proposed for the research.

PART B

EVALUATION OF THE STABILITY, METABOLISM AND ANTI-CALCIFYING PROPERTIES OF THE PHOSPHOCITRATE ANALOGUES

CHAPTER 5

THE INHIBITORY EFFECTS OF N-PHOSPHO-2-AMINO TRICARBALLYLATE AND N-SULPHO-2-AMINO TRICARBALLYLATE ON CALCIFICATION IN VITRO

5.1 INTRODUCTION

The formation of renal calculi is a complex and poorly understood phenomenon involving intricate interrelationships between a variety of inhibitors and precipitating salts occurring in a physiological medium. Calcium oxalate and calcium phosphate represent by far the two most common salts that occur as components of stones, and therefore factors that affect their precipitation would be expected to largely influence stone formation. Accordingly, a variety of test systems have been developed to study the various stages of crystallization of these salts *in vitro*, and how these stages are affected by inhibitors. Whilst it is generally well accepted that there is often a lack of correlation between the degrees of effectiveness of calcification inhibitors *in vitro* and *in vivo*, it is nevertheless recognized that an effect on calcification *in vitro* is an essential requirement if a compound is to arrest calcification *in vivo*. For this reason, *in vitro* assays have in the past provided valuable guidelines for determining what new compounds warrant future testing in biological systems with regard to their possible potential as stone-preventing agents. The availability of two new potential inhibitors, namely PAT and SAT, requires then that these compounds undergo extensive testing in well defined systems, which is the basis of the work described in this chapter.

The crystallization of calcium phosphate is a multiphase process, wherein a number of transformable phases exist, differing in the stoichiometric ratios of calcium to phosphorous. Amorphous calcium phosphate (ACP) has been suggested as the precursor of the more crystalline forms both *in vitro* (Meyer and Eanes, 1978) and *in vivo* (Dougherty, 1978; Posner and Betts, 1975). Model systems have been developed therefore to study the inhibition of this transformation. One such system determines the rate of ACP hydrolysis to hydroxyapatite. The developing acidity in the solution accompanying this process can be neutralized by measured base titrimetry. Similarly, models have also been developed to study the effects of inhibitors on the stages of calcium oxalate crystallization. The model

proposed for the present study was the inhibition by test substances of seeded crystal growth from a stable supersaturated solution of calcium oxalate (Meyer and Smith, 1975a).

The mechanism of action of crystallization inhibitors appears to be related to their binding onto preformed crystal surfaces where they inhibit induction of new crystals, growth and aggregation (Fleisch, 1978). The affinity of inhibitors to binding sites, however, does not appear to be directly related to their calcium chelating properties, since EGTA, a strong chelator of calcium, is a poor inhibitor of hydroxyapatite formation (Sutor and Percival, 1978). Nevertheless, past studies have exemplified that certain structural features are common to potent inhibitors of calcification. Whilst early workers proposed that the possession of a minimum of two phosphate groups for potent inhibition of hydroxyapatite formation was essential (Robertson and Fleisch, 1970; Termine and Conn, 1976), the demonstration of the potent inhibition by phosphocitrate (PC) of this transition has since caused modification of such a structure-activity proposal. The minimum requirement for a compound to potently inhibit hydroxyapatite formation, as defined by Williams and Sallis (1982), is the possession of a phosphate group and at some other proximal position, an acidic group. Once this criteria is met, additional factors contribute to the degree of potency of the molecule such as the number of chelating groups, their proximity and stereochemical arrangement, steric factors and lability of the molecule.

One theory recently presented suggests that the hydrophilicity of the molecule and its functional groups are important determinants of the potency of inhibitors of hydroxyapatite formation (Moreno et al., 1984). It is proposed that the adsorption of an inhibitor is dependant on its ability to displace water molecules at the hydration layer of the crystal surface. This might explain, at least in part, why the phosphate moiety, being much more hydrophilic than carboxyl, is a far more powerful contributor to the overall inhibitor potency of a molecule.

Whilst structure-activity relationships of inhibitors of hydroxyapatite formation have been well studied, similar factors relating to calcium oxalate crystallization have been less well formulated. Nevertheless, it does appear that similar requirements exist. Hence PC, PP_i and bisphosphonates have all proven to be potent inhibitors of calcium oxalate and calcium phosphate crystallization in vitro (Williams and Sallis, 1982; Fleisch et al., 1968; Meyer et al., 1977; Meyer and Smith, 1975b).

From all structural and stereochemical considerations of the two

new compounds available, it was expected that PAT would potently inhibit both calcium oxalate and calcium phosphate crystallization. To what degree SAT would inhibit was unpredictable, since no low molecular weight compound containing a sulphamate (or sulphate) has been proven to cause significant inhibition of these processes. Nevertheless, the only structural differences between SAT and citrate (the latter being a weak inhibitor), is the presence of a sulphamate group in SAT substituting for the hydroxy group of citrate. The sulphamate group, which is a more powerful chelator than a hydroxy group should therefore make SAT a more potent inhibitor than citrate. Whether SAT would be as potent as PC or PAT was nevertheless speculative.

The work described in this chapter, therefore, is aimed primarily at establishing the inhibitor potential of these two new PC analogues, and comparing their activity to well-known inhibitors such as PC, PP_i and 1-hydroxyethylidine-1,1-bisphosphonate (HEBP). In addition, the availability of other molecules sharing the tricarballylate skeleton as well as deacetylated derivatives of PAT and SAT, enabled additional investigation into their activity with a view to providing further insight into the structure-activity relationship of calcification inhibitors.

5.2 MATERIALS

PAT and SAT were prepared by methods described in Chapters 3 and 4 of this text. PC was synthesized by the method of Williams and Sallis (1980), and 1-hydroxyethylidine-1,1-bisphosphonate (HEBP) was a gift from the Proctor and Gamble Co., Cincinnatti, Ohio. Other compounds tested were prepared as herein documented or by similar methods to those described for the synthesis of PAT and SAT. AT was formed by hydrolysis of 5,5 di(ethyloxycarbonyl methyl) hydantoin (see Chapter 2) by the method of Dornow and Rombusch (1955). Subjecting trimethyl 2-nitro tricarballylate to base hydrolysis, followed by neutralization [AG 50W-X8 (H^+)] and crystallization led to the production of 2-nitro tricarballylic acid. N-phospho aspartate was prepared from reaction of aspartate and $POCl_3$ in the presence of MgO (Winnick and Scott, 1947) and subsequently purified and characterized by methods described in Chapter 3. N-sulpho aspartate was obtained and characterized in identical fashion to SAT. All remaining compounds studied in the test systems described (including citrate, tricarballylate and PP_i) were obtained from commercial sources, as were other chemicals that were utilized.

5.3 METHODS

5.3.1 INHIBITORY ASSAY - CALCIUM PHOSPHATE

The ability of a test substance to inhibit the transformation of amorphous calcium phosphate (ACP) to hydroxyapatite was assessed using titrimetric base uptake as the measured parameter (Williams and Sallis, 1979). With this method 5.0ml portions of 0.50M $CaCl_2$ and 0.50M NaH_2PO_4 were added with stirring to 600ml of decarbonated distilled water to give a total calcium phosphate concentration of 4.2mM, thus providing the immediate generation of ACP. The pH was quickly adjusted to 7.4 with 0.2M NaOH and maintained during the conversion of ACP to hydroxyapatite by automatic base titration (0.05M NaOH) using a pH-stat (Radiometer, Copenhagen, Denmark). A plot of base uptake against time provides a parameter termed induction time (I_t) as outlined by Meyer and Eanes (1978). In the presence of an inhibitor this value is increased; the difference in induction time with or without an inhibitor being designated ΔI_t (Fig. 5.1). The larger the value of ΔI_t for a given concentration of a compound, the greater is its inhibitory activity. In the present study, values of ΔI_t were evaluated over the concentration range 0-120μM for the different test compounds (Fig. 5.2). Compounds showing little or no inhibition over this range were further tested at a concentration of 200μM. All responses were measured at 25°C.

5.3.2 INHIBITORY ASSAY - CALCIUM OXALATE

A comparison of the potency of test substances to inhibit calcium oxalate crystal growth was made using the method described by Meyer and Smith (1975a). The basis of this technique is the initial generation of a metastable solution of calcium oxalate, followed by the promotion of crystal growth after addition of calcium oxalate seed crystals. In the presence of an inhibitor the rate of soluble calcium depletion (due to calcium oxalate crystal growth) is diminished. The degree of this effect reflects the relative potency of the particular inhibitor. In this study, the compounds tested were PC, PAT, SAT, HEBP and PP_i.

Seed crystals of calcium oxalate were generated as outlined by Meyer and Smith (1975a), following the simultaneous dropwise addition of 100ml 0.4M $CaCl_2$ and 100ml 0.4M potassium oxalate over 2h to 300ml of distilled water with vigorous stirring at 70°C. The suspension was maintained at 70°C for a further 5h before allowing to cool overnight to room temperature. The solid was collected by centrifugation and washed

Fig. 5.1 Inhibitory effect of SAT on the transition of amorphous calcium phosphate (ACP) into hydroxyapatite, and calculation of ΔI_t.

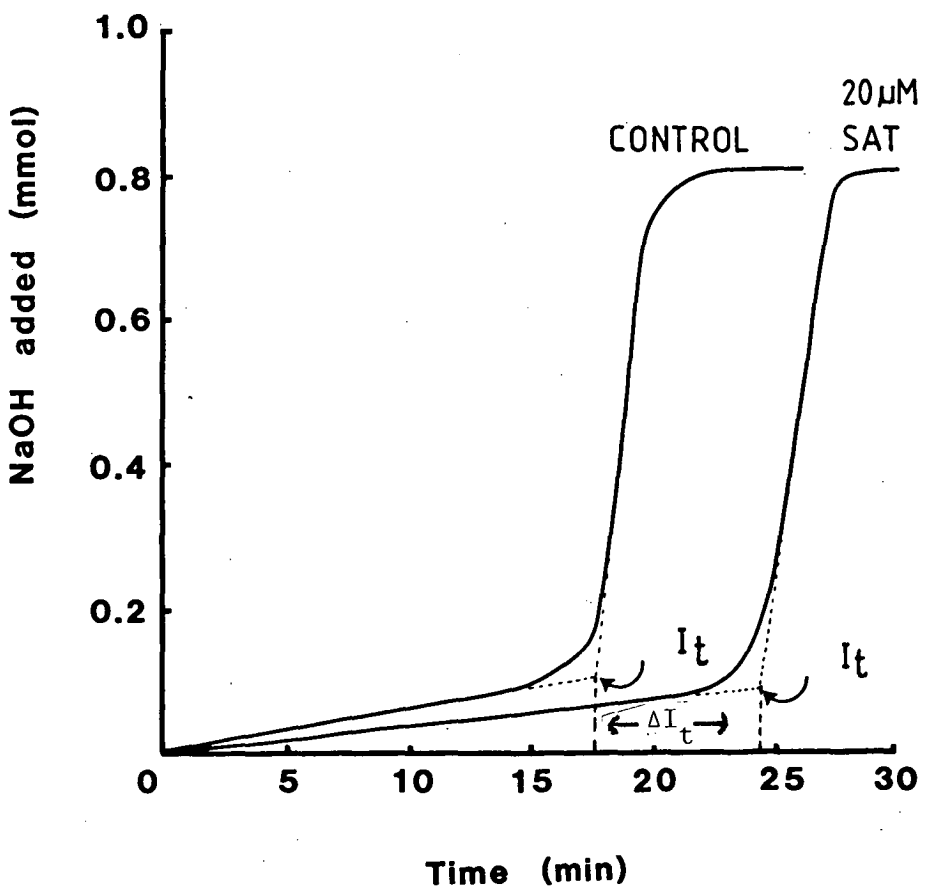
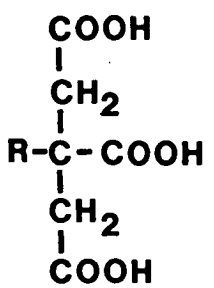


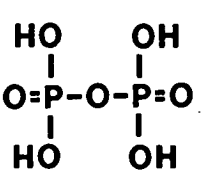
Fig. 5.2 The range of derivatives that were tested for inhibition of calcium phosphate phase transformation and calcium oxalate crystal growth.

TRICARBALLYLATE DERIVATIVES:

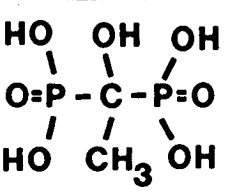


R = -H : Tricarballylate
-OH : Citrate
$-NH_2$: 2-Amino tricarballylate
$-NH-CO-NH_2$: 2-Ureido tricarballylate
$-NO_2$: 2-Nitro tricarballylate
$-O-PO_3H_2$: Phosphocitrate
$-NH-PO_3H_2$: PAT
$-NH-SO_3H$: SAT

PYROPHOSPHATE DERIVATIVES:

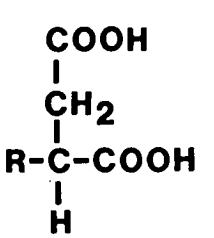


Pyrophosphate



HEBP

ASPARTATE DERIVATIVES:



R = $-NH-PO_3H_2$: N-phospho aspartate
$-NH-SO_3H$: N-sulpho aspartate

repeatedly with distilled water until the supernatant was free of chloride, (as tested by the absence of AgCl formation following the addition of concentrated $AgNO_3$ solution). The solid was dried thoroughly in an oven at 100°C before resuspension in water (18mg/ml). Stock solution required for subsequent experiments was retained and the seed crystals were matured for at least 10 days at 37°C before using in the test system.

To assay, 0.5ml $CaCl_2$ (50mM) and 0.5ml of potassium oxalate (50mM) were added to a stirred solution of 49ml NaCl (0.15M) at 37°C to generate a metastable solution of calcium oxalate. The inhibitor was then added (1.0ml) and the pH adjusted to 6.0. An aliquot (0.4ml) of the above described seed crystal suspension was added to the solution and duplicate samples (2.0ml) immediately removed and filtered through Millipore (0.45μM). Further duplicate samples were taken at appropriate time intervals and calcium in the filtrates was determined by Atomic Absorption Spectrophotometry (Varian Techtron Model 1000, Vic, Australia). Aliquots from the filtrates (0.7ml) were mixed with a lanthanum chloride solution (0.4ml of 50,000ppm La^{3+}), 5M $HClO_4$ (0.2ml) and water (0.7ml). Concentrations were determined on a scale calibrated with standards ranging from 0 to 6μg Ca^{2+}/ml.

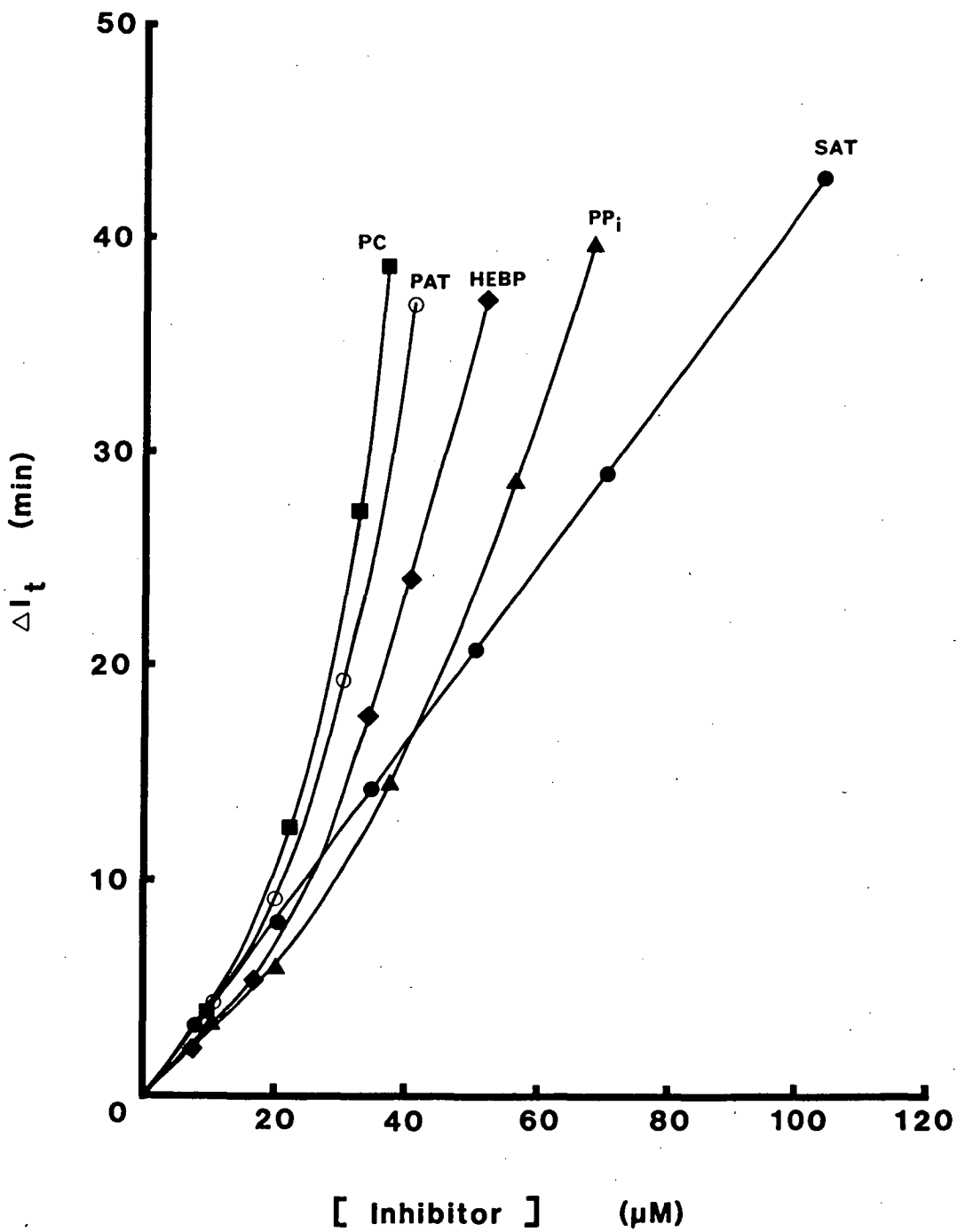
In initial experiments, the level of inhibitor was held constant (27μM) and the rate of calcium depletion measured as a function of time. Samples were taken at 0, 5, 10, 15, 30, 45, 60 and 75min. In subsequent experiments, it was necessary to measure the degree of inhibition in relation to inhibitor concentration; hence samples were taken only at 0 and 60min and the difference found in the soluble calcium concentration was compared then with inhibitor concentration over the range 0-100μM.

5.4 RESULTS

5.4.1 INHIBITION OF THE CONVERSION OF AMORPHOUS CALCIUM PHOSPHATE TO HYDROXYAPATITE

A comparison of potency, as reflected by ΔI_t, between the test compounds found to significantly inhibit the conversion of ACP to hydroxyapatite over the concentration range 0-120μM, is represented in Fig. 5.3. Whilst PC, HEBP and PP_i are already well established as potent inhibitors of this process, of significant note was the inhibition observed by the two new compounds PAT and SAT. In particular, PAT proved extremely potent, in fact almost as potent as PC and significantly more potent than the other inhibitors tested. SAT, as predicted, also proved to be a strong inhibitor.

Fig. 5.3 The effect of different inhibitors at varying concentrations on the transition (ΔI_t) of amorphous calcium phosphate (ACP) to hydroxyapatite.



Although somewhat less powerful than the other inhibitors at high concentrations, SAT nevertheless appeared to be a more effective inhibitor than either HEBP or PP_i at lower concentrations. Hence with this system, at concentrations greater than 40μM, the order of potency was found to be PC ⩾ PAT > HEBP > PP_i > SAT. At levels lower than 40μM, the trend was more complex; nevertheless it was clear that PC and PAT were more potent than the other inhibitors.

In relation to other compounds examined in this system, of the tricarballylate derivatives, only citrate gave some inhibition (ΔI_t = 6.4min) at a concentration of 200μM. Tricarballylic acid and its 2-amino, 2-ureido and 2-nitro derivatives all failed to inhibit at this level. With respect to the deacetylated derivatives of PAT and SAT (i.e. N-phospho aspartate and N-sulpho aspartate), N-phospho aspartate was found to significantly inhibit the conversion of ACP at a level of 100μM (ΔI_t = 20 min). Although much weaker than SAT, it was far more potent than citrate at a similar concentration. N-sulpho aspartate, however, failed to show any inhibition, even at a level of 200μM.

5.4.2 INHIBITION OF CALCIUM OXALATE CRYSTAL GROWTH

The inhibition of calcium oxalate crystal growth by the various inhibitors was studied as a function of time at a set concentration (Fig. 5.4), and as a function of inhibitor concentration, following a fixed incubation period (Fig. 5.5). The results clearly indicate a reversal of some trends noted from studies of the inhibition of ACP to hydroxyapatite conversion. Of major interest was the relatively poor inhibition by PAT, which was shown to be the weakest inhibitor of the five tested over the selected concentration range. Of note, with the time course study, (Fig. 5.4), PAT proved to be an effective inhibitor over the initial phase of incubation, but as time elapsed, its degree of inhibition relative to the other tested inhibitors markedly diminished. This trend was consistent with the chemical breakdown of the molecule under the conditions of the assay (i.e. pH 6.0, 37°C), a factor which was later substantiated from assays determining the chemical labilities of PAT and SAT over the pH range (Chapter 6).

SAT again proved to be an effective inhibitor, although not as potent as the established inhibitors. Nevertheless, as was also evident with the ACP to hydroxyapatite conversion, at low concentrations, SAT appeared to be more powerful than PP_i. The other major observation from the results was that HEBP was by far the strongest inhibitor of calcium oxalate

Fig. 5.4 Effect of inhibitors (27μM) on the rate of depletion of calcium from a seeded metastable solution of calcium oxalate. Results are mean values from duplicate assays.

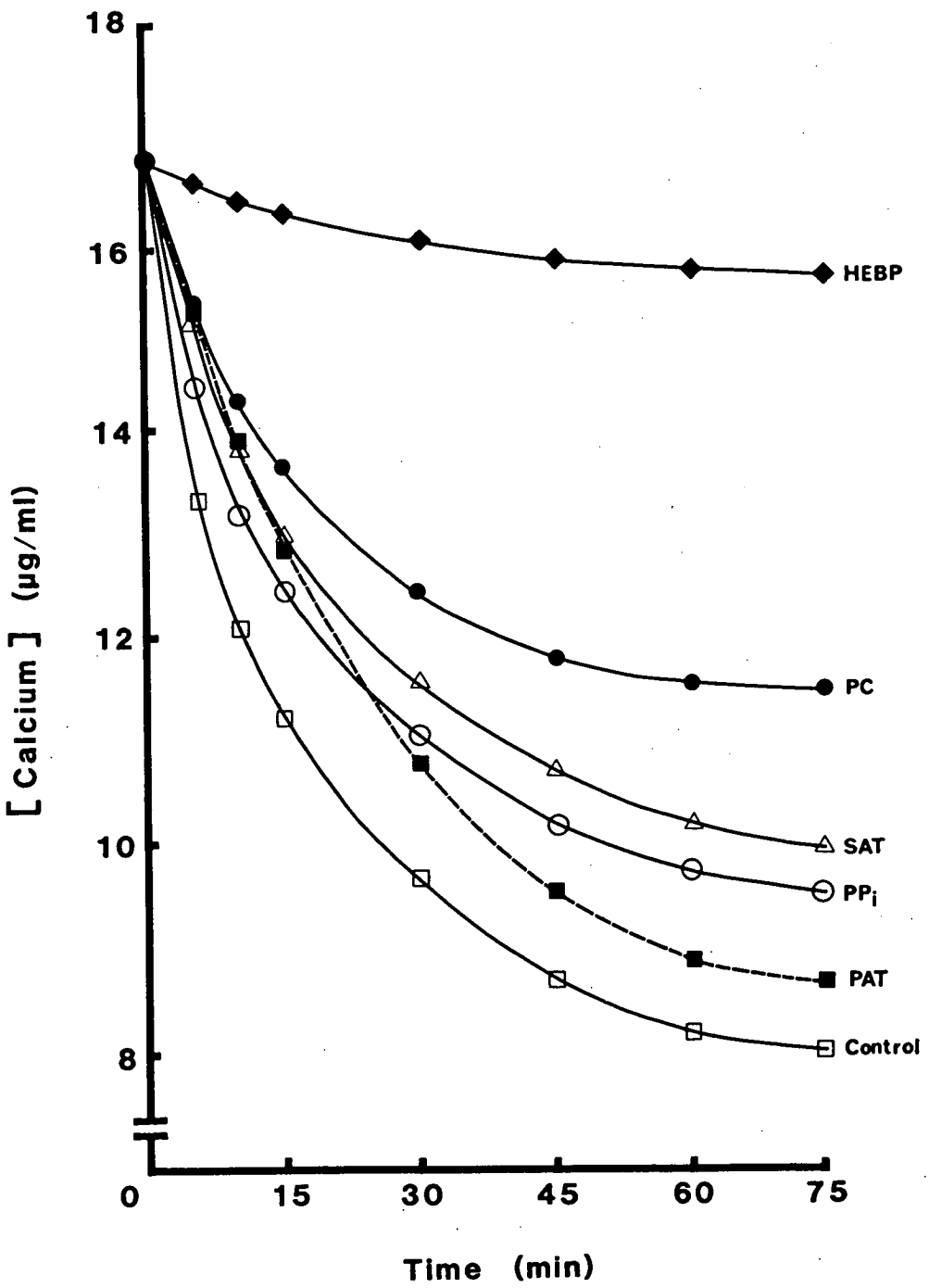
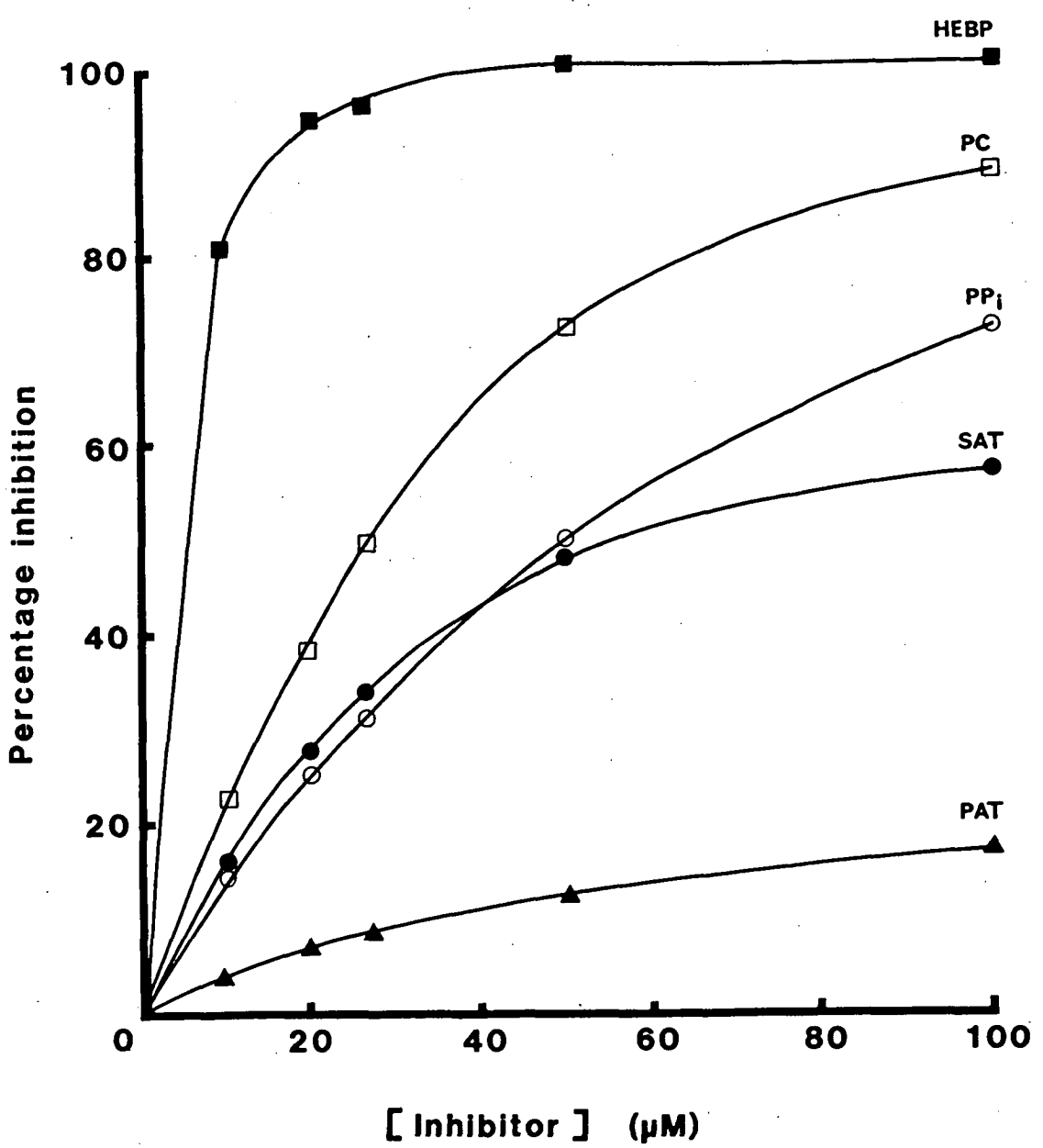


Fig. 5.5 Effect of inhibitors on the depletion of calcium from a seeded metastable solution of calcium oxalate. Aliquots were taken for assaying 0 and 60min after the addition of the seed crystals and the percentage inhibition due to inhibitor calculated by reference to the change in calcium in the control sample over the same time period. Results are mean values from duplicate assays.



crystal growth, being much more potent than the next most effective inhibitor, PC. Again, this was a reversal of the results established previously with the calcium phosphate system. The indicated order of inhibitor potency then was given by HEBP >> PC > $PP_i \geqslant$ SAT >> PAT.

5.5 DISCUSSION

The data presented in this chapter has clearly established the ability of the two new PC analogues PAT and SAT to prevent calcium salt crystallization *in vitro*, and their degree of inhibition relative to well known calcification inhibitors. SAT proved to be an effective inhibitor of both calcium oxalate crystal growth and the conversion of ACP to hydroxyapatite. Although not as good as PC and HEBP, nevertheless its inhibition over a concentration range was generally comparable to PP_i, which itself is also recognized as a strong inhibitor of calcification *in vitro*. PAT proved to be the least powerful compound of those studied with respect to prevention of calcium oxalate crystal growth. This observation was due, at least in part, by the hydrolysis of PAT under the conditions of the assay (pH 6.0, 37°C) as determined in subsequent studies. Under conditions where PAT was more stable, as was the case with the calcium phosphate system (pH 7.4, 25°C), the compound did however prove to be a potent inhibitor, almost as effective as PC. Nevertheless, the lability of PAT under the conditions of the former system, which would more nearly approximate the physiological state expected in normal urine, would limit its probable usefulness *in vivo*.

The degree of inhibitory activity that was attained by PAT in a calcium phosphate crystallization system clearly indicated that the substitution of the oxygen bridge in PC for the secondary amino grouping of PAT, had not significantly altered the properties of the active groups within the molecule in relation to their contribution towards inhibitor potency, proximity or stereochemistry. Robertson and Fleisch (1970) have made a similar observation in comparing the inhibitor potency of PP_i (P-O-P bond) and imidodiphosphate (P-N-P). Further, it has been found experimentally that the P-O-P bond angle is remarkably similar to the P-N-P between these two molecules (Larsen *et al.*, 1969). By analogy it would be anticipated that the P-O-C bond of PC would be similar to that of the P-N-C of PAT.

The further substitution of the PC moiety, i.e. replacement of P for S, results in the formation of SAT, and as experimentally determined, a concomitant decrease in inhibition potential relative to PC with respect to

both calcium oxalate crystal growth and ACP phase transformation. This decrease in potency cannot be rationalized in terms of any change in the stereochemistry of the molecules or steric factors, since by consideration of the hybridization states and theoretical values for covalent radii of nuclei (Kneen et al., 1972) one would expect the S-N-C bond of SAT to be of similar angle and dimensions to that of the P-O-C bond of PC. Further, the size and stereochemistry of the sulphamate and phosphate groups would be similar. Whilst changes in chemical properties of both the carbonyl and hydroxy groups may occur due to the substitution of P for S, perhaps a more significant difference between the two molecules is that the phosphate possesses two ionizable hydroxy groups, whereas the sulphamate possesses only one. It is likely that this factor allows PC to bind to crystal growth sites more efficiently than SAT, and thus makes it a superior inhibitor.

In fact, studies by other workers using bisphosphonates have demonstrated, at least with calcium oxalate crystallization, that inhibitor potential can vary considerably with pH. Meyer et al. (1977), for example found that the amount of HEBP required for 50% inhibition of the described calcium oxalate crystal growth system at pH 5.0 was 200 times more than that required at pH 7.0. Since these molecules contain acidic groups that ionize in this region, it is likely that these observed changes in activity reflect correspondingly different states of ionization of the molecules. Despite this, Meyer and Nancollas (1973), suggest that other subtle structural and electrostatic considerations may be of equal or greater importance in the binding of phosphonates to seeding crystals over the pH range.

The effect of substituting P for S in the basic structure of an inhibitor was further demonstrated by the comparison of N-phospho aspartate and N-sulpho aspartate as inhibitors of ACP transformation. N-phospho aspartate inhibited significantly (although less than SAT) at 100μM, whereas N-sulpho aspartate failed to inhibit even at a concentration of 200μM. The other interesting comparison that could be made was that these compounds could be considered as "deacetylated" derivatives of PAT and SAT respectively. The loss of this extra chelating group in both cases appeared to severely affect the degree of inhibition, i.e. PAT >> N-phospho aspartate, whereas with SAT, loss of this group rendered a product completely devoid of inhibitory power.

Whilst the loss of acetyl groups from PAT or SAT was shown to dramatically reduce the inhibitor potency, the substitution by a hydrogen atom of one of the other chelating arms, i.e that containing either the

phosphoramidate, phosphate or sulphamate linkage of PAT, PC or SAT respectively, gave an even more pronounced effect. Hence tricarballylate showed no inhibition whatsoever, substantiating results of others (Tew et al., 1980). Further substitution at this position to give additional tricarballylate derivatives failed to restore inhibitor potency; citrate, in fact, was the only other compound to show any inhibition, albeit weak. Lack of inhibition after substitution of an amino or ureido moiety at this position reinforces other reports that primary amino groups abolish (Williams and Sallis, 1982; Moreno et al., 1984), or at least in this case, do not enhance inhibitor potential. While the oxygen atoms of nitro groups may have some acidic nature when bound to non-tertiary carbon atoms due to resonance stabilization, in the case of 2-nitro tricarballylate this is not likely as the nitro is bound to a tertiary carbon. Therefore, any chelating capacity of the group would be lost and the group would be unlikely to contribute to the inhibitor potency of the molecule. Hence, 2-nitro tricarballylate was found to have no effect on the inhibition of ACP transformation. However, other factors may also contribute, such as the trigonal stereochemistry or other physical or chemical properties of the nitro group.

Another significant finding from the results presented here was that PC proved a markedly more potent inhibitor than HEBP in the transformation of calcium phosphate, whereas with calcium oxalate crystal growth, their relative potencies were reversed. This indicates that certain inhibitors have greater affinity for specific calcium salts; yet it remains hard to rationalize this observation in terms of structure. This factor is further highlighted by the recognition that structurally similar compounds may act by inhibiting different phases of crystal transformation and/or bind to different sites upon the same crystal surface. For example, Williams and Sallis (1982), have made a clear distinction between two types of inhibitors of ACP transformation, on the basis of how their increasing concentrations affect the parameters defining the transition. While potent inhibitors from both these classes shared the minimum structural requirements as previously defined, no common structural difference was found between these two types despite the fact that the types appeared to inhibit through a different mechanism. It was proposed that the "type I" inhibitors elicit their affect by stabilizing hydroxyapatite embryos and inhibiting further growth by binding to active growth sites, whereas type II inhibitors are considered to act mainly by binding to and decreasing the lability of ACP. Examples of type I inhibitors were PC, HEBP and PP_i, and

"type II" inhibitors ATP and phosphonoformate. No attempt was made here to separate the two new compounds into these two inhibitor type categories, i.e. the overall degree of inhibition by test compounds of the transformation of ACP to hydroxyapatite was considered more relevant than the precise mechanism of action of these inhibitors.

The present study therefore has been restricted to examining the inhibitory effects in two models; one involving the calcium phosphate phase transformation and the other, calcium oxalate crystal growth. It should be stressed that no attempt was made to look at the affect of inhibitors on nucleation or crystal aggregation. Whilst it is clear that these are important aspects of stone development and maturation in vivo, the problem lies in the difficulty associated with studying these stages independantly in vitro, i.e. it is difficult to separate nucleation and crystal growth, and crystal growth and aggregation. Nevertheless, it does appear that the particular models studied were pertinent. Hydroxyapatite is itself a major stone constituent and a precursor to other more mineralized forms that are also major stone constituents. Therefore transformation of ACP to hydroxyapatite is often an important factor influencing stone formation. Crystal growth, which was the studied parameter with the calcium oxalate system, may possibly be the most important factor in the formation of a calculus, because if growth is arrested, the initially precipitated small crystalline nuclei seem to pass harmlessly to the urine (Meyer and Smith, 1975a).

An attempt has been made to compare both the structural and stereochemical features of a variety of compounds, especially the two new compounds PAT and SAT, with inhibitor potency. Results presented here clearly exemplify the importance of the unique stereochemical configuration that PC, PAT and SAT all share; that is, the possession of a carbon atom from which four strong chelating arms radiate in a tetrahedral fashion. This becomes evident when the structure is disturbed. Removal of only one of these chelating groups, or even replacement of a group by another of lower chelating capacity, was shown to markedly diminish, if not abolish, the degree of inhibition observed. For this reason, SAT, although proven as a powerful inhibitor in its own right, was less potent than PC, since the sulphamate moiety is a weaker chelator than phosphate. It is likely that the tetrahedral arrangements of these molecules confers upon them special properties giving them extra affinity for crystal growth sites, and hence make them equal if not better inhibitors of calcification than compounds containing two phosphate groups, such as the PP_i derivatives. In relation to any future possible therapeutic potential deriving from the new

compounds, PAT would appear to be restricted on the grounds of chemical lability. SAT, on the other hand, even if proven here to be a less potent inhibitor *in vitro* than PC and HEBP, nevertheless might be useful if it could be demonstrated that a) it were more stable to hydrolysis than PC and b) it was less toxic and better absorbed than HEBP.

5.6 SUMMARY

1. PAT was found to inhibit potently the transformation of ACP to hydroxyapatite, almost to the same degree as PC. SAT, although somewhat weaker in this system was nevertheless comparable to PP_i at lower concentrations.

2. The ability of PAT to inhibit calcium oxalate crystal growth appeared limited; this was later found to be attributable to its hydrolysis under the conditions of the assay. SAT proved to be more potent than PAT under these conditions, and again at low concentrations was equipotent relative to PP_i.

3. Any severe disruption of the unique structure of PC by removal or modification of any of the four radiating chelating groups caused a marked reduction in inhibitor potential. This was most evident when the phosphate group was substituted; hence for this reason SAT was less active than PC, and tricarballylate had no activity. Removal of an acetyl group also caused a marked, but less severe decrease on the degree of inhibition.

4. Results presented here did confirm that the sulphamate moiety can contribute to the inhibitor potency of a molecule.

5. PC was found to be the most potent inhibitor tested in the transformation of ACP into hydroxyapatite, whereas with calcium oxalate crystal growth, HEBP was found to be by far the most effective inhibitor.

CHAPTER 6

THE CHEMICAL AND BIOLOGICAL STABILITY OF N-PHOSPHO-2-AMINO TRICARBALLYLATE AND N-SULPHO-2-AMINO TRICARBALLYLATE

6.1 INTRODUCTION

With the establishment of the inhibitory power of the PC analogues in vitro, it remained to be determined whether these compounds possessed properties that would make them suitable as anti-calcifying agents in vivo. Factors that needed to be considered following administration of the molecule were absorption, tissue distribution and metabolism, clearance to urine, and any indication of toxic effects that could arise, eg. at the bone site following prolonged administration. It is the purpose of this chapter to gain insight into these areas by studying such properties of SAT and PAT.

Although radiolabelled PAT was not available, it was hoped to gain insight into the stability of the phosphoramidate linkage of this molecule by studying its hydrolysis in vitro under varying conditions of temperature, pH and in the presence of hydrolytic enzymes. Radiolabelled SAT was however available, and in addition to its value for routine in vitro assays which have just been described, the [^{35}S]-label made it possible to study some of the pharmacokinetic properties of SAT, in particular its absorption following oral administration, stability, binding to the bone site and clearance to the urine. This chapter also describes analytical techniques that were developed to aid in the separation and quantitation of [^{35}S]-SAT and its possible metabolic products from body tissue.

6.2 MATERIALS

N-phospho-2-amino tricarballylate (PAT), unlabelled N-sulpho-2-amino tricarballylate (SAT) and [^{35}S]-labelled SAT were prepared by methods described in the preceding chapters. [^{35}S]-SAT was diluted to the appropriate specific activity prior to use with unlabelled SAT. Calf intestine alkaline phosphatase (E.C. 3.1.3; 400 units/0.17ml), and limpet sulphatase (E.C. 3.1.6.1; 14.5 units/mg solid) were supplied by Boehringer-Mannheim, Melbourne, Australia and the Sigma Chemical Company, Sydney, Australia respectively. The scintillation fluid consisted of 18ml of a mixture of 0.6% 2,5-diphenyloxazole (PPO) in toluene and 2-ethoxy ethanol (10:6, v/v).

6.3 METHODS

6.3.1 THE CHEMICAL STABILITY OF PAT

The chemical stability of N-phospho-2-amino tricarballylate (PAT) was determined under various conditions of pH and temperature. Hence the hydrolysis of the molecule was investigated at pH values of 3.0, 6.0, 7.4 and 10.4 (the buffers were glycine/HCl, citrate/NaOH, Tris/HCl and glycine/NaOH respectively at 0.1M final concentration) at 37°C, pH 7.4 and 0.1M HCl at 25°C and 1.0M NaOH at 100°C. The rate of hydrolysis was monitored by the method of Winnick and Scott (1947), which is based on the strong absorbance of phosphoramidate linkage at 225nm in aqueous solution. At this particular wavelength the hydrolytic products have negligable absorbance. To perform the assay, 10mg of PAT dissolved in 0.5ml of water was added to 2.5ml of buffer, acid or base (final concentration of PAT = 7.5mM), the solution was thoroughly mixed and then scanned for a decrease in optical density at 225nm with time. Solutions containing an equivalent amount of P_i and AT (i.e. hydrolyzable products) were used as blanks. Unfortunately the limited availability of PAT restricted duplicate experiments being performed.

6.3.2 HYDROLYSIS OF PAT BY ALKALINE PHOSPHATASE

Winnick and Scott (1947) have reported that the phosphoramidates, phosphoglycine and phosphocreatine, are cleaved by alkaline phosphatase *in vitro*. From further investigations (Winnick, 1947) it appeared that alkaline phosphatase was a major enzyme accountable for phosphoramidate hydrolysis, and so for the present studies, it seemed essential that the rate of hydrolysis of PAT could be determined in the presence of this enzyme. To monitor this process, the spectrophotometric method described above was unsuitable, because the blank reading due to enzyme alone was too high. The method that was adopted was essentially that of Winnick (1947), whereby aliquots from the incubation were taken at various time intervals, and P_i determined by the method of Lowry and Lopez (1946). Hence, PAT (10mg; 22µmol) was added to 0.1M glycine/NaOH buffer pH 10.4 (4.5ml) at 37°C, and the reaction quickly initiated by the addition of 1 unit of alkaline phosphatase in the same buffer (0.5ml). The mixture was immediately stirred, and an aliquot taken (0.2ml) for P_i analysis and further aliquots taken every 5min. The aliquots were quickly deproteinized after removal by the addition of saturated ammonium sulphate in 0.1M sodium acetate (pH 4.0; 0.8ml), and after rapid centrifugation, the supernatants

were diluted to 5ml with 0.1M acetate buffer pH 4.0. Analysis for P_i was then carried out in identical fashion to that described by Lowry and Lopez (1946). Blanks including enzyme alone and PAT alone in the same buffer were assayed in parallel runs. Unfortunately, restrictions on the amount of PAT available, did not permit duplicate experiments.

6.3.3 THE CHEMICAL STABILITY OF SAT

The assessment of the chemical stability of SAT was made from studies at pH 7.4 and 37°C using 0.1M Tris/HCl buffer, 0.1M NaOH (100°C), and 0.1M HCl (100°C and 25°C). SAT (4.5mg; 10μmol) was dissolved in 1ml of water and then added to the appropriate solution (4ml) to give a final concentration of base, acid or buffer as specified above. In the case of acid or base, aliquots (0.5ml) were taken at 0, 5, 15 and 30min, and 1, 2, 6, 12 and 24h from the initiation of the incubation, and frozen until all aliquots were collected. The hydrolysis of SAT at pH 7.4 and 37°C was determined over a longer time interval; hence aliquots were collected every day for 8 days and frozen until all samples were ready. The degree of hydrolysis of SAT was then determined by estimating for free sulphate present in the aliquots using the turbidimetric assay outlined in section 4.3.2.1.

6.3.4 THE ACTION OF ENZYMES ON SAT IN VITRO

The actions of rat spleen aryl sulphatase, sulphamatase and limpet sulphatase on SAT were investigated.

6.3.4.1 The Isolation of Aryl Sulphatase from Rat Spleen and its Action on SAT

Aryl sulphatase was purified from rat spleen by the method of Friedman and Arsenis (1974). In brief, this method consisted of homogenization of a rat spleen, treatment with Triton X-100 (0.1%), centrifugation and fractionation of the supernatant by iso-electric focusing for final purification. Fractions collected were assayed for aryl sulphatase activity using p-nitrocatechol sulphate (p-NCS) as substrate by the method of Roy (1953). The fraction with highest activity (and specific activity) was isolated, dialyzed to remove ampholine and lyophilized. The dried product was redissolved in 0.5M sodium acetate buffer, pH 5.0 (200μl) to give the final enzyme preparation.

At the time of this assay [^{35}S]-SAT was unavailable, so unlabelled SAT had to be used as substrate. Hence SAT (9mg; 20μmol) or p-NCS (5μM)

dissolved in 1.0ml was added to 0.5M acetate buffer, pH 5.0 (1.0ml) and the tubes were brought to 37°C before the addition of 20µl of enzyme preparation. Aliquots (0.1ml) from the tubes containing p-NCS were taken after 30 and 60min, and the hydrolysis determined by the amount of p-nitrocatechol released (Roy, 1953). Aliquots (0.3ml) from tubes containing SAT were taken at 1, 12 and 24h after initiation of the experiment and assayed for inorganic sulphate (4.3.2.1).

6.3.4.2 Sulphamatase

The method described above for the isolation of aryl sulphatase from rat spleen has also been utilized by Friedman and Arsenis (1974) to isolate heparin sulphamatase, an enzyme capable of cleaving the sulphamate linkage of heparin. Attempts to recover such a fraction in this work were unsuccessful; i.e. no fraction recovered contained any measurable heparin sulphamatase activity. This could be due in part, to the fact that the same investigators had the availability of [^{35}S]-heparin for assaying, whereas in the present study the less sensitive technique of detection of liberated sulphate by chemical assay was used. In addition, the activity of heparin sulphamatase, although higher in spleen than any other tissue, is still relatively low, i.e. its activity is 0.2% to that of aryl sulphatase in this tissue, and hence is only detected following prolonged incubations with substrate.

The failure to isolate this enzyme, coupled with the fact that sulphamatase enzymes are not commercially available, led to the submission of a sample of SAT for analysis elsewhere. Dr. John Hopwood, Department of Chemical Pathology, The Adelaide Childrens Hospital, Adelaide, South Australia kindly performed the following assay. SAT was incubated at 37°C with human liver sulphamatase at pH 5.0 for 4 days; under these conditions total hydrolysis of standard sulphamatase substrates (eg. glucosamine-, galactosamine-sulphate) normally occurs within 8h. The reaction was monitored using an amino acid analyzer to detect liberated AT.

6.3.4.3 Sulphatase

To determine whether SAT was degraded by limpet sulphatase, [^{35}S]-SAT (1mg; 0.5µCi/mg) was incubated in 0.2M sodium acetate buffer pH 5.0 (0.2ml) with or without limpet sulphatase (7.2 units/0.5mg) for 6h at 37°C. At the end of the incubation, aliquots (10µl) from both test and control incubations were subjected to high-voltage electrophoresis for analysis of the radiolabelled products (section 4.3.4). The availability of

[^{35}S]-SAT made this assay more sensitive than the previous one using aryl sulphatase.

6.3.5 METABOLISM OF SAT IN VIVO

6.3.5.1 Administration of [^{35}S]-SAT to Rats and Collection of Samples

The metabolic fate of the [^{35}S]-SAT isotope was studied in 100-200g male Wistar rats following administration either by intravenous, intraperitoneal or oral means. In all experiments, rats were maintained on normal dietary and drinking rations throughout the study. Unless otherwise specified, results were obtained from duplicate experiments and were expressed as average values. Limitations on the amount of radiolabel available restricted the number of experiments possible.

Intravenous administration was found to be the best route for studying the properties of SAT in terms of the compound's uptake from the blood into tissues, subsequent release from tissue back into circulation, and its clearance to urine. The isotope (3µCi/6mg/100µl) was administered through the tail vein of rats anaesthetized with ether and whose ureters were ligated. At predetermined times (0-2h), rats were exsanguinated and blood collected in heparinized tubes. The body cavity was opened and the kidneys and liver removed, weighed and freeze-clamped. The bladder plus contents (which was taken as a measurement of the counts cleared from the kidney to urine) were excised and placed in a vial. Bone was taken from fore and hind limbs, the cartilaginous ends removed, and the marrow blown out. All samples were quickly frozen in liquid nitrogen and then stored in vials at -15^{o}C until analyzed.

In further experiments when the distribution and clearance of [^{35}S]-SAT was determined 24h after administration, urine was collected by placing rats in metabolic cages rather than through ureter ligation.

For studies utilizing intraperitoneal administration, the samples were collected in identical fashion to that described for intravenous administration.

To evaluate the absorption of SAT, rats were administered orally via a blunted tuberculin syringe 3µCi/6mg SAT/0.4ml, and placed in metabolic cages for 3 days. Urine and faeces were collected every 24h, and at the end of the 3rd day the rats were sacrificed and tissues collected as before.

6.3.5.2 Measurement of Total Counts

6.3.5.2.1 Tissue, Blood and Urine

Frozen tissue collected previously was ground to a fine powder using a mortar and pestle previously chilled in liquid nitrogen; the liquid samples were simply allowed to thaw. The total counts present were then determined following digestion by the method of Mahin and Lofberg (1966). Urine (200µl) (or entire bladder plus contents) and blood (200µl) or powdered tissue (200mg) were added to glass vials followed by the addition of 70% $HClO_4$ (200µl) and 30% H_2O_2 (400µl). Vials were sealed with tops resistant to digestion conditions, and then heated at 80°C for 4-6h to ensure complete digestion. Upon cooling, scintillation fluid (18ml) was added to the vials, and the radioactive counts measured.

Knowing the weight of liver, kidneys, or the volume of the urine, together with the total skeletal weight and blood volume calculated from the body weight and sex of the animal used (Donaldson, 1920; Altman and Dittmar, 1974); the measured counts could be converted to whole tissue counts. When bladder plus contents were measured instead of urine, total counts were determined directly.

6.3.5.2.2 Faeces

Total counts in faeces were estimated in the following manner. Faeces were collected from metabolic cages, dried (100°C), ground to a powder and then extracted by the addition of 1M $HClO_4$ (20ml/10g faeces) with vigorous stirring for 2min. The suspension was allowed to stand for 30min, shaken again and then centrifuged. An aliquot from the supernatant (2.0ml) was taken and added to scintillation fluid (18ml) for counting.

6.3.5.3 Analysis of the Specific Components Contributing to the Radioactivity in Samples

Prior to any ultimate analysis or quantitation, there was a requirement to recover the radiolabelled metabolites from tissue samples and also to remove interfering substances. It was essential too that treatment did not modify the metabolites (e.g. hydrolyze SAT), that the technique was rapid and capable of handling a large number of samples simultaneously. A variety of methods were tested for the fulfilment of these criteria. The methods were evaluated following the addition of [^{35}S]-SAT and/or [^{35}S]-sulphate to blood, urine or ground tissue from untreated rats and ultimately from rats administered [^{35}S]-radioisotope.

The yield of radioisotope was determined by comparison with the counts obtained from whole tissue oxidation.

Three different methods were tested that subsequently proved unsuitable for the analysis and are summarized as follows.

(i) Extraction of samples by trichloroacetic acid (TCA): Samples were deproteinized by ice-cold TCA, and the TCA and other interfering substances removed by extraction into an organic phase containing tri-N-octylamine. This method had been used successfully by Williams and Sallis (1981) for the recovery of [^{32}P]-PC and derived metabolites from biological tissue. However its application here was unsatisfactory since the recovery of [^{35}S]-SAT was < 40%.

(ii) Deproteination by HCl: Deproteination of samples was effected with ice-cold 1M HCl, and excess HCl was removed by lyophilization. Whilst recovery of isotope was acceptable with this method (80%), the final sample had a high salt concentration which interfered with the ultimate electrophoretic analysis of the radiolabelled products.

(iii) Homogenization and ultrafiltration of samples: This was based on the method described by Acuff and Smith (1981) whereby [^{35}S]-sulphate is extracted quantitatively from tissue. Tissue was homogenized in a NH_4OH/EDTA medium, centrifuged to remove cellular debris, and the supernatant deproteinized by ultrafiltration. However several practical limitations were apparent with this procedure. Firstly, each ultrafiltration took 30min and since only one ultrafiltration unit was available, this meant it would take in excess of 12h to ultrafilter all the samples. Further, the recovery of added [^{35}S]-SAT with this technique was no more than 70%, due to the large amount of liquid volume not recovered. In addition, the final sample had a high salt concentration that also proved to interfere with the analysis of the radiolabelled products.

The method of sample treatment finally adopted and now detailed made use of the already established chromatographic system described in Chapter 4 which had been shown capable of the excellent resolution of both SAT and sulphate from other possible contaminants. Hence with this method tissue samples (0.8-1.0g) or blood and urine samples (1.0ml) were initially deproteinized with ice-cold 1M $HClO_4$ (1.5ml). Dropwise addition of $HClO_4$ was accompanied by vigorous stirring and the resultant suspension immediately centrifuged. The supernatant was retained and the precipitate washed with ice-cold water (1.0ml) before further centrifugation. The supernatants were combined, neutralized by the addition of 0.5M K_2CO_3 (0.8ml) and cooled to 4^{o}C. Precipitated $KClO_4$ was removed by centrifugation

and the supernatant was retained for ion-exchange chromatography.

The neutralized extract was applied to a column of AG 2-X8 (HCO_3^- form; 1cm x 5mm) and after a preliminary washing with 0.15M $NaHCO_3$ (20ml), radiolabelled components were eluted with 0.60M $NaHCO_3$ (35ml) at a flow rate of 0.5ml/min. The eluant was desalted by the addition of excess AG 50W-X8 (H^+) with stirring at 4°C for 10min, the suspension then filtered and neutralized before lyophilization. This process was repeated to ensure complete removal of bicarbonate.

In early work, treatment was stopped following perchloric acid deproteinization and neutralization with K_2CO_3. The neutralized extract was then lyophilized and redissolved for analysis by electrophoresis. Unfortunately, as was found previously, a high salt concentration in the sample restricted the movement of spots from the origin, and this led to the further purification of the sample by the described ion-exchange chromatography system.

The overall recovery for this procedure proved to be 85-90% relative to that determined by whole tissue oxidation and analysis. This was true for all tissues studied and the recoveries were identical both for [^{35}S]-SAT and [^{35}S]-sulphate. When a mixture of [^{35}S]-SAT and [^{35}S]-sulphate was added to a tissue homogenate and then treated in this fashion, clear resolution of the radiolabelled components was obtained by electrophoresis of the final sample. Further, electrophoretic analysis showed that following addition of either [^{35}S]-SAT or [^{35}S]-sulphate only to a homogenate, no chemical modification (e.g. hydrolysis) occurred by the technique described. In relation to the recovery of radioactivity from biological material following administration of [^{35}S]-SAT <u>in vivo</u>, again the radioactivity recovered following this treatment was 85-90% of that determined for whole tissue oxidation.

6.3.5.4 <u>Analysis of Products by High-Voltage Electrophoresis</u>

The proportion of counts attributable to [^{35}S]-SAT was determined by high-voltage electrophoresis. Lyophilized extracts were redissolved in 150µl, an aliquot (50µl) was taken for counting and a further aliquot (20µl) was subjected to analysis by high-voltage electrophoresis (4.3.4). Standards (i.e. standard [^{35}S]-SAT, [^{35}S]-sulphate and unlabelled sulphamate dissolved in tissue extracts from untreated rats) were included in parallel runs to determine their migration, and by comparison, the proportion of counts attributable to each component determined in the test sample.

6.4 RESULTS

6.4.1 THE CHEMICAL STABILITY OF PAT

PAT was shown to be extremely labile at neutral or acid pH (Fig. 6.1). Under conditions that approach the normal physiological state, i.e. pH 7.4 and 37°C, PAT underwent 60% hydrolysis within 1h. At pH values below this, PAT became more labile with increasing acidity, and at 0.1M HCl and 25°C, complete hydrolysis was evident within 20 seconds (not shown). At alkaline pH, PAT became increasingly more stable with only 2% hydrolysis evident in 4h at pH 10.4 and 37°C. Similarly, when PAT was incubated at 100°C in 1.0M NaOH, the amount of hydrolysis accountable after 4h was 5%.

6.4.2 HYDROLYSIS OF PAT BY ALKALINE PHOSPHATASE IN VITRO

Although PAT was shown to be chemically stable at pH 10.4 and 37°C, under these conditions alkaline phosphatase was shown to readily lyze the phosphoramidate linkage of PAT (Fig. 6.2). Results show that total hydrolysis was evident within 1h, and the reaction rate for this hydrolysis was approximately 18 times that found for the hydrolysis of PC by the same enzyme under similar conditions (Williams, 1981).

6.4.3 THE CHEMICAL STABILITY OF SAT

SAT proved to be far more stable than PAT over the pH range, as shown in Fig. 6.3. As was found with PAT, SAT appeared to be more stable at alkaline rather than acid pH. At 0.1M NaOH and 100°C, only 5% hydrolysis was evident after 24h, whereas quantitative hydrolysis occurred within 5min at 0.1M HCl and 100°C. However, under the same acid conditions but at a temperature of 25°C, the compound was relatively stable, with only 40% hydrolysis after 24h. At 37°C and pH 7.4 (not shown) SAT appeared completely stable, with no detectable hydrolysis of SAT occurring, even after 8 days.

6.4.4 THE ACTION OF ENZYMES ON SAT IN VITRO

SAT was shown to be resistant to the actions of both aryl sulphatase derived from rat spleen and limpet sulphatase in vitro. The aryl sulphatase preparation proved to be extremely active in cleaving p-NCS (20units/20µl), but when SAT was used as substrate (20µmol) there was no evidence of liberated sulphate from chemical analysis after 24h. The availability of [^{35}S]-SAT with the assay using limpet sulphatase made detection of SAT cleavage products more sensitive. Nevertheless after

Fig. 6.1 The hydrolysis of PAT under varying conditions of pH and temperature. Values are plotted from individual experiments and calculated following the scanning of solutions at 225nm, as described in the text.

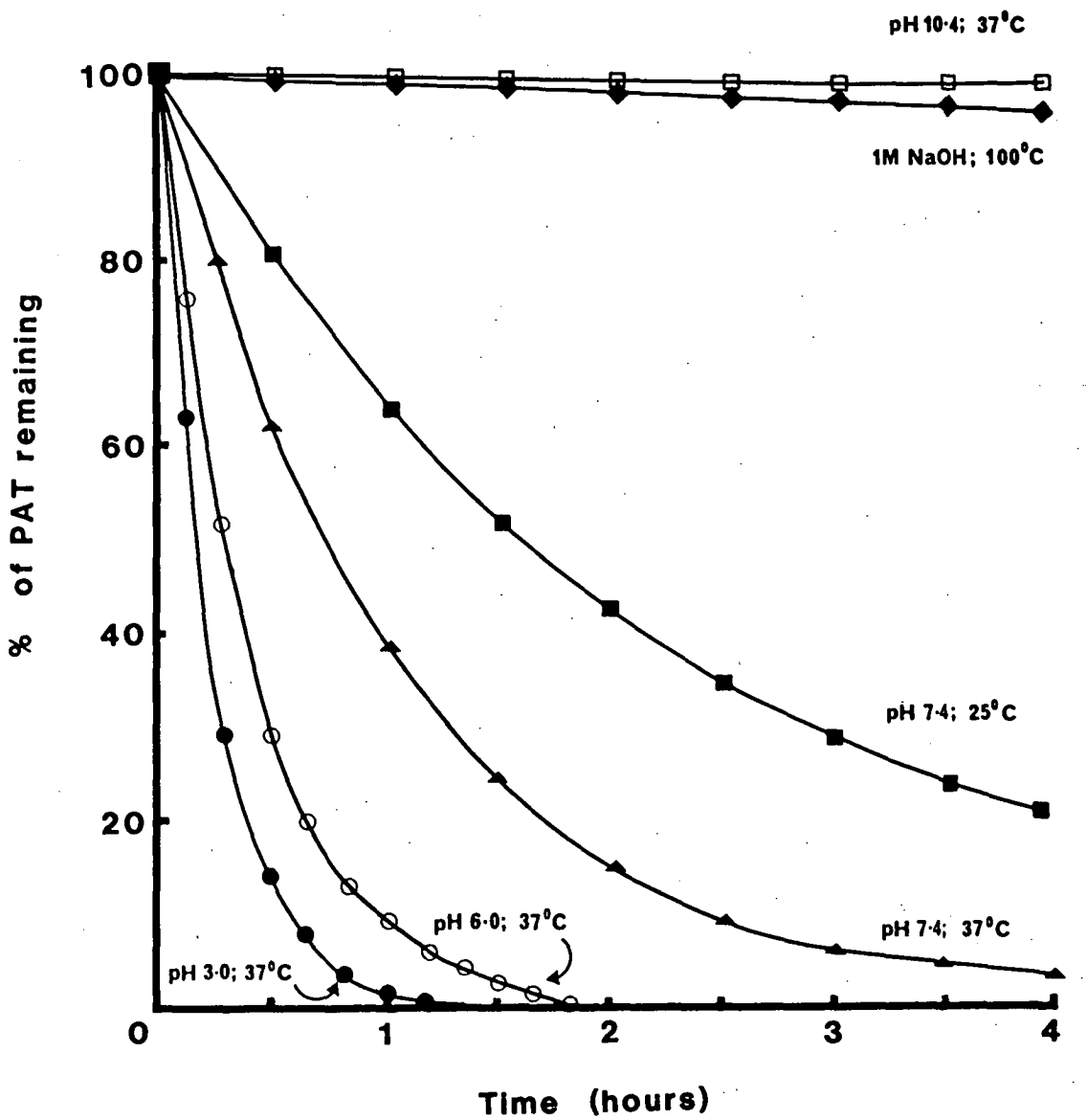


Fig. 6.2 The degradation of PAT by alkaline phosphatase. PAT (22µmol) was incubated with 1 unit of alkaline phosphatase at pH 10.4, and at specified time intervals, aliquots were taken and the extent of hydrolysis determined by the quantitation of free phosphate present.

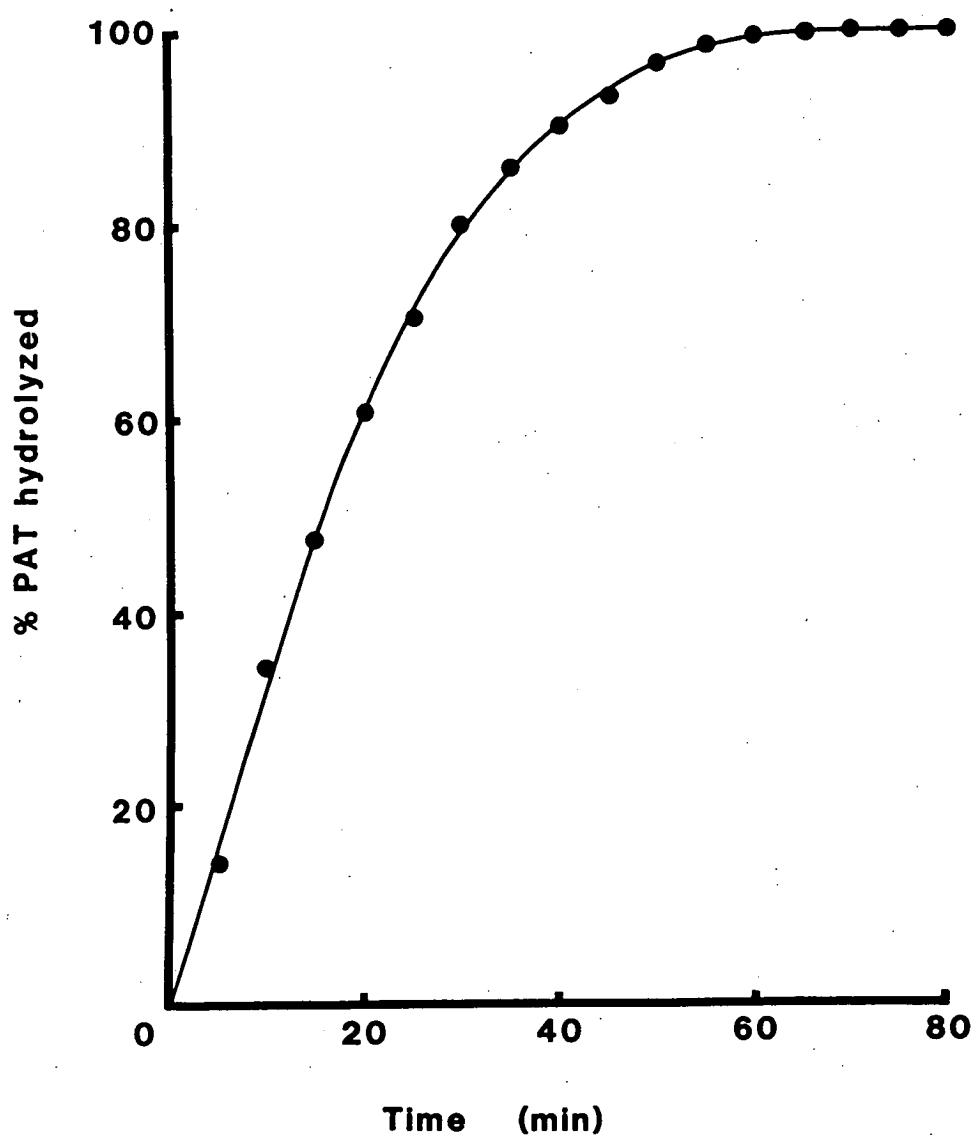
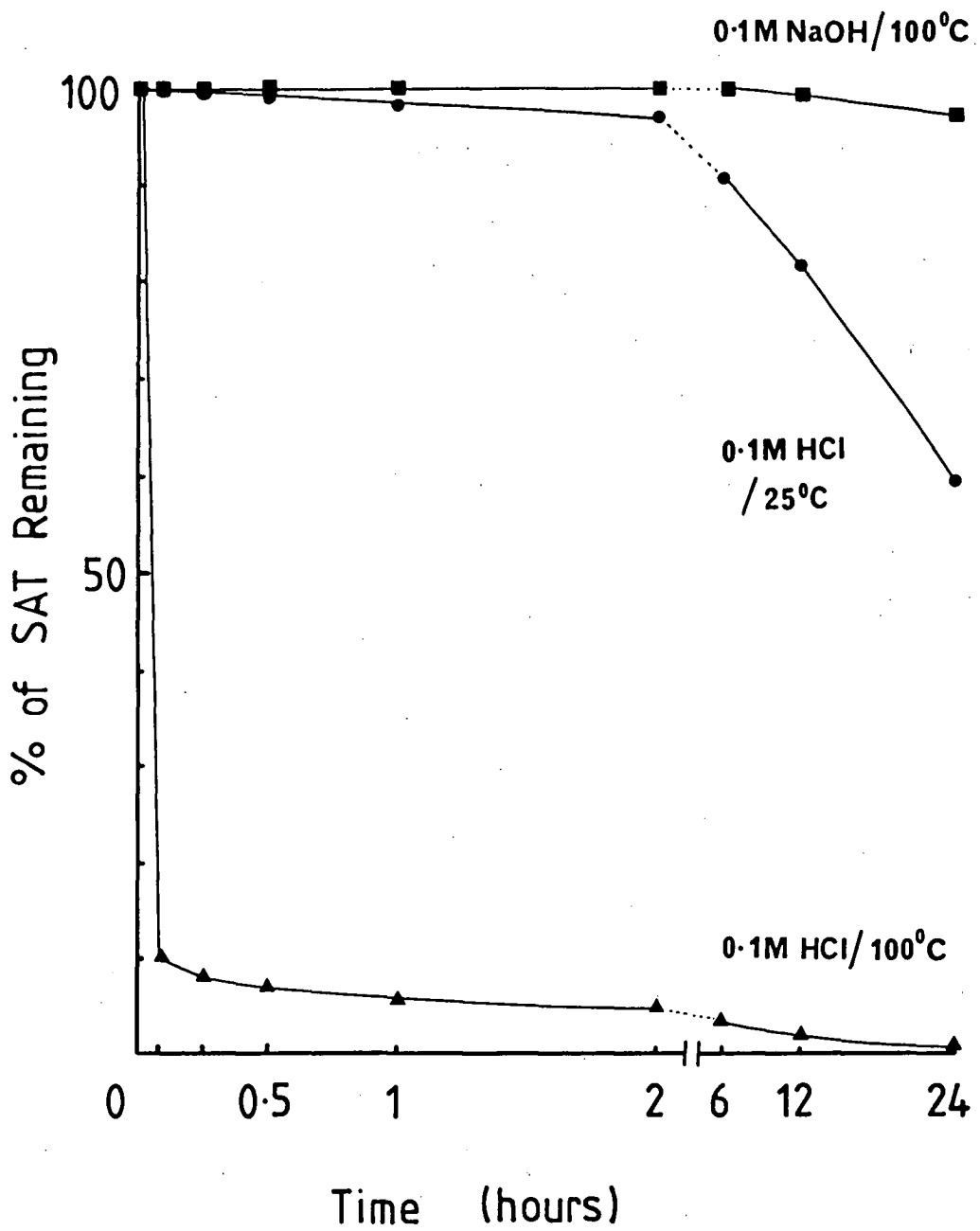


Fig. 6.3 The hydrolysis of SAT under alkaline and acid conditions; as determined by the amount of free sulphate in aliquots. Mean values from duplicate experiments are plotted.



incubation of 2μmol of [^{35}S]-SAT for 6h with sulphatase (1 unit), it again was clear that, as determined by electrophoretic analysis, no hydrolytic products were produced.

Furthermore, after incubation of SAT with human liver sulphamatase for 4 days, no evidence was produced that SAT had been in anyway cleaved by this enzyme (John Hopwood, pers. comm.).

6.4.5 METABOLISM OF SAT IN VIVO

The distribution of radioactivity following the intravenous administration of [^{35}S]-SAT to rats, as determined by tissue oxidation (i.e. total counts) is represented in Fig. 6.4. Results show that after an initial uptake, the radiolabel was rapidly cleared from tissues and blood, and within 2h, 25% of the initial radioactivity appeared in the urine. When rats were placed on metabolic cages and sacrificed 24h after radiolabel administration, the collected urine contained 80% of the initial radioactivity. Apart from bone, where a small proportion of radioactivity remained (1%), only trace amounts (< 0.01%) were associated with other tissues.

In relation to the analysis of the radiolabelled products, samples taken 60min after initial administration were deproteinized, purified and analyzed by high-voltage paper electrophoresis. In every instance, total radioactivity appeared to be associated with [^{35}S]-SAT with no detectable [^{35}S]-sulphate present. A typical electrophoretic pattern is represented in Fig. 6.5.

Intraperitoneal administration of [^{35}S]-SAT gave a similar distribution of radioactivity after 1, 2 and 24h as compared to intravenous administration (Fig. 6.6). Tissue samples analyzed by electrophoresis confirmed the previous findings supporting the stability and resistance to breakdown of SAT.

The measurement of radioactivity present in faeces and urine following oral administration of [^{35}S]-SAT gave an indication as to its absorption. The compound was proven to be well absorbed. After 24h, only 35% of the total counts had passed through unabsorbed to the faeces. This indicated that approximately 65% of the administered dose had been absorbed. The majority of the absorbed dose was rapidly cleared to the urine; i.e within 24h, 50% of the total counts were present in the collected urine. A further 2% was passed to the urine in the following two days. When rats were sacrificed after three days, all tissues had trace amounts with the exception of bone, which was found to retain a small

Fig. 6.4 Distribution of radioactivity following the intravenous administration of [^{35}S]-SAT to rats. At designated times rats were sacrificed, samples collected and counted. Values plotted are mean values from duplicate experiments.

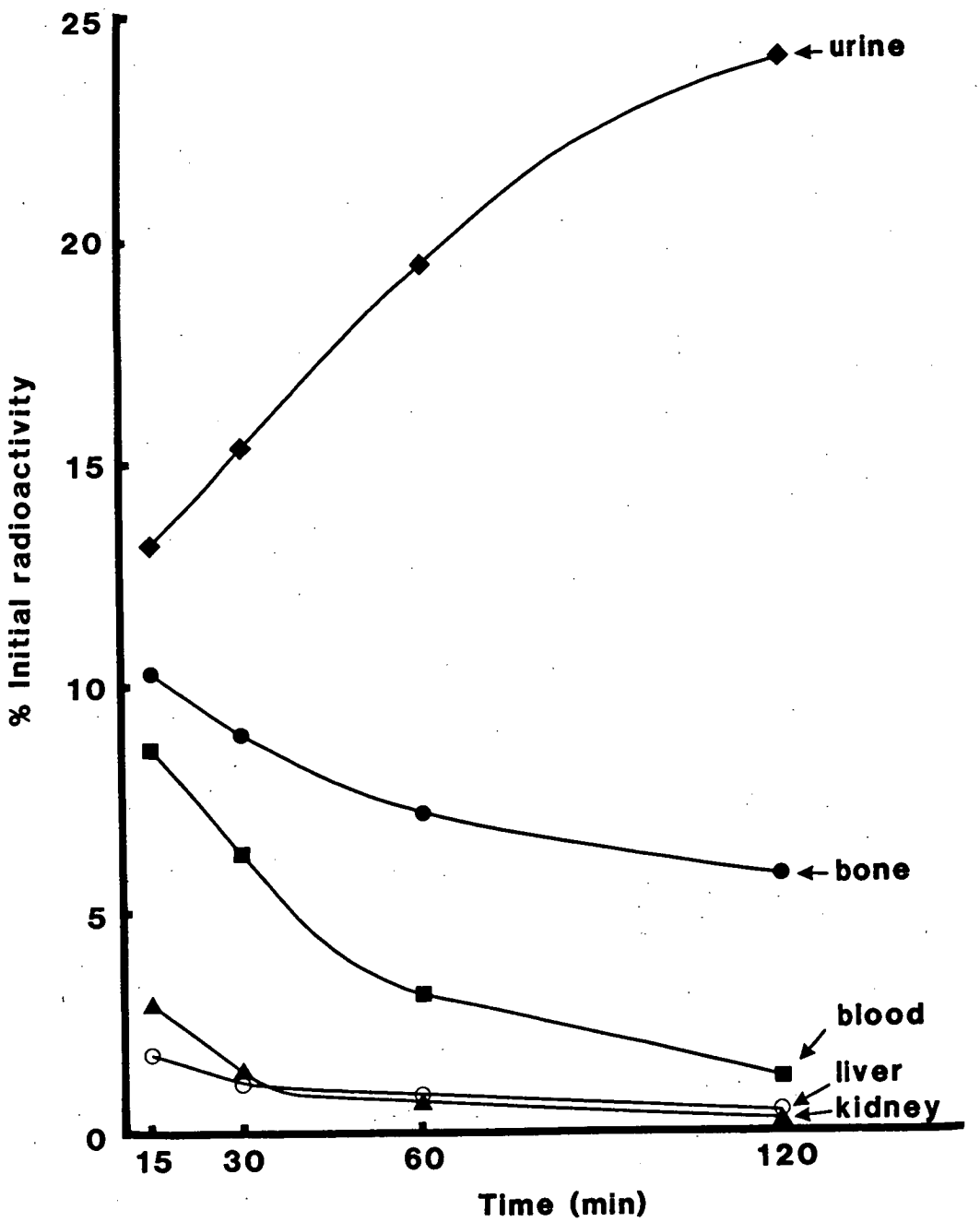


Fig. 6.5 Analysis by electrophoresis of [^{35}S]-labelled compounds (■) present in kidney extract 1h after giving [^{35}S]-SAT intravenously. Comparison is made to reference standards (▩).

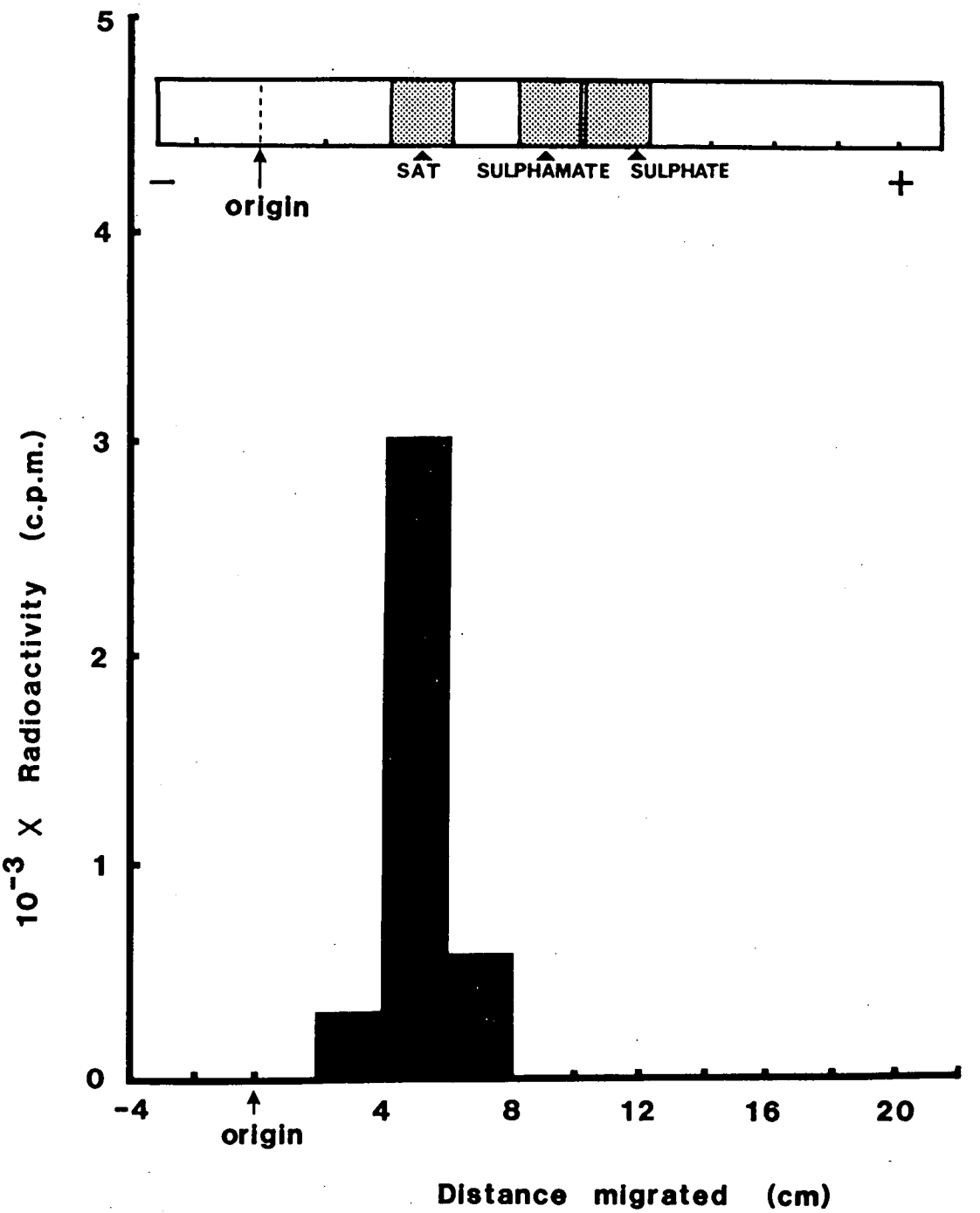
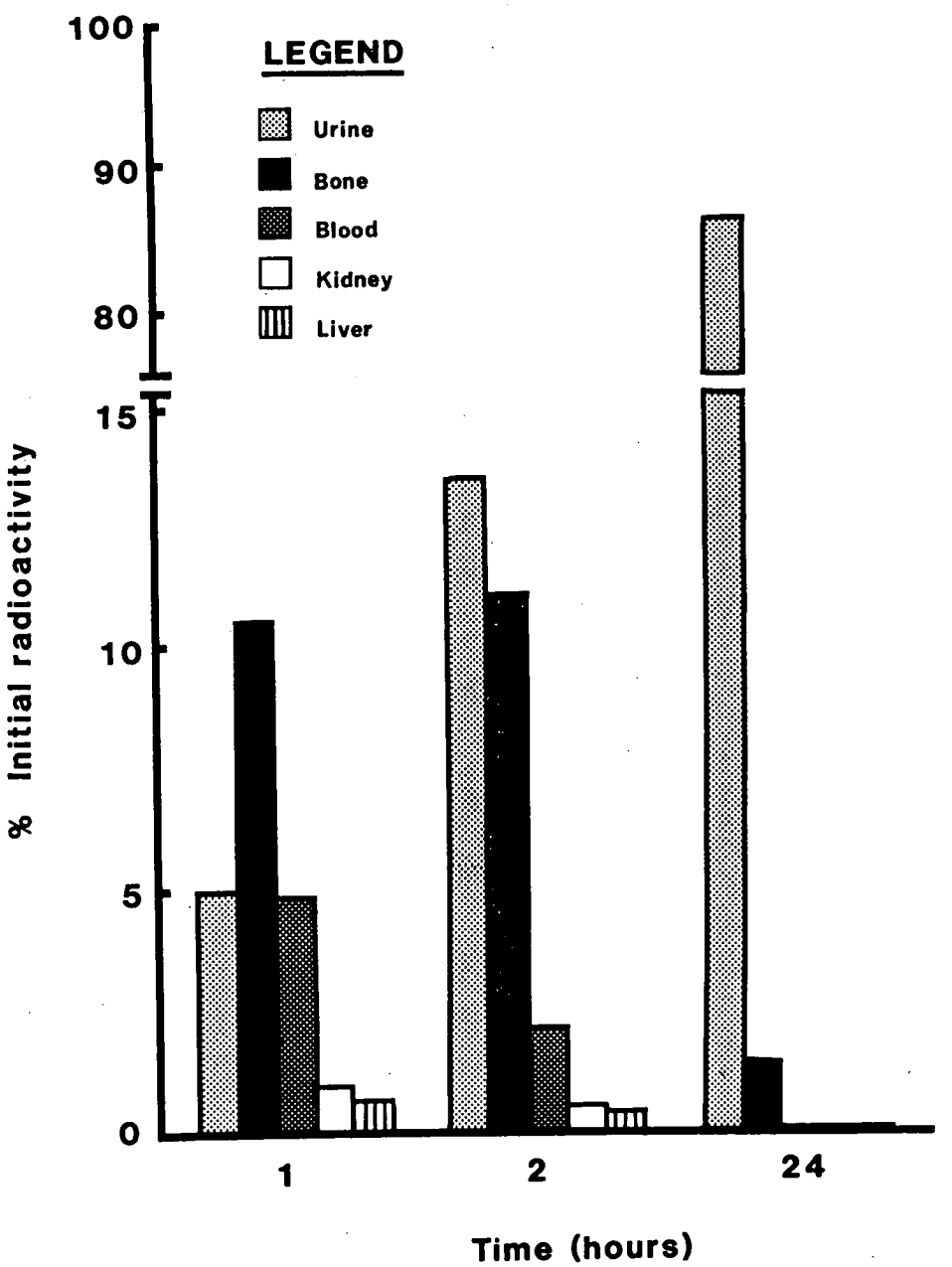


Fig. 6.6 Distribution of radioactivity following the intraperitoneal administration of [^{35}S]-SAT to rats. At designated times rats were sacrificed, samples collected and counted. Values plotted are mean values from duplicate experiments.



fraction (1%) of the initial administered dose. The results are shown in Table 6.1. Electrophoretic analysis of urine and bone samples after tissue treatment again proved that SAT was resistant to degradation, with no radiolabelled components other than [^{35}S]-SAT being evident in these fractions.

6.5 DISCUSSION

Whilst work described in the previous chapter established the potent anti-calcifying activity *in vitro* of the two new PC analogues, PAT and SAT, it was the purpose of this chapter to assess the stability and metabolic properties of the compounds in biological systems. Metabolic studies were assisted in the case of SAT, by the availability of a [^{35}S]-radiolabel. Since radiolabelled PAT was not available, the stability of this compound in biological systems had to interpolated from *in vitro* assays.

PAT was extremely labile under acidic conditions, which suggested that PAT would be totally unsuitable for oral administration due to the acid nature of gastric contents (pH 1-2). Nevertheless, intravenous or intraperitoneal administration would not circumvent this problem, since even at pH 7.4 and 37^{o}C (normal physiological conditions) PAT was shown to readily undergo hydrolysis. This implies that, unless protected, PAT would be likely to be rapidly cleaved in blood and renal tissue. Further, the high phosphatase activity in kidney, which reportedly accounts for major lysis of administered [^{32}P]-PC (Williams and Sallis, 1981), would further promote the breakdown of any PAT reaching renal tissue. Data presented here indicates that PAT is readily destroyed by alkaline phosphatase *in vitro*, and based on results from similar *in vitro* hydrolytic studies by Williams (1981), PAT appears far more susceptible to cleavage than PC. The chemical lability of PAT at pH 6.0 and 37^{o}C was somewhat intermediate between that at pH 3.0 and 7.4 at the same temperature, and indicated that even if some PAT did reach urine, its existence would be short-lived due to further chemical lysis.

These findings were in contrast to the reported stability of the P-N bonds in imidodiphosphate (P-N-P). Presumably, the presence of two phosphoryl groups may confer upon this molecule added stability due to increased resonance stabilization (occurring through the P=O and P-N bonds) than would be the case with only one P-N bond present (i.e. PAT).

A literature survey on the chemical stability of other N-phospho amino acids reveals scanty information on values for the relative rates of

Table 6.1 The absorption and clearance of [^{35}S][a]-SAT, and its adsorption to bone following oral administration.

TIME (DAYS)	% EXCRETION	% IN FAECES	% IN BONE [b]
1	50 ± 4	35 ± 3	n.d.
2	1.1 ± 0.3	< 0.1	n.d.
3	0.5 ± 0.2	< 0.1	1.0 ± 0.2
TOTAL	51.6 ± 4.5	35 ± 3	1.0 ± 0.2

[a] Values given are as mean ± 1 S.D. % of the administered dose; 3 rats/ experimental group.

[b] No counts were present in blood, liver or kidneys after 3 days.

n.d. = not determined

hydrolysis of these compounds at varying pH and temperature values. Nevertheless, reports have suggested that such compounds are stable in alkali, fairly stable in a neutral medium, but particularly sensitive to acid (Zervas and Katsoyannis, 1955; Preobrazhenskaya, 1972). These trends were generally observed with PAT, with the degree of susceptibility observed at neutral and acid pH being even more striking than that expected from these reports. Whilst it is possible that the steric strain surrounding the phosphoramidate linkage might contribute to added chemical lability, it was hoped that the crowded environment surrounding this bond might have made the compound more resistant to enzymatic attack than other phosphoramidates. Unfortunately this proved not to be the case, and the susceptibility of PAT to alkaline phosphatase was of a similar degree to that shown by such compounds as phosphoglycine and phosphocreatine (Winnick and Scott, 1947).

In contrast to PAT, SAT was proven to be chemically stable, and virtually inert to breakdown by enzymes both *in vitro* and *in vivo*. Chemically, SAT was completely stable at pH 7.4 and 37°C; no hydrolytic products were evident even after 8 days. The molecule was also resistant to hydrolysis under alkaline conditions. Although susceptible to acid hydrolysis, the degree of lability was nowhere near that observed for PAT. The compound was rapidly cleaved in acid at 100°C, but at a milder temperature of 25°C, the compound was relatively stable. Similar properties of the sulphamate linkage *in vitro* have been found by other workers (Jorpes *et al.*, 1950; Reitz *et al.*, 1946).

SAT was resistant to the action of both sulphatase and aryl sulphatase *in vitro*. Whilst the actions of both these enzymes were assessed, it is however recognized that they may have similar substrate specificity. In fact, Roy (1976) has suggested that the name aryl sulphatase is incorrectly used in regard to mammalian systems, and has categorized such enzymes as sulphatases A, B and C. Generally, all these enzymes (in addition to limpet sulphatase) catalyze the hydrolysis of sulphate esters where the parent compound may be an alcohol, phenol or carbohydrate, but do not touch the sulphamate linkage (Roy, 1976). Therefore the inability of the enzymes tested here to lyze SAT was not surprising. Furthermore, it was shown that sulphamatase was incapable of cleaving SAT *in vitro*. Again, this was the predicted result, since such enzymes are highly specific for natural substrates.

The resistance of the sulphamate linkage to breakdown *in vivo*, at least in the case of certain cycloalkylsulphamates, has been previously

reported (Spillane and Benson, 1978; Spillane et al., 1979). Following oral administration these workers demonstrated a recovery of 15-50% of the parent sulphamate unchanged in urine, with the proportion of metabolic by-products being < 0.3%. These findings are ratified by those produced here; SAT was found to be well absorbed following oral administration, resistant to breakdown in the body and to have a high clearance to urine. Studies involving the metabolism of [^{35}S]-SAT following intravenous administration were consistent with these trends, whereby SAT was rapidly cleared to the urine and stable in tissue and in circulation. In addition, these experiments provided clearer insight into the uptake and release of [^{35}S]-SAT from blood and various tissues. Again, whilst it was evident that counts were quickly removed from blood, liver and kidney, it appeared that counts were less readily released from the bone site, where a small proportion of [^{35}S]-SAT remained after 24h. A similar pattern of radiolabel distribution was apparent following intraperitoneal administration of the isotope.

In relation to any in vivo anti-calcifying activity of SAT, it is perhaps pertinent at this stage to make some evaluation on its likely potential based on the information given in the last two chapters. If one assumes an aqueous volume of 2ml for the kidneys, and a random distribution of SAT, then the concentration of SAT 1h after administration of 3mg (6.7µmol) in kidney would be 25µM. This level would appear to be capable of inhibiting calcification, on the basis of the in vitro data. Whilst this does give some encouragement to pursue in vivo studies, two points should be raised. It is unlikely that the distribution of SAT would be random, i.e. the compound may be concentrated in particular intra- or extra-cellular compartments. The second factor suggests that the uptake of SAT by kidney may in fact be much greater when crystallization within the kidney is occurring. This is based on the observations with bisphosphonates whereby the kidney would normally be exposed to these compounds briefly following administration. However under conditions of ectopic calcification a greater uptake of bisphosphonates occurs due to adsorption either onto depositing mineral or onto already formed mineral, thus inhibiting the calcification process (Fleisch, 1983).

The retention of a small proportion of SAT at the bone site reflects possibly this last factor and may be related to its binding to hydroxyapatite at this site. Uptake by bone is strongly evident with certain bisphosphonates, leading to the toxic effects following long-term administration. Whilst such a long-term study would have been desirable

here to fully assess any side-effects SAT might have, it was nevertheless impractical due to the restraints on the amount of the compound available. Further, the expertise and facilities required for the proper examination of bone were not readily available.

It is salient perhaps to make certain comparisons between the metabolic properties of SAT, as determined by work recorded in this chapter, and the properties of the well characterized synthetic bisphosphonate, HEBP. Clearly SAT has superior absorptive properties (65%), whereas only 3% of HEBP is absorbed following oral administration. Whilst both compounds are stable in circulation, the other significant factor is that when absorbed, approximately one half of the HEBP finds its way to bone and the majority of the remainder reaches urine. With SAT however, only a small fraction reaches bone, whereas the majority is cleared to urine. Clearly then, SAT has better absorptive properties, and is less likely to have adverse effects on bone metabolism on the basis that far less SAT reaches the bone than HEBP. Nevertheless, it is recognized that major research has been undertaken in recent years to develop bisphosphonates that are more readily absorbed and less toxic to bone (Shinoda *et al.*, 1983), and it is not precluded that in the future these also may be likely candidates for therapeutic use against stone disease. Certainly however, if SAT were proven to be a potent inhibitor of calcification *in vivo*, the long term effects of SAT on bone would need further investigation in the future.

6.6 SUMMARY

1. PAT was shown to readily undergo chemical hydrolysis at acid and neutral pH, although stable in alkaline solution. In addition, the compound underwent rapid hydrolysis in the presence of alkaline phosphatase.

2. These observations eliminated any possible role that PAT might have had in arresting biological calcification.

3. In contrast, SAT was relatively stable in acid medium and extremely stable both at neutral and alkaline pH. SAT was also resistant to the actions of sulphatases and sulphamatase *in vitro*.

4. Analytical methods established also confirmed that SAT was completely stable *in vivo*, in contrast to the lability reported for PC.

5. SAT was shown to be well absorbed following oral administration; with absorption being far superior to that reported for the bisphosphonate HEBP.

6. All forms of administration (oral, intravenous and intraperitoneal) demonstrated that after initial uptake, SAT was rapidly cleared from tissues to the urine.

7. A small proportion of SAT remained in bone following administration; unfortunately the long-term effects of SAT on bone could not be studied due to certain restrictions. Nevertheless, the compound had far less affinity for bone than has been reported for HEBP, and this factor might make SAT less toxic than the bisphosphonates following chronic administration.

8. The amount of SAT reaching the kidney following oral administration was considered sufficient to arrest calcification *in vivo* through physicochemical means.

CHAPTER 7

THE ABILITY OF N-PHOSPHO-2-AMINO TRICARBALLYLATE AND N-SULPHO-2-AMINO TRICARBALLYLATE TO ARREST CALCIFICATION IN VIVO

7.1 INTRODUCTION

Whilst methods in the precedings chapters have been directed at establishing some of the metabolic properties and effects on *in vitro* calcification of the PC analogues, it is still to be established whether the compounds would prove effective in preventing calcification *in vivo*. To study this parameter, models have been described which involve the administration of lithogenic materials to laboratory animals to induce renal calcification; thus the effectiveness of the administration of an inhibitor toward calcium deposition can then be determined. The methods however, do have obvious limitations. Natural stone formation is multifactorial and may occur as an imbalance between factors such as levels of inhibitors and precipitating salts. Since parameters affecting such factors are poorly understood and difficult to reproduce in a test system *in vivo*, test systems adopted generally rely on the administration of a single lithogenic agent. In addition, natural stone formation may result after long exposure to conditions conducive to stone formation, whereas in the case of experimental models, studies have tended to be more acute, with crystallization occurring in time periods as short as 15min after initiation of lithogen administration (Khan *et al.*, 1979). Hence the quantities of lithogen administered to experimental animals to induce calcification often represents a far greater challenge of that component than is ever encountered in a human stone disease situation.

Another consideration that should be highlighted is that the effectiveness of crystal growth and aggregation inhibitors is dependant on their efficient binding and coverage of crystal surfaces. Whilst this is possible in the natural situation, in acute model systems where crystals are continuously forming and growing at a rapid rate, such a situation may not be truly attainable. Given these shortcomings, nevertheless such models have served as useful tools of study for the testing of potential *in vivo* inhibitors providing information as to how they affect early formation of crystals that might then be extrapolated to clinical conditions.

As emphasized in previous discussions, calcium oxalate and calcium phosphate represent by far the two most common components of renal stones; therefore if an inhibitor is to have widespread application as an anti-calcifying agent it is essential for it to inhibit the crystallization of both these salts potently *in vivo*. Work described in this chapter relates to the ability of PAT and SAT to arrest the crystallization of calcium phosphate and calcium oxalate in renal tissue *in vivo*. Two specific models using laboratory rats were employed, and the choice of these acute models was governed by the restricted availability of the compounds PAT and SAT. For calcium oxalate, crystallization was induced by a single intraperitoneal challenge of sodium oxalate, essentially as described by Khan *et al.* (1979). Under these conditions crystallization is evident within 30min. For calcium phosphate, the model studied was that described by Schneider *et al.* (1960), whereby hydroxyapatite deposition occurring in renal tissue after daily administration of calcium gluconate is examined after 9 days. The effectiveness of the PC analogues in preventing the crystallization of these salts is compared with PC, and the well documented inhibitor HEBP. From the data generated and the data reported from earlier experimentation, the potential of these compounds to act in a biological system is assessed.

7.2 MATERIALS

SAT and PAT were synthesized by methods outlined in Chapters 3 and 4. PC was prepared by the method of Williams and Sallis (1980). HEBP was a generous gift from the Proctor and Gamble Co., Cincinnatti, Ohio. Calcium gluconate (10% w/v) was supplied by the Boots Company, North Rocks, N.S.W., Australia. Oxalate decarboxylase was purchased from Calbiochem (San Diego, California).

7.3 METHODS

7.3.1 CALCIUM OXALATE CRYSTALLIZATION

7.3.1.1 Administration of Inhibitor and Sodium Oxalate Challenge

The administration of a sodium oxalate challenge was used to induce renal calcium oxalate crystallization as described by Khan *et al.* (1979), and the effect of prior administration of inhibitor on the extent of deposition was the tested parameter. Hence rats (100g) were given an intraperitoneal dose of inhibitor (3.3-50μmol/0.5ml of 0.9% saline) or

placebo (0.5ml of 0.9% saline) 1h prior to an intraperitoneal challenge of sodium oxalate (7mg/0.5ml). The inhibitors tested were PAT, SAT, PC and HEBP, and throughout the experiment there was no restriction placed on the availability of food or water. The rats were sacrificed 4h after the challenge, kidneys were removed, weighed and quickly frozen by immersion in liquid nitrogen.

7.3.1.2 Examination of Kidneys Following Inhibitor and Oxalate Administration

7.3.1.2.1 Histochemical Examination

Thin radial central sections of kidney (8μm) were prepared using a microtome. The sections were placed on microscope slides previously smeared (and still sticky) with 5% egg albumin in 0.5% NaCl/glycerol (50:50). The slides were then gently heated (30-40°C) until dry. Napthalhydroxamic acid was used to stain as described by Roscher (1971) and the stained sections examined by light microscopy. Crystals of calcium oxalate stained yellow against a blue cellular background.

7.3.1.2.2 Calcium Analysis

Frozen kidney tissue was ground to a fine powder using a mortar and pestle which had previously been chilled in liquid nitrogen. Samples of ground tissue (100mg) were then taken, placed into scintillation vials together with 200μl of 70% $HClO_4$ and 400μl of 30% H_2O_2. After sealing the vials with tops resistant to digestion conditions, the samples were digested by heating at 80°C for 4-6h. Upon cooling to room temperature, the contents were diluted to 10ml with $LaCl_3$ solution (50,000ppm La^{3+}) and calcium in the samples was determined by Atomic Absorption Spectrophotometry using a calcium standard scale calibrated between 0 and 10μg Ca^{2+}/ml.

7.3.2.2.3 Oxalate Analysis

After extraction of powdered kidney tissue (1.0g; as described above) oxalate in kidney samples was estimated according to the method of Sallis et al., (1977) based on the conductometric measurement of enzyme-liberated CO_2 from oxalate.

7.3.2 CALCIUM PHOSPHATE CRYSTALLIZATION

The experimental model for kidney calcification chosen in this study was one that has been extensively used by other investigators and

known to produce severe calcification in renal tissue within a few days by the daily administration of calcium gluconate (Schneider et al., 1960; Tew et al., 1981).

Male rats (50g) were given daily an intraperitoneal dose of either inhibitor (3µmol in 0.5ml of 0.9% NaCl) or placebo (0.5ml of 0.9% NaCl) 1h before an intraperitoneal dose of calcium gluconate (1.0ml; 10%). Treatment was continued for 9 days, during which time the rats were maintained on normal rations of food and water. Twenty-four hours after the ninth injection the rats were sacrificed, kidneys removed and weighed, and then quickly freeze-clamped. Frozen tissue was ground to a fine powder, digested with H_2O_2 and $HClO_4$ and assayed for calcium in identical fashion to that described above (7.3.1.2.2).

7.4 RESULTS

7.4.1 EFFECT OF INHIBITORS ON CALCIUM OXALATE CRYSTALLIZATION

7.4.1.1 Histochemical Examination

In initial experiments, light microscopy was used to examine renal tissue sections of control and inhibitor treated groups, noting in particular any quantitative differences in crystal numbers. Specifically, the extent of crystal deposition was examined in terms of the average number of crystals per field of view and comparison also made of the mean crystal size between groups. When SAT was administered at a dose of 6.7µmol/100g rat there was no discernable difference between tissue sections from these rats and control rats, i.e. in both instances a marked deposition of calcium oxalate had occurred (Fig. 7.1). It was apparent that any proper statistical evaluation of differences between experimental groups by this method would have involved preparing many uniform sections from each kidney of each rat, completing a large number of random counts from each section and overall analyzing data obtained from many animals. Whilst the histological examination was useful in proving that a) rats had responded to the oxalate challenge, and b) a drastic reduction in crystal deposition had not occurred due to the administration of SAT, nevertheless for routine quantitative analysis, the extent of deposition of calcium oxalate was better evaluated by assaying for tissue calcium and oxalate.

7.4.1.2 Calcium Analysis

Kidney calcium concentration in the different experimental groups has been represented in Fig. 7.2. Results clearly showed that the rats had

Fig. 7.1 Typical kidney sections stained by the Roscher method (1971) derived from A) normal rat, and B) rat receiving a sodium oxalate challenge. Calcium oxalate crystals stained yellow. Magnification: x 300.

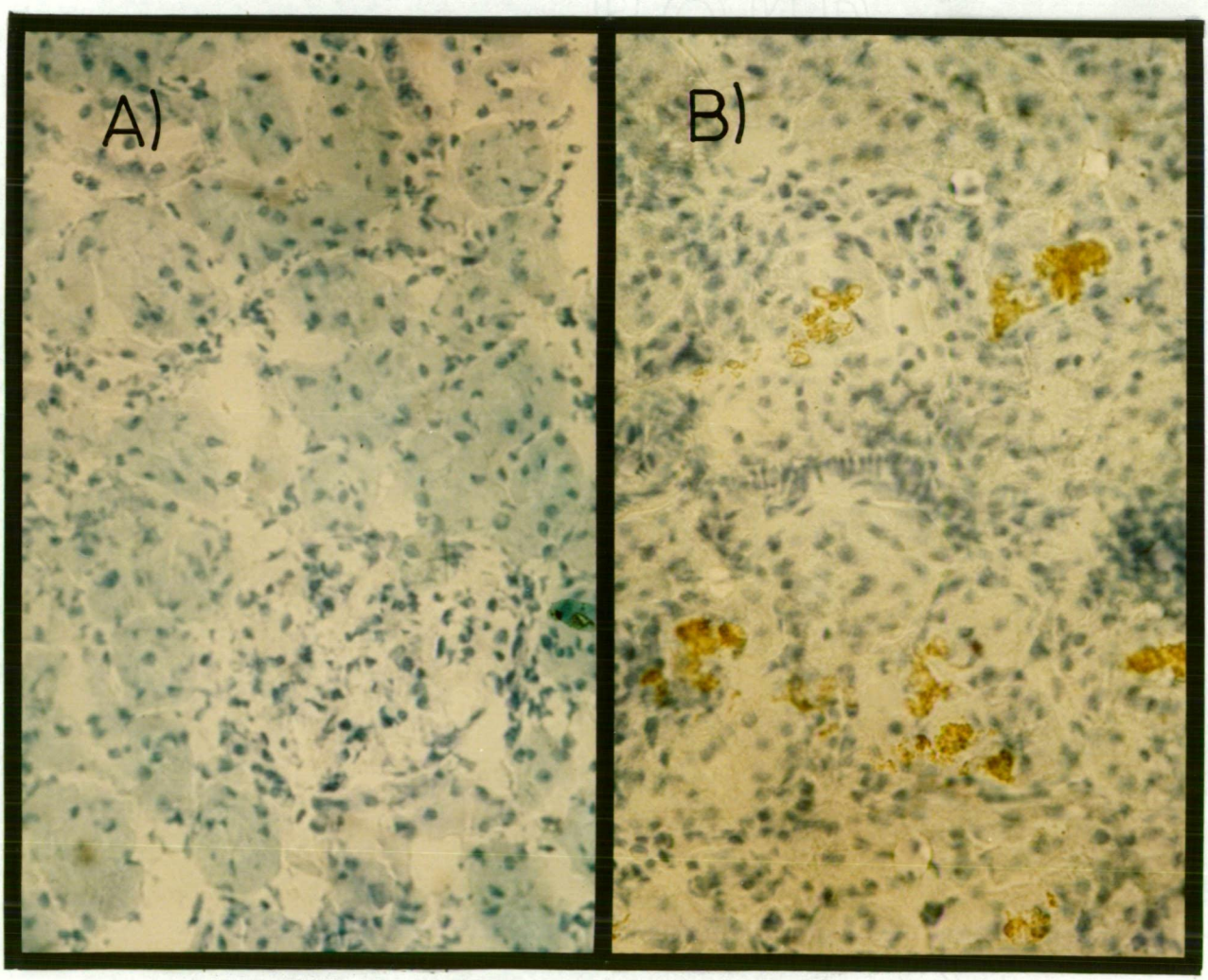
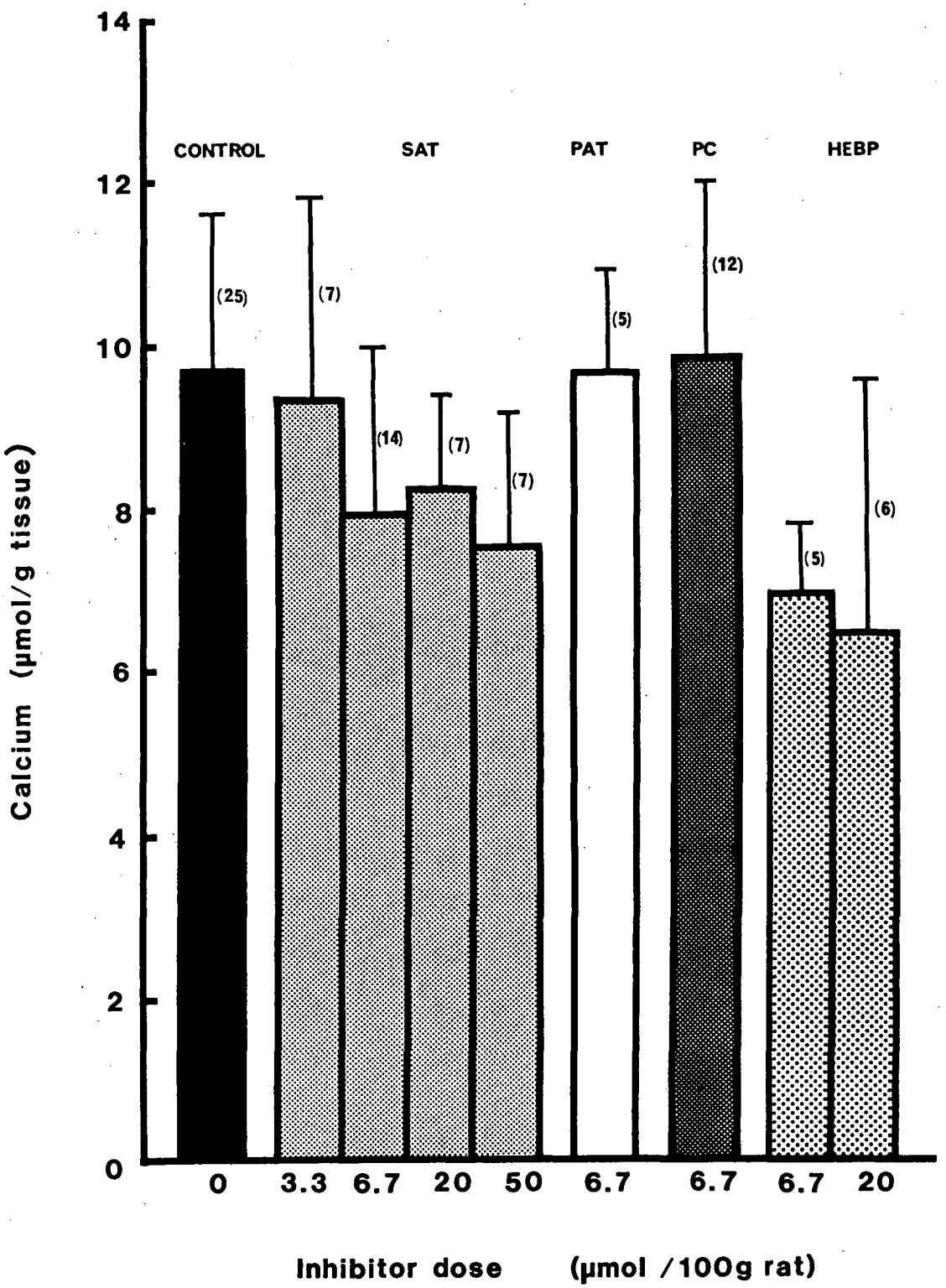


Fig. 7.2 Effect of inhibitors on the deposition of calcium oxalate in kidney tissue. Rats (100g) received inhibitor 1h prior to a challenge of sodium oxalate (7mg). Rats were sacrificed 4h later and kidney calcium determined. Figures shown are mean values ± 1 s.d. with the number of rats/experimental group given in parentheses.



responded to the oxalate challenge. Rats administered oxalate but no inhibitor (i.e. control) had five times the amount of calcium present as untreated rats; by inference from histological examination, this was due to the complexing of calcium with oxalate. The administration of different inhibitors prior to the oxalate challenge had varying effects on the amount of calcium deposited in the tissue. When PAT or PC were administered at a level of 6.7μmol, there was no significant effect on the extent of calcium deposition. SAT however at this dose did cause a reduction of kidney calcium in the order of 25%. This degree of inhibition was only slightly increased by higher concentrations of SAT, with 50μmol SAT resulting in a 30% overall reduction. HEBP was the most potent of inhibitors tested giving 35% inhibition at the 6.7μmol level, and again, as was evident with SAT, this effect was not markedly enhanced by higher concentrations.

7.4.1.3 Oxalate Analysis

Assays for kidney oxalate confirmed that deposition of calcium oxalate had occurred within this tissue following the oxalate challenge. The levels of oxalate found in kidneys from rats in the control group was approximately seven times the normal value. PAT and PC had no effect on the level of oxalate present in kidney relative to control. SAT and HEBP produced a similar degree of inhibition (as to that determined by calcium analysis) while HEBP appeared the most potent (Table 7.1).

7.4.2 EFFECT OF INHIBITORS ON CALCIUM PHOSPHATE CRYSTALLIZATION

Results from the kidney calcium concentrations following daily administration of the various inhibitors in conjunction with the calcium gluconate challenge are shown in Fig. 7.3. Some contrasting trends were evident with this model compared to the more acute model involving calcium oxalate. SAT had no inhibiting effect on hydroxyapatite formation, in fact the values for kidney calcium were slightly elevated relative to that of the control. PAT produced a similar response. PC, however, did have a pronounced effect causing a 45% reduction in hydroxyapatite formation, confirming trends established by other workers (Tew et al., 1981). HEBP, as a comparison inhibitor, was again the most potent of the compounds tested, inhibiting by 65% the deposition of hydroxyapatite.

7.5 DISCUSSION

The work described in this chapter has attempted to provide some insight into the anti-calcifying activity within the kidney of the two PC

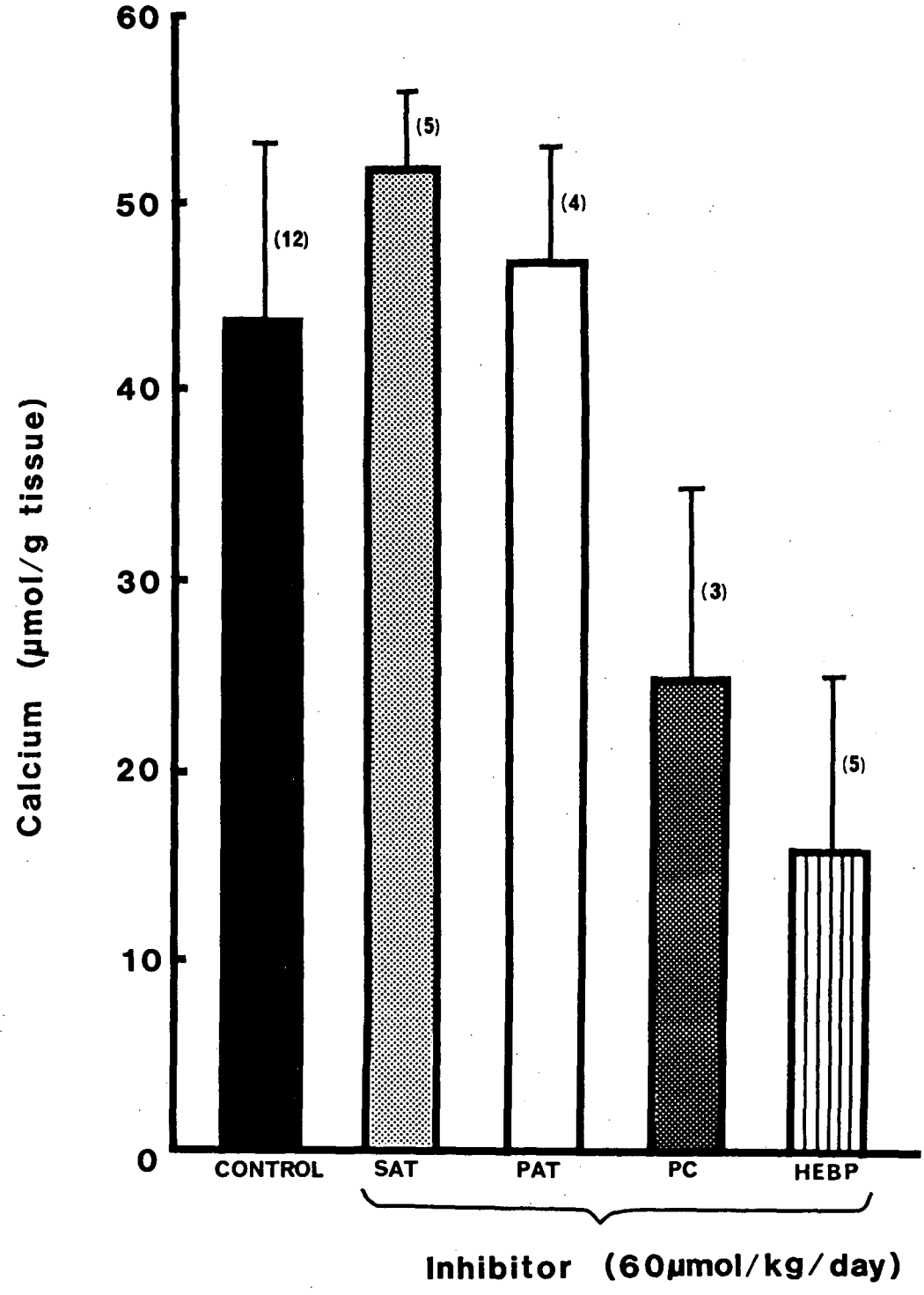
Table 7.1 **Effect of inhibitors on the deposition of oxalate within rat kidney tissue. Rats (100g) received an intraperitoneal dose of inhibitor (6.7µmol) 1h prior to an intraperitoneal challenge of sodium oxalate (7mg). The rats were sacrificed 4h later and kidney oxalate determined.**

INHIBITOR	KIDNEY OXALATE (µmol/g TISSUE)[a, b]
None (Control)	6.6 ± 2.0
SAT	5.7 ± 1.5
PAT	6.4 ± 1.8
PC	6.8 ± 2.9
HEBP	5.4 ± 2.1

[a] n = 10 (5 rats/group; both kidneys analyzed from each rat).

[b] Normal value for rat kidney oxalate was found to be 0.90 ± 0.21µmol/g tissue.

Fig. 7.3 The deposition of calcium within renal tissue following the daily administration of calcium gluconate (1.0ml; 10%) in conjunction with inhibitor (60μmol/kg/day) to rats for 9 days. Figures shown are mean values ± 1 s.d. with the number of rat/experimental group in parentheses.



analogues, PAT and SAT. To study this parameter two specific models were utilized, one involving the crystallization of calcium oxalate and the other hydroxyapatite. Both models proved to be viable as it was possible to demonstrate inhibition by HEBP, a compound chosen as a reference inhibitor, and in this respect so confirm the findings of others (Potokar and Schmidt-Dunker, 1978; Fleisch et al., 1970). Of the other proven in vitro inhibitors, PAT, as predicted, failed to inhibit either the formation of calcium oxalate or hydroxyapatite. PC however, while failing to inhibit deposition of calcium oxalate, did prove to be a potent inhibitor of hydroxyapatite formation. With SAT, this trend was reversed; SAT did inhibit to a significant extent calcium oxalate crystallization, but failed to reduce the deposition of hydroxyapatite.

To interpret and rationalize the varying responses of the inhibitors, it is necessary to understand the fundamental differences between the two models. The first factor is the time difference required in the studies using either model. This arises from the fact that calcium oxalate is a far more insoluble salt than hydroxyapatite. Thus, under the oxalate challenge given here, crystallization occurs at the kidney within 30min (Khan et al., 1979). This contrasts to the response time arising from calcium gluconate administration, as significant deposition of hydroxyapatite occurs only after 2-3 days (Tew et al., 1981). Another consideration is the fact that inhibitors may preferentially inhibit the crystallization of a particular salt both in vitro and in vivo. This was highlighted in the in vitro studies discussed in Chapter 5, where PC proved to be a far more potent inhibitor of hydroxyapatite formation than HEBP, whereas for an inhibition of calcium oxalate crystal growth, their relative potencies were reversed.

A further significant difference between the two experimental models is the actual site of calcium deposition. Using the calcium oxalate model, histological studies have shown that deposition of crystals at the kidney site occurs primarily occurs within the lumen of tubules (Khan et al., 1979; Dykstra and Hackett, 1979). The model involving calcium gluconate administration however has been shown to promote the deposition of calcium as hydroxyapatite in the tubular cells inside mitochondria, and secondary to this deposition (but to a lesser degree), in the extra-mitochondrial cytosol (Tew et al., 1981). Clearly, since the major site of calcification is different with the two models, factors such as transport properties of the inhibitors across cellular and mitochondrial membranes, and their stability intraluminally or within the various sub-cellular compartments would affect their localization within renal tissue. This in

turn would be a major influence on the relative inhibition they might exert on the crystallization induced by these models.

In the light of the preceding considerations, the responses of the various inhibitors may now be discussed in more depth. The inability of PAT to inhibit in either system clearly results from its extreme susceptibility to both chemical and enzymatic hydrolysis. HEBP on the other hand, a compound highly resistant to the action of enzymes (Fleisch, 1981, 1983), and a potent inhibitor of calcification *in vitro*, proved to be the most potent inhibitor of those tested. Other workers have also found a close relationship between the ability of various bisphosphonates to inhibit crystal growth *in vitro* and their effectiveness *in vivo* in the prevention of soft tissue calcification (Fleisch *et al.*, 1970; Shinoda *et al.*, 1983), and as suggested by Fleisch (1983), this latter effect is explicable by a physicochemical mechanism.

Two factors may contribute to the fact that PC failed to inhibit the calcium oxalate system, but did prove a potent inhibitor of hydroxyapatite accumulation. The first arises from the observation *in vitro*, whereby PC, although useful in preventing calcium oxalate crystallization, appeared far more powerful in its action to prevent the conversion of ACP to hydroxyapatite. The second factor may be the distribution of exogenous PC following administration. By inference to the sites of crystallization with the two models, one explanation of the results is that exogenous PC does reach mitochondria, but little is retained at the intraluminal site. These interpretations do have some basis. Endogenous PC, for example, has been shown to be localized predominantly within mitochondria where it is thought to be biosynthesized (Williams, 1981). Compartmentalization may protect the phosphate ester from the actions of lytic enzymes present in the cytosol. In this regard, it is possible that exogenous PC is accumulated and stable within the mitochondria of the kidney. Although the uptake of PC by mitochondria has yet to be established, it is likely that endogenous PC might be transported via the tricarboxylate carrier, which has specificity for citrate analogues (Palmieri *et al.*, 1972). Whilst it is inevitable that a proportion of PC is hydrolyzed before reaching mitochondria [suggested by Williams and Sallis (1981)], results here, and those reported by Tew *et al.* (1981) do suggest indirectly that a significant proportion of exogenous PC probably does reach this site.

Williams (1981) has proposed that the results by Tew *et al.* (1981) may be as a consequence of the selective uptake of PC by the kidney, where

it is rapidly hydrolyzed to P_i and citrate, and the latter compound, by virtue of its weak anti-calcifying activity inhibits nephrolithiasis. Nevertheless, the amount of PC that was shown to be taken up by the kidney by this author is not sufficient to fully support this view. Whilst it is clear that partial hydrolysis of PC accounts for a reduction in the inhibitory power, as reflected by results presented here whereby PC is a more potent inhibitor of hydroxyapatite formation than HEBP *in vitro*, whereas *in vivo* the trend is reversed, an interpretation of the results is that PC may not be as labile as previously thought.

One mechanism that has yet to be considered that could account for these apparently contrasting observations, is synergism between PC and citrate, a well documented phenomenon *in vitro* (Williams and Sallis, 1982; Tew *et al.*, 1980). If it were true that a major fraction of the PC reaching kidney is rapidly hydrolyzed at this site, leading to a local elevation of the levels of citrate, then strong synergistic effects between citrate and unhydrolyzed PC might partially offset the loss in inhibitor power attributable to PC hydrolysis. Presumably, PC hydrolysis may occur in the cytoplasm of the cell, whereas any PC reaching mitochondria (the major site of calcification with this model) may be protected against further lysis, and there synergistically act with citrate to potently inhibit the transformation of ACP to hydroxyapatite.

The hydrolysis of PC at other cellular or intercellular sites may explain the lack of inhibition by PC of calcium oxalate crystallization, which with this model occurs at the intraluminal site. The fact that very little exogenous PC is retained at the intraluminal site, again, is supported by results from Williams and Sallis (1981), who found that after administration of [^{32}P]-PC, no unhydrolyzed ester was passed into urine.

The differing actions of SAT with the two models are difficult to rationalize either in terms of lability at different sites or in specificity towards the inhibition of calcium oxalate. The compound has proven totally stable *in vivo*, and appears to inhibit calcium phosphate and calcium oxalate crystallization to approximately the same extent *in vitro*. Perhaps one answer might be in the differential distribution of the compound following administration. Data from the previous chapter show that administered [^{35}S]-SAT is rapidly cleared to the urine. In fact, a figure indicates that 30min after administration of the isotope, 15% passes to the urine unchanged. Similar experiments with [^{32}P]-PC (Williams, 1981) show that only 5% of total counts are cleared after an identical time lapse. This could suggest that SAT may be less readily reabsorbed by tubular cells

than PC, i.e. during the time course of the calcium oxalate model a larger proportion of SAT is associated intraluminally, which is the site of crystal formation. Another observation of interest associated with the inhibition of calcium oxalate crystallization by SAT was the effect of different concentrations of SAT. Significant inhibition occurred at a level of 6.7µmol, but much higher concentrations (50µmol) resulted in only a slight increase in inhibition. One explanation of this trend could be due to the saturation of binding sites on the crystal surfaces by SAT.

The possibility that SAT might be less permeable to mitochondria than PC could further explain the failure of this compound to prevent hydroxyapatite deposition within this organelle. Unfortunately, several restrictions did not permit a detailed study of the transport phenomenon associated with PC and its analogues. These were time limitations, the specific activity and amount of radioisotope available, and the recognition that proper evaluation might possibly involve elaborate techniques and/or produce data difficult to interpret.

Whilst the aforegoing discussion has offered ideas to explain the observed results, it is important to stress that they are to a large degree speculative and lack evidence. Additional explanations may contribute to these trends, such as further unrecognized differences between the experimental models or even secondary effects that these molecules have upon administration.

Although the shortcomings of the two models have been discussed and are thoroughly recognized, nevertheless the results presented do allow some evaluation of the potential clinical values of the inhibitors. PAT has no apparent therapeutic value due to its lability, but it would seem that PC and SAT do have some promise. In regard to PC, its potent inhibition of hydroxyapatite formation shown here and by Tew et al. (1981), indicate that PC may in the future, have a role as a stone preventing agent. SAT, whilst not inhibiting hydroxyapatite formation, was effective in retarding the crystallization of calcium oxalate and may also have a role in arresting stone growth.

Based on the results, it might be naively suggested that PC may be more active in the prevention of stones consisting of hydroxyapatite, whereas with stones comprised mainly of calcium oxalate, SAT may prove more useful. Whilst this may well be the case, it may not be valid to extrapolate fully to the situation of natural stone formation. The problem lies in the fact, that apart from other considerations, in the case of natural stone formation (a) the nucleator for calcium stones, and (b) the initial

site of nucleation are controversial features that still remain to be clearly established by investigators. In this regard, whilst the ability of SAT and PC to inhibit in the test systems described here where the identity of the nucleator and the site of initial deposition are clearer, because these factors are unknown in normal stone formation, the effects of PC and SAT in this situation may not be necessarily as straightforward. For example, if brushite were the nucleator for calcium oxalate stones [as has previously been suggested (Pak et al., 1971)], then PC, being a potent inhibitor of calcium phosphate crystallization might prove a more effective agent than SAT for oxalate stones. Similarly, other arguments could be presented. Unfortunately, as argued here, and recognized by most authors, at present there exists no totally suitable experimental model to study the relative effectiveness of inhibitors in their prevention of kidney stone disease.

Whilst some of the effects of PC and its analogues on kidney calcification have been studied, the compounds may have different or even similar actions at other locations within the urinary tract where stone formation may occur. The bladder, for example, is another major site of stone formation; hence the experimental compounds may prove useful in retarding stone growth at this site. Obviously a different relationship would exist between the inhibitors and precipitating salts here, depending on such factors as secretion and reabsorption of components and hydrolysis of inhibitors that might occur both during the transit of fluid to the bladder and within the bladder itself. Also the rate of clearance of urine from the bladder, which would determine the time of exposure of inhibitors to the forming crystals or preformed stone, would be influential. Whilst it was considered beyond the scope of the current study to investigate this area, the potential of the compounds to also have anti-calcifying activity at non-renal sites is nevertheless recognized.

In summary, the results suggest (but not confirm) that SAT and PC may have roles as therapeutic agents. Specifically, it is likely that PC may be effective in preventing calcium phosphate stones, whereas SAT may be more useful for calcium oxalate stones.

7.6 SUMMARY

1. Two models were utilized to study the anti-calcifying properties of PC and its analogues. These models, which involved calcium oxalate and hydroxyapatite crystallization, were proven responsive to inhibition since HEBP, a recognized calcification inhibitor, was shown capable of inhibiting crystallization in both cases.

2. With both systems, PAT failed to show any degree of inhibition, whereas HEBP proved the most potent inhibitor.

3. PC inhibited potently the deposition of hydroxyapatite, but failed to inhibit calcium oxalate crystallization. SAT, on the other hand, did inhibit to a significant extent the crystallization of calcium oxalate, but did not inhibit the formation of hydroxyapatite.

4. The differing effects of SAT and PC may be rationalized in terms of differences in the specificity of the molecules for the crystallizing salts, localization within kidney tissue, lability or other unrecognized differences between the models.

5. Whilst the limitations of the experimental models are recognized, as are the dangers of extrapolation of data to the clinical situation, results do suggest that both PC and SAT may have future roles as therapeutic agents for the treatment of calcium stones in specific cases.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The underlying causes of stone formation are still, in a majority of cases, a mystery, and successful treatment has yet to be formulated. In the last decade, whereas major advancements have been achieved in surgical procedures for the removal of formed stones, progress has been much slower in developing therapeutic compounds to prevent or even control stone formation. Although a number of agents, including thiazides, magnesium oxide, orthophosphates, cellulose phosphate, allopurinol and citrate, have proven useful under defined conditions, none to date have been universally effective.

Whilst many of the above agents act by causing modest increases in urinary inhibitory activity and/or effecting minor reductions in the concentrations of lithogenic salts, measures that would lead more substantial increases in the levels of potent inhibitors in urine would seem superior. The most potent inhibitor compounds recognized from in vitro studies have included a number containing the phosphate moiety. Investigations therefore have been directed at determining whether such compounds can be excreted unchanged following oral administration, because, if so, a major increase in the urinary inhibitor activity should prevail. Pyrophosphate (PP_i), was one of the first natural urinary inhibitors recognized, but following administration, this compound suffers the disadvantage of being labile to enzyme attack, thus reducing its effectiveness.

The development of the bisphosphonates, compounds characterized by an enzyme resistant P-C-P bond, at first sight would appear to overcome the problems associated with PP_i. Unfortunately, although these compounds have proven to be potent in their inhibition of soft tissue calcification, adverse actions at the bone has limited their use as stone preventing agents. Work is still continuing however, to try and modify the basic structure in an attempt to minimize the side-effects.

One of the more promising phosphorylated compounds that has emerged in recent times as a potential controller of calcification is phosphocitrate (PC). This compound, a potent inhibitor of calcification in vitro, occurs naturally, and therefore its therapeutic use is unlikely to produce any damaging side-effects. One in vivo study has indicated that admini-

stered PC has no detectable effect on the mineralization of cartilage and bone leading to the conclusion that the compound should be regarded as non-toxic to these sites. HEPB on the contrary, at a similar dose does lead to a marked inhibition of these processes (Reddi et al., 1980). The available evidence then would suggest that exogenous PC might be an effective inhibitor for preventing abnormal calcification in soft tissue, provided the compound is stable at the particular site of calcification. Whether or not the compound could be used to arrest urolithiasis would depend very much upon its rate of breakdown in biological tissues and fluids. There is some evidence however, that PC does undergo hydrolysis in renal tissue following administration (Williams and Sallis, 1981).

The aim of the present research was to consider the development of new compounds, in particular analogues of PC, which might prove more useful than the aforementioned compounds in the prevention of calcium urolithiasis. Past research with bisphosphonates and PC of course provided guidelines for such studies. Specifically, the criteria sought in the new compounds were that they a) shared a similar structure to PC, so as to have comparable anti-calcifying activity and b) were more resistant to lysis than the parent molecule, with none of the adverse side-effects reported to be associated with the bisphosphonates. In these respects, a number of potential analogues of PC were envisaged, differing from PC in their pattern of substitution at the P and O atoms of the P-O-C linkage. Many were eliminated on the basis of the degree of difficulty in preparation, their predicted stability, and ultimately, the possibility of toxic effects. Two analogues did appear to have potential, namely N-phospho-2-amino tricarballylate (PAT) and N-sulpho-2-amino tricarballylate (SAT), the phosphoramidate and sulphamate derivatives of PC respectively. Further, the synthesis of these analogues did appear readily achievable. From existing information, PAT was expected to be as potent an anti-calcifying agent as PC in vitro. SAT, although more difficult to predict, was expected to have potency somewhere intermediate between citrate and PC. In relation to the lability of these molecules, PAT was predicted to be cleaved by phosphatase enzymes, although whether it would be more stable to such lysis than PC was uncertain. SAT, in contrast, was foreseen to be highly resistant to breakdown. No evidence was available to suggest that compounds with such linkages might prove toxic at the bone site.

The synthesis of these particular analogues was therefore undertaken. Initially, it was necessary to synthesize the relevant precursors in 2-amino tricarballylate (AT), and its tri-ester. A successful preparation

of AT was achieved by following documented methods (Dornow and Rombusch, 1955). A tri-ester of this compound had not been reported, but a similar compound, trimethyl 2-nitro tricarballylate, had been prepared (Kaji and Zen, 1973). Hydrogenation of this latter compound was found here to yield two main products, namely the desired product in trimethyl 2-amino tricarballylate, and its deaminated derivative. After investigating purification methods, these two products were successfully separated by solvent extraction. Subsequently, trimethyl 2-amino tricarballylate was purified by distillation, and the new compound fully characterized by a number of techniques including [^{1}H]-NMR, mass spectroscopy, GC and nitrogen analysis.

With the availability of both AT and its trimethyl ester, it was then possible to investigate a number of synthetic routes for the preparation of PAT. Attempts to phosphorylate AT by selective agents, which included PMI, P-γ-P, and MgO + $POCl_3$, were initially undertaken in preference to routes that utilized the trimethyl ester of AT. Such methods had more appeal because, if successful, PAT would have been produced directly, without the need to eliminate protecting groups by further treatment. However, as the yields produced with these reagents were poor and not sufficient to meet the requirements of the project, the non-selective phosphorylants were then considered. o-Phenylene chlorophosphate appeared the most promising; nevertheless, despite the high yield of initial coupling with trimethyl 2-amino tricarballylate, major difficulties were encountered in the removal of the aromatic protecting group afforded with this phosphorylant. Whilst a number of attempts were made to overcome this problem, none proved satisfactory. Ultimately, another phosphorylant, CEP proved the most effective agent for producing PAT. The synthetic route was based on the method of Williams and Sallis (1980), although subtle variations were incorporated relating to the specific nature of the different reagents. With the synthesis reported herein, approximately equimolar proportions of phosphorylant and amine were utilized [c.f. Williams and Sallis (1980); large excess of alcohol] and use of this ratio allowed the omission of an ion-exchange purification step for partially purifying an intermediate coupled product. Although the yield was low for the overall synthesis (1%), this was still in excess of that produced by any other agent, and of importance, the final product was shown by subsequent analysis to be of high-purity.

The development of a new specific assay for detecting the presence of the AT moiety greatly aided in the detection of PAT eluting in column

fractions. For the characterization of PAT, it was also a requirement to develop analytical systems that would clearly resolve PAT from contaminants in order to assess the purity of the final product. TLC, high-voltage electrophoresis and isotachophoresis were employed and all supported the purity of the final sample. Further evidence of the structure of PAT was provided from analyses of constituents following hydrolysis, by the aforementioned analytical techniques in addition to chemical assays for phosphate and nitrogen. Definitive structural proof was provided by [^{1}H]-NMR spectroscopy.

In view of the difficulties and low yield encountered with the synthesis of PAT, it was clear that for the preparation of SAT the selection of a powerful sulphonating agent was essential. After examination of the literature, it was evident that pyridine-sulphur trioxide was such a compound. This agent, which was readily prepared by the reaction of chlorosulphonic acid with pyridine, offered two further advantages in that a) the compound was capable of selectively sulphonating amine groups, and b) using this reagent a route for the incorporation of a [^{35}S]-radiolabel was apparent. Subsequently, SAT was prepared directly in a 10% yield using this reagent to couple with AT. The reaction conditions were essentially as described by Warner and Coleman (1958) for the sulphonation of other amines, but superior methods were developed for the ultimate separation and purification of reaction components. Hence, after removal of excess AT by cation-exchange chromatography, and selective removal of sulphate by precipitation as a barium salt at pH 3.0, SAT could be precipitated as a barium salt at alkaline pH. Further purification by anion-exchange chromatography proved by subsequent analysis to yield the desired compound.

Analytical systems were developed to determine the purity of SAT. These systems, which included TLC, high-voltage electrophoresis and isotachophoresis, had features distinct from those used for PAT. Further, to aid in the detection of SAT resolved by the former two systems, a stain was developed that was specific for organic sulphate esters present as N-sulphate. Sulphate and nitrogen assays provided information on the chemical composition of the compound and were supportive of the proposed structure. The presence of specific functional groups within the molecule was supported by infra-red spectroscopy and [^{1}H]-NMR analysis again provided additional proof of the structure of the new compound. The basic method employed for the synthesis of SAT was also adapted to yield [^{35}S]-radiolabelled SAT and the identity and purity of the product derived from this synthesis was confirmed using the same techniques that were

utilized to characterize non-radiolabelled SAT.

Thus the preparation, purification and characterization of the two new PC analogues PAT and SAT was successfully achieved, and in the case of SAT, the incorporation of a radiolabel accomplished.

The next stage of the project was to evaluate whether the new compounds did share a similar in vitro anti-calcifying potential to that shown by PC. In assessing this parameter, two well established test systems were chosen that involved amorphous calcium phosphate being transformed to the hydroxyapatite phase, and secondly, the growth of calcium oxalate crystals. As predicted, both compounds proved capable of inhibiting in these systems, although varying degrees of effectiveness were evident with the different test systems. PAT was as potent as PC in preventing hydroxyapatite formation, but was a poorer inhibitor of calcium oxalate crystal growth; a fact later ascribed to the chemical lability of PAT under the operating pH of the latter system. SAT was demonstrated to be a strong inhibitor in both systems, although not as potent as PC.

The availability of other derivatives of tricarballylate, namely compounds differing in substitution at the 2-position, and "deacetylated" derivatives of PAT and SAT, enabled the testing of their inhibition of hydroxyapatite formation. Whilst all derivatives failed to produce the same degree of inhibition seen by PC and its analogues, nevertheless some interesting trends were revealed. Replacement of the phosphate group of PC (or similarly the sulphamate or phosphoramidate groups of SAT or PAT) by functional groups of less chelating power in -H, $-NH_2$, $-NH-CO-NH_2$ or $-NO_2$ in all cases rendered a molecule completely devoid of activity. Further, a decline in inhibitory power was noted with the loss of the acetyl chelating arm of SAT and PAT, to yield N-sulpho aspartate and N-phospho aspartate respectively. N-sulpho aspartate failed to show any inhibition over the concentration range studied. N-phospho aspartate was significantly less potent than PAT, with inhibitory power somewhat intermediate between SAT and citrate.

Early proposals for the structural requirements of potent inhibitor molecules suggested that possession of at least two proximal phosphate groups was a necessity (Robertson and Fleisch, 1970). PC however, has subsequently proven an exception to this rule, and has caused a reappraisal of the structure-activity relationship of inhibitor molecules (Williams and Sallis, 1979, 1982). It has been suggested that the ability of PC to inhibit potently in the absence of a second phosphate group is attributable to its unique stereochemistry; i.e. the possession of four strong chelating

arms radiating in a tetrahedral configuration from a central carbon atom (Williams and Sallis, 1982). Results here have reinforced this suggestion, whereby the removal or replacement of any chelating group of PC by a group with less chelating power, produces a marked reduction, if not total abolishment, of inhibitory power.

Delineation of the precise mechanism of action of the new compounds was not a primary objective in these studies. However, based on the general mechanistic ideas proposed for inhibitors of a similar structure to those studied here, it is probable that the new compounds act in a similar manner, i.e. through binding to specific sites on the crystal lattice where they inhibit further nucleation, growth and aggregation.

One unique finding was the demonstration that the sulphamate group can contribute to the inhibitory potency of a molecule. While not as strong as phosphate (or phosphoramidate), it is nevertheless far more effective than an hydroxyl group. The data obtained do not permit any valid comparison to be made between the sulphamate and carboxyl groupings. However, on the basis of the suggestion by Moreno et al., (1984), whereby the affinity of a functional group for hydroxyapatite is related to its dehydrating power, one would expect the sulphamate to be more active. In any event, the demonstrated contribution of this group toward inhibition now adds more flexibility with regard to the incorporation of functional groups into potential inhibitor molecules. In the future, this may aid in the development of further inhibitors even more potent than those currently tested.

Whilst a necessary requirement for any test compound to have potential as a therapeutic agent is its ability to potently inhibit calcification in vitro, other influences in vivo may ultimately determine its true effectiveness. Factors that warrant consideration are the absorption of the compound following oral administration, its metabolism, toxicity and ultimately its clearance to urine. Since radiolabelled PAT was unavailable, it was not possible to study these parameters with PAT. Even if it had been possible, in vitro assays indicated that the compound was more readily hydrolyzed by alkaline phosphatase than had been reported for PC by Williams (1981). Further, the compound also underwent rapid hydrolysis in the absence of enzyme at neutral pH, with the rate of hydrolysis becoming more marked with increasing acidity. These studies suggested that, even in the absence of enzyme hydrolysis, PAT would be rapidly degraded following oral administration by the acidity within the gut.

In contrast, SAT was generally shown to have suitable metabolic properties. The availability of [^{35}S]-radiolabelled SAT made it possible to

study a number of the pharmacokinetic properties of SAT. This compound was found to be well absorbed, rapidly cleared to urine, with no evidence of degradation occurring. These results were consistent with those of other workers studying similar properties of other sulphamate compounds, namely cycloalkylsulphamates (Spillane et al., 1979; Spillane and Benson, 1978), although possibly the absorption of these more lipophilic compounds may be mediated through a different mechanism than that which occurs with SAT. There was some evidence that a minor percentage of SAT was binding to bone, but the amount bound was certainly markedly less than has been reported following the administration of bisphosphonates (Michael et al., 1972). Whilst it was not possible to fully study the long-term effects that SAT might have had at the bone site due to the restricted availability of the compound, nevertheless, if SAT were to have a future role as a therapeutic agent, this factor would warrant more extensive investigation.

Having determined both the in vitro activity and some metabolic properties of the PC analogues, it remained for experimental verification whether the compounds would inhibit calcification in vivo. Two well documented experimental models were chosen for study. The deposition of calcium oxalate or hydroxyapatite within renal tissue was induced by administration of suitable lithogenic agents, and the effects of administration of inhibitor on the extent of calcification was studied. Comparison with the new inhibitors was made with both PC and HEBP. Both models proved suitable for study since HEBP was capable of inhibiting the induced calcification. Not surprising, PAT failed to inhibit in either system. SAT however, was shown to significantly reduce the deposition of calcium oxalate, but to have no effect on hydroxyapatite formation. With PC, the situation was reversed. A number of postulated mechanisms have been presented to explain the reverse actions of SAT and PC, including differences in a) specificity of the molecules for the crystallizing salts, b) localization within kidney tissue, c) lability, and d) any secondary effects associated with the molecules.

Although the reported rapid destruction of PC in kidney tissue following intravenous administration (Williams and Sallis, 1981) provided the initial basis for this project, the since demonstrated ability of PC to prevent the induced hydroxyapatite deposition within renal tissue by Tew et al. (1981) (and also now confirmed here) has thrown doubt on the original rationalization. Possible interpretations of these apparent contradictions are that a) PC may not be as labile as previously thought, and/or b) PC may undergo a selective hydrolysis at the kidney site, but the ensuing

elevation in levels of citrate might strongly interact synergistically with unhydrolyzed PC to partially offset the loss of inhibition due to PC hydrolysis. A more detailed pharmacokinetic study may clarify these and other uncertainties regarding the metabolic fate of administered PC. For example, an additional aspect that has yet to receive investigation is the fate of the orally administered compound. These studies may be of particular importance in providing information on the absorption of the compound or whether the compound undergoes hydrolysis prior to absorption. Information so gained may have major influence on its therapeutic usefulness.

In relation to future possible applications of PC and/or its analogues, the lability of PAT clearly rules out its use. The demonstrated ability of PC to inhibit however, suggests that a reappraisal of its therapeutic potential is required. The simplest interpretation of the results of Tew et al. (1981) and the data reported herein, is that PC may be effective in the prevention of calcium phosphate stones. SAT, on the other hand, by virtue of its ability to inhibit calcium oxalate crystallization with the model studied here, may be more useful in the treatment of stones comprised mainly of this salt. These interpretations are probably oversimplified, since the limitations of the experimental models have been discussed previously and obviously do not truly reflect what may happen in the natural situation.

Additional avenues for investigation for the uses of SAT and PC are apparent. As oral administration of citrate has been shown to increase urinary citrate levels and prove effective in stone prevention in some specific cases (Pak, 1983), one perhaps logical form of treatment that should be considered is the co-administration of PC and citrate. The strong synergistic actions between PC and citrate evident in vitro may make this form of treatment effective if such a relationship occurs in vivo. Since the biosynthetic pathway associated with PC synthesis and degradation is not fully elucidated, one further advantage of co-administration of these agents might arise if a specific enzyme was responsible for the major lysis of PC in the kidney. A property shared by many enzymes is the phenomenon of product inhibition, whereby the product of the enzyme reaction inhibits the enzyme and thus may regulate its activity. If citrate were to act in a similar fashion on an enzyme responsible for PC lysis, then co-administration of citrate may limit the hydrolytic action of the enzyme, thus sparing PC. Whilst this is speculative, establishment of the processes involved with PC metabolism could provide insight into ways not only to

extend the biological half-life of administered PC, but also to increase the endogenous production of PC. In either case, such methods would then prove an effective treatment.

Results suggest that, in vivo, PC and SAT may be selective in their inhibitory actions on calcium salt crystallization. If so, administration of both these agents simultaneously may be more effective in preventing the growth of stones of mixed composition than the administration of either agent alone. This therapy may have additional advantages if a synergistic relationship can be shown to exist between PC and SAT (c.f. PC and citrate).

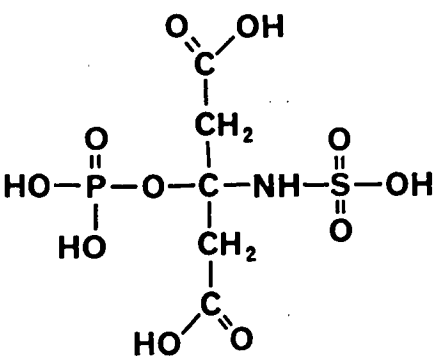
Whilst the application of anti-calcifying agents (specifically PC and its analogues) for the prevention of renal calcification has been considered, a number of other diseased states associated with abnormal calcification exist where the use of such agents may prove effective. Bisphosphonates, for example, whilst unsuitable for stone disease, have in some reports proven to be effective in the treatment of certain bone disorders such as Paget's disease (Fleisch, 1983) and rheumatoid arthritis (Bijvoet et al., 1980). With respect to PC and its analogues, a recent investigation by Shankar et al. (1984) suggests that these agents may in future have clinical importance in the treatment of arteriosclerosis. These latter workers demonstrated that PC was potently effective in preventing aortic medial calcification, a process that is thought to have a significant contribution to the development and acceleration of atherosclerosis (Ross and Glomset, 1976). SAT was also shown to inhibit, although less effectively. Investigations are also currently in progress to evaluate the potential of these two agents in the prevention of bladder stones.

Major discussion in this thesis has focused on the role of inhibitors and other factors affecting renal calcification, but some comment is possibly required on the mechanisms associated with calcification at other sites. Lehninger (1977) has proposed a function of mitochondria in the regulation of normal controlled calcification (e.g. bone and cartilage) and also in initiating certain forms of pathological calcification. This property of mitochondria appears to be related to their ability to accumulate calcium in high concentrations, such that the solubility product of amorphous calcium phosphate may be exceeded. Under these conditions, the level of inhibitors within the mitochondria may have a crucial role in determining whether the calcium phosphate is stabilized in the amorphous phase, or whether it is transformed to the more mineralized form of hydroxyapatite. Whilst PC has been implicated as being present

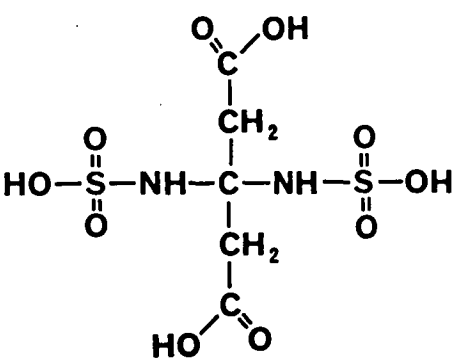
in urine, nevertheless, intracellularly, the compound has been found to be exclusively contained within mitochondria. This has led to the conclusion that a major role of the compound is in the maintenance of a soluble form of calcium phosphate within mitochondria. In this regard, Lehninger (1977) has suggested that PC is a major inhibitor responsible for the regulation of mitochondrial associated calcification. Confirmation of this idea has yet to be made. Whilst mitochondrial calcification has been suggested as a precursor to a number of forms of pathological calcification (Anderson, 1981), not all authors agree as to its contribution in initiating the formation of calcium containing renal stones (Ganote et al., 1975). Therefore, whether any relationship exists between the levels of inhibitors (such as PC) in mitochondria, and the pathogenesis of renal stones is uncertain.

Information gained here on the structure-activity relationships of inhibitors, and metabolic properties of the sulphamate group, together with existing information, also allows a prediction of what direction future research could take to develop superior PC analogues. Fig. 8.1 indicates a range of analogues that might be considered. The preparation of compounds I and II (see Fig. 8.1) might require the investigation of alternate synthetic strategies utilizing distinct precursors from those employed here, although compounds III and IV might be derived from PC, and similarly compound V from SAT. All compounds would be expected to be potent inhibitors of calcification in vitro. Whether they would prove useful in vivo would be dependant upon a number of other factors previously mentioned, including absorption, stability, tissue selectivity and possible toxic effects. In relation to stability, compound II, on the basis of the demonstrated stability of the sulphamate linkage, should be resistant to enzyme action. The inclusion of an imidodiphosphate group (P-N-P bond), reportedly a group with greater stability than the pyrophosphate moiety, could make compound III more stable than, for example, PC. Compounds I, IV and V are derivatives whereby both phosphate and sulphamate (or sulphate) are substituted at various positions within the basic molecular skeleton. If the incorporation of the sulphamate (or sulphate) groups were to have a stabilizing action on the normally labile phosphate ester linkages, this effect would also make these compounds useful. Whilst this latter suggestion is to a large degree supposition, nevertheless in the case of compounds III-V, an additional factor might compensate if the compounds were not as stable as desired. While the compounds might be strong inhibitors in their own right, if they were cleaved, the liberated products would include PC in the case of compounds III and IV, and SAT with V. Therefore, both the

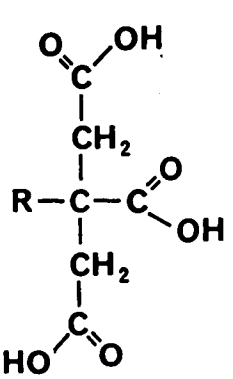
Fig. 8.1 A range of derivatives expected to be potent inhibitors of calcification.

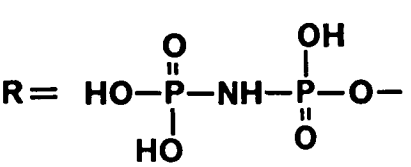


(I)

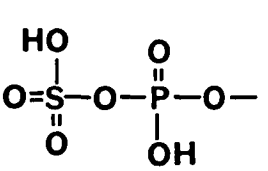


(II)

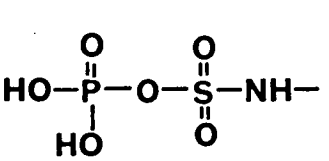




(III)



(IV)



(V)

compounds themselves and their hydrolytic products would be potent inhibitors. In effect, following administration, these compounds might therefore have a greater biological half-life in terms of inhibitory activity than would administration of PC or SAT alone.

In conclusion, based on the studies presented here, future research on urolithiasis and other forms of pathological calcification could be directed into two important areas. There is a clear need to gain an understanding of the biosynthesis and metabolism of PC. By doing so, control over the endogenous production of PC might be realized which could be of benefit. Further, continued investigations of structure-activity relationships should help in the development of more powerful anti-calcifying compounds, which may also provide an effective treatment method for the prevention of diseases associated with abnormal calcification.

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N-SULFO-2-AMINO TRICARBALLYLATE, A NEW ANALOG OF PHOSPHOCITRATE: METABOLISM AND INHIBITORY EFFECTS ON RENAL CALCIFICATION.

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INTRODUCTION

Although the role and precise mechanism of action of urinary inhibitors has not been fully elucidated, most authors agree that measures leading to an increase in endogenous or exogenous inhibitors will prevent kidney stone disease. To warrant investigation as a therapeutic agent an inhibitor should be well absorbed when given orally, potently effective with minimal or no side effects, target specific and rapidly cleared. Few compounds meet these strict criteria. Perhaps the most useful of the synthetic inhibitors have been the diphosphonates, which are proven potent anti-calcifying agents. Nevertheless their value is reduced by restricted absorption and known secondary changes to bone metabolism. Of the new inhibitors, phosphocitrate (PC), a naturally occurring compound and a potent inhibitor of calcification in vitro appears the most promising. However, the nature of the chemical bonds of PC make this molecule susceptible to enzyme hydrolysis. This has led us to develop a more stable PC analog, namely N-sulfo-2-amino tricarballylate (SAT) (Fig.1). Some characteristics of this enzyme resistant sulfamate analog are described.

MATERIALS AND METHODS

SAT and PC were prepared by methods previously documented [1, 2]. Radiolabeled (^{35}S) SAT was synthesized in a similar fashion to that of non-radiolabeled SAT (unpublished). Ethane-1-hydroxy-1, 1-diphosphonate (EHDP) was a generous gift from Procter and Gamble.

The stability of SAT in vivo was determined following the intravenous administration of ^{35}S-SAT (10umol, sp. act. 0.24mCi/ mmol) to rats (200g). The ureters were ligated and at predetermined times the rats were sacrificed. Liver, kidneys and bladder plus contents were removed, as well as samples from blood and bone. Total counts in tissue were measured following the oxidation of samples by a $HClO_4$/ H_2O_2 mixture. Counts attributable to ^{35}S-SAT were determined separately after the preparation of an acid extract from tissues and further removal of contaminating ions from radiolabeled products by ion-exchange chromatography. The eluant was desalted, lyophilized then redissolved in a minimum volume before diagnostic analysis of the radio-labeled products by paper electrophoresis.

The absorption of SAT following oral administration was established using a similar dose of ^{35}S-SAT given to rats (200g) in metabolic cages. Urine and faeces were collected after 1, 2 and 3 days and were treated as above following acid extraction.

The effects of SAT on calcification in vivo was studied using two different models:

A) Rats (100g) were given an intraperitoneal dose of inhibitor (3.3-50umol) 1h prior to an intraperitoneal challenge of sodium oxalate (7mg). In the absence of inhibitor

this model has been shown to result in the massive deposition of calcium oxalate crystals within the kidney [3]. The rats were sacrificed 4h later and the extent of calcium oxalate deposition in renal tissue determined by calcium analysis (Atomic Absorption Spectrophotometry) after tissue digestion.

B) Rats (50g) were given an intraperitoneal dose of inhibitor (3umol) 1h prior to an intraperitoneal dose of calcium gluconate (1.0ml; 10%) daily for 9 days. Treatment in the absence of inhibitor has been shown by others to result in the massive deposition of hydroxyapatite (HA) within the kidney [4]. Kidneys were removed 24h after the final injection and calcium determined by methods previously described.

RESULTS

The distribution of radioactivity following the intravenous administration of ^{35}S-SAT is shown in Fig. 2. Following the initial uptake of counts by tissues from blood, radioactivity was cleared rapidly to the urine. Few counts were present in tissues after 24h (not shown) with the exception of bone which retained 1% of the initial dose for at least 3 days. Electrophoretic analysis proved that SAT was resistant to enzymic degradation in all of the tissues and body fluids studied (Fig.3). When SAT was given orally and faeces and urine collected by the use of metabolic cages, data indicated that there was 70% absorption with 80% of the absorbed SAT being cleared to the urine within 24h.

In relation to the studies on the effects of PC and SAT on calcification in vivo, the inhibitors had differing effects depending on the particular model studied. With the model involving calcium oxalate deposition, SAT (6.7umol) reduced by 25% kidney calcium relative to control whereas PC at this

level had no effect. The degree of inhibition was only slightly increased by higher concentrations of SAT; 50umol of SAT resulted in only a 30% reduction (Fig.4). Oxalate analyses also confirmed these trends (not shown). With the other model, phosphocitrate was very effective in preventing HA formation reducing kidney calcium by 50% relative to control confirming the results produced by other authors [4]. SAT however, failed to cause any reduction at the same concentration (Table 1). EHDP, a well documented calcification inhibitor but possessing adverse long-term side effects, was used as a reference inhibitor in these studies and with both models proved more potent than either PC or SAT.

CONCLUSION

Previous studies have shown that SAT is capable of inhibiting potently both calcium oxalate crystallization and HA formation *in vitro*. Although not as good as PC, nevertheless its activity was comparable to that of pyrophosphate [1]. This information has promoted our further investigations into the properties of this new inhibitor to evaluate its therapeutic potential as a treatment for nephrocalcinosis. In this study, SAT was found to be well absorbed, resistant to enzymic degradation, and rapidly cleared to the urine. In the short term there was no evidence of toxicity in rats given large doses. The data are suggestive that SAT may be more effective in preventing calcium oxalate nephrolithiasis than PC, whereas with the deposition of HA, the reverse action of inhibitors seemed to occur. The response differences may be related to the sites of crystallization and distribution of inhibitors within tissues, metabolism of inhibitors, mechanism of action of the inhibitors in relation

to the different crystal types or various other factors. In view of the encouraging responses obtained with SAT, other analogs which may be more potent are currently being investigated. Such studies may help to extend knowledge of the structure-activity relationships of this class of inhibitors in relation to inhibition of biological calcification.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Health and Medical Research Council.

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Table 1 The deposition of calcium within renal tissue following the daily administration of calcium gluconate (1.0ml; 10%) in conjunction with inhibitor (3umol/ day) to rats for 9 days.

Inhibitor	Number of Rats / Group	Kidney Calcium* (umol/ g tissue)
None (control)	10	45±12
SAT	5	52±4
PC	3	25±10

* Left and right kidney were analyzed separately.

Fig.1 Structures of N-sulfo-2-amino tricarballylate (SAT) and phosphocitrate (PC).

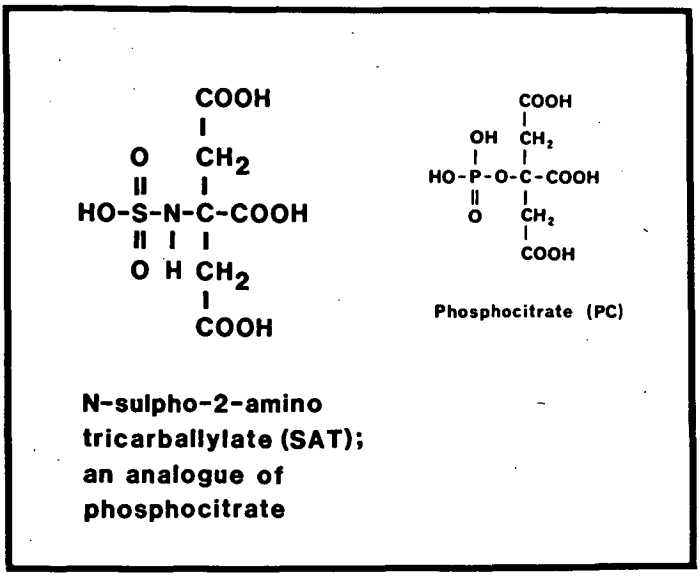


Fig.2 Distribution of radioactivity following I.V. administration of ^{35}S-SAT to rats. At designated times rats were sacrificed, samples collected, oxidized and counted.

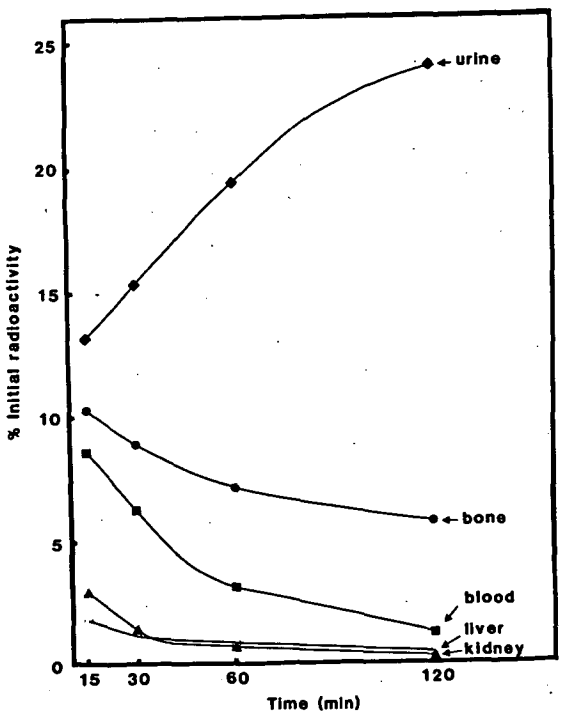


Fig.3 Analysis by electrophoresis of ^{35}S-SAT labeled compounds (■) present in kidney extract 2h after an I.V. dose of ^{35}S-SAT. Comparison is made to reference standards (▨). Running conditions: 3% formic acid - 2.5% acetic acid at 2000V for 40min.

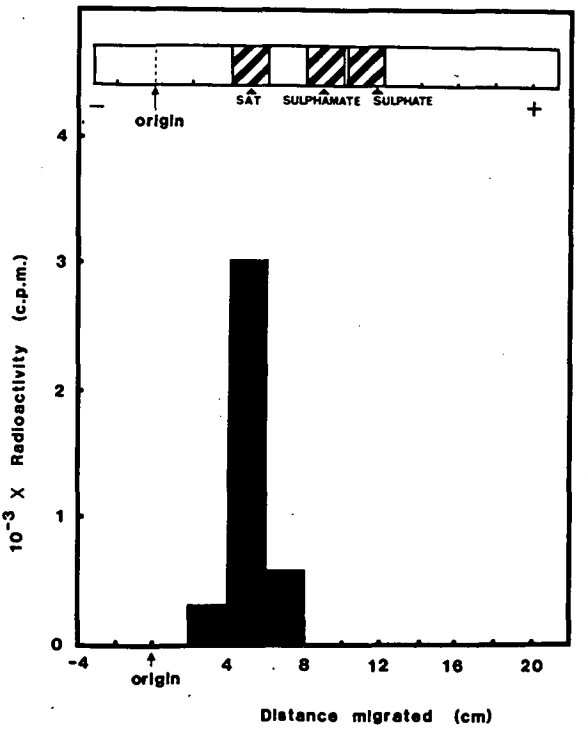
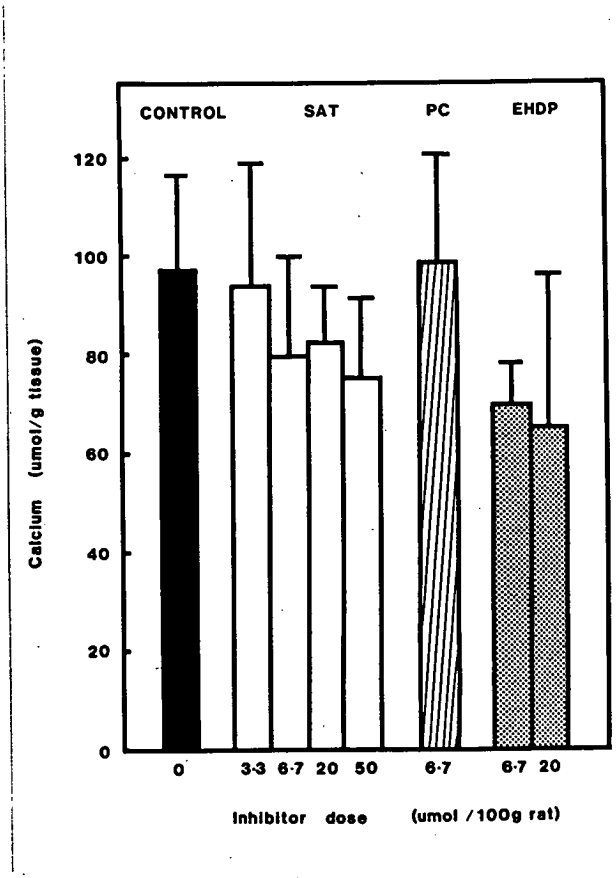


Fig.4 Effect of inhibitors on the deposition of calcium oxalate in kidney tissue. Rats (100g) received an intraperitoneal dose of inhibitor 1h prior to an intraperitoneal challenge of sodium oxalate (7mg). The rats were sacrificed 4h later and kidney calcium determined.



J. of Labelled Compounds & Radiopharmaceuticals - in Press

SYNTHESIS OF N-[^{35}S]-SULPHO-2-AMINO TRICARBALLYLATE

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SUMMARY

The synthesis of [^{35}S]-labelled N-sulpho-2-amino tricarballylate (SAT) and methods for its purification and analysis are described. In the first stage of the two-step synthesis, chloro-[^{35}S]-sulphonic acid is reacted with pyridine to yield pyridine-[^{35}S]-sulphur trioxide. This agent is then utilized to sulphonate 2-amino tricarballylic acid yielding the ultimate product [^{35}S]-SAT, which was required for pharmacokinetic studies.

Key Words: N-sulpho-2-amino tricarballylate, [^{35}S]-label, sulphonation, sulphamate, phosphocitrate analogue, calcification inhibitor

INTRODUCTION

In recent years, a variety of both natural and synthetic compounds capable of inhibiting calcification *in vitro* have been investigated as therapeutic agents for the prevention and treatment of kidney stone disease. However, the majority of inhibitors tested to date have proven unsuitable as they are either a) destroyed rapidly in the body, b) unsuitable for oral administration due to poor absorption, c) insufficiently potent, or d) toxic when administered over long periods. We have recently described the synthesis of a new compound, N-sulpho-2-amino tricarballylate (SAT), a sulphamate analogue of the naturally occurring inhibitor phosphocitrate (1). SAT has proven to be a potent inhibitor of calcification *in vitro* although not as good as phosphocitrate, nevertheless preliminary studies have indicated that the bonds of SAT are much more resistant to enzymic degradation than the labile phosphate ester bond of phosphocitrate (2). To study the stability and pharmacokinetics of SAT *in vivo*

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and therefore evaluate its true therapeutic potential, the incorporation of a radiolabel into the molecule was clearly a necessity.

The method proposed for this synthesis was based on existing methods for preparing non-radiolabelled pyridine-sulphur trioxide (3) and SAT from this latter agent (1). These methods were scaled down and modified to achieve maximum utilization of the radiolabel. Hence, chloro-[^{35}S]-sulphonic acid was reacted with pyridine to yield pyridine-[^{35}S]-sulphur trioxide, which in turn was coupled with 2-amino tricarballylate to give [^{35}S]-SAT (Fig. 1).

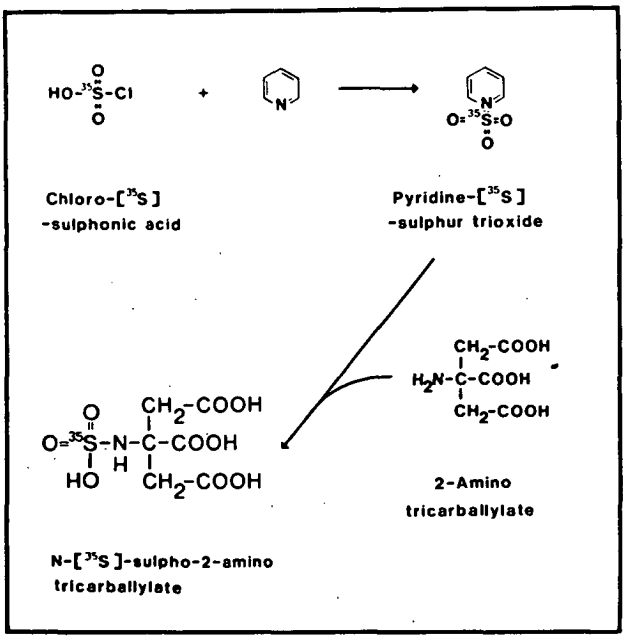

Fig. 1 Synthetic route for the preparation of N-[^{35}S] -sulpho-2-amino tricarballylate

MATERIALS

Chloro-[^{35}S]-sulphonic acid and [^{35}S]-sodium sulphate were obtained from Amersham, Buckinghamshire, England. The resins AG 2-X8 (Cl^-; 100-200 mesh) and AG 50W-X8 (H^+; 200-400 mesh) were products of the Bio-Rad Laboratories, Richmond, California, U.S.A. The AG 2-X8 resin was converted to the bicarbonate

form before use. All chemicals were reagent grade and obtained from commercial sources. The scintillation fluid comprised of a mixture of 0.6% 2,5 diphenyloxazole in toluene and cellusolve (10:6 v/v).

METHODS

Preparation of Pyridine-[^{35}S]-Sulphur Trioxide

To a standard scintillation vial equipped with a magnetic stirrer bar was added 3.0 ml of anhydrous chloroform and 160 µl anhydrous pyridine. The vial was sealed with a rubber cap and cooled to 0° C by means of an ice-bath. Chloro-[^{35}S]-sulphonic acid (2 mCi/5 µl) was then diluted to 100 µl with non-radiolabelled chlorosulphonic acid, and drawn up into an airtight syringe fitted with teflon tubing. The rubber cap of the reaction vial was pierced, the tip of the teflon tubing introduced, and the syringe was clamped in an upright position above the level of the vial as depicted in Fig. 2.

The chloro-[^{35}S]-sulphonic acid was then added dropwise from the syringe over a 10 min period with stirring while the temperature was maintained at 0° C. At the end of this addition, the ampoule originally containing the chloro-[^{35}S]-sulphonic acid was rinsed with 100 µl of dry chloroform. This was again drawn up into the syringe, and the contents added to the reaction vial as before. The reaction mixture was allowed to stir for a further 5 min at 0° C before filtration under suction. The collected pyridine-[^{35}S]-sulphur trioxide was then washed with ice-cold anhydrous chloroform (2.0 ml), which had been used to rinse the reaction vial. The

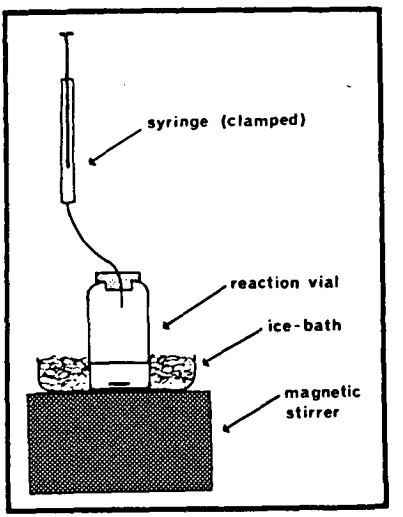

Fig. 2 Apparatus used for pyridine -[^{35}S]-sulphur trioxide synthesis

solid was dried thoroughly *in vacuo* over concentrated sulphuric acid in a desiccator for 2 h. The scintillation vial was retained for use in the next stage of the synthesis, and also dried in this fashion. The radiochemical yield of pyridine-[^{35}S]-sulphur trioxide was 27% (65 mg; sp. act. 8.3 μCi/mg).

Preparation of N-[^{35}S]-Sulpho-2-Amino Tricarballylate

To the scintillation vial used for the previous preparation was added 500 mg of 2-amino tricarballylic acid [prepared from diethyl acetone-dicarboxylate by the method of Dornow and Rombusch (4)] in 10 ml of water; the temperature was lowered to 10° C and the pH adjusted to 9.6 with 2 M NaOH. The pyridine-[^{35}S]-sulphur trioxide (65 mg) was added in 5 portions with continuous stirring over a 10 min period. The pH was maintained at 9.6 with 2 M NaOH using a pH-stat and the temperature held constant during this time. The mixture was then allowed to stir for a further 30 min with the pH still maintained at 9.6 while allowing the temperature to rise to 20° C. The resulting clear yellow solution was then passed through a 1.5 x 5.5 cm column of AG 50W-X8 (H^+) at 4° C to remove unreacted pyridine and 2-amino tricarballylate. After washing the column with a further 10 ml of water, the combined eluants were adjusted to pH 9.0 and loaded onto a 2.2 x 1.5 cm column of AG 2-X8 (HCO_3^-). After a preliminary wash with 250 ml of 0.35 M $NaHCO_3$ to remove [^{35}S]-sulphate, the stronger binding [^{35}S]-SAT was recovered by elution with 250 ml of 0.60 M $NaHCO_3$ collected in 5 ml fractions. The presence of [^{35}S]-sulphate and [^{35}S]-SAT was determined by measuring the radioactivity of aliquots (100 μl eluant/10 mls scintillant) taken from the 5 ml fractions collected (see elution profile, Fig. 3). Relevant tube contents containing [^{35}S]-SAT were pooled and decarbonated by stirring at 4° C for 10 min with an excess of AG 50W-X8 (H^+). The suspension was filtered, and the filtrate was adjusted to pH 7.0 with NaOH and lyophilized to give a white powder (25 mg; sp. act. 3 μCi/mg). The yield of radiochemical for this step was 15%, with the overall radiochemical yield for both steps being 4%.

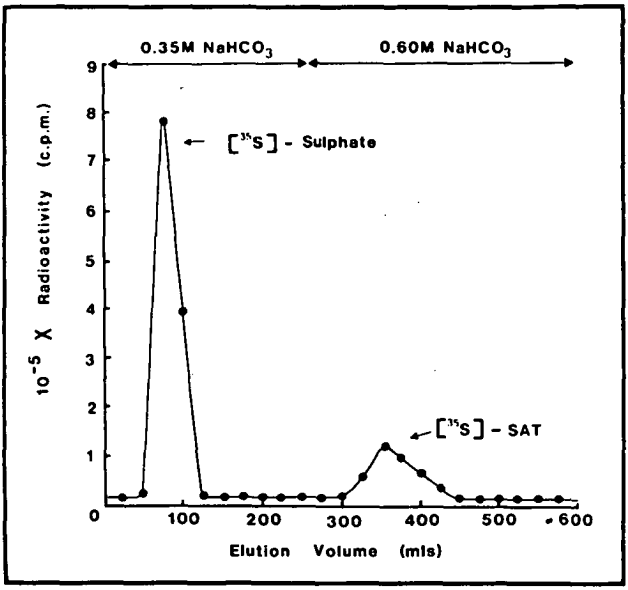

Fig. 3 Elution of [^{35}S]-SAT from AG 2X-8 resin

Analysis

Inorganic and N-hydrolyzable sulphate analyses (1), nitrogen determination (5) and high voltage paper electrophoresis were utilized to confirm the purity of [^{35}S]-SAT. Electrophoresis was performed by applying sample (1 µl; 1 mg/ml) of [^{35}S]-SAT and [^{35}S]-sulphate standard diluted to the same specific activity to Whatman No.3 MM paper and running at 2000 V for 1 h in an acid buffer [2.5% (v/v) acetic acid and 3% (v/v) formic acid]. The paper was dried and marked in a grid fashion before cutting into sections (3 cm wide and 1 cm long) incorporating the migration path of the compounds. These sections of paper were extracted with 2.0 ml of 1 M HCl, and the extracts were added to vials with 18.0 ml of scintillation fluid for counting.

RESULTS AND DISCUSSION

Radiolabelled [^{35}S]-SAT was successfully prepared from chloro-[^{35}S] -sulphonic acid with an overall radiochemical yield of 4%. The low yield was

largely attributable to the poor coupling of pyridine-[^{35}S]-sulphur trioxide with 2-amino tricarballylate (15%) a factor encountered with the synthesis of non-radiolabelled SAT (1) and presumably due to the steric hindrance surrounding the amino group of 2-amino tricarballylate. Nevertheless, the product was found to be of high purity as determined by sulphate analyses (free sulphate <0.1%; hydrolyzable N-sulphate 2.36 µmol/mg) and nitrogen analysis (2.41 µmol N/mg). This gave a sulphate to nitrogen ratio of 0.98 (theoretical value 1.00). Electrophoretic analysis (Fig. 4) confirmed these findings, showing the final product to be free of inorganic sulphate and any other detectable radiolabelled contaminants. In addition, the R_f of [^{35}S]-SAT in this system and its elution profile from AG 2-X8 resin was identical to non-radiolabelled SAT which had been fully characterized by physical ([^{1}H]-N.M.R., infra-red spectroscopy) as well as the abovementioned chemical techniques (1).

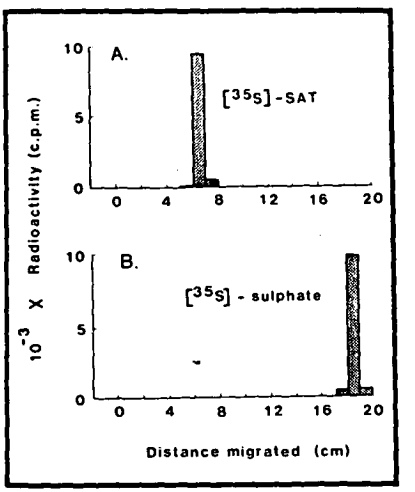

Fig. 4 Electrophoretic analysis of A) synthetic [^{35}S]-SAT, and B) standard [^{35}S]-sulphate

In summary, despite the low yield, N-[^{35}S]-sulpho-2-amino tricarballylate was produced in high purity by this simple and rapid two-step synthesis. The product obtained was of sufficient activity to support 20-30 pharmacokinetic experiments with laboratory rats, and these studies are currently being undertaken to evaluate the role that this new compound might have as a future drug in the treatment of kidney stone disease.

ACKNOWLEDGEMENTS

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ANALYTICAL BIOCHEMISTRY 132, 115–123 (1983)

The Synthesis and Characterization of *N*-Sulfo-2-amino Tricarballylate: An Analog of Phosphocitrate and Inhibitor of Calcification

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The synthesis of *N*-sulfo-2-amino tricarballylate, a sulfamate analog of phosphocitrate, is described using pyridine–sulfur trioxide to sulfonate 2-amino tricarballylate. The product was purified using selective precipitation and chromatographic techniques and characterized by a variety of physical and chemical means. In particular, its ability to inhibit hydroxyapatite formation and calcium oxalate crystallization was assessed by comparison with known inhibitors including phosphocitrate and pyrophosphate. On the basis of these results, this new compound may ultimately have a role in preventing abnormal biological calcification.

KEY WORDS: *N*-sulfo-2-amino tricarballylate synthesis and purification; sulfonation; phosphocitrate analog; inhibitor; calcium oxalate; calcium phosphate.

Phosphocitrate (PC),[2] a naturally occurring compound (1), is now recognized as an extremely potent inhibitor of calcification (2–4). However, its true potential as an *in vivo* inhibitor may be dependent upon its ultimate lability in body tissues and fluids. While the compound remains relatively stable in circulation, it is rapidly degraded by the kidney (1). As PC can be cleaved to phosphate and citrate by phosphatases *in vitro* (5), degradation may be due to phosphatases, or even specific phosphohydrolases. Whereas this may be an advantage in controlling the concentrations of phosphate and citrate for biological calcification, this could severely limit the usefulness of PC as a therapeutic agent in the prevention of calcification.

From these considerations it is clear that there is a need to develop a new compound, structurally similar to PC to ensure inhibitory activity, yet with increased resistance to enzymatic degradation. Such a compound might therefore prove to be a more useful and equally potent inhibitor of calcification in biological tissue than PC. In this report, we describe the synthesis of a sulfamate analog of PC, namely *N*-sulfo-2-amino tricarballylate (SAT). Pyridine–sulfur trioxide, a complex previously shown to be capable of selectively sulfonating various polyfunctional amines (6), was used to sulfonate 2-amino tricarballylic acid to achieve this synthesis. Characterization of the compound by physical and chemical means is detailed, and the compound's possible therapeutic value is discussed.

MATERIALS

All chemicals used were reagent grade and obtained from commercial sources. The ion-exchange resins AG 2-X8 (Cl^-; 100–200 mesh) and AG 50W-X8 (H^+; 200–400 mesh) were products of Bio-Rad Laboratories, Richmond, California. The resins were cycled before use and the AG 2-X8 resin was converted to the bicarbonate form. Sodium bicarbonate buffers used for column chromatography were adjusted to a pH of 7.2 by bubbling CO_2 gas through them for 10 min.

[1] To whom correspondence should be addressed.

[2] Abbreviations used: PC, phosphocitrate; SAT, *N*-sulfo-2-amino tricarballylate; AT, 2-amino tricarballylate; HPMC, hydroxypropylmethylcellulose; HCB, hexachlorobutadiene; ACP, amorphous calcium phosphate; HA, hydroxyapatite.

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ethanol, acetone, and ether, respectively, before air drying. A yield of 2.7 g of the partially pure product was obtained. Before final purification by ion-exchange chromatography, the barium was removed. This was done by stirring the solid with AG 50W-X8 (H^+; 50-ml resin volume) for 10 min before pouring as a slurry onto a column of AG 50W-X8 (H^+; 3.5 × 4.0 cm). After the column was washed with 50 ml of water, the combined eluants were adjusted to pH 7.5–8.0 before loading onto a 3.5 × 6-cm column of AG 2-X8. After a preliminary wash with 500 ml of 0.35 M $NaHCO_3$ to remove residual sulfate, SAT was recovered by elution with 0.60 M $NaHCO_3$, and collected in 100 fractions of 10-ml volume. The presence of SAT was determined by analysis of sulfate before and after hydrolysis. Relevant fractions found to contain SAT were pooled and an excess of AG 50W-X8 (H^+) was added with stirring at 4°C for 10 min to remove $NaHCO_3$. The suspension was filtered and pH adjusted to 7.0 before lyophilization. This process was repeated with the freeze-dried product to ensure complete decarbonation. The subsequent yield was 500 mg (approximately 10%).

Analysis

Sulfate. Two methods were employed for the analysis of free sulfate and *N*-sulfate. For column chromatography, free sulfate was detected in 0.5-ml aliquots from fractions by first decarbonating with 100 μl of acetic acid and then assaying using the sodium rhodizonate method outlined by Terho and Hartiala (10). To detect *N*-sulfate using this procedure, 0.5-ml aliquots were incubated with 100 μl of 15% $NaNO_2$ and 100 μl acetic acid for 30 min at room temperature before assaying. Under these conditions, sulfate is quantitatively released from *N*-sulfate linkages (11). While this method was the most sensitive found for the detection of inorganic and organic sulfate, a somewhat less sensitive turbidimetric assay was found to be more reliable for the quantitative assay of sulfate in the final product. This was a modification of that described by Inoue and Nagasawa (11) and was as follows. For *N*-sulfate, 0.5 ml of a sample or standard (0.5–2.5 μmol) was added to 0.5 ml 5% $NaNO_2$ and 0.5 ml of 33% (v/v) acetic acid, shaken, and left standing for 30 min at room temperature. Trichloroacetic acid (2.0 ml; 8% w/v) was added and the tubes were shaken, followed by the addition of 1.0 ml of barium chloride–gelatin reagent (1 g $BaCl_2$ and 0.5 g gelatin/100 ml) with immediate shaking. The solution was allowed to stand for 20 min, and its turbidity was then measured at 500 nm. This method was made applicable to free sulfate determinations by the substitution of 0.5 ml of 5% $NaNO_2$ and 33% acetic acid with 1.0 ml of water.

Nitrogen. The nitrogen content of the product was determined as ammonium following digestion with concentrated sulfuric acid, as outlined by Johnson (12).

Thin-layer chromatography. Samples (1 μl; 10 mg/ml) of SAT, Na_2SO_4, or sodium sulfamate were applied to plates coated with cellulose (0.25 mm; MN 300HR from Machery–Nagel, Düren, West Germany) and developed in a solvent mixture of isobutyric acid: methanol:7.5% (w/w) ammonia:10% (w/v) trichloroacetic acid (40:100:10:15 v/v) for 3 h. Plates were air-dried, and trichloroacetic acid was removed by careful dipping in diethyl ether. Following ether evaporation, the plates were sprayed with a mixture containing equal volumes of 5% $NaNO_2$ and 33% acetic acid and placed in an enclosed tank containing the same mixture in such a manner that the plates did not come in contact with the liquid. This was done to effect the hydrolysis of the *N*-sulfate linkage in SAT. After 30 min the plates were removed and dried at 60°C. Identification was made by spraying with a solution of equal volumes of Reagent A (20 ml 10 *M* acetic acid, 4 ml 0.025 *M* $BaCl_2$, 16 ml 0.1 *M* $NaHCO_3$ made up to 100 ml with ethanol) and Reagent B (10 mg sodium rhodizonate, 100 mg ascorbic acid in 20 ml of water made up to 100 ml with ethanol). Sulfate appears as a white spot on a pink background.

pounds tested were SAT, PC, and pyrophosphate at a concentration of 27 μM.

Calcium in the filtrates was determined by atomic absorption spectroscopy (Varian Techtron Model 1000, Victoria, Australia). Aliquots from the filtrates (0.7 ml) were mixed with a lanthanum solution (0.4 ml of 50,000 ppm), 5 N $HClO_4$ (0.2 ml), and water (0.7 ml). Concentrations were determined on a scale calibrated with standards ranging from 0 to 6 μg Ca^{2+}/ml.

RESULTS AND DISCUSSION

Following partial purification by chromatographic and selective precipitation techniques, *N*-sulfo-2-amino tricarballylate was separated from the other reaction components by anion-exchange chromatography using AG 2-X8 (HCO_3^-) resin. After removal of free sulfate and other contaminating ions with a batch elution of 0.35 M $NaHCO_3$, the stronger binding SAT was recovered by elution with 0.60 M $NaHCO_3$. A typical elution profile for sulfate and SAT is shown in Fig. 2.

Considering the possibility that impurities not detected from ion-exchange chromatography were coeluting with SAT, a variety of systems were employed for the characterization of the final product to confirm both its purity and identity. Sulfamate was recognized as a potential impurity in the final product, derived possibly through ammonium contamination in the reaction mixture or breakdown of SAT. For this reason, sodium sulfamate was included as a reference in the characterization techniques. High-voltage electrophoresis gave definitive separation of SAT, sulfate, sulfamate, and AT, and with this system, synthetic SAT was shown to be free of these components as well as any other detectable impurities (Fig. 3). This was also the case with thin-layer chromatography (R_f values: sulfate, 0.09; SAT, 0.17; and sulfamate, 0.30) and isotachophoresis (step height in mm: chloride, 5; SAT, 39; AT, 66; and glutamate, 90).

Sulfate assays indicated that less than 0.1% of free sulfate was present in the final sample of SAT. Following hydrolysis, 2.36 μmol of sulfate was produced per milligram of SAT. Since nitrogen digestion revealed 2.41 μmol of nitrogen present per milligram of SAT, a sulfate to nitrogen ratio of 0.98 was obtained. This was in good agreement to the theoretical value of 1.00.

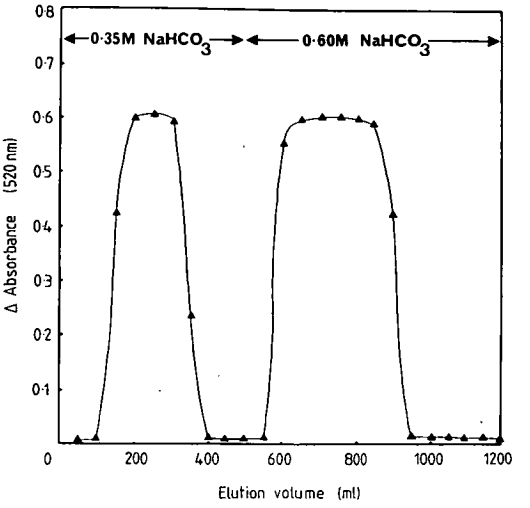

FIG. 2. Elution of SAT from AG-2X8 (HCO_3^-) resin. Following removal of sulfate with 0.35 M $NaHCO_3$, SAT was eluted with 0.60 M $NaHCO_3$. All fractions were treated with $NaNO_2$/acetic acid and then assayed for free sulfate by methods described in the text.

The quantitative hydrolysis of SAT by nitrous acid shown by the sulfate analysis as well as electrophoresis and thin-layer chromatography was diagnostic for the presence of an N—S bond in the molecule. This method of hydrolysis is specific for the cleavage of *N*-sulfate whereas it does not significantly hydrolyze the *O*-sulfate linkage (11).

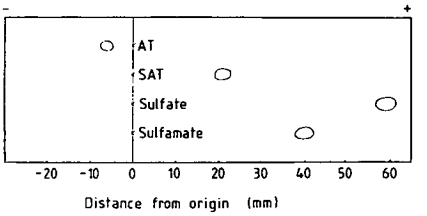

FIG. 3. Electrophoretic analysis of SAT and other reaction components. Running conditions and staining techniques were as described in the text.

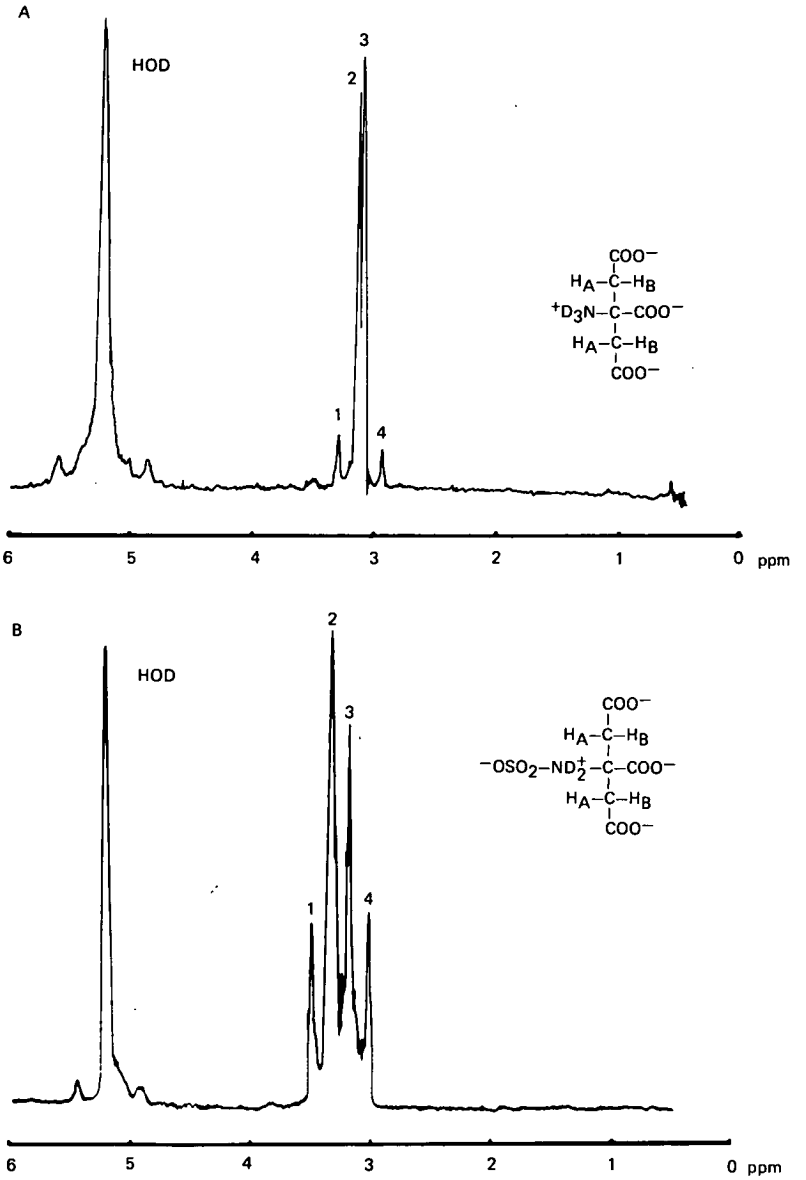

FIG. 5. ^{1}H NMR spectra of (A) 2-amino tricarballylate and (B) *N*-sulfo-2-amino tricarballylate determined at pH 7.0 (ambient temperature).

hydroxyapatite formation, has been discussed in detail by Williams and Sallis (2,3). From their studies, they proposed a certain minimum structural requirement for a molecule to inhibit, suggesting that a molecule should possess at least a phosphate group and an acidic group, the latter being either a carboxylic acid or phosphate group. SAT, however, does not fall into this category and is a case where potent inhibition exists in the absence of a phosphate moiety. It is of interest, however, that *N*-sulfoaspartate (prepared in analogous fashion to SAT), a compound similar to SAT, showed no inhibitory action with respect to hydroxyapatite formation even at a 100 μM level (unpublished). In comparing the

published), whereas Williams and Sallis (1) found from similar experiments that PC is rapidly destroyed in the kidney. For this reason SAT could prove to be more useful than PC as a therapeutic agent in the prevention and treatment of diseases associated with the abnormal accumulation of calcium.

The present studies have paved the way for a closer examination of the effects of the substitution of the PC molecule in relation to inhibitor potency. Other analogs involving subtle changes in the basic PC structure are also under investigation and hopefully will add further information to that already obtained on the structure–activity relationship of PC and SAT.

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