THYROID HORMONE DEIODINASES

by

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

E Q Clayton.
5/7/1982

PREFACE AND ACKNOWLEDGEMENTS

The experimental work described in this thesis was performed part-time during my employment as a lecturer in the Department of Biochemistry, University of Tasmania between 1976 and 1981. During that period some work was performed jointly with two B.Sc. (Hons.) candidates: Mr P.G. Stanton (1978) and Mr N.P. Swan (1980) who were under my supervision.

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In the presentation of the thesis, wherever possible, the recommendations of O'Connor and Woodford (1975) have been followed.

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SUMMARY

Assay systems involving iodine-125 labelled iodothyronine substrates have been established which can detect deiodination with high sensitivity. A HPLC separation has also been established which complements the radiochemical assays.

Using these assays, 5'-deiodinase enzyme activities have been localized to the plasma membranes, to the endoplasmic reticulum and to the lysosomes prepared from liver and kidney homogenates. A further activity in the cytosol of these tissues was also found, but evidence suggested, in part, that its presence there may be due to an artefact of the homogenisation procedures.

Purification studies were performed on the cytosol 5'-deiodinase (because of its solubility) and on the combined microsomal 5'-deiodinase activity. Substantial purification (300 fold) of the 5'-deiodinase from cytosol was achieved using thyroxine as the ligand in affinity chromatography. Gel electrophoresis of this purified product suggested the presence of only a small number of proteins which combine in a regular fashion to produce aggregates of related structures.

The combined plasma membrane and endoplasmic reticulum microsomal activity was significantly purified (3-10 fold) using iopanoic acid (which is a 5'-deiodinase inhibitor) as the ligand in affinity chromatography. The degree of purification compares favourably to that achieved by other workers.

A model for deiodination of T_4 is suggested in which the production of both T_3 and rT_3 occurs predominantly at the plasma membrane. The rT_3 produced is then subject to rapid catabolism by deiodination in the lysosome.

The rate of rT_3 deiodination by lysosomes is controlled by the inhibition or stimulation of lysosomal autophagy or changes in the lysosomal membrane or both by drugs, hormones or aminoacids. This leads to alterations in the intracellular levels of rT_3 which in turn feeds-back negatively on the plasma membrane enzyme (and also perhaps the endoplasmic reticulum deiodinase) to inhibit the production of T_3. This then leads to a rise in plasma rT_3 followed soon after by a fall in plasma T_3.

It is further postulated that the final common pathway for dietary changes of rT_3/T_3 levels is via the intracellular levels of aminoacids in the liver, especially those (e.g. Asn, Gln, Leu) which are gluconeogenic or anabolic.

Finally drugs which are known to be lysomotrophic and markedly affect the stability of lysosomes are predicted to have effects on the ratio of rT_3 to T_3 found in plasma.

CHAPTER 1

Introduction to Thesis and Review of the Literature

1.1 A Brief Review of the Development of our Understanding of Thyroid Physiology

1.1.1. Historical Introduction

The thyroid gland's ability to take up iodine from the blood and incorporate that iodine into the hormone released by the gland has long excited a wide range of medical and scientific interest.

Medically, the thyroid gland has always been of great interest; both in the nature of the gland itself and in the effects of its secretions. Normal, elevated or low secretions are described in clinical situations as eu-, hyper- or hypo-thyroidism. Failure of homeostatic control of thyroid hormone levels leads to characteristic syndromes which in turn can be explained by the reduced or exaggerated effects of the changed levels of thyroid hormone on growth, metabolism, and the nervous system.

The synthesis of thyroid hormone and its release represent many interesting features: the uptake and metabolism of a trace element present in the diet; the first described example of the incorporation of a halogen element into a biological compound; the synthesis within a very large protein of an unusual aminoacid; (and in retrospect) the first example of side chain (or post-translational) modification of a protein following its synthesis; and the first described example of the proteolysis of a protein to give an active hormone.

Several good historical reviews exist on the development of our understanding of the nature of the thyroid gland and its functions, e.g. Kelly (1961), Stevenson (1963) and Trotter (1964).

The modern chemical study of thyroid function was initiated by the work of Baumann (1896 a, b) in which he described not only the presence of iodine in the thyroid gland, but also that the iodine was contained predominantly in the protein fraction. This protein fraction after hydrolysis, yielded an iodine containing substance which he named in German: "thyrojodin" or in English "iodothyrin".

Following Baumann's discoveries, Kendall (1915) reported that he had obtained "thyroxin" as a crystalline solid after hydrolysis of bovine glandular material. Harrington (1926a), improved the method of isolation of thyroxin so that larger yields could be obtained, and was thus able to characterise (1926b) and finally synthesize thyroxine (1927).

The actual nature of the circulating hormone in the plasma remained controversial. It was thought that thyroglobulin or a thyroxine containing peptide was the circulating hormone because of the co-precipitation of thyroxine with plasma proteins. However the work of Taurog and Chaikoff (1948), Laidlaw (1949) and Rosenberg (1951) confirmed that it was the small molecular weight molecule thyroxine which was the iodine containing substance circulating in the bloodstream.

The significant highlights of the debate for and against the possibility of free or non-covalently bound or covalently bound thyroxine have been succinctly reviewed by Pitt-Rivers (1978). The arguments have come eventually to favour a non-covalently protein-bound thyroxine with only a very small fraction of "free" or non-protein-bound hormone circulating in the plasma.

The work of Harrington in elucidating the nature of the active principle in the colloid of the thyroid gland, was followed by Smith and

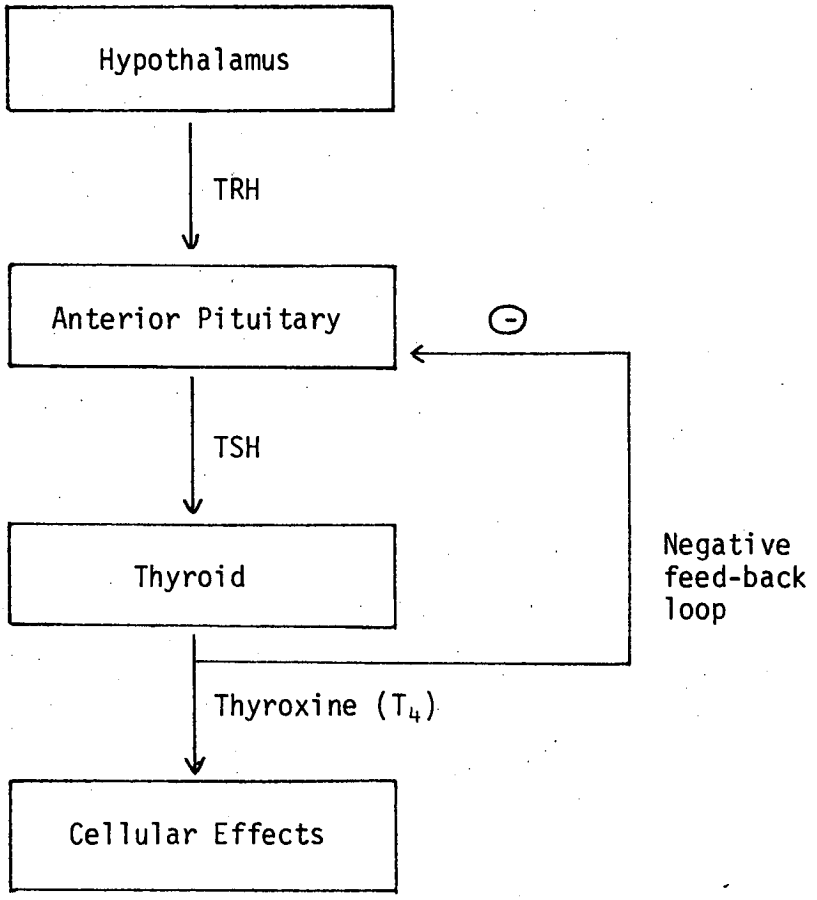


FIGURE 1.1 The Control of Thyroid Hormone Secretion (Circa 1952).

fellow workers who showed that there was an anterior pituitary principle which stimulated thyroid growth and secretion. This provided a further level of understanding of the thyroid gland (Sebert and Smith, 1930; Smith, 1930).

The concept of feedback inhibition of thyroid secretion on the pituitary was proposed by Aron et al. (1931) and further refined by Hoskins (1949). Modification of the feedback concept came with the work of Green and Harris (1947) whose work implied that the hypothalamus could modulate the activity of the thyroid separately from the effect of the feed back control of thyroxine. This was confirmed by Greer (1951, 1955) who showed that the hypothalamus and hence, the then putative, TRH was necessary for the production of TSH and its trophic effect on the thyroid.

So that by the early 1950's, most endocrinologists felt that thyroid physiology could be described (see Fig. 1.1) by the interaction of hypothalamus, pituitary and thyroid.

1.1.2 The Role of Triiodothyronine

A further level of control was then postulated by Gross and Pitt-Rivers (1953a)when they suggested that thyroxine (T_4) was the precursor hormone activated outside the thyroid by deiodination to 3,4,3', triiodothyronine (T_3).

The evidence for their postulate was as follows:

(i) T_3 circulated in the blood (Gross and Pitt-Rivers, 1952; Roche, Lissitzky and Michel, 1952a, b).

(ii) On a molar basis T_3 was three to four times more potent than T_4 (Gross and Pitt-Rivers, 1953a; Lerman, 1940).

(iii) Injected radiolabelled ^{131}I-T_4 was converted to ^{131}I-T_3 (Gross and Pitt-Rivers, 1951, 1952a).

(iv) T_3 could completely alleviate myxoedema in people (Gross et al., 1952) and rats and mice (Gross and Pitt-Rivers, 1953b).

This work was reviewed in retrospect by Pitt-Rivers (1978).

1.1.3 The Loss of Interest in the Peripheral Conversion of Thyroxine to Triiodothyronine

The postulate that triiodothyronine was the "effective" circulating hormone remained disputed on several points. Firstly, there were conflicting reports as to whether T_4 could be converted to T_3. Pitt-Rivers et al. (1955) had again found, by using paper chromatography, that T_3 was derived peripherally from T_4, but Lassiter and Stanbury (1958), from the same laboratory, were unable to show such conversion following a single injection of ^{131}I-T_4 when using an improved chromatographic separation of iodothyronines from serum. Secondly, the initial in vitro supporting evidence for peripheral conversion of T_4 to T_3 by Albright and Larson and co-workers (Albright, Larson and Tust, 1954; Larson, Tomita and Albright, 1955; Albright and Larson, 1959) and by Sprott and MacLagan (1955) and by Cruchaude et al. (1955) was challenged by others who, although they were able to show deiodination of thyroxine by various in vitro systems, were unable to show formation of T_3 (Etling and Barker, 1959; Galton and Ingbar, 1961; Tata et al., 1957; Tata, 1957; Plaskett, 1961; Stanbury et al., 1960; Wynn et al., 1962; Yamazaki and Slingerland, 1959).

Tata (1959, 1960a, b, 1961) extended his work on deiodinases and partially purified (70-fold) from the soluble fraction of rabbit muscle,

an "enzyme" which required both Fe^{2+} and a flavin and which was inhibited by chelating agents including EDTA, 8-hydroxy-quinoline, αα' bipyridyl, atebrin, acriflavine, and chlorpromazine. The deiodinases of brain homogenates were also inhibited by mercurials (Tata et al., 1957). The "enzyme" was assayed by using ^{131}I-T_4 as a substrate and the products were separated by paper chromatography. Tata (1960b) found that whilst dehalogenation was occurring, non-specific protein in the reaction vessel could be "transiodinated" at the same time. This work on muscle deiodinase was confirmed by Lissitzky et al. (1961, 1962) who also showed that light was necessary for the flavin to have its effect on the deiodination.

1.1.4 Non-enzymic Deiodination

Lissitzky et al. (1961) and Galton and Ingbar (1962) then showed that deiodination of thyroxine by light and flavin could occur in a tissue-free reaction. These authors showed that boiled tissue at times markedly accelerated deiodination. This led Morreale de Escobar et al. (1962, 1963) to investigate the reaction using a miscellaneous selection of purified exogenous proteins and adding these fresh or after boiling to a mixture of FMN and T_4 which were then exposed to light. The effects of these proteins were varied. Some would increase deiodination (e.g. lysozyme), others would have no effect (e.g. insulin and protamine), or would be inhibitory (e.g. albumin). After boiling, the properties of the proteins could change to cause increased deiodination (e.g. trypsin), or to have no effect (e.g. lysozyme), or to decrease deiodination (e.g. urease) or for deiodination to appear when the non-denatured

protein was inactive (e.g. ribonuclease and β lactoglobulins). Aminoacids tested had generally little effect except for histidine and tryptophane which markedly stimulated deiodination.

The number of factors affecting non-enzymic deiodination were extended by Reinwein and Rall (1966a) who confirmed that labelled thyroxine and triiodothyronine could be deiodinated by light plus flavin mononucleotide (FMN) and showed that this phenomena could be accelerated by di- and tri-valent metals especially in the presence of some chelators of biological origin such as amino acids and proteins. This accelerating effect was however inhibited by stronger chelators such as quinoline-8-carboxylic acid. The effect of EDTA was variable; promoting deiodination when no metal was present and inhibiting when some cations, especially Fe^{2+} were present in certain molar ratios.

Most authors agreed that the action of light was probably via the generation of free radicals.

Another possible source of non-specific deiodination was demonstrated by Galton and Ingbar (1963) in that T_4 could be deiodinated by tissue peroxidases and the process inhibited by the exogenous addition of catalase, or activated by addition of Fe^{2+} or haemoglobin. This deiodination could be stimulated in unboiled media by the addition of glucose and glucose oxidase (a known H_2O_2 generating system).

The same authors (Galton and Ingbar, 1966) later observed that the very active deiodination brought about with boiled liver homogenate, was not reproduced by boiled tissue slices unless the reaction was carried out in the preparation medium. They showed that a factor

responsible for deiodination was rapidly diffusible from tissue, readily dialysable and could be added back to the tissue to restore activity. This seemed to implicate a small molecular weight factor or factors.

Reinwein and Rall (1966b) extended the observations on non-enzymatic deiodination of thyroxine by hydrogen peroxide in phosphate buffers by showing that light was not necessary if H_2O_2 and one of a set of certain metal ions were provided. A completely metal-free system was inactive. Metals varied in their action, some required EDTA for activity (e.g. Fe^{++}, Fe^{+++}), others did not and were in fact inhibited (e.g. Cu^{++}).

All these contributions to the study of non-enzymic deiodination led to a swing in opinion that favoured a non-enzymic process that was somehow associated with protein structure or aminoacid side chains per se and activated in the presence of metals and flavins, rather than being the effects of an active catalytic site of a true enzyme. These findings cast a great deal of doubt on those preceding papers showing deiodination as an enzymic process. This applied especially to those papers in which the authors could not show the presence of T_3 or other intermediates, but had depended simply on measuring deiodination by the production of iodide. This doubt was so strong, that interest in the peripheral deiodination of T_4 declined.

The other (and perhaps more persuasive) line of evidence which discounted T_3 was that the qualitative measurement of the total concentrations of the two hormones in serum showed that T_4 was in very much higher concentration than T_3; see for example Pitt-Rivers et al.(1955). The large amount of T_4 relative to T_3 was confirmed, when reasonably accurate quantitative estimates could be made. Pind (1957) and MacLagan et al. (1957) found that the circulating

concentration of T_3 was between 50 and 100 times less than that of T_4. So that, whilst most authors conceded the extra potency of T_3 in bioassay, it was considered to be of much lesser importance relative to T_4.

In retrospect, a more balanced assessment of the evidence for T_4 to T_3 conversion would have found great difficulty in dismissing the strong evidence that peripheral deiodination must occur: e.g. that exogenous ^{131}I-T_4 led to the production of $^{131}I^-$ in the urine *in vivo* in man (Riggs, 1952; Pochin, 1951), in rats and or mice (Kalant *et al.*, 1955; MacLagan and Wilkinson, 1954; Flock and Bollman, 1955; and Gross and Leblond, 1951), and in the dog (Flock *et al.*, 1956). In addition, Ford *et al.* (1957) had shown by the *in vivo* administration of ^{131}I-T_4 the formation of T_3 from T_4 in various tissues of the guinea pig by a careful, autoradiographic and chromatographic study.

Other strong supporting evidence came from *in vitro* organ perfusion studies on the isolated rabbit kidney (Glitzer *et al.*, 1956), and rabbit liver (Becker and Prudden, 1959) which showed that both organs would convert T_4 to T_3. These studies however lacked a quantitative measure of the specific activity of the radio-tracers and hence it was not possible to assess the importance of the T_4 to T_3 conversion.

1.1.5 The Levels of Free (or Non-Protein) Thyroid Hormones

Other developments had been taking place in thyroid physiology which helped eventually to lead to a reconsideration of the role of T_3. Recant and Riggs (1952) showed that it was the level of non-protein bound or free T_4 which could maintain nephrotic patients in a euthyroid

state despite a lowered serum protein and a lowered PBI (i.e. total T_4). These authors argued from the concepts of Goldstein (1949) who had reviewed drug action and found that the biologically effective concentration was the non-protein or "free" drug level and of McLean and Hastings (1935) who showed that ionized or "free" Ca^{2+} was constant but that the protein bound Ca^{2+} could vary considerably *in vivo*.

Robbins and Rall (1957) calculated the free T_4 levels in a large number of subjects with various hyper- and hypothyroid states and found that it correlated linearly with thyroid hormone degradation rates, and with the BMR. Later this argument was extended by Robbins and Rall (1960) who, when reviewing the literature, found that free T_4 levels correlated better than total T_4 levels with euthyroidism in various states such as pregnancy, nephrosis, estrogen or androgen treated subjects, and in people with idiopathically raised TBG levels. They calculated that the concentration of free T_4 was of the order of 4×10^{-11} M.

Sterling *et al.* (1962) measured the binding constant of albumin for thyroxine (as it is one of the three binding proteins for T_4) and then calculated a value for free T_4 of 0.6×10^{-10} M based on the physiological concentration of albumin and the then accepted total T_4 value.

A better estimate of free T_4 was later obtained by Sterling and Hegedus (1962) using a dialysis technique with serum to estimate the dissociation constant for T_4 and this allowed an estimate of free T_4 of 1.3×10^{-10} M in serum to be made. These values immediately suggested that the biological active fraction of T_4 was only a minute

fraction of the total T_4. The other important observation that had been made by several authors was that T_3 was less avidly bound than T_4 by TBG and not at all by TBPA. These are the main thyroid hormone binding proteins. Albumin is thought to bind no more than about 10% of T_4. The binding of T_4 and T_3 by plasma proteins was reviewed by Tata (1962) who obliquely made the point that as T_3 was less tightly bound the free concentration could be comparable to that of T_4 and it might after all be important even though the same author had dismissed the enzymic production of T_3.

1.1.6 The Renaissance of Interest in Triiodothyronine

The renaissance of interest in T_3 began with the work of Nauman *et al.* (1967) who were able to measure serum T_3 reasonably accurately by a chromatographic method and found a euthyroid value of 0.33 ± 0.07 μg/dl with a free T_3 value of 1.51 ± ng/dl (or about 0.05%). Total T_3 was found to vary more significantly in thyroid states than did T_4 . Their calculated ratio of free T_3 : T_4 was 1:3 and was found to vary with hypo- and hyper-thyroidism showing a decrease in the ratio in hyper-thyroidism and an increase in hypothyroidism. Although Nauman *et al.* (1967) made no mention of the potency of T_3 relative to T_4, they discussed the metabolic clearance of T_3 and its distribution value based on the work of Fisher and Oddie (1964) and suggested that the flux through this pool implied that there was preferential release of T_3 by the thyroid. They thus concluded that T_3 had an equal metabolic role to that of T_4. Prior to Nauman *et al.* (1967), Shinaoka and Jansini (1965), although unable to measure absolute amounts, had accurately measured the ratio of T_4:T_3 in patients with thyrotoxicosis or thyroid tumour and concluded T_3 might provide half the metabolic effect.

This work was extended by Sterling et al. (1969) who changed and improved the extraction and chromatographic method of Nauman et al. (1967) which had overestimated the T_3 level due to the formation of the methyl ester of T_4 during the extraction with acid methanol. The methyl ester of T_4 co-chromatographed with T_3. However they confirmed qualitatively the observations of Nauman et al. on T_3 and T_4 in various thyroid states although finding a lower level of T_3 in euthyroid subjects (227 ± 27 ng/dl) and extended this work by finding in a series of clinically euthyroid patients, who had been treated for hyperthyroidism, a low T_4 which seemed to be compensated for by a normal or high-normal T_3. They also found in some rare cases, thyrotoxicosis apparently due to an elevation of T_3 with a normal T_4. They pointed out that this T_3 toxicity had been found in one case much earlier by MacLagan et al. (1957).

Although not explicitly stated, I believe that these observations and those of Nauman et al. (1967) stimulated Sterling's group to re-examine the question of whether peripheral deiodination of T_4 to T_3 occurred in vivo. Sterling's group then found that athyreotic patients maintained on T_4, had T_3 in their blood. Then, rather than giving a single injection of ^{131}I-T_4 as had been done by Lassiter and Stanbury (1958), they gave repeated multiple daily injections of ^{125}I-T_4 and showed that ^{125}I-T_3 was formed from T_4 in vivo (Braverman et al., 1970).

Similar findings were made with normal subjects and using techniques to exclude deiodination of T_4 to T_3 during chromatography (Sterling et al., 1970; Pittman et al., 1971) and were confirmed in similar experiments using rats by Schwartz et al. (1971).

Following these papers, peripheral deiodination of T_4 to T_3 was accepted as an important physiological mechanism in providing T_3 *in vivo*. Thus the postulate of Gross and Pitt-Rivers (1953) was, at least in part, accepted again. The question of intrinsic activity of T_4 was still under question and remains so at the time of writing this thesis.

The whole body studies quoted above did not indicate the site of this deiodination. This led to a flurry of papers, and subsequently a reconsideration of the early work done after the time of Gross and Pitt-Rivers. This early work on peripheral deiodination is discussed below.

1.1.7 A Reappraisal of Research into the Deiodination of Thyroxine by Peripheral Tissues.

It is difficult to assess the often contradictory early papers which deal with *in vitro* deiodination that were published before the watershed paper of Braverman *et al.* (1970). Clearly deiodination had been shown to occur in the whole animal *in vivo* using I^{131}-thyroxine in physiological amounts.

Pitt-Rivers and Tata (1959), Ingbar and Galton (1963) and Galton (1968) in extensive discussions written before the paper of Braverman *et al.* (1970), tackled the controversy over peripheral deiodination in two ways. Firstly, they examined the likely methodological weaknesses, in particular, the mis-identification of products and secondly, they examined likely or possible non-enzymatic mechanisms for deiodination which would or could have been operative in the published systems. In retrospect, their criticisms have not necessarily proved to be valid.

Pitt-Rivers and Tata (1959) made the point that deiodination of labelled compounds can only be said to have occurred if the presence of iodide is established by unequivocal methods and to this end listed the following criteria:

(i) chromatographic identification of radioactive I^- in more than one solvent in the presence of carrier iodide;

(ii) extraction of I_2 into CS_2 after oxidation with ferric ions;

(iii) electrophoretic mobility;

(iv) isotopic dilution to constant specific activity;

(v) adsorption on suitable ion exchange resins;

and suggested that false conclusions would be drawn if nonspecific tests such as water solubility were used; as in this case, a water soluble thyroxine glucuronide conjugate could be formed by liver and kidney homogenates which is not precipitated by acid conditions. Thus Pitt-Rivers and Tata (1959) criticised the work of Sprott and MacLagan (1955) who had extracted their iodide fraction into a water phase; even though the latter authors for part of their work with non-radioactive thyroxine had used a AgS/Ag electrode for the measurement of the iodide released. A stronger criticism of Sprott and MacLagan was made by Lissitsky et al. (1956) who believed that the "deiodinase" was entirely non-enzymic because deiodination occurred equally in boiled and unboiled preparations, and was most active at the extremes of pH; viz 3.5 and 9.5. The work of Morreale de Escobar et al. (1962, 1963) on boiled proteins would also tend to discredit the claim by Sprott and MacLagan (1955) on the prolonged heat stability of the enzyme.

One should note in passing, however, that more modern work of Hesch et al. (1976) has shown a similar, but not as extreme, bimodal pH optima for the deiodination of thyroxine as determined by RIA. Pitt-Rivers and Tata (1959) also criticised the work of Albright et al. (1954) and Larson et al. (1955) on the grounds that their chromatographic solvent used to separate T_4 and its products after incubation with kidney slices did not distinguish between T_3 and TA_4 and did not measure iodide. The former authors felt that production of TA_4 to be a more likely product. However Allbright and Larson (1959) showed,with an improved solvent system which adequately separated T_4, T_3, TA_4 and TA_3, that kidney slices did produce T_3.

Taurog (1963a, b) had shown spontaneous deiodination of ^{131}I-thyroxine and other iodophenols during the drying period when applied in aqueous buffers or in butanol to filter paper or silica plates. The deiodination was markedly inhibited by whole serum, albumin, ethanol or propylene glycol but not butanol. It was partially inhibited by propylthiouracil and by non-radioactive carriers. No decrease was observed with added iodide, EDTA or methimazole. Paper electrophoresis did not cause deiodination. With this work Taurog cast doubt on all the previous studies with ^{131}I-T_4 and paper chromatography.

Further, Morreale de Escobar et al. (1963) had shown that the deiodinase activities isolated by Tata (1963) and by Lissitzki et al. (1962) were most likely a non-enzymic photochemical deiodination of of thyroxine catalysed by iron and flavin. So that, in her review Galton (1968) could not accept that there had been any convincing demonstration of in vitro deiodination.

In retrospect it seems to me that only the whole cell preparations of Albright, Larson and co-workers and Becker and Prudden (1959) and the pituitary homogenates of Volpert et al. (1966) gave a valid demonstration of enzymic deiodination but quantitatively, were likely to be in error in view of Taurog's work.

1.2 A Review of Recent Studies on the Peripheral Deiodination of Thyroxine

1.2.1 Background

Following the unequivocal demonstration by Braverman et al. (1970) that T_4 could be converted to T_3 in vivo, there has been an explosion of interest in peripheral deiodination. Many early papers were concerned with the identification of the organ(s) responsible for this transformation.

In vitro T_3 production was shown using human fibroblasts in culture (Refetoff and Matalon, 1970), with the perfused rat heart (Rabinowitz and Hercker, 1971), and using cultured human kidney and liver cells (Sterling et al., 1973). Woeber et al. (1972) showed by paper chromatography, that intact leucocytes, when stimulated to phagocytose, would deiodinate thyroxine using hydrogen peroxide to produce I^- and an iodinated protein material at the origin. This reaction was thought to be by a different mechanism than that of other tissues. Haibach (1971) showed that thyroid and kidney minces would deiodinate T_4 to T_3 by using an enxyme which was not iodotyrosine deiodinase.

It should be noted that all these preparations consisted of whole, or a mixture of whole and broken cells. It was some time before the first convincing evidence of T_4 to T_3 production was demonstrated by Hesch et al. (1975) and Visser et al. (1975) using the broken cells of rat liver homogenates. To do so, they employed sensitive and specific radioimmuno-assays (RIA).

The development of RIA for thyronines was the final factor which has allowed the renewed interest in metabolism of thyroid hormones both *in vitro* and *in vivo* to be so quickly productive since 1970. A final impetus for the development of RIA for thyroid hormones had come from Larsen (1971) and Fisher and Dussault (1971) who, when using the Sterling (1969) procedure for measuring serum T_3 found that the paper chromotographic step still led to a small but significant artefactual deiodination of T_4 as had been first observed by Taurog (1963a, b). Thus, the work of Brown *et al.* (1970) in showing that specific antibodies could be raised to T_3 led many laboratories to raise their own specific antibodies to T_4 and to T_3 and developed RIA to these thyronines. The early development of the radioimmunoassays with a comparison to the Sterling technique are reviewed by Larsen (1972a) and again by Burke and Eastman (1974).

The sensitivity of RIA for T_3 allowed Larsen (1972b) to confirm Sterling's clinical findings for T_3 but lowered the normal value of total T_3 from 2.2 ng/dl to 1.1 ng/dl. Larsen also reported that levels of T_3 in newborn cord blood appeared to be in the hypothyroid range (0.5 ng/dl) whilst pregnant women were in the normal range.

Sullivan *et al.* (1973) reported selectively low T_3 and elevated free T_4 levels in the serum of dying patients who had low T_3 in tissues at necropsy. Carter *et al.* (1974) reported a wide range of patients who had chronic liver, lung, or kidney illnesses or who had desseminated carcinoma in whom blood concentrations of T_3 were low. Since these early reports, many different conditions and drugs have been shown to be associated with low plasma T_3 (see Table 1.1).

TABLE 1.1

(Modified from Chopra et al., 1978)

Clinical Situations with Low-Serum Total and/or Free T_3 and Normal Total T_4 and/or Normal or High Serum Free T_4 (Low T_3 Syndrome)

1. Age related
   - Fetus and the neonate at the time of birth
   - Advanced age
2. Caloric deprivation states
   - Complete fasting
   - Protein-caloric malnutrition
   - Anorexia nervosa
3. Systemic illnesses
   - Febrile states
   - Hepatic cirrhosis
   - Renal failure
   - Miscellaneous illnesses
   - Toxaemia of pregnancy
   - Burns
4. Surgical operations
5. Drugs
   - Dexamethasone
   - Radiocontrast agents for cholecystography
   - Antianginal Agent (Amiadarone)
   - Propylthiouracil
   - Propranolol

Pharmaco-kinetic studies of metabolism of T_3 and T_4 have been conducted in some of these situations where T_3 levels were low, e.g. sheep fetus (Chopra et al., 1975a), complete fasting (Portnay et al., 1974a; Suda et al., 1977), and hepatic cirrhosis (Nomura et al., 1975, Chopra, 1976a). These studies suggest that the low T_3 is due to a decreased production of T_3 whilst that of T_4 is normal, implying an inhibited peripheral conversion of T_4 to T_3 rather than an increased loss. This mechanism is thought to apply to many states of low plasma T_3.

1.2.2 Reverse T_3 and the 5-Deiodination of Thyroxine

The work of Chopra (1974) in developing a radioimmunoassay for 3,3',5' triiodothyronine (termed by that author "reverse-T_3" and variously abbreviated as RT_3 or rT_3) has opened up a much more extensive viewpoint on peripheral thyroid hormone metabolism. Chopra was able to show that rT_3 could be found and measured in human blood thus confirming and extending the early chromatographic work of Roche et al. (1956a, b) who had found rT_3 in rat thyroid and blood. Chopra found that the ratio of rT_3 to T_3 or to T_4 was markedly in excess to the ratio found in proteolytic or Pronase hydrolysates of thyroid glands. Also, hypothyroid and athyretoic patients receiving T_4 were found to have normal levels of rT_3 suggesting that like T_3, a large proportion of rT_3 was derived by peripheral deiodination of T_4, although some direct secretion by the gland was not excluded. Chopra et al. (1975d, 1976a) using their RIA for rT_3 made the further discovery that total and free rT_3 levels were elevated in most disease states having a low T_3 and was normally elevated in the cord blood of newborn humans and sheep.

This work of Chopra was the first to show that thyroxine could be deiodinated from either the beta ("phenolic" or "outer") ring or from the alpha ("tyrosyl" or "inner") ring in an apparently controlled and reciprocal manner. Alpha or 5'-deiodination converted the thyroxine to T_3 and beta or 5-deiodination converted thyroxine to rT_3 which is inactive on bioassay as a thyroid hormone (Stasilli et al., 1959; Money et al., 1960; Goldfine et al., 1976) but may have activity in other cell systems (reviewed by Chopra et al., 1978; 1980).

These discoveries have led Chopra and other workers to look for other lesser-iodinated iodothyronines in plasma (see Fig 1.2) which would potentially be derived by the sequential deiodination of both rings of thyroxine. These have all now been identified in blood although their respective levels are disputed (Chopra, 1980; Burger, 1980). The rate of metabolism through various pathways is not clear, nor is it clear whether various products have inherent biological roles or merely represent metabolic breakdown products.

1.2.3 The Control of In Vivo Deiodination

Surks and Oppenheimer (1971) performed studies using exogenous T_4 labelled in either ring in normal humans and rats and suggested that the deiodination of thyroxine was random. In fact, peripheral production rates to T_3 and rT_3 may approach equality in normal euthyroid subjects (see data Chopra et al., 1978). However it is now clear that the production of T_3 and rT_3 and their subsequent metabolism are not random events but are in fact controlled in some fashion. Thus our overall view of the thyroid axis has been extended from Fig. 1.1 to that in Fig. 1.3.

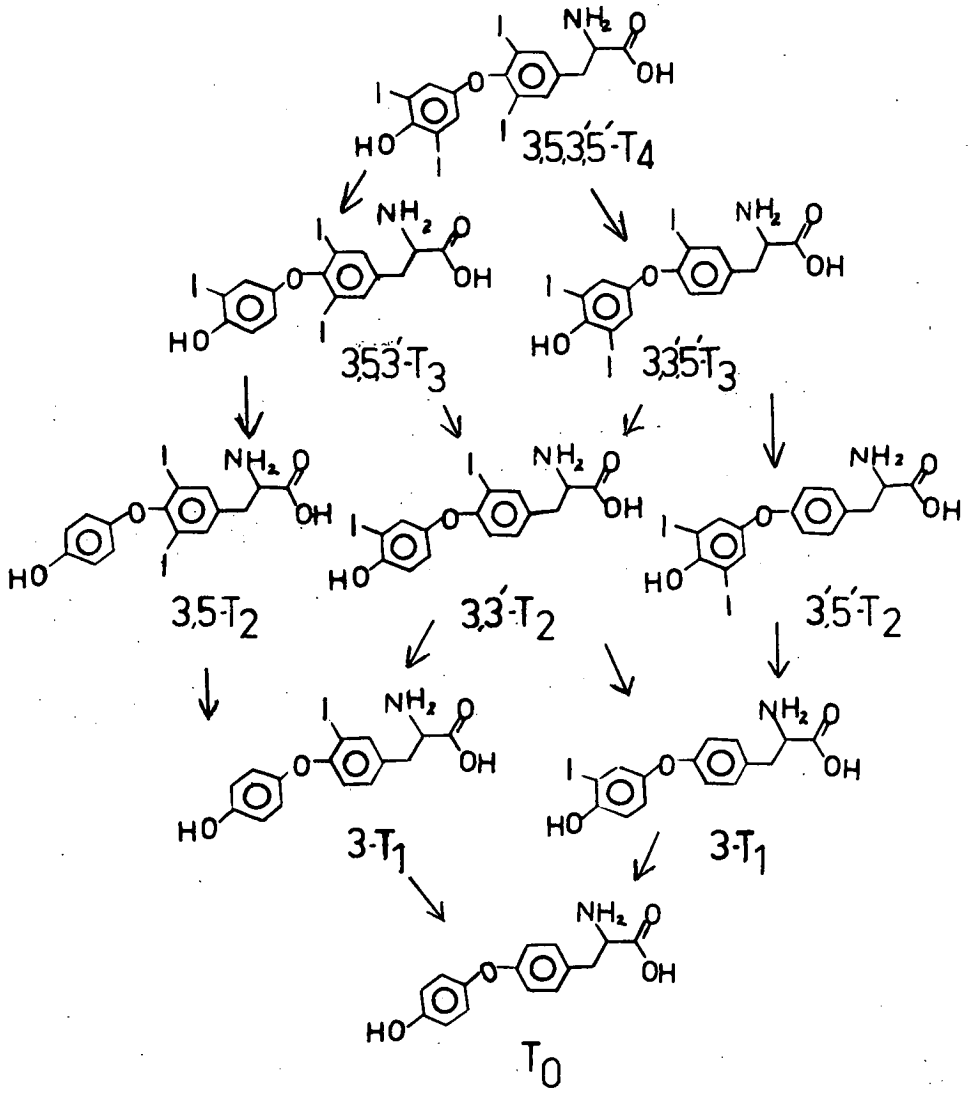


FIGURE 1.2 The Successive Steps in the Complete Deiodination of Thyroxine (redrawn from Cody, 1981).

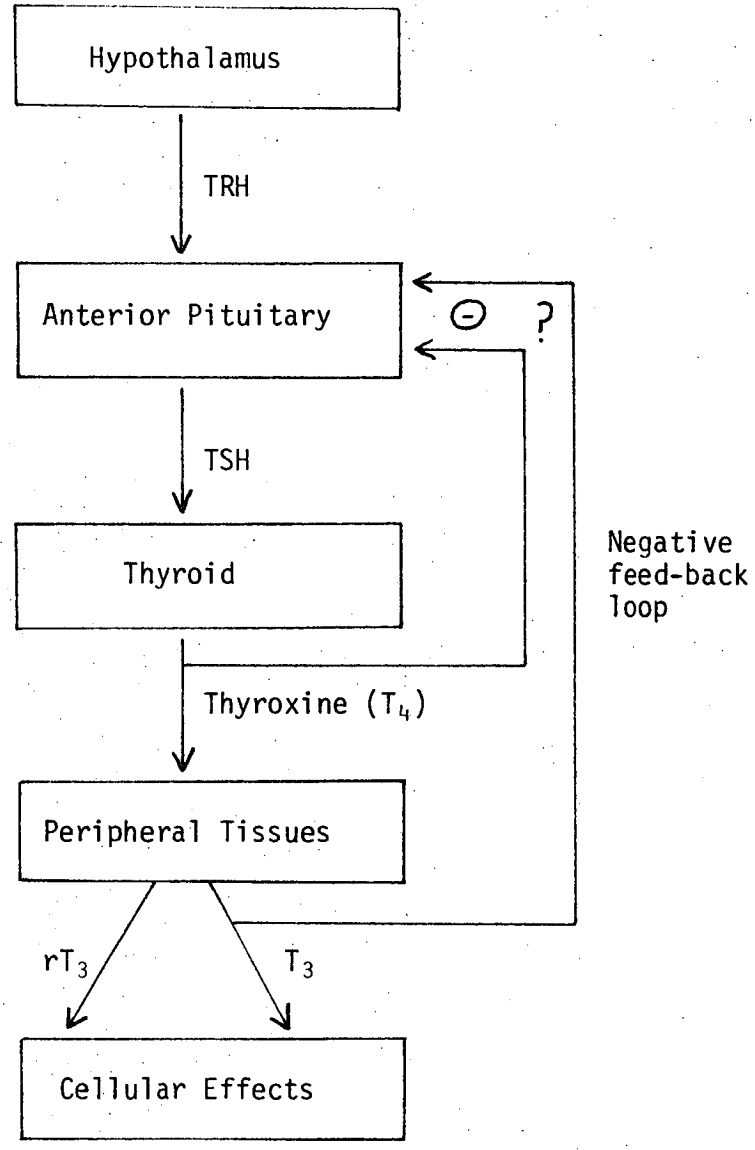


FIGURE 1.3 Control of Thyroid Hormone Secretion (Circa 1980).

What is not clear is how all the factors known to affect T_3/rT_3 ratios exert their effects at the peripheral tissues to cause the altered levels of T_3/rT_3 and how they are adjusted to ensure euthyroidism in normal subjects.

The modifying effect of food intake has been particularly studied since it was shown by Vagenakis et al. (1975) that a total fast led to a measurable increase in rT_3 with depressed T_3 levels within 24 hours, reaching a maximal effect at about 5 days. A similar pattern was soon found in other calorie deprived states including protein-caloric malnutrition (Chopra et al., 1975d) and anorexia nervosa (Nicod et al., 1976). Refeeding restored thyronine levels to normal (Chopra et al., 1975d). In most reports T_4 was little changed. Refeeding after total starvation with as little as 100 Kcal of carbohydrate caused a partial return to normal with 800 Kcal of carbohydrate causing a complete return to normal (Azizi, 1978). Fructose is just as effective as glucose (Burman et al., 1979). Westgren et al. (1977) have reported that oral carbohydrate but not intravenous glucose is effective in restoring the ratio. Pure protein refeeding does not restore T_3 to normal but substantially returns rT_3 to normal (Azizi, 1978). In fact rT_3 may return to almost normal after about four weeks despite continuing a partial fast (300 Kcal/day), whilst T_3 remains low (Visser et al., 1978). Otten et al. (1980) found that fasting per se was not necessary since a diet completely devoid of fat but isocaloric to a normal diet may cause a fall in T_3 and rise in rT_3. Danforth et al. (1979), conversely, have shown that voluntary overeating of carbohydrate is associated with elevated T_3 and low rT_3 although a rise in T_3 was not found when the diet contained the extra calories as fat. Studies in diabetes tend to confirm the link of thyroid hormones with carbohydrate

metabolism. Firstly, untreated insulin-dependent diabetic patients show a low plasma T_3 (Suda et al., 1977). These values return to normal sometime after treatment with insulin. Richmond et al. (1980) have shown in bacterial sepsis that total parenteral nutrition but not i.v. glucose can reverse the change in T_3/rT_3 ratio back to normal. (However the work of Westgren et al. (1977) on i.v. versus oral glucose mentioned above needs to be borne in mind). Richmond et al. suggest that in many sick patients the low T_3/high rT_3 ratio might be due to inadequate nutrition and not necessarily to the disease state per se. In this regard the finding that high doses of synthetic glucocorticoids such as dexamethasone also induce this change in ratio (Duick et al., 1974; Chopra et al., 1975c; and Burr et al., 1976) is of great interest since it is well known that in many serious illnesses both the levels and the need for endogenous glucocorticoids rise (Selye, 1946).

The information linking levels of T_3 and to a lesser extent rT_3 to carbohydrate ingestion suggest that it may be the hormones (e.g. insulin, cortisol glucagon) or the substrates (e.g. glucose, aminoacids) mobilized during gluconeogenesis that lower the T_3/rT_3 ratio and not pituitary TSH. However the finding of Visser et al. (1978) and Otten et al. (1980) that plasma rT_3 levels often correlate well with plasma urate levels but not as well to creatinine suggests that factors other than carbohydrate metabolism may be operative.

The changes in rT_3 and T_3 appear not to be always truly reciprocal nor synchronous. As mentioned above, Visser et al. (1978) showed a long term effect in partial fasting whereby protein refeeding restored rT_3 but not T_3. McLarty et al. (1976) showed that some patients with severe illness and who subsequently died had very low or absent T_3 and elevated rT_3 levels that decreased one or more days before death.

Loos et al. (1980) repeatedly sampled the blood of six thyroidectomised patients who were receiving constant infusions of T_4 over a 24 hr period. This study showed that T_3 and rT_3 levels have a circadean rhythm induced by peripheral mechanisms. Both T_3 and rT_3 levels show two maxima and two minima which were not reciprocal in time nor proportional in change.

1.2.4 In Vitro Studies on Deiodination

The demonstration by Visser et al. (1975) and of Hesch et al. (1975) that liver homogenates could deiodinate T_4 to T_3 has been followed by a large number of papers documenting the properties of in vitro deiodination.

1.2.4.1 Comparison of the In Vitro Deiodination by Various Tissues

Albright and Larson (1959) showed that in vitro human kidney slices were the most active of all tissues in the deiodination of T_4 to T_3 with possibly some activity in the liver and heart. Similar findings had been made in the rat by Albright et al. (1954) but had been subsequently challenged by Tata (1959) who felt Albright et al. had misidentified T_4-glucuronate as iodide.

Chopra (1977a) surveyed the tissues of the rat for deiodinase activity and found that kidney and liver were far more active than all the other tissues tested (muscle, heart, spleen, lung, brain, gut) which had showed little activity. The thyroid gland was not tested.

Galton (1975) using phosphate homogenising buffers, found that the rat pituitary would deiodinate T_4 but not produce T_3. The chromatography system employed however did not resolve rT_3 and T_4 nor any of the diodothyronines. Galton appears to have overlooked the work of Grinberg et al. (1963) and Volpert et al. (1966) who had shown the conversion of T_4 to T_3 by mouse and human pituitary tumors could be demonstrated using Eagles' medium for incubation rather than using phosphate buffers which lead to a high deiodination rate without T_3 production.

Newer studies from Larsen's laboratory have confirmed that the rat pituitary can convert T_4 to T_3 both in vivo and in vitro (Silva and Larsen, 1977, 1978; Silva et al., 1978; Larsen et al., 1980; Kaplan, 1980). This work implies that the anterior pituitary may need to be considered as a peripheral tissue in Fig 1.3. It is of interest that, in this new work, T_4 to T_3 deiodination by pituitary in vitro could only be shown in the presence of 5mM dithithreitol. This concentration is much higher than had been used by Visser et al. (1976) to obtain maximal-activity with liver microsomes. Deiodination of T_4 to T_3 by pituitary has been confirmed in vitro by El-Zaheri et al. (1980) in neonatal rats and in vivo by Lewis et al. (1980) and Obregón et al. (1980).

Recent work by Kaplan and Yaskoski (1980) has confirmed and extended the observations that the brain is a site of deiodination. These authors found that both cerebral and cerebellar homogenates would deiodinate both rings of T_4 but required from 10 to 100 mM DTT for full activity, depending on the thyroid status of the rats. There was little or no

activity between 1-5 mM DTT. This explains the previous inability of Chopra or Kaplan (Chopra, 1977a; Kaplan and Utiger, 1978; and Kaplan, 1980) to show deiodination by brain homogenates. Early work by Tata et al. (1957) had shown brain homogenates to deiodinate T_4 to I^- without forming T_3. It was however considered by Tata and Pitt-Rivers (1959) and Galton (1968) as photochemical rather than enzymic activity even though it was inhibited by mercuric chloride and heat labile; properties which suggest a sulphydryl-requiring enzyme.

The deiodination of T_4 to T_3 in thyroid minces has been shown by Haibach (1971) and confirmed by Erickson et al. (1980). Using a 100,000g pellet of thyroid homogenate, the latter authors showed a requirement of 5 mM DTT for activity.

In summary liver, kidney, anterior pituitary, thyroid, and brain, broken-cell preparations all cause deiodination of T_4 but require DTT or other sulphydryl compounds to show activity.

1.2.4.2. The Sub-cellular Localization and the Co-factor Requirements

Both Hesch et al. (1975) and Visser et al. (1975) in their original papers found 5'-deiodinating (T_4 to T_3) activity to be associated with the microsomal fraction of liver cell homogenates. Visser et al. (1976) extended their observations to show that a sulphydryl compound from the cytosol was necessary for maximal activity of the microsomes. Cytosol could be replaced by dithiothreitol and to a lesser extent

mercaptoethanol. The deiodinase was not inhibited by CN^- thus distinguishing it from the possible enzyme systems described by Stanbury et al. (1960) which had required Fe^{2+}, O_2 and a sulphydryl compound but were inhibited by CN^-.

Production of reverse T_3 from T_4 in subcellular fractions was not shown until Cavalieri et al. (1977) found a cytosolic enzyme with an alkaline pH optima (8.5) in rat liver. It was thought to be a distinct enzyme from the microsomal T_4 to T_3 deiodinase of Hesch et al. (1975) or of Visser et al. (1975).

Hüffner et al. (1977) and Hüffner and Grussendorf (1978) suggested that rT_3 production might not be easily detected as it was very rapidly deiodinated in vitro. The latter authors found rT_3 formation in the 100,000g pellet and not in the soluble fraction. Hüffner et al. (1977) found a rT_3 producing system in microsomes with a pH otpimum of 8.5 and a T_3 producing system in the same preparation at a pH optimum of 6.5.

Sinsheimer et al. (1978) using model, iodinated-ring compounds (not true thyroid hormones) found three deiodination systems: a minor component in the microsomes and two systems in cytosol; one was dependent on gluththione, the other was independent of sulphhydryl compounds.

Leonard and Rosenberg (1978a) extended the microsomal findings by showing with studies of "marker" enzymes associated with various cell fractions, that the T_4 5'-deiodinase seemed to be a plasma membrane enzyme in rat kidneys. This work

prompted a re-examination by several groups of the sub-cellular site of the deiodinating enzymes because until Leonard and Rosenberg (1978a), most workers had depended predominantly on sedimentational criteria to specify their sub-cellular localization of the deiodinase.

Chopra and co-workers (Maciel et al., 1979) confirmed Leonard and Rosenberg's finding that most T_3 forming activity was associated with the plasma membranes of liver but there was still significant separate microsomal activity. On the other hand, Auf dem Brinke et al. (1979) found T_4 to T_3 activity associated only with endoplasmic reticulum and not plasma membranes of rat liver. This was supported by Fekkes et al. (1979) who also found that liver deiodinase activity for both the inner and outer rings was present only in rat liver parenchymal cells and not Küpfer cells and that after sub-cellular fractionation, the deiodination was associated with the endoplasmic reticulum and not the plasma membranes. They pointed out that the activities from microsomes were very much higher than those found by Cavalieri et al. (1977) in their cytosolic rT_3-forming reaction and for this reason implied that the latter's work might not be relevant.

In summary, most authors feel that both 5- and 5'-deiodinase activities are associated with the endoplasmic reticulum in rat liver but there is strong evidence for an alternative plasma membrane localization in rat kidney. Difficulty has been found in showing 5-deiodination in any viscus *in vitro* because of the rapid deiodination of rT_3. Both T_3 and rT_3 formation seems to have been clearly shown to occur in the

brain by Kaplan et al. (1981) who used paper chromatographic methods.

1.2.4.3. The Role of Glutathione

The work of Visser et al. (1976) directed attention to a cytosolic factor which was necessary for the enzymic conversion of T_4 to T_3. This factor could be inhibited by Hg^{2+} ions and p-chloromercuriphenylsulphate and replaced by thiol compounds such as dithiothreitol and mercaptoethanol. Chopra (1978) showed that foetal tissues were not as active in T_4 to T_3 conversion but the activity could be brought up to adult levels by the addition of thiol compounds. The activity in adult liver homogenates could be inhibited by thiol reagents ($HgCl_2$, N-ethylmaleimide and diamide) and activated by reduced thiols (dithiothreitol, mercaptoethanol, and reduced glutathione). Kaplan (1979), Balsam and Ingbar (1979) and Harris et al. (1979) found that fasting led to a decrease in the cytosolic thiol compounds of rat liver and these were restored by prior administration of oral glucose before killing. Several authors have suggested that activity of the pentose shunt by producing NADPH and in turn reducing G-SS-R to GSH may be a mechanism for controlling T_4 to T_3 production (Visser, 1978; Harris et al., 1979a). In support of this assertion, Balsam and Ingbar (1979) found that the activity of cytosol derived from starved liver or kidney on liver microsomes could be increased by adding NADPH or GSH to the cytosol. However NADPH when added directly to microsomes had no effect whereas GSH was active. Imai (1980)

by assaying extracts of cytosol, separated by column chromatography, has produced strong confirmatory evidence that the endogenous thiol which activates deiodination is glutathione.

The question of whether changes in reduced glutathione levels are associated with *in vitro* states of reduced T_3 formation has been re-investigated in the light of its probable importance as a cofactor. Chopra (1978) has found decreased non-protein sulphhydryl groups (NPSH) in the foetal sheep with decreased *in vitro* deiodination of T_4 which is restored by *in vitro* addition of thiols. Harris *et al.* (1979) found that in starved foetal, neonatal or thyroidectomised rats, as well as in the hypopituitary dwarf mouse, the levels of liver NPSH were all reduced, as were the levels of production of T_3 from T_4 *in vitro*. Further the addition of thiols *in vitro* only partially restored liver deiodination of T_4 to T_3 in the foetal and neonatal liver up to day 5, but after day 5 activity was completely restored. Dithiothreitol did not restore deiodination activity *in vitro* from livers taken from rats thyroidectomised 60 days previous to the *in vitro* experiment. Deiodination of T_4 to T_3 activity in these livers was restored to normal by administration of T_3 *in vivo* for 10 days prior to assays. This treatment did not restore liver NPSH levels. Kaplan (1979) also found only partial restoration of deiodinase activity by DTT *in vitro* using livers from fasted rats.

These findings have in part been confirmed and extended by Chopra (1980) who noted that NPSH in rat liver fell quickly

after a day of fasting and returned to normal over 4 days. The whole period of fasting was associated with decreased *in vitro* deiodination of T_4 and rT_3 (but not of T_3) which could be restored fully by DTT only on day 0 and day 1, and then only partially on the remaining days of the fast.

Chiraseveenuprapund *et al.* (1975, 1978) were unable to show an increased deiodinase activity in renal homogenates by the addition of thiols, but thiol blocking agents (e.g. N-ethylmaleimide) inhibited the reaction. Later work on kidney microsomes showed that DTT would increase the activity (Leonard and Rosenberg, 1978b). However Kaplan *et al.* (1979) and Balsam and Ingbar (1979) have shown no decrease in deiodinase activity of the kidney in the fasting rat.

1.2.4.4. Sex Differences in Tissue Deiodination

Harris *et al.* (1979b) have shown T_4 to T_3 production by liver homogenates derived from both male or female were similar from days 1-24. However at age 30 days, a significant decrease in T_3 production was observed in the female rat. This decrease reached approximately 50% by day 90 and remained constant thereafter. *In vitro* addition of thiols, e.g. DTT did not eliminate the difference Castration of females before puberty prevented these differences and if performed after puberty led to a gradual restoration to male values over about 40 days.

1.2.4.5 Effects of Hypo- and Hyperthyroidism

Albright et al. (1954) showed that kidney slices from thyroidectomised rats had decreased T_4 to T_3 production whereas there was an increase in T_3 production following prior administration of exogenous T_4. Kaplan and Utiger (1978), after prior administration of T_4 to rats in vivo, found an increase in deiodination of T_4 to T_3 and rT_3 to T_2 in homogenates from liver but they observed no such change in kidney homogenates. Thyroidectomised rats showed reduced deiodination of T_4 and rT_3 in both liver and kidney homogenates.

Balsam et al. (1978), using liver and kidney slices from either hypophysectomised or thyroidectomised rats, found very similar decreases in deiodination of T_4 to T_3, and in the kidney decreased formation of TA_4. The changes after removal of either gland were fully restored by administering T_3 in the diet but not by giving adrenal or sex steroids.

1.2.4.6 Inhibitors and Enzyme Mechanism

Various substances inhibit peripheral deiodination both in vivo and in vitro in both man and rat. Examination of their structures and of their reaction kinetics has given insight into the possible reaction mechanisms of deiodination. Chopra et al. (1978) found that T_4 to T_3 deiodination in rat liver was not inhibited by large doses of sodium iodide, iodotyrosines, 3,5,-T_2, methylated, brominated or chlorinated analogues of T_3 and rT_3 nor by methimazole (methylmercapto-

imidiazole); however it was inhibited in a dose dependent manner by rT_3, $3',5'T_2$, TA_4, $3,3'T_2$, 6-propylthiouracil, iodoacetic acid and some iodinated X-ray contrast agents, in particular ipodate and iopanoic acid. Other substances known to inhibit *in vitro* deiodination are propanalol and quinidine on isolated rat kidney tubules (but not homogenates) (Heyma *et al.*, 1980) and sodium salicylate (Chopra and Solomon, 1980; Chiraseveenuprapund *et al.*, 1978).

In vivo inhibition of deiodination is found with PTU and other six membered thionamide derivatives but not methimazole or other five membered thionamides (Hershman and Van Middlesworth, 1962; Geffner *et al.*, 1975; and Saberi *et al.*, 1975). Propranalol (Wiersinga and Tauber, 1976), X-ray contrast agents Bergi *et al.*, 1976; Wu *et al.*, 1978), and dexamethasone (Duick *et al.*, 1974; Chopra *et al.*, 1975c) also inhibit *in vivo*; often with a rise in rT_3. Infusion of rT_3 in pharmacological quantities to rats (Coiro *et al.*, 1980), but not in physiological doses to man, inhibits T_4 to T_3 deiodination.

Recent studies by Visser (1979, 1980a, 1980b), using a rat liver preparation, have indicated that the 5'-deiodination of iodothyronines of both T_4 and rT_3 follows a ping-pong mechanism implying the formation of an enzyme-sulfenyl iodide (E-SI) intermediate. The function of thiols (cofactor) is to regenerate free enzyme by reduction of this intermediate. Thiouracils inhibit deiodination by forming a mixed disulfide (dead-end complex) by reaction with the -SI group. Thus,

binding of thiouracils to the 5'-deiodinase with the concomitant loss of enzyme activity only occurs under conditions where enzymic deiodination has taken place and therefore requires the presence of substrate. Similar findings have been made by Leonard and Rosenberg (1980) in rat kidney who have further observed that the binding of PTU to enzyme prevents denaturation by sulphhydryl reagents, e.g. iodoacetamide and the activity can be later restored by adding high levels of DTT which reduce the enzyme-PTU mixed disulphide.

1.2.4.7 Multiplicity of Enzymes

There is strong evidence that there are at least two deiodinase enzymes which each act on the different rings of thyroxine. In vivo evidence is derived firstly from sick and fasting subjects where T_3 and rT_3 levels alter independently of each other (or at least not synchronous nor reciprocal). Secondly, patients who have been given PTU have depressed T_3 levels and elevated rT_3 levels which suggests a differential sensitivity of the two putative enzymes to the inhibitor and thirdly, the changes at birth; when T_3 levels rise rapidly but those of rT_3 fall much more slowly, this suggests at least two enzymes.

In vitro evidence for more than one deiodinase comes from their different properties in solution. Liver homogenates when incubated with T_4 at pH 6.5 produce predominantly T_3 and at pH 8.5 produce rT_3 with much less or no T_3. It is possible

that rT_3 is produced at lower pH although this is not certain, since exogenous rT_3 is extremely quickly deiodinated below pH 8.0 in liver homogenates (Hüfner and Grussendorf, 1978).

The differential effect of PTU and of fasting, both of which inhibit T_3 production far more than rT_3 by in vitro microsomal enzyme preparations, is circumstantial evidence of more than one enzyme (Visser, 1980). Auf dem Brinke et al. (1979) have suggested that liver microsomes show kinetics and bimodal pH dependence which might be explained by the existence of two 5'-deiodinases and that there might also be a third activity associated with the lysosomes of liver.

Visser et al. (1979) have examined the sequential deiodination of T_4 to $3,3'T_2$ via either T_3 or rT_3. They concluded the simplest explanation was that there were only two enzymes, one each for the 5 and 5' positions, which could act in sequence. They also concluded that the reason for the variation in the reported pH optimum for 5' deiodination of T_4 was due in part to the alteration of K_m and V_{max} with pH and the use of non-saturating substrate conditions by previous authors including themselves. Most authors have concluded that proof of the existence of one or more enzymes awaits their eventual isolation and study.

1.2.4.8 Purification of Deiodinases

Several authors have described attempts at purification. Because the enzyme is probably membrane bound a major difficulty has been in the adequate solubilization of the enzyme, together

with its lability once solubilized. Takaishi et al. (1979) have used deoxycholate (DOC) for solubilization with a small increase in specific activity, Fekkes et al. (1980b) used cholate and W 1 ether and then applied the enzyme to either ion exchange or thiol covalent chromatography but again obtained only 2 to 3 fold increase in specific activity. Köhrle et al. (1980) have also used DOC or cholate. They discarded the detergents acetyl-sulphate, SDS, Triton X-100 as unsatisfactory since they caused either loss or inhibition of enzyme activity. The use of CM or DEAE ion exchange cellulose did not improve specific activity nor did it separate the T_4 and rT_3 5'-deiodinases.

1.3 Conclusion and Aims

This survey of the literature includes some reported work which is contemporaneous with my own part-time research undertaken in the period from 1976 to 1981. The work is included because of the rapid advances made in this field of research during the last five years. Not to do so, would leave an unbalanced introduction with many deficits when compared with current knowledge.

The overall aim of this work was, if possible, to separate and purify to homogeneity from kidney or liver, any of the enzymes catalysing the deiodination of the iodothyronines. The subsequent study of the separated or purified enzymes might then provide valuable information in that:

(1) it might well answer the vexed question of how many enzymes catalyse deiodination;

(2) it might clarify the role of sulphydryl compounds either as co-factors or as substrates or both;

(3) it would allow investigation of potential activators and inhibitors and their molecular mechanism;

(4) it would allow studies on the size and structure of the enzyme;

(5) it would allow kinetic studies on the enzymes and provide knowledge on the reaction mechanisms.

CHAPTER 2

Measurement of Iodothyronine Deiodination

2.1 Introduction

For the purpose of monitoring the purification of an enzyme, a rapid method of measuring activity is desirable even if it is not as accurate as another more accurate assay but which is slower, or more difficult to perform (Dixon and Webb, 1962).

However if such a rapid method is approximate or if it does not discriminate between possible products, it is necessary to have an additional assay which measures in an accurate and unambiguous manner any product formed. This is relevant to thyroxine as a substrate, as deiodination might occur from either of its two rings leading to two different sequences or pathways as shown in Fig. 1.2 (reviewed by Chopra et al., 1978).

Potentially there could be attack on the inner or on the outer ring (alternative nomenclature: tyrosyl or phenolic; 5- or 5'-deiodination).

Consideration of the reaction mechanism for deiodination of thyroxine:

$$T_4 \rightarrow T_3 + I^- \qquad (2.1)$$

$$T_4 \rightarrow rT_3 + I^- \qquad (2.2)$$

shows that at least two lesser iodothyronines could be formed, namely T_3 or rT_3 and I^-, although the possibility needs to be considered that $\frac{1}{2}I_2$ might be formed. This is unlikely however as Visser et al. (1976, 1978) have shown that T_4 to T_3 deiodinase

activity in liver homogenates and fractions *in vitro* is dependent on the presence of a reduced sulphydryl compound. *In vitro*, dithiothreitol (DTT) is the most effective sulphydryl cofactor or substrate (section 1.2.4) but it is thought that glutathione (GSH) is the active compound *in vivo* (Visser, 1976, 1979; Imai *et al.*, 1980; Chopra, 1978).

Thus equations 2.1 and 2.2 may be written as:

$$T_4 + 2RSH \rightarrow T_3 + R\text{-}S\text{-}S\text{-}R + HI \qquad (2.3)$$

$$T_4 + 2RSH \rightarrow rT_3 + R\text{-}S\text{-}S\text{-}R + HI \qquad (2.4)$$

Other enzymic mechanisms of deiodination may exist which do not require a reducing compound as a cofactor or substrate. Sinsheimer *et al.* (1978), using synthetic iodinated ring compounds as models, found that they were deiodinated *in vitro* by rat liver cytosol by one of two mechanisms. The first, similar to the mechanism in equations 2.3 and 2.4 required glutathione (GSH) but the second mechanism not only did not require GSH, it was inhibited by GSH.

As was discussed in Chapter 1, the early workers studying the peripheral deiodination of thyroxine had great difficulty in showing that deiodination by cell fractions was the result of enzyme action rather than a non-enzymic (e.g. photochemical) reaction.

It was therefore essential to establish that the assays devised in Sections 2.2 and 2.3 for the measurement of iodide-125 reflect the enzymic production of iodide-125 from the substrate rather than non-enzymic processes.

To show that incubations with enzyme preparations are revealing enzymic rather than non-enzymic deiodination it was necessary to establish a suitable control.

In general, the in vitro determination of any enzyme activity requires a number of conditions to be met before meaningful results are obtained.

(1) A source of enzyme must be found, e.g. a tissue or tissue fraction.

(2) An appropriate reaction medium, e.g. one with defined pH, salt concentration, temperature.

(3) The timed commencement of reaction by the addition of sufficient substrates.

(4) The use of controls to measure the non-enzymic reaction rate.

(5) The measurement of change in substrates or products or both, either by continuous measurement or by stopping the reaction in aliquots at a predetermined time and then measuring the changes.

(6) The extraction and separation of substrates or products or both may be necessary before their measurement.

The experimental evolution of such as assay however, usually proceeds in the reverse order of the above points. That is, a means is established for the measurement of the substrates or of the products and then the means of fulfilling the other criteria listed above are experimentally verified. Hence the development of the assays used will be discussed in approximately the reverse order in the subsequent sections, beginning with estimation of iodide and then of thyronines.

The establishment of an assay for deiodination became a problem of the measurement of individual iodothyronines and of the I^- ions

produced by deiodination. A less suitable alternative for the measurement of deiodination is the increase in the amount of oxidized sulphydryl compound formed. However it would be less specific having a low signal to noise ratio. That is, the likely concentration of GSH utilized of approximation 10^{-8} mol/l when compared to that of the total GSH in the reaction mixture of 10^{-3} mol/l.

The physiological concentration range of thyroxine in plasma in man is about 10^{-7}mol/l and that of T_3 10^{-9} mol/l and rT_3 0.5 x 10^{-9} mol/l (reviewed by Di Stefano and Fisher, 1976). The two in vitro methods which seemed capable of use were:

(i) saturation analysis, e.g. radioimmunoassay of T_4 and its products;

(ii) chromatographic separation of radiolabelled ^{125}I-T_4 and its products followed by measurement of the radioactivity of the separated iodothyronines.

Other workers, for example Hesch et al. (1975), Chopra (1977) and Visser et al. (1975) chose to employ RIA in measuring T_4 disappearance and T_3 production by enzyme action without measuring the production of I^- and that of other lesser iodinated thyronines. Although subsequently diiodo- and monoiodothyronines have excited much interest by many workers, e.g. Chopra et al. (1978).

The overwhelming advantage of RIA is its simplicity in performance (but not necessarily in its development) and its sensitivity and suitability to the very low levels of iodothyronine for both in vivo and in vitro studies. RIA of thyronines has several requirements. It needs an antibody of sufficient sensitivity and specificity and a labelled hormone (tracer), usually labelled with iodine-125.

At the time of the commencement of my investigations, only T_4, T_3 and later rT_3 were commercially available as radiochemicals either as ^{125}I (or ^{131}I) labelled chemicals, and as animal facilities were not then available to me for raising antibodies. I therefore decided not to try to use RIA but to employ ^{125}I labelled thyroxine or other labelled iodothyronines incubated with *in vitro* systems and separate the products formed by chromatography. This would have two advantages, firstly, the I^- (which is not measurable by RIA) and the iodothyronines liberated,would be radioactively labelled, thus allowing measurement of very small concentrations (depending on specific activity of starting material) but far below the 10^{-9} mol/l limit. Secondly, it would potentially allow the measurement in the one assay of all of the products formed, given that they could all be separated chromatographically and the iodine -125 measured by a gamma-counter which was available. The counter was a Packard Model 3001 Tricarb Scintilation Spectrometer which gave for iodine-125 an apparent efficiency of 70%. The use of aqueous solutions, chromatographic paper, or TLC backing sheets of plastic or aluminium foil, had no significant effect on the efficiency of the counting by the counter.

Therefore the problem of measurement was readily solved, the major problem remaining being the one of separation of iodine and the iodothyronines to allow their measurement.

There were many published methods for the separation and measurement of iodide and iodoaminoacids, for example using paper chromatography (Roche *et al.*, 1964), thin layer chromatography (Osborn and Simpson, 1969; Cahnmann, 1972), ion exchange column chromatography (Sorimachi and Ui, 1975) and gas-liquid chromatography (Richards and Mason, 1966).

None of these methods, except perhaps for gas chromatography, would separate all of the iodothyronines of interest in a single run, but almost all these compounds could be resolved by the use of two-dimensional chromatography, especially TLC (West et al., 1965).

However when I came to use the methods of Roche et al. (1964), I was unable to reproduce any of their published separations of T_4 and rT_3, nor of the diiodothyronines. This was confirmed by Dr H. Rokos of Henning GMBH, Berlin (personal communication) who was also unable to reproduce the separations of Roche et al. (1964). In a review, Plaskett (1964) has pointed out that highly variable R_f values had been obtained by various authors using apparently the same chromatographic systems in separating the iodoamino acids on paper. He further suggested that very small changes in the physical dimensions of tanks or of their vapour concentrations, especially that of ammonia, were in part responsible for the wide variations noted and which had sometimes led to the non-reproducible results between different laboratories. Pitt-Rivers and Tata (1959) also mention the great variability of solvent systems on the migration of iodothyronines.

Because of the variability in paper and thin layer chromatograhy it was necessary to devise other methods for the measurement of iodide and iodothyronines. This work is described in the subsequent sections.

2.2 Ion Exchange Chromatography

Backer et al. (1967) introduced the use of sulphonated polystyrene cation-exchange resins (e.g. Dowex 50W) for the extraction of thyroxine

from serum samples which at the same time allowed the removal of inorganic iodide. This ion-exchange method was modified by Tajuddin and Elfbaum (1973) for the extraction and clean up of serum iodothyronines prior to their derivatization for gas chromatography. This ion-exchange method was then combined with an ethanol precipitation and extraction for the assay of the $^{125}I^-$ liberated enzymically from ^{125}I-iodothyronines (see Fig. 2.2).

The same method allows separation of iodothyronines as a group suitable for their separation by HPLC. Recoveries can be estimated by counting both I^- and thyronine fractions.

2.3 Butanol Extraction

Although the ion exchange method is reliable it is slow, so that another more rapid method is desirable. The use of butanol as a separating agent was examined in the following experiments because of its long history in improving the specificity of PBI (protein bound iodide) as the BEI (butanol extractable iodide) from serum in the estimation of circulating thyroxine.

I felt that a hydrophobic solvent, when added to butanol or another solvent, would help to exclude water and hence I^- from the solvent phase. The protocol is shown in Fig. 2.2 and the extraction data is displayed in Table 2.1 and Fig. 2.3.

Table 2.1 shows that a good retention of iodide in the aqueous phase occurs with several solvents, and that this separation is improved by the addition of petroleum spirit as a strong hydrophobic solvent. In particular, the combination of BuOH/Pet.Spt. between 50/50 to 25/75

FIG. 2.1 Ion-Exchange Extraction for Iodide and Iodothyronines.

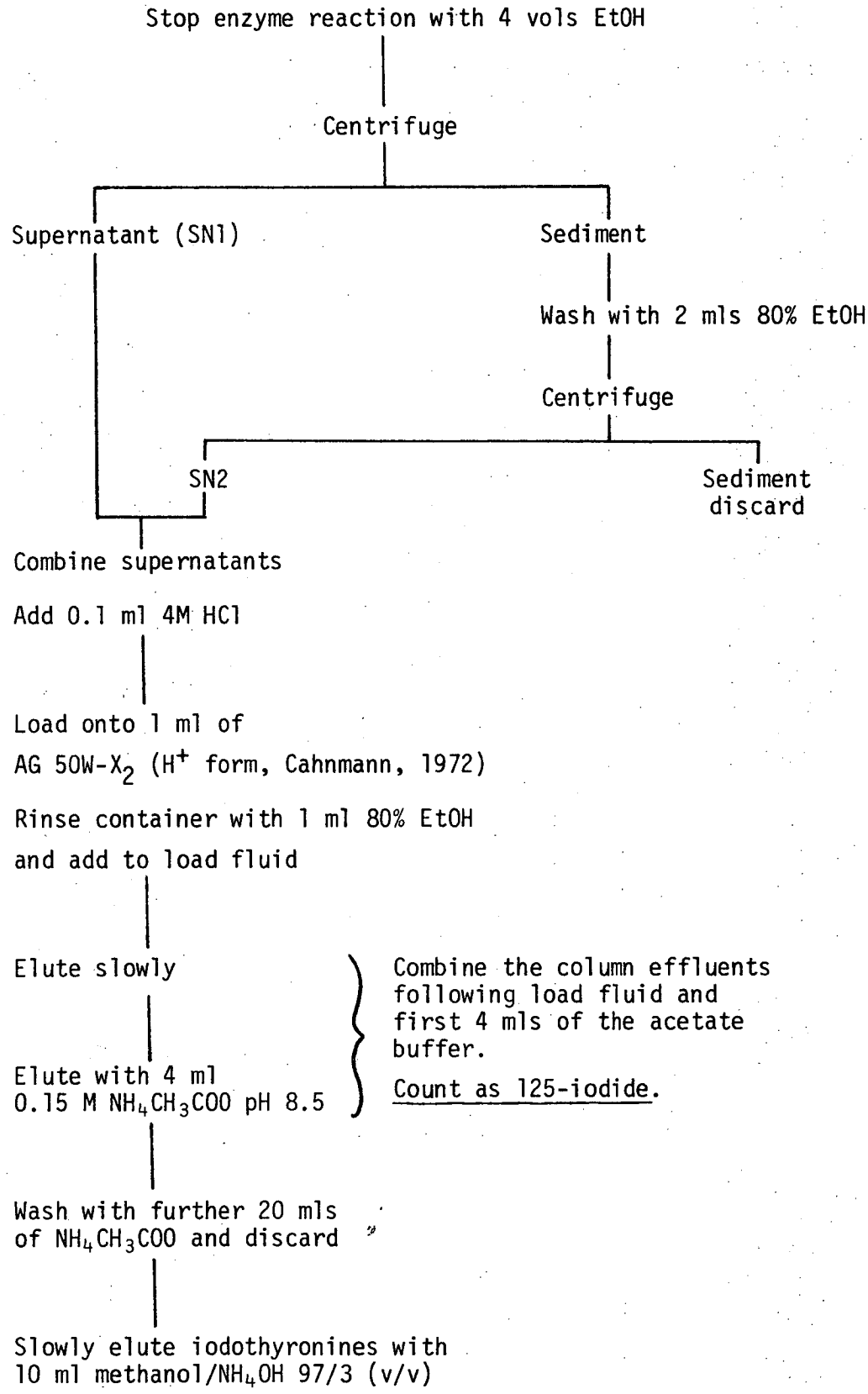
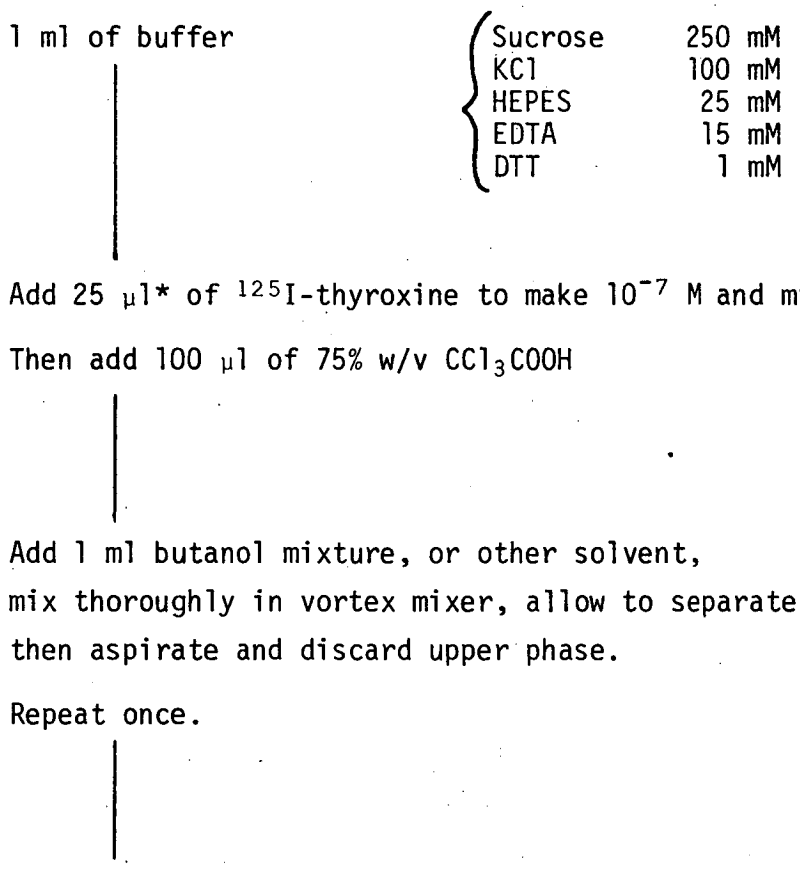


FIG. 2.2 Protocol for Solvent Extractions.



*This sample of 25 µl of [3'5'-^{125}I] thyroxine contained a total of 220,000 cpm of which ion-exchange determinations showed 51,579 ± 5667 cpm present as free iodide anion.

TABLE 2.1 Iodide Separation by Solvent Extraction.

Solvent Composition (parts by volume)		Iodine-125 in Aqueous Layer (% of total radioactivity added)		
		Mean	s.d.	(n)
n-butanol (BuOH)	100	16.0	1.2	7
/Pet.Spt.(60°-80°)	75/25	19.9	2.2	5
	50/50	20.6	1.5	5
	25/75	25.1	2.7	5
	0/100	81.2	5.5	5
/Pet.Spt.(80°-100°)	75/25	17.9	2.3	5
	50/50	17.7	2.0	5
	25/75	18.9	2.1	5
	0/100	82.5	6.4	5
benzyl alcohol	100	72.8	10.4	10
/Pet.Spt.(60°-80°)	75/25	31.4	3.6	5
/Pet.Spt.(80°-100°)	75/25	34.6	2.6	5
isoamylalcohol	100	20.3	2.6	10
/Pet.Spt.(60°-80°)	50/50	19.1	3.4	5
/Pet.Spt.(80°-100°)	50/50	21.2	3.1	5
t-amylalcohol	100	19.6	3.4	10
/Pet.Spt.(60°-80°)	50/50	20.8	1.6	5
/Pet.Spt.(80°-100°)	50/50	20.3	2.3	5

Total cpm added for each extraction 212,282 ± 20,807 cpm (100 ± 9.8%)
and when assayed by ion-exchange indicated
that T_4 was 160,703 ± 20,021 cpm (75.7 ± 9.4%)
and I^- was 51,579 ± 5,667 cpm. (24.3 ± 2.7%)

appears best in this regard. However inspection of a plot of this set of data for BuOH/Pet.Spt. in Fig. 2.3 suggests that some thyroxine may be excluded by the petroleum spirit and remain in the aqueous phase. This certainly occurs with 100% Pet.Spt. The addition of petroleum spirit also markedly improved the speed at which the two phases separated after mixing. By itself, butanol often needed centrifugation to separate the two phases. Butanol/Pet.Spt. has a further advantage of being a cheaper solvent mixture than the other alcohol mixtures which give equivalent separations.

The possibility of thyroxine being retained in the aqueous phase was investigated in the following experiment. A sample of $^{125}I^-$ was prepared from an old batch of ^{125}I-thyroxine by the ion-exchange method and then diluted to give 4474 cpm/ml. As well, 10 µl of ^{125}I-thyroxine (containing 70,058 cpm of which 13,420 cpm were shown to be $^{125}I^-$) was added to 1 ml of water. Multiple samples of both I^- and thyroxine containing I^- were then extracted using Butanol/Pet.Spt. combinations. The data is presented in Fig. 2.4 and the calculated aqueous distribution of T_4 is shown in Fig. 2.5. The two figures suggest that using butanol/Pet.Spt. (50/50 v/v) gives 86% I^- and 5% T_4 in aqueous phase.

The value of 5% T_4 in aqueous phase might be of concern if the degree of deiodination were of that order, however in practice more than 80% of T_4 is protein bound after TCA precipitation (Nakagawa & Ruegamer, 1967) so that in an actual assay it would be anticipated that T_4 will be markedly decreased by its affinity for the precipitated protein which occurs on stopping the reaction and less than 1% of T_4 will be present in the aqueous layer. This was tested by employing the protocol in Fig. 2.6 using a 10% w/v renal homogenate in TRIS buffer pH 7.4. Five determinations of each group were made and the data is shown in Table 2.2.

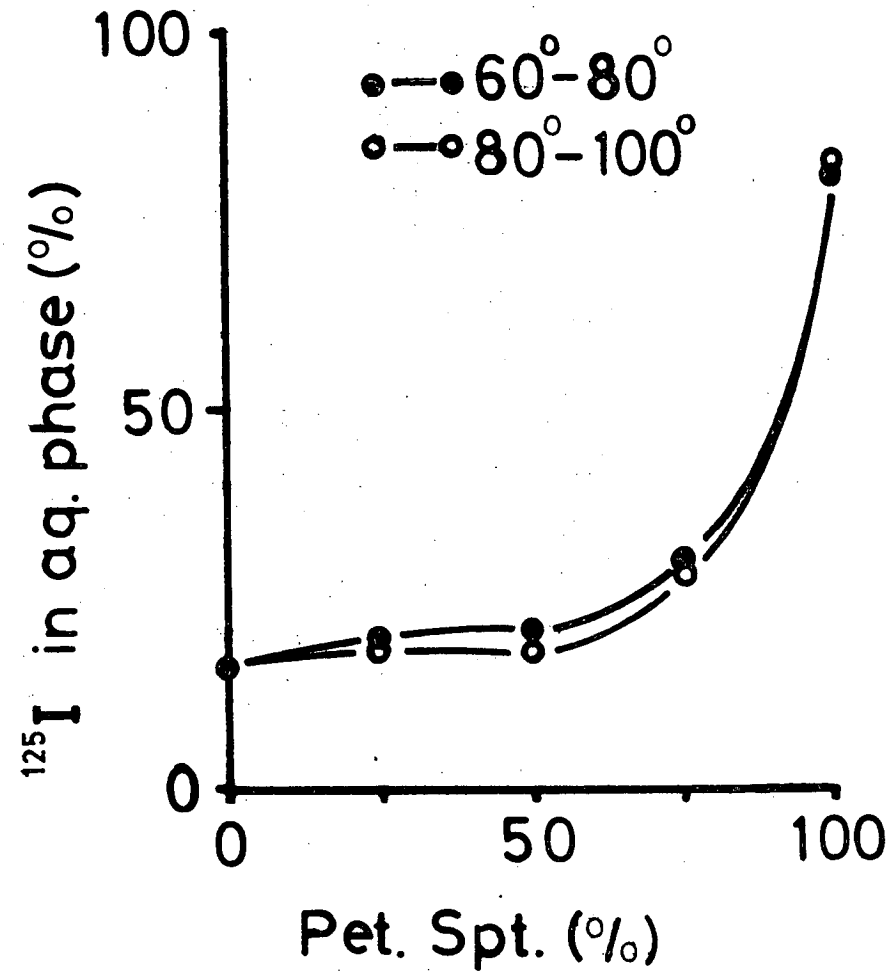


FIGURE 2.3 The Effect of Pet. Spt. on the Butanol Extraction of T_4 and I^-.

The data plotted is taken from Table 2.1.

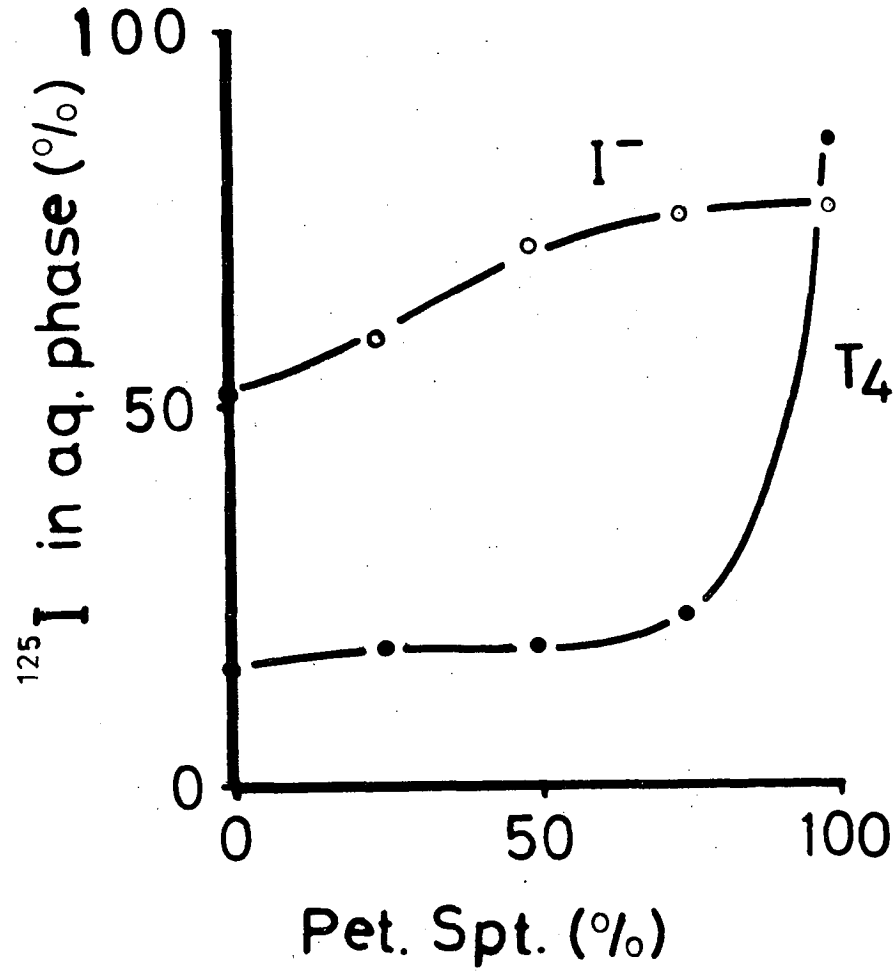


FIGURE 2.4 Distribution of T_4 and I^- between Water and Butanol/ Pet. Spt. Mixtures.

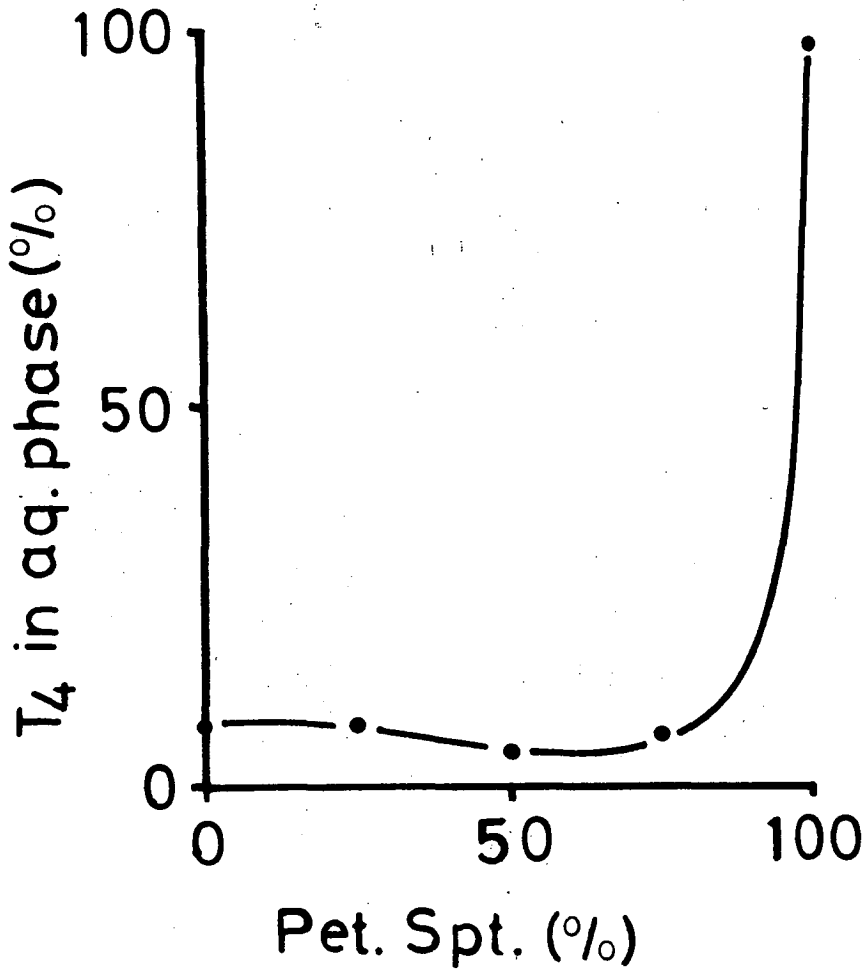


FIGURE 2.5 Corrected Data for the Distribution of T_4 between Water and Butanol/Pet. Spt. Mixtures.

FIG. 2.6 Protocol for Butanol/Pet.Spt. Extraction in the Presence of Protein.

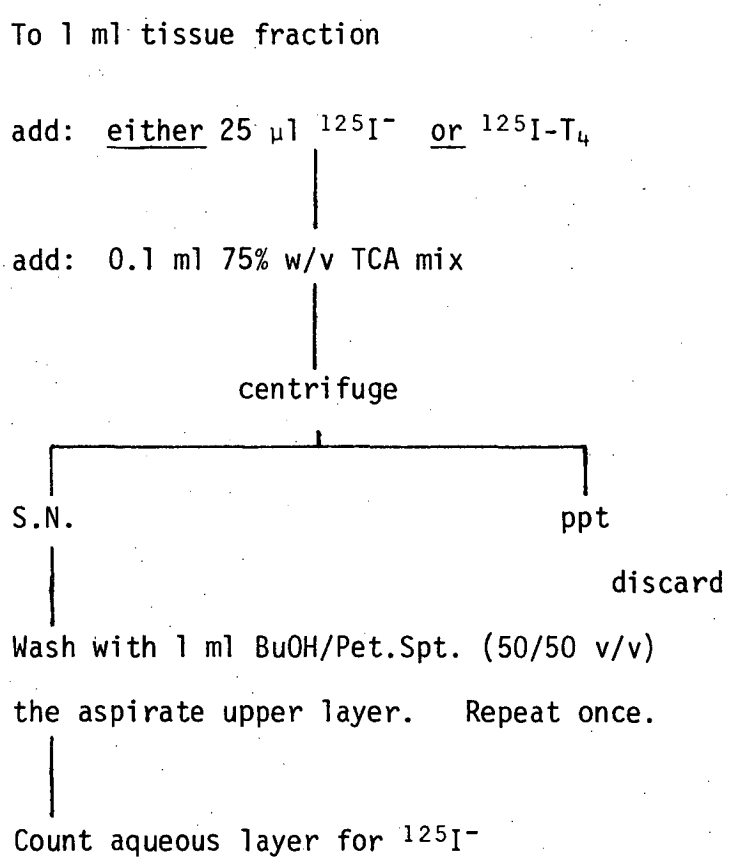


TABLE 2.2 Radioactivity (%) in Aqueous Layer and Protein Precipitate after Butanol/Pet.Spt. Extraction.

Fraction	Buffer		Homogenate	
	$^{125}I^-$	^{125}I-T_4	$^{125}I^-$	^{125}I-T_4
Aqueous layer	93 ± 3	2 ± 0	80 ± 3	1 ± 0
Protein precipitate	Not Applic.	Not Applic.	27 ± 1	66 ± 1

As the data in Table 2.2 have not been corrected for endogenous I^- present in the ^{125}I-T_4 used, it indicates that $\leq$1% of T_4 will be found in the aqueous layer when the extraction is used on protein solutions, and that a likely extraction efficiency for $^{125}I^-$ will be about 80% which is an acceptable figure for routine assay of the enzyme. The above method was also tested for effects of strong salt (0-1000 mM KCl), detergents (0.1% Triton X100 and 0.1% SDS) and sucrose (250 mM), all had an effect of $\leq$5% on the extraction of iodide, and had $\leq$1% effect on residual aqueous T_4.

In conclusion, the butanol/petroleum spirit extraction method gives a rapid, cheap, and reproducible method of separation of iodide from thyroxine. It has about 80% recovery of iodide with $\leq$1% contamination by thyroxine in the aqueous layer under the conditions of assay. It therefore fulfils the requirement for a rapid assay (Dixon and Webb, 1962). However it does not discriminate between various iodothyronine products. The separation and measurement of iodothyronines is discussed in the next section.

2.4 Separation of Iodoaminoacids by High Performance Liquid Chromatography (HPLC)

2.4.1 Introduction

High performance liquid chromatography (HPLC) is also known as high pressure liquid chromatography (HPLC) or as high speed liquid chromatography (HSLC). These terms are synonyms for the same sort of general chromatographic system. Such systems are all characterised by the above adjectives, viz: speed, performance and pressure. The latter condition of high pressure being needed to achieve the former two. HPLC is in fact no different in principle to any other form of chromatography. It does, however, often possess the advantages of speed or resolution.

The fundamental theory for HSLC was developed by Giddings (1965) and Hamilton (1966) and has since been improved and extended by many workers (reviewed by Kirkland, 1971; and by Brown, 1973). It has been widely introduced in the mid 1970's with continuing improvement in performance, especially of pumps and column packings, and in reliability and ease of use (see reviews by Perry et al., 1972; Done et al., 1974; Johnson and Stevenson, 1972, 1978; and Simpson, 1976).

A marked increase in the use of stationary hydrophobic ("reversed-phase") packings through which a more polar mobile phase is pumped, has occurred and such systems now provide the majority of HPLC separations being reported (reviewed by Karger and Giese, 1978; and by Brown and Krstulovic, 1979).

2.4.2 Application to Iodoaminoacids

The use of HPLC to separate both derivatized (De Vries et al., 1975; Bollet and Caude, 1976) and non-derivatized aminoacids (Hancock et al., 1976) has been rapidly developed. Many of these approaches are therefore relevant to the separation and quantitation of the thyronines as they are phenolic iodoaminoacids. In fact, the two manufacturers, Waters and Merck, have both published instructions for using their reversed phase (C_{18}) column packings to separate and quantitate the T_4 and T_3 in thyroxine tablets. Both systems employed aqueous methanol. Waters used KH_2PO_4 and Merck used proprionic acid as the polar solvent modifiers. Karger et al. (1974) published a normal phase separation of T_4 and T_3 using perchlorate ions pairs bound to a silica column. However none of these systems adequately separate other iodothyronines, especially the two pairs rT_3 and T_4, and 3,3'-T_2 and 3'5'-T_2. Hancock et al. (1978) introduced 0.1% phosphoric acid in methanol or acetonitrile mobile phases with C_{18} reversed phase packings which could separate rT_3 and T_4 but not 3,3'-T_2 and 3'5'-T_2. The phosphoric acid provides a counter-ion or ion-pair of opposite polarity to the molecule of interest and it is this "ion-pair" that is separated, leading to improved separation, sharpened peaks and reduced retention times (Hearn and Hancock, 1979).

The detection of iodoaminoacids is possible by their light absorption in the U.V. range. They have the generalised maximum common to aminoacids in the low U.V. range at about 210-220 nm with additional specific maxima (depending on ionization state of

phenolic ring) between 280 and 330 nm (Robbins and Rall, 1967). However the sensitivity is insufficient to measure less than about 10^{-6}M when using a mercury lamp detector at 254 nm.

Nachtmann et al. (1978) have coupled a post column detection system to a RPLC separation using the redox reaction between cerium (IV) and arsenic (III) catalysed by trace amounts of iodine. This method, although troubled by an erratic baseline, has a very high sensitivity and may eventually allow detection of physiological concentrations of T_4 and T_3 of 10^{-9}- 10^{-7}M.

Another detection system suitable only for in vitro work is to use labelled (^{125}I) iodothyronines and collect the column effluent into fractions and count these in a gamma-counter.

The thyronines are hydrophobic in nature, being quite insoluble in water, but soluble in alcohols and strong bases. Solubility is often reduced by the formation of insoluble chelates with divalent cations (Kendall and Osterberg, 1920). Because of these properties, together with the preliminary separations established by Waters and by Merck, it was decided to attempt to improve the separations using reversed-phase HPLC (RPLC).

The potential of this approach has been confirmed by Hearn et al. (1978) who have established separations of the thyronines but who failed to adequately separate 3,3'-T_2 and 3'5'-T_2.

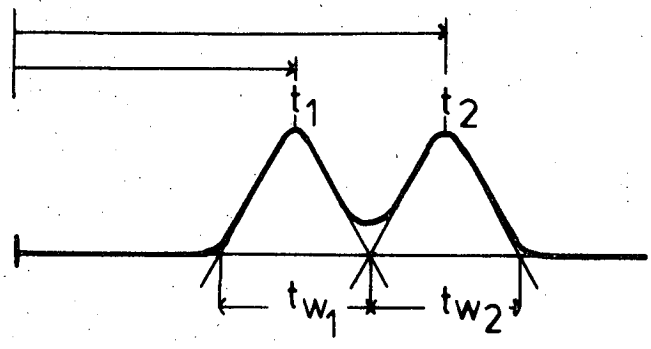
2.4.3 Basic Theory of HPLC Separations

The theoretical basis for establishing a separation and subsequent measurement of band height on HPLC is well discussed by Snyder and Kirkland (1974) and Karger et al. (1973), from whom the following condensed treatment is taken.

The resolution (R_s) of two bands on a chromatogram is defined as

$$R_s = \frac{t_2 - t_1}{\frac{1}{2}(t_{w1} + t_{w2})} \qquad (2.5)$$

and can be measured on the chromatogram (see Fig. 2.7)



To control the resolution of two bands in an empirical sense, resolution can be shown to be related to measurable variables on the chromatogram so that for two closely migrating bands

$$R_s = \underset{\text{(i)}}{\left(\frac{1}{4}\right)}\underset{\text{(ii)}}{\left(\alpha - 1\right)}\underset{\text{(ii)}}{\sqrt{N}}\underset{\text{(iii)}}{\left(\frac{k'}{k'+1}\right)} \qquad (2.6)$$

where k' (the capacity factor) is given by

$$k' = \frac{t_x}{t_x - t_o}$$

and α (the selectivity factor) is given by

$$\alpha = \frac{k_2}{k_1}$$

where k_1 and k_2 are the capacity factors for bands 1 and 2. For two close bands $k' = k_1 = k_2$

and N (the theoretical plate count) of a band is given by

$$N = 16\left(\frac{t_x}{t_w}\right)^2$$

The concept of N or theoretical plate count was introduced by Martin and Synge (1941) who showed that

$$N = \frac{H}{L}$$

where L is length of column and H is height equivalent of theoretical plates, a concept which in turn was derived from distillation theory.

Equation 2.6 is a fundamental relationship in HPLC which allows the empirical control of resolution by manipulating the three terms α, N, k which are essentially independent of each other. This means that it is possible to optimise these variables one after the other.

The separation selectivity as measured by α in term (i) is varied by changing either the composition of either the moving or stationary phase or both. The separation efficiency as measured by N is varied by changing the column length or solvent velocity. The capacity factor k' is varied by changing solvent strength to increase k' (weaker solvent) or decrease k' (stronger solvent). These manipulations are shown diagramatically in Fig. 2.8.

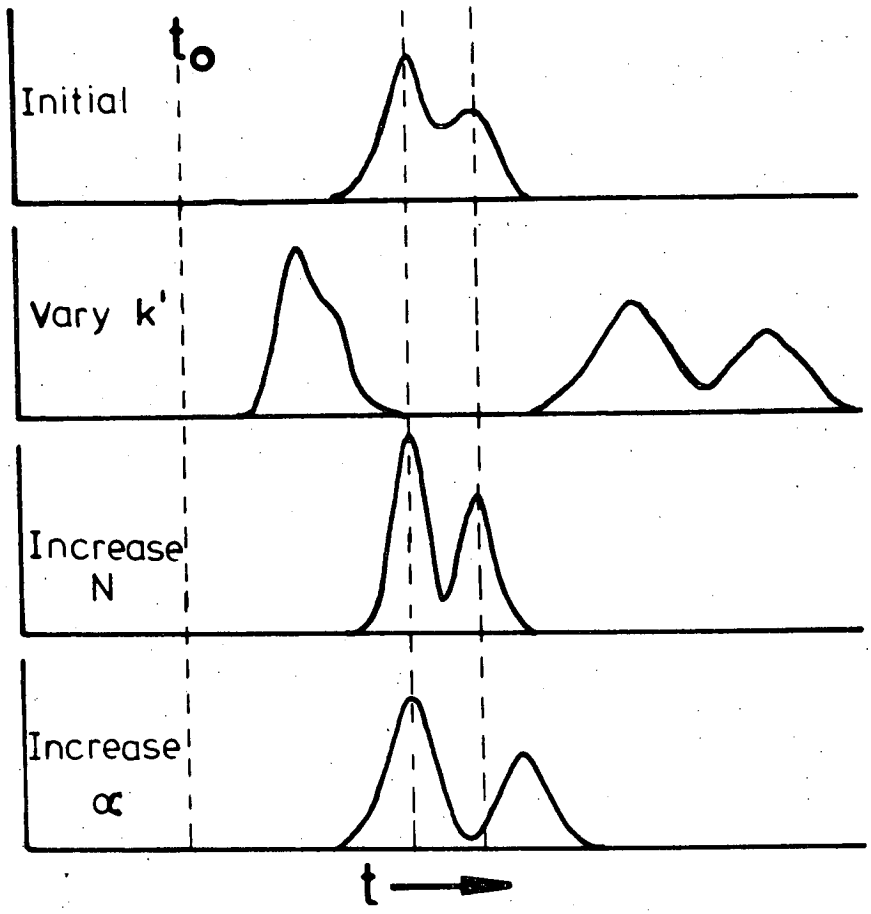


FIGURE 2.8 The Effect on Resolution of Manipulating the Chromatographic Parameters for HPLC.

2.4.4 Chromatographic Equipment and Solvents

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which consisted of two M-6000 A solvent delivery pumps, a U6K universal liquid chromatograph injector and a M-660 solvent programmer coupled to a series 440 uv absorbance detector (254 nm) and a Linear Instruments double channel pen recorder. A μBondpak C_{18} (10 μm) of 300 x 3.9 mm was used for all separations.

The column material consists of 10 micron diameter porous silica particles, the silanol groups of which have been reacted as follows (reviewed by Grushka and Kikta, 1977):

$$\begin{array}{c}|\\ -\mathrm{SiOH}\\ |\end{array} + \mathrm{ClSi(CH_3)_2(CH_2)_{17}CH_3} \rightarrow \begin{array}{c}|\\ -\mathrm{SiO}-\\ |\end{array}\mathrm{Si(CH_3)_2(CH_2)_{17}CH_3} + \mathrm{HCl} \quad (2.7)$$

for steric reasons not all silanol groups will react ($\leq$45%), the silica surface thus consists of a mixture of silanol and bonded groups. The free silanol groups can then be reacted with trimethylchlorosilane to block them and prevent their interference with column reactions.

The use of the μBondpak C_{18} column of constant length virtually fixes N for any separations using the column except for variations in flow rate. However k' and α can be varied widely. These experimental variations in k' and α are discussed in the following sections.

Iodoaminoacids were all purchased from Henning GMBH, Berlin except for 3,5-T_2 (Koch-Light, Haverhill, U.K.) and MIT and DIT (Sigma, St. Louis, U.S.A.). KH_2PO_4 (Ajax, Sydney). Methanol either Merck (AR) or Waters (HPLC grade), n-propanol (AR) (Ajax, Sydney). Ethanol (nominally 95%, Berri Cooperative, Adelaide) and redistilled over ferrous sulphate through a 30 cm column packed with glass-wool to remove ultraviolet absorbing material. This meant that each batch of ethanol varied slightly.

As alcohol-water mixtures shrink on mixing, mixed solvents were always made by adding the aqueous component last to bring the solution to the final volume. All the solvents were filtered

through a 0.5μm Teflon filter (Millipore, Sydney) and degassed under partial vacuum before use.

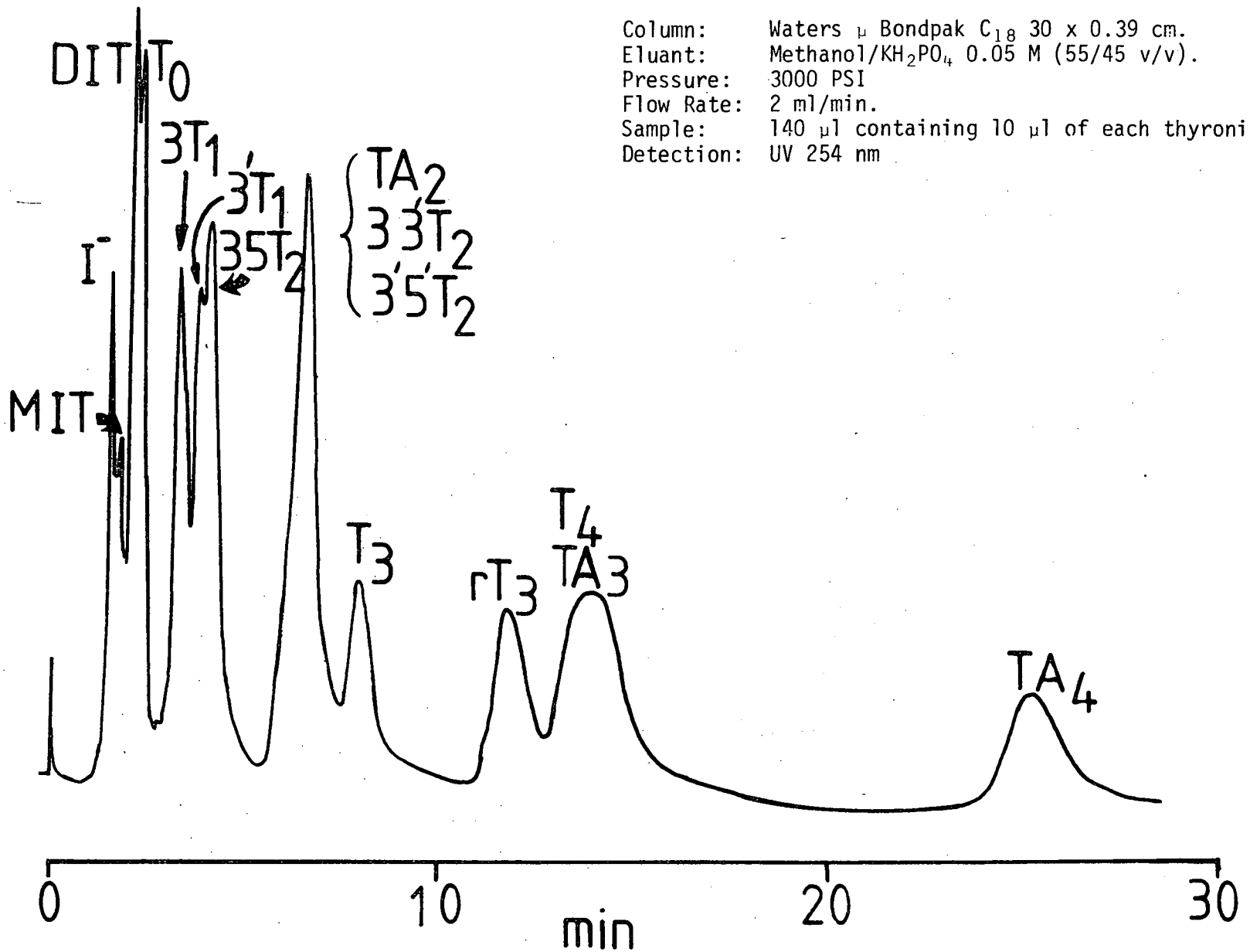
Standards (25 μg/ml) were prepared in either 50% v/v methanol or 30% v/v ethanol and then filtered and degassed similarly. The standards were kept at -5°C in the dark to prevent deiodination. When in use, the standards were kept at room temperature in the dark near the apparatus.

2.4.5 Manipulations of the Mobile Phase

Methanol is an often used solvent for the mobile phase in reversed phase HPLC, and as discussed earlier, has been used in the separation of thyronines. Accordingly, aqueous mixtures of methanol with 0.05 M KH_2PO_4 in various proportions were tried, the best separation, in terms of k' and R_s, was obtained with 55% methanol (see Fig. 2.9). Table 2.4 lists t_x (elution time) and t_w (band width duration) for each thyronine when chromatographed separately under these conditions. Most thyronines are resolved, but the triplet TA_2/3,3'-T_2/3',5'-T_2 and the pair TA_3/T_4 are not resolved. Of these, the most important in terms of deiodination reactions is the pair 3,3'-T_2 and 3',5'-T_2 which are not resolved at all. By further reducing the proportion of methanol in the mobile phase it is possible to partially separate 3,3'-T_2 and 3',5'-T_2 but at the expense of prolonged elution time (t_x), increased k', markedly reduced band height, and increased band width and tailing (see Fig. 2.10). Table 2.4 compares the separation of 3,3'-T_2 and 3',5'-T_2 at 55% and 40% methanol.

FIGURE 2.9 The Optimum Separation of Iodothyronines Obtained with Methanol.

Column: Waters μ Bondpak C_{18} 30 x 0.39 cm.
Eluant: Methanol/KH_2PO_4 0.05 M (55/45 v/v).
Pressure: 3000 PSI
Flow Rate: 2 ml/min.
Sample: 140 μl containing 10 μl of each thyronine.
Detection: UV 254 nm



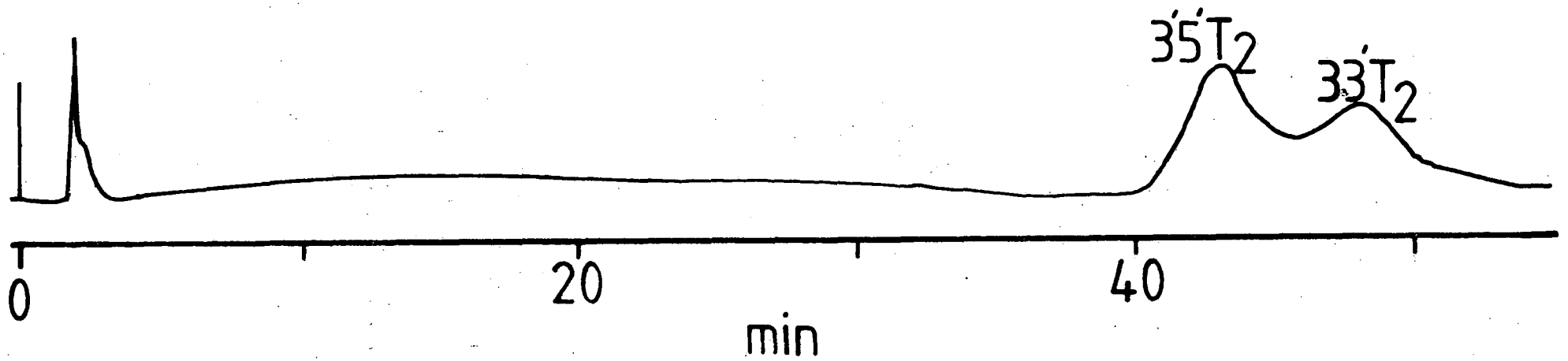


FIGURE 2.10 Partial Separation of 3',5'-T_2 and 3,3'-T_2 by Methanol.

Column: Waters μ Bondpak C_{18} 30 x 0.39 cm.
Eluant: Methanol : KH_2PO_4 0.05 M 40:60
Pressure: 3000 PSI
Flow Rate: 2 ml/min
Sample: 20 μl of each.
Detector: 254 nm.

TABLE 2.3 Elution of Thyronines by Methanol 55/45 (v/v)

KH_2PO_4 0.05M.

Thyronine/ Iodoaminoacid	Chromatographic Variable		
	t_x (time of elution; min)	t_w (width of band in time; min)	$k' (= \frac{t_x - t_o}{t_x})$
MIT	1.6	0.15	0.45
DIT	2.05	0.15	0.86
T_o	2.05	0.15	0.86
$3T_1$	3.1	0.2	1.81
$3'T_1$	3.6	0.3	2.27
$3,5\text{-}T_2$	3.9	0.3	2.54
$3,3'\text{-}T_2$	6.4	0.5	4.81
$3'5'\text{-}T_2$	6.5	0.4	4.91
$3,5,3'\text{-}T_3$	7.75	0.55	6.05
$3,3'5'\text{-}T_3$ (rT_3)	11.75	0.95	9.68
$3,5,3'5'\text{-}T_4$	14.3	0.8	12.00
DIAC	6.15	0.45	4.59
TRIAC	13.9	0.8	11.64
TETRAC	25.8	1.4	22.45

TABLE 2.4 Alteration of Methanol Concentration and R_s of 3',5'-T_2 and 3,3'-T_2.

Methanol (vol. %)	Chrom. variables	3,'5'-T_2	3,3'-T_2
55%	t_x (mins)	6.5	6.5
	k'	4.91	4.91
	α	1.00	
	R_s	0.00	
40%	t_x (mins)	30.1	33.45
	k'	19.07	21.30
	α	1.12	
	R_s	0.67	

The use of 40% v/v methanol/KH_2PO_4 although partially resolving 3,3'-T_2 and 3',5'-T_2 would at the same time so prolong the elution times (and k') for other iodothyronines such as T_3, rT_3 and T_4 that their band height would approach the baseline as predicted by $N = 16\left(\frac{t_x}{t_w}\right)^2$.

Instead, an alteration in α is required without markedly affecting k'. This can be achieved, as noted in the introduction by altering either the column packing or the solvent. It was decided to try alterations of solvent as the first approach.

Various strengths of aqueous solutions of acetonitrile were tried but failed to separate 3,3'-T_2 and 3',5'-T_2. Such mixtures

were, however, able to markedly reduce elution times and hence k' when compared to aqueous methanol mixtures.

Ethanol and propanol are not often used in HPLC because of the difficulties firstly, in getting spectroscopically clean ethanol and secondly, the increased viscosities of both, when compared to methanol, require higher pump pressures. Nevertheless I felt that they might be useful solvents to vary α and allow the separation of the diiodothyronines.

In order to allow comparison of ethanol and propanol with methanol, various aqueous mixtures of each were used to separate 3,3'-T_2 and 3',5'-T_2. Solvent strengths were chosen to give overlapping solvent polarity index values (Snyder, 1974). The separation achieved with varying ethanol concentration is shown in Fig. 2.11. No resolution was obtained with n-propanol. The chromatographic variables for both solvents are shown in Table 2.5.

Ethanol is obviously the superior solvent in terms of higher selectivity (α) and resolution (R_s). Thus the alcohols do not form a series of increased resolution with increased molecular weight. Ethanol is fortunately higher in its selectivity for 3,3'-T_2 and 3',5'-T_2.

Manufactured columns may vary in performance both between columns initially and during their lifetime of use so that empirically for each column, the separation must be optimised. This became particularly apparent during this work, for example, the R_s for 3,3'-T_2 and 3',5'-T_2 was initially 1.25, one month later

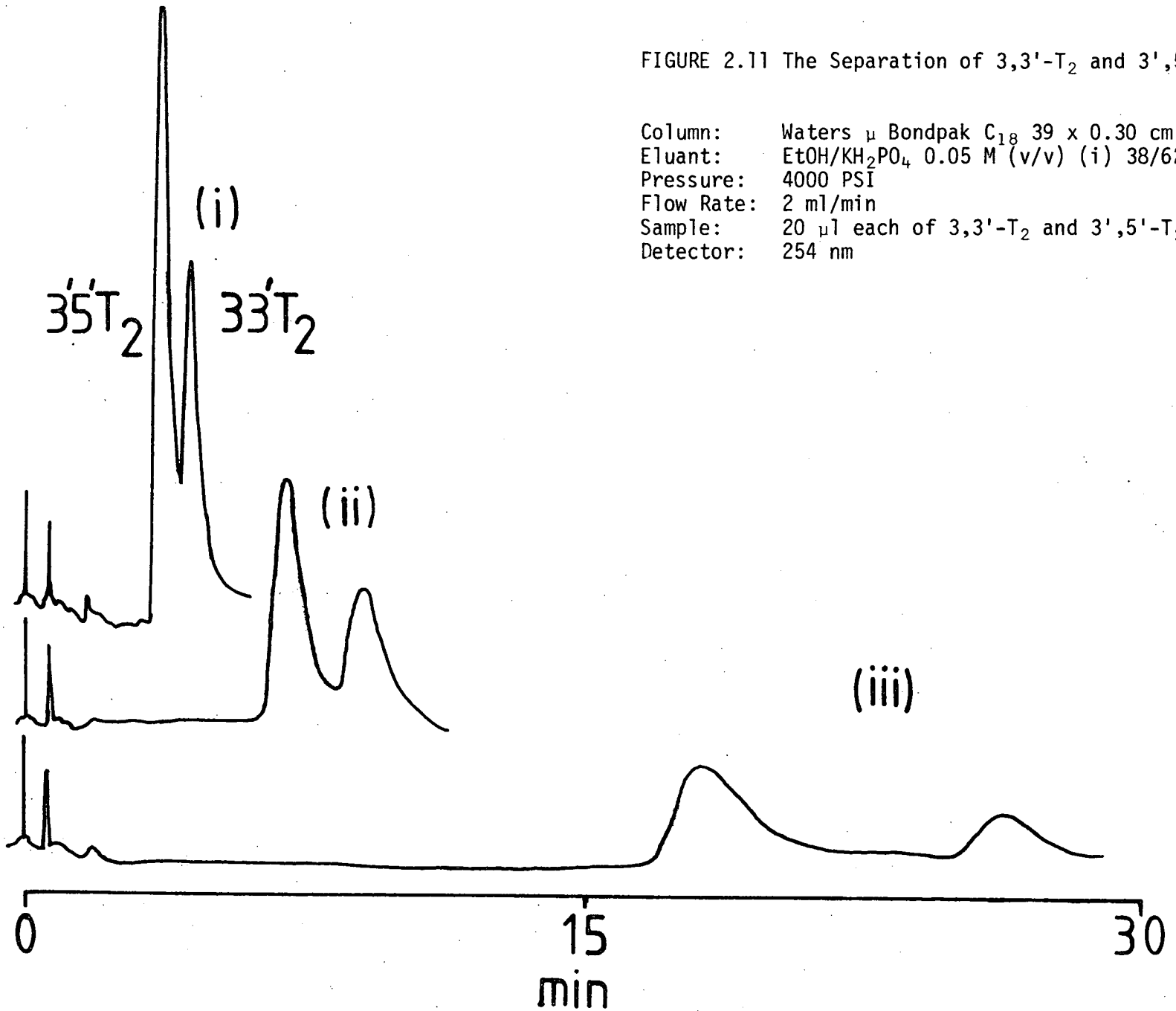


FIGURE 2.11 The Separation of 3,3'-T_2 and 3',5'-T_2 by Ethanol Mixtures.

Column: Waters μ Bondpak C_{18} 39 x 0.30 cm.
Eluant: EtOH/KH_2PO_4 0.05 M (v/v) (i) 38/62; (ii) 32/68; (iii) 25/75.
Pressure: 4000 PSI
Flow Rate: 2 ml/min
Sample: 20 μl each of 3,3'-T_2 and 3',5'-T_2
Detector: 254 nm

TABLE 2.5 Effect of C_2 and C_3 Alcohols on the Separation of 3,3'-T_2/3',5'-T_2.

% Solvent/KH_2PO_4			
Ethanol		3',5'-T_2	3,3'-T_2
38%	t_x (min)	3.65	4.65
	k'	4.21	5.64
	α	1.34	
	R_s (min)	1.25	
32%	t_x	6.85	8.9
	k'	8.78	11.7
	α	1.33	
	R_s	1.37	
25%	t_x	18.1	26.05
	k'	24.9	36.21
	α	1.45	
	R_s	2.86	
Propanol			
23.5	t_x	3.90	3.90
	k'	2.90	2.90
	α	1.00	
	R_s	0.00	
19.6	t_x	7.00	7.00
	k'	6.00	6.00
	α	1.00	
	R_s	0.00	

it had dropped to 0.81 and the next day 0.30 with marked skewness and band trailing. The column was discarded and a new one installed. The optimal separation for all the iodothyronines using ethanol is shown in Fig. 2.12 and the chromatographic variables tabulated in Table 2.6. However R_s for 3,3'-T_2 and 3',5'-T_2 is much reduced when compared to the previous column but because the peaks are sharp and have very low skew, the resolution is more than adequate for band height determination.

TABLE 2.6 Separation of Iodothyronines with 39%v/v Ethanol/KH_2PO_4 0.025 M.

	t_x	k'	R_s
T_o	19.0	1.11	
			3.56
$3T_1$	31.0	2.44	
			0.93
$3'T_1$	34.5	2.83	
			1.23
$3,5T_2$	40.5	3.5	
			3.29
$3',5'T_2$	61.5	5.83	
			0.37
$3,3'T_2$	64.0	6.11	
			2.56
T_3	87.0	8.67	
			3.17
rT_3	127.5	13.2	
			1.66
T_4	153.75	16.1	

The plate value N, $N = 16\left(\frac{t_x}{t_w}\right)^2$ is in part a measure of band broadening. Its value, especially for the later bands, can be improved by means of a gradient as shown in Fig. 2.13. However, although the bands are sharpened and the N value is improved, the use of a gradient creates several problems. Firstly a long period of equilibration is needed to obtain stable conditions and if injection occurs before true equilibrium is

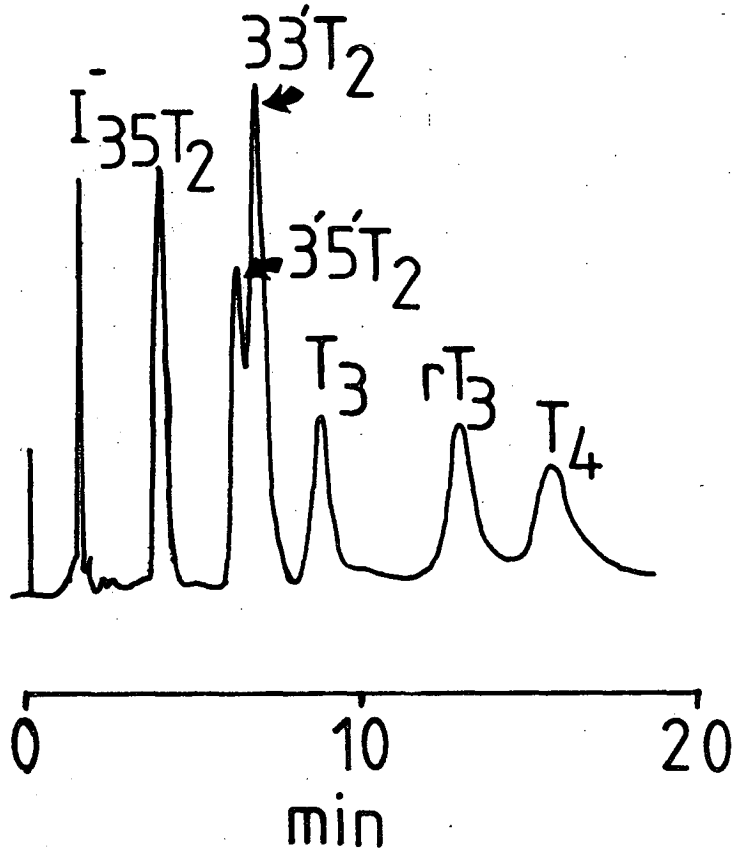


FIGURE 2.12 The Separation of the Iodothyronines by Ethanol.

Column: Waters μ Bondpak C_{18} 30 x 0.9 cm.
Eluant: EtOH:KH_2PO_4 0.05 M (38/62 v/v)
Pressure: 4000 PSI
Flow Rate: 2 ml/min
Sample: 50 μl containing 125 ng of each thyronine.

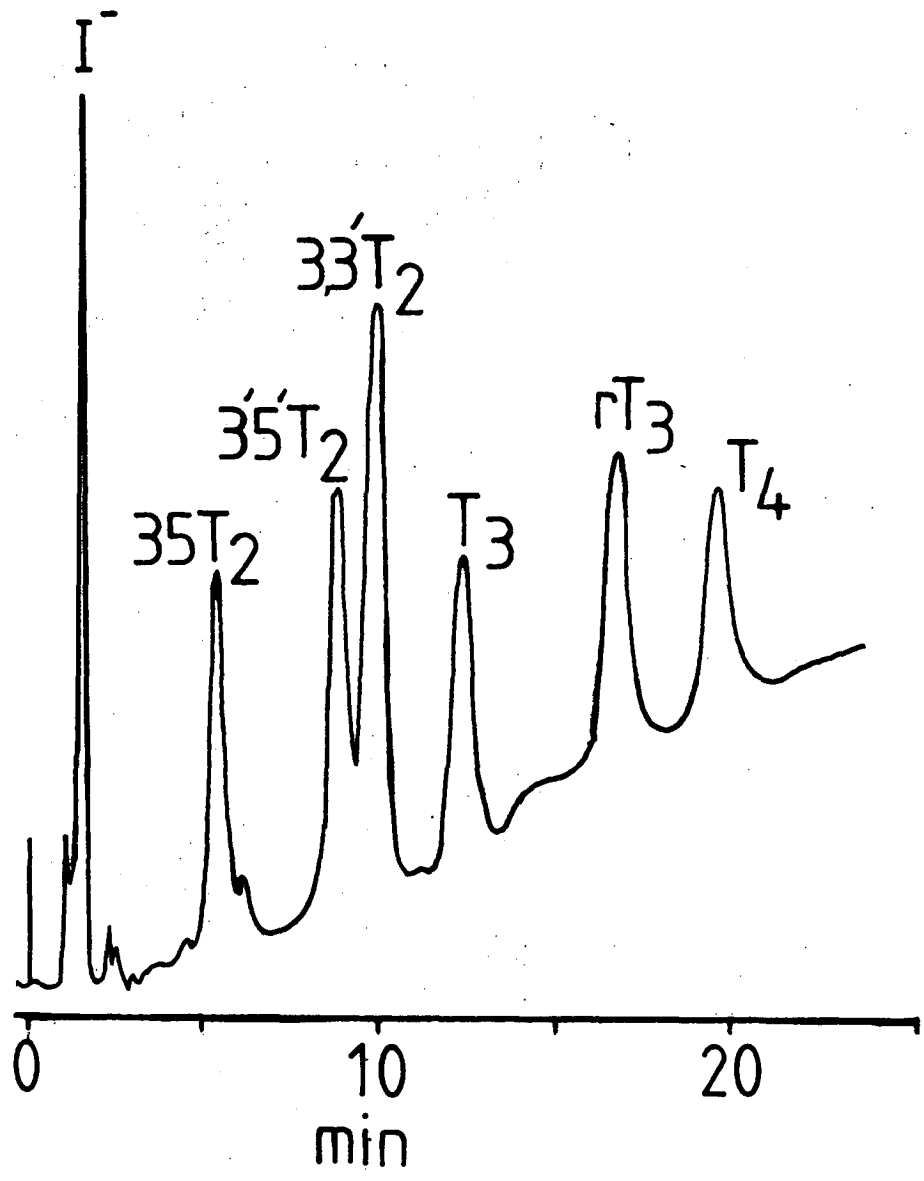


FIGURE 2.13 Gradient Elution of Iodothyronines.

Column: Waters μ Bondpak C_{18} 30 x 0.9 cm.
Eluant: 35/65 linear gradient over 60 min to 50/50 EtOH/KH_2PO_4 0.05 M.
Pressure: 3800 → 4000 PSI.
Flow Rate: 2 ml/min.
Sample: 50 μl containing 125 ng of each thyronine.

obtained, the variation in elution time alters band heights, making their quantitation difficult. A rising baseline also makes band height measurement difficult. Isocratic conditions are usually preferred for these reasons.

Potassium dihydrogen phosphate (0.05 M) was used in all separations following the separation devised by Waters Association for T_4 and T_3 and the work of Hancock and Hearn (1976) who found that 0.01% v/v phosphoric acid improved resolution, band height and reproducibility with many peptides. However the low pH of 0.1% H_3PO_4 in solution is not recommended by Waters Associates (personal communication) who suggested that a pH ≤ 2 attacks the silicon-carbon bands of the reverse phase packing. I therefore continued to use KH_2PO_4which has an approximate pH of 4.0, instead of H_3PO_4. The effect of KH_2PO_4 on k' was assessed by measuring the resolution (R_s) of 3,3'-T_2/3',5'T_2 in the absence of KH_2PO_4 whilst varying the proportion of EtOH. The results are shown in Table 2.7. This data is graphically compared to the presence of 0.05 M KH_2PO_4(Table 2.5) in Fig. 2.14 which suggests that similar resolutions may be obtained with or without KH_2PO_4 but that in the absence of KH_2PO_4 equivalent resolution is achieved at lower ethanol concentrations. However, Fig. 2.14 suggests that separation with KH_2PO_4 is less critically dependent on the concentration of ethanol; i.e., the curve of R_s between 1.0 - 1.5 is flatter than the very steep curve for ethanol alone.

The added KH_2PO_4 was varied between 0 and 0.05 M and the resolution of 3,3'-T_2 and 3',5'-T_2 was measured keeping the proportion of EtOH at 35% and a flow rate of 2 ml/min (see Table 2.8).

TABLE 2.7 Separation of 3,3' and 3',5' Diiodothyronines by EtOH Without KH_2PO_4.

EtOH % v/v		3'5'-T_2	3,3'-T_2
35	t_x	94.5	134
	k'	9.5	13.9
	α	1.46	
	R_s (mins)	2.52	
37.5	t_x	77	96
	k'	7.56	9.67
	α	1.28	
	R_s	1.56	
41.25	t_x	59	64.5
	k'	5.56	6.17
	α	1.11	
	R_s	0.7	
42.5	t_x	54	58
	k'	5.00	5.42
	α	1.08	
	R_s	0.56	

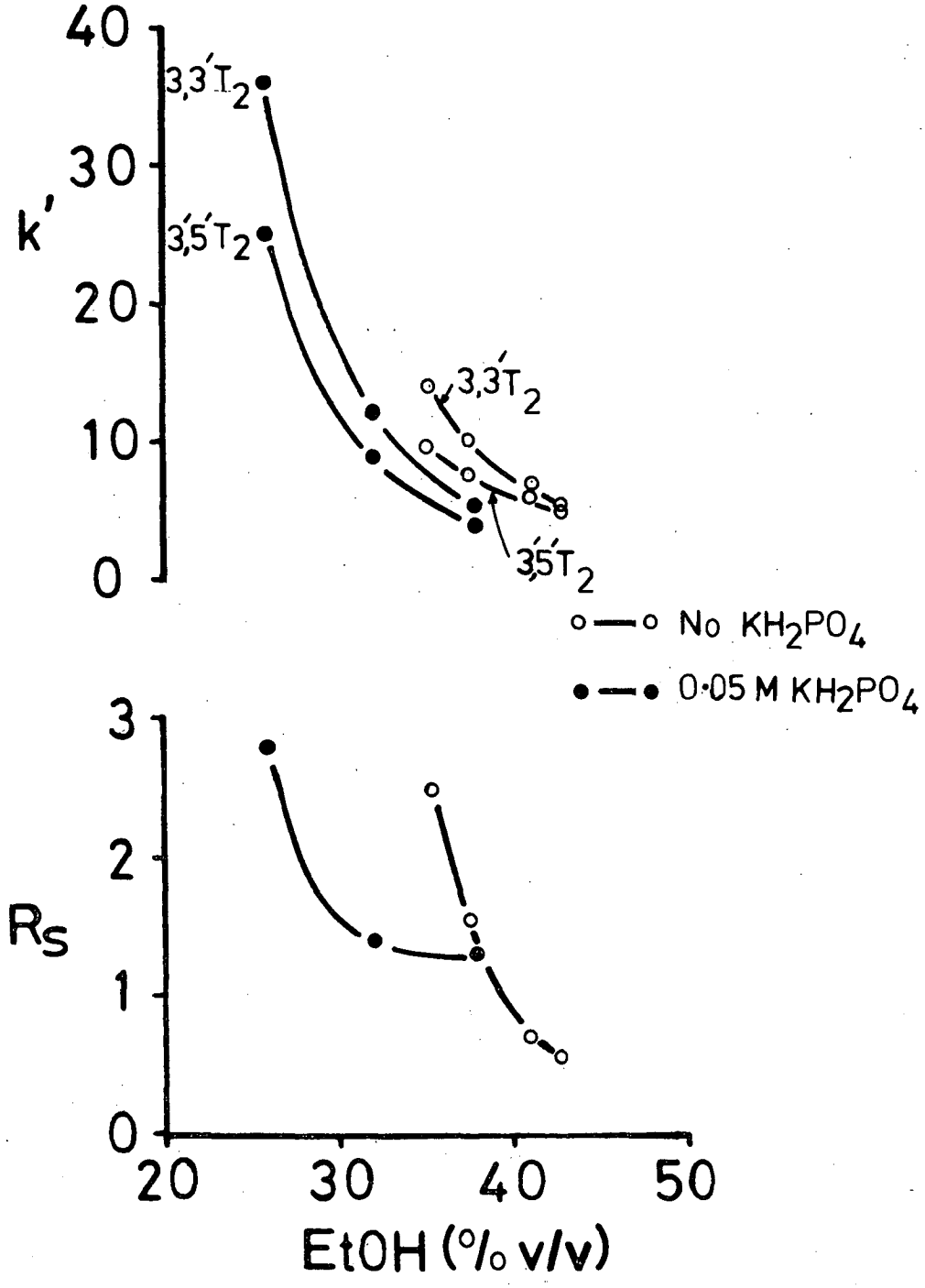


FIGURE 2.14 The Effect of KH_2PO_4 in the Mobile Phase on the k' and R_s for the Separation of 3,3'-T_2 and 3',5'-T_2 by HPLC.

TABLE 2.8 Effect of KH_2PO_4 on the Resolution of 3,3'-T_2 and 3',5'-T_2.

KH_2PO_4 (mol/l)	Elution time (t_x) 3'5'-T_2	Elution time (t_x) 3,3'-T_2	R_s:3,3'-T_2/ 3',3'-T_2
0	6.9	7.9	1.7
0.01	6.9	7.9	2.0
0.025	6.9	7.7	1.6
0.05	7.0	8.0	2.0

Table 2.8 shows that there is very little effect of KH_2PO_4 on retention time. However the addition of KH_2PO_4 may allow greater variations in solvent composition without deterioration of resolution.

Deming and Turoff (1978) made a systematic study of the effects of pH on the mobilities of weak organic acids, adapting the graphical "window" technique of Laub and Purnell (1975, 1976) who had devised their method for the optimization of the separation of complex mixutres by gas-liquid chromatography. The "window" method is quite simple; all the components of interest are run at three or more variations of the mobile phase (in this case pH) and a plot (for every pair of chosen substances) of the relative retention $\alpha = \frac{k_1}{k_2}$ is made against the change in mobile phase (i.e. pH). The method is particularly useful in allowing one to choose conditions for maximising the resolution of all the bands, especially those which migrate across each other with change in mobile phase.

The pH of the mobile phase was therefore adjusted in the aqueous component by adding sufficient orthophosphoric acid to make it 0.01 M and then adjusting the pH with KOH (5M). The range was kept between 2.5 and 6.0 to prevent damage to the silica based packing. The aqueous phase was then degassed and combined with degassed ethanol to give a final concentration of 39% v/v which was run at 2 ml/min. The void time was 0.9 min. The k' for all iodothyronines and the R_s for adjacent pairs are plotted against pH in Figs. 2.15 and 2.16.

It is in fact not necessary to plot a window diagram as the components all maintain the same relative position as shown by Fig. 2.15. The difficult pair 3'5'/3,3' have the lowest resolution (R_s) and were unaffected by changes in pH.

However pH does have effects on retention time, especially of the later components rT_3 and T_4. So that, for processing a large number of samples a pH of about 3.0 would give the fastest elution time for the whole chromatogram of about 14 min. compared to 18 min. at pH 2.5 or pH 6.0.

In conclusion, for best resolution of T_4 and all its potential iodothyronine products, the optimal system is 39% v/v ethanol in 0.01 M H_3PO_4 adjusted to pH 3.0 with KOH.

The linearity of response and calibration for each iodothyronine was checked by the injection of a constant volume (20 µl) containing various amounts of each compound (Fig. 2.17). A linear response is obtained for all thyronines.

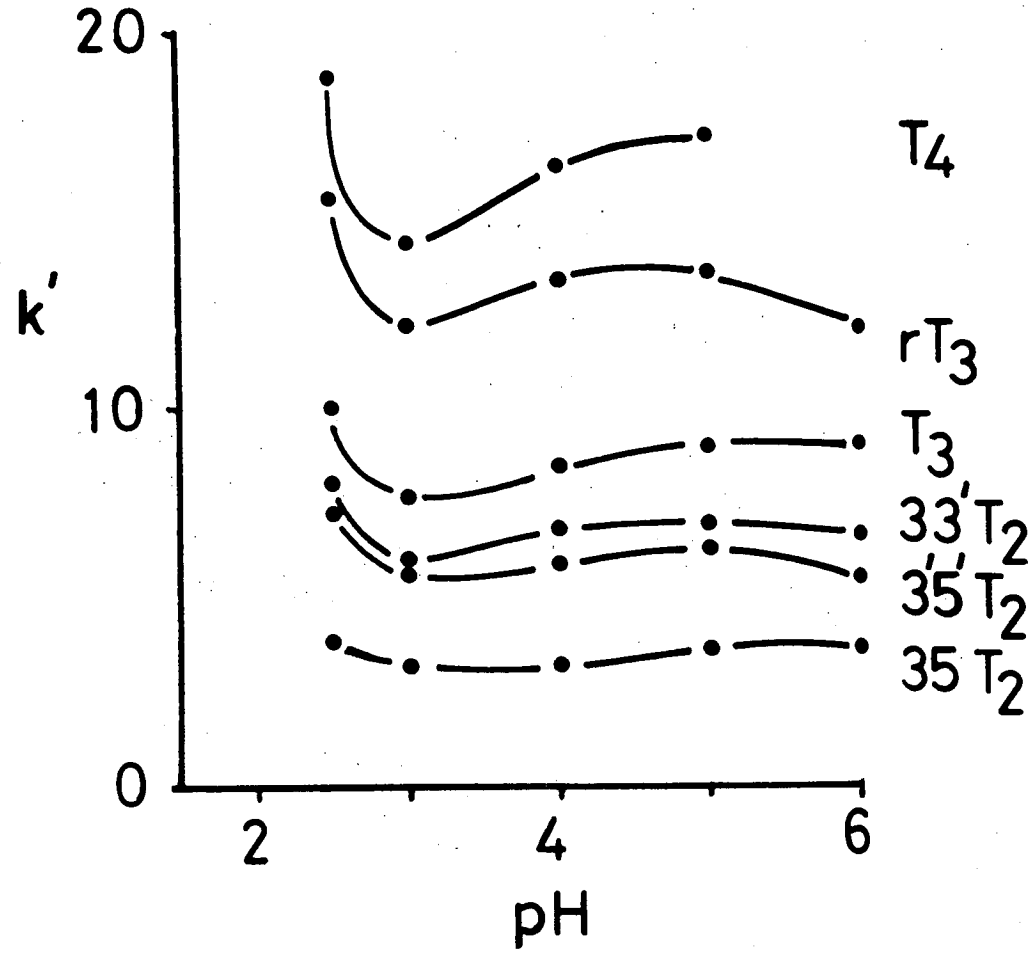


FIGURE 2.15 The Effect of pH on k' for the Separation of the Iodothyronines by HPLC.

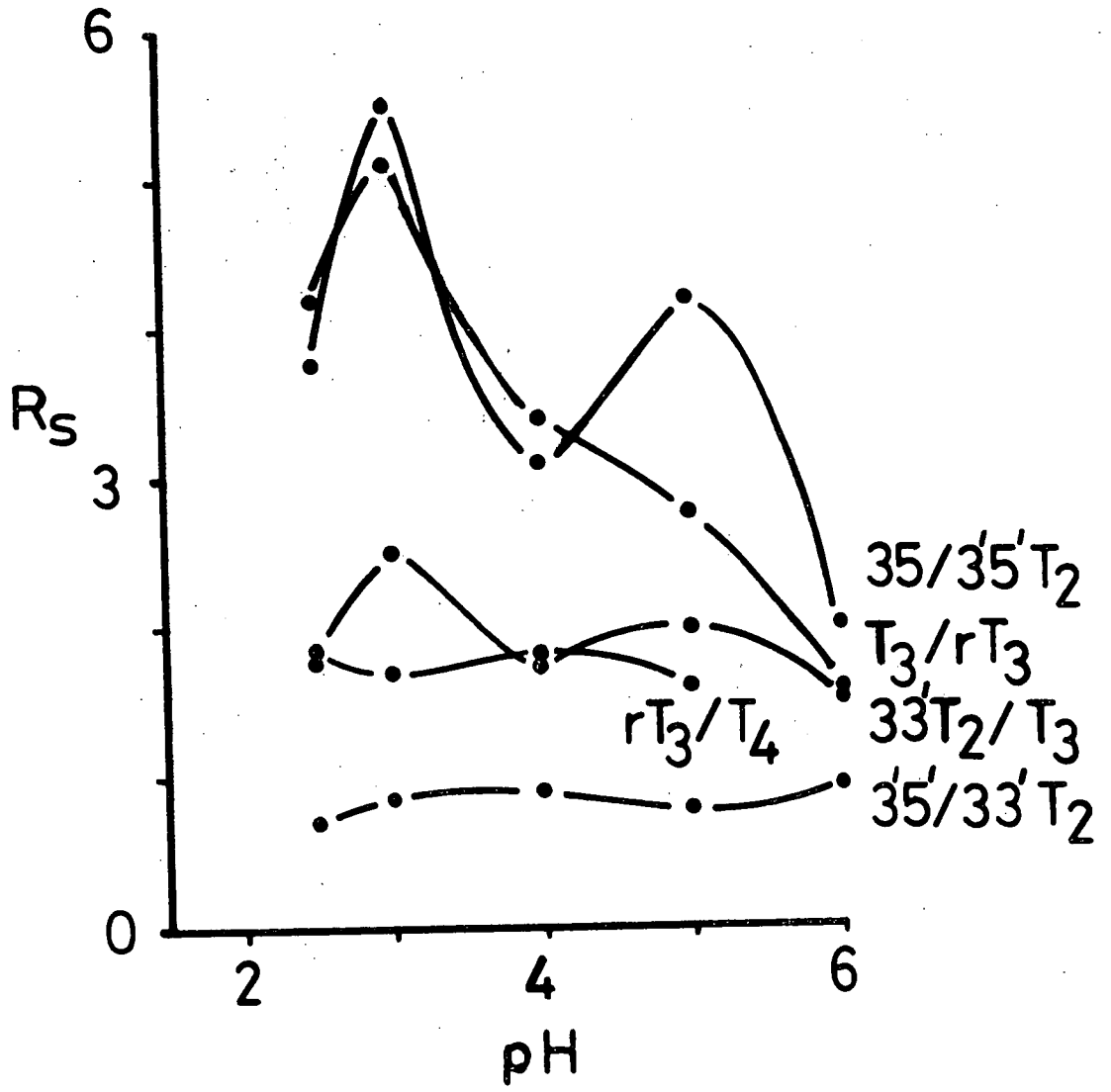


FIGURE 2.16 The Effect of pH on R_s for Adjacent Pairs of Iodothyronines Separated by HPLC.

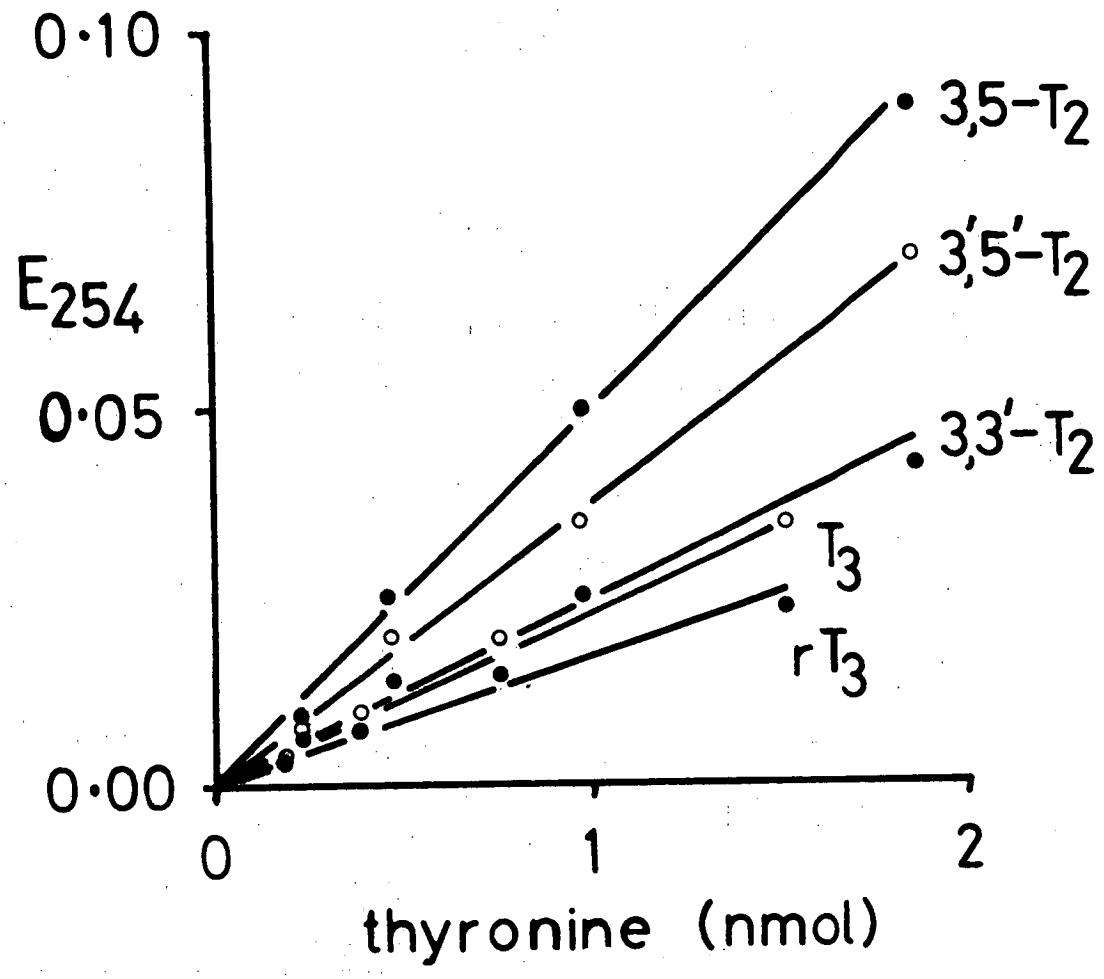


FIGURE 2.17 The Detector Response at E_{254} with Varying Amounts of Iodothyronine Injected onto HPLC.

2.4.6 Internal Standard and Extraction of Thyronines from Incubations.

To improve the validity of peak measurement derived from tissue extracts the synthetic thyronine 3'-isopropyl-3, 5-diiodothyronine (gift from Dr. E.C. Jorgenson) was used. It had a retention time between that of rT_3 and T_4 (see Fig. 2.18). The internal standard was added at the end of the incubation. The incubation was then extracted as in section 2.2 and the methanol/ammonia eluant dried under air at 40°C in dim light. It was then taken up in 120 µl of mobile phase and 100 µl injected into the HPLC.

2.5 Preparation of Radioactive Substrate

The detection of deiodination by the methods in section 2.2 and 2.3 requires the use of iodine-125 labelled thyroxine (or other thyronine). In this study the thyroxine (and other thyronines) were obtained commercially with the iodine-125 only in the outer ring of the thyronine so that the thyroxine was labelled as follows: [3',5'-^{125}I]-thyroxine, rT_3 was labelled in the same positions, and T_3 was labelled as [3'-^{125}I]-3,3'5'-triiodothyronine.

These substrates were purchased from the Radiochemical Centre, Amersham and from the New England Nuclear Corporation, Boston. They were supplied at different times in 50% aqueous 1,2 propanediol, 50% ethanol or 50% propanol.

For use, each substrate was diluted with 50% aq. propanediol and non-radioactive thyroxine (or other thyronine) was added so that

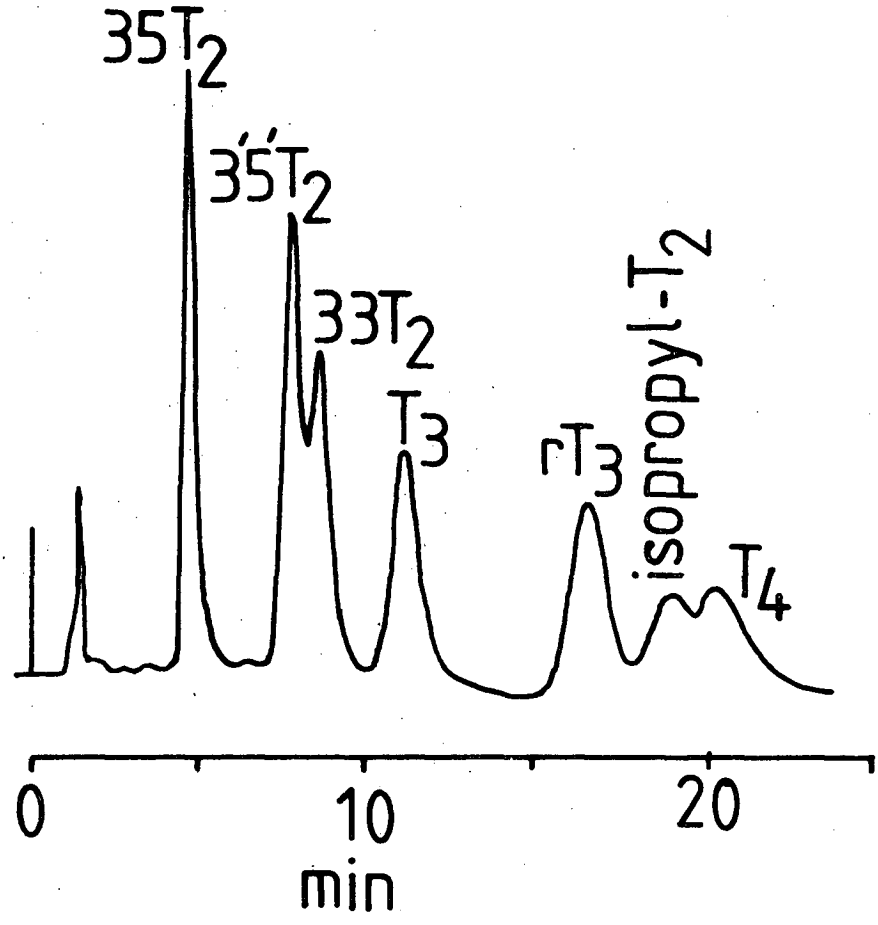


FIGURE 2.18 The Migration of Isopropyl Diiodothyronine (internal std.) on HPLC.

Conditions were the same as in Figure 2.12.

25 µl of the substrate when added to a 1 ml reaction volume contained between 50,000-300,000 cpm and a final concentration of T_4 of 10^{-7} mol/l (or for T_3, rT_3 10^{-8} mol/l). These values were chosen to be close to physiological serum concentrations, and still provide an adequate specific acitivity of the substrate to allow detection of deiodination.

As the radioactive iodine-125 atoms are present at the 3' or 5' positions in the outer (or phenolic) ring of the thyronines, they allow only the detection of deiodination from this outer ring. For brevity, this deiodination is described as 5'-deiodination. However this assumes that in solution the steric hindrance due to the bulky iodine atoms is overcome, and the phenolic ring is able to rotate around the angled oxygen bridge and allow the 3' and 5' iodine atoms to become equivalent substitutes for the enzyme (reviewed by Cody, 1981). Of course, "5'-deiodination" of T_3 is not really a correct description as a single iodine atom in the outer ring is, by convention, always in the 3' position.

2.6 The Selection of Buffer Conditions

2.6.1 Introduction to the Selection of Buffer Components

As was reviewed in Chapter 1, early workers had used radio-chemical techniques to demonstrate T_4 deiodination by peripheral tissues. Later however, many instances of deiodination were shown to be non-enzymically induced either by flavin nucleotides activated by light or by hydrogen peroxide. Characteristic of many of these non-enzymic deiodinations, was their authors' inability to demonstrate any products of T_4 deiodination other than the end product iodide. Thus the assays

in sections 2.2 and 2.3, which depend on the measurement of iodide released from T_4 or other thyronine, must be shown to maximise the enzymic deiodination rate and to minimise the non-enzymic rate.

Recent workers on thyroxine deiodination have used a variety of buffer components; for example, Hesch et al. (1975) used triethanolamine or Krebs-Ringer bicarbonate; Visser et al. (1976) used TRIS/EDTA; Chopra (1977) used phosphate buffers; as did Leonard and Rosenberg (1978).

Although the exact pH optima for T_3 production from T_4 varies between authors, all have found the pH optimum to lie between 6.5 and 7.5. Hence a neutral pH (7.2-7.4) was chosen for initial studies. Of the potentially suitable buffers, HEPES (N-2 hydroxyethylpiperazine-N'-2-ethane sulphonic acid, Calbiochem, Sydney) pKa 7.55, TRIS (tris(hydroxymethyl) aminomethane, Sigma, St. Louis) pKa 8.1 and Na_2HPO_4 (Ajax, Sydney) pKa 7.2 were chosen as appropriate substances to buffer at pH 7.4.

With respect to other components of the incubation medium, Visser et al. (1976) using liver cell homogenates and microsomes have shown that DTT is the most active of the essential sulphydryl compounds required in stimulating the conversion of T_4 to T_3 in vitro. Conversion was further stimulated by the addition of EDTA with DTT but not by EDTA alone.

These observations on EDTA are of great interest as Kobayashi et al. (1966) found that metal chelators including EDTA are very effective in the prevention of trace-metal induced non-enzymic deiodination. On the other hand, Reinwein and Rall (1966) had

shown that non-enzymic deiodination by metal ions was either stimulated or inhibited by EDTA and which depended on the concentrations of both the EDTA and the cation (e.g. Fe^{2+}). Other cations which induced deiodination (e.g. Cu^{2+}) were only inhibited by EDTA. These authors showed a similar increased or decreased deiodination with different aminoacids, particularly those that would chelate metal ions.

With respect to non-enzymic deiodination, those authors who have used thiol compounds to stimulate T_4 to T_3 conversion (e.g. Visser et al., 1976; Chopra, 1978; and Imai et al., 1980) do not record if they have investigated whether the reduced thiol compounds have an effect on deiodination not mediated by enzymes as catalysts; their published works used as a control the omission of the sulphydryl compound. However, of relevance is that cysteine is known to inhibit the rapid non-enzymic deiodination of radiolabelled thyroxine on storage (Braverman et al., 1970).

Nevertheless, a priori it seemed possible that if the thiols were substrates in the reductive enzymic deiodination of thyroxine, then they may cause deiodination at an appreciable rate without the catalytic enzyme being present. Also, as HEPES and TRIS are amino compounds, they might have similar actions to the amino acids, in either stimulating or inhibiting non-enzymic deiodination.

2.6.2 The Interaction of Buffer Components on the Non-Enzymic Deiodination of Thyroxine

The effects of EDTA, DTT, GSH, HEPES, TRIS, and NaH_2PO_4 were examined both singly and in combination, to determine their effects, if any, on non-enzymic deiodination. The data and experimental method are shown in Table 2.9 and its caption.

TABLE 2.9 The Effect of Buffer Ions and Sulphydryl Compounds on the Non-Enzymic Production of Iodide ($^{125}I^-$) from ^{125}I-Thyroxine in the Dark.

Buffer Component	Addition of TCA (420 mM)	Addition of EDTA (15 mM)	Sulphydryl Addition		
			None	GSH (10 mM)	DTT (1 mM)
NONE			-0.4	4.8	2.1
	+		-1.3	1.3	10.4
		+	1.4	2.8	2.3
	+	+	-0.3	1.1	1.8
HEPES (50 mM)			-0.7	4.5	2.3
	+		-0.3	1.3	7.2
		+	0.1	2.3	4.0
	+	+	0.3	1.1	0.9
TRIS (50 mM)			-0.6	2.1	1.7
	+		-1.3	1.1	1.4
		+	-0.7	2.3	1.8
	+	+	-1.4	0.8	0.8
NaH_2PO_4 (50 mM)			0.2	4.1	2.0
	+		-1.5	1.2	9.2
		+	0.5	1.9	0.9
	+	+	-0.4	0.6	1.3

Each value shown is the mean value of 5 determinations of $^{125}I^-$ liberated from ^{125}I-T_4 and is expressed as the percentage of the total radioactivity added with correction for $^{125}I^-$ already present. To simplify presentation, standard deviations are not shown but were all less than 15% of their respective means. The observations were made as follows. To a series of glass tubes at 0°C, concentrated solutions prepared from glass distilled water, were added to make, in a volume of 1 ml, the final concentrations shown in the table. All the solutions, except the water and the trichloracetic acid, were adjusted to pH 7.2 with either HCl or NaOH before dispensing. The ^{125}I-T_4 (10^{-10} mol and 322,500 cpm) in 25 µl was then added just prior to the transfer of the tubes for 2 hours into a 37°C water bath in the dark. After which time, 100 µl of TCA was added to the tubes not already containing 0.42 M TCA. $^{125}I^-$ was then measured after butanol extraction (Section 2.3).

From Table 2.9 it can be seen that in the presence of either TCA or EDTA or both, there is no measurable deiodination. This remains true when HEPES, TRIS or NaH_2PO_4 are present.

The only statistically significant (t-test >95% c.l.) increase in deiodination occurs when sulphydryls are present as follows. Glutathione is associated with a small increase in non-enzymic deiodination in neutral (pH) conditions in the absence of EDTA and TCA, but not when either EDTA or acid conditions (TCA) or both are present. HEPES and NaH_2PO_4 have no effect on this deiodination but TRIS inhibits the GSH associated effect.

Dithiothreitol on the other hand is associated with a marked increase (7-10%) in non-enzymic deiodination in acid conditions (TCA) but not when EDTA is also present nor in neutral conditions with or without EDTA. HEPES and NaH_2PO_4 have little or no effect, but TRIS again inhibits the non-enzymic deiodination.

In summary, glutathione stimulates non-enzymic deiodination in neutral conditions and DTT in acid conditions. Both processes are inhibited by EDTA and TRIS.

2.6.3 The Effect of Light on Non-Enzymic Deiodination

The effect of light on the non-enzymic deiodination of thyroxine was examined in the presence of HEPES buffer with additions of DTT, EDTA and KCl singly and in combination. The methods and results are shown in Table 2.10 and its caption. KCl was chosen as potassium and chloride ions are the predominant intracellular ions and might conceivably have an effect on

TABLE 2.10 Effect of Light on the Non-Enzymic Deiodination of ^{125}I-Thyroxine in HEPES Buffer.

Lighting	TCA present (420mM)	Additions to HEPES (50 mM) buffer pH 7.4				
		None	EDTA (15mM)	KCl (100mM)	DTT (1mM)	EDTA/KCl/DTT (15/100/1mM)
DARK		2.0	0.4	2.1	2.3	2.4
	+	1.8	0.3	2.0	2.7	4.2
LIGHT		1.1	0.0	1.9	2.4	2.4
	+	1.5	-1.0	2.0	2.4	3.0

Each value shown is the mean of duplicate determinations of the percentage $^{125}I^-$ liberated from ^{125}I-T_4 during the incubation of two identical sets of tubes either in the light or in the dark for 2 hours. The methods used were the same as is described in the caption to Table 2.9. The tubes were prepared using HEPES as the buffer with the added components and their final concentrations as shown in the table.

non-enzymic deiodination either by chemical exchange of chloride for iodide or because K^+ is not complexed by EDTA.

It is apparent from Table 2.10 that light does not increase the non-enzymic deiodination in the buffers tested, in particular, there is no additive effect with DTT.

2.6.4 The Deiodination of ^{125}I-Thyroxine by Renal Homogenates

The percentage of iodide released from thyroxine (10^{-7}M) by the kidney homogenates in the different buffers is shown in Table 2.11. The method is described in the caption to Table 2.11.

Consideration of Table 2.11 in comparison with Tables 2.10 and 2.9 shows that in the presence of trichloracetic acid, the denatured homogenate protein has no effect on deiodination which

TABLE 2.11 Deiodination of $^{125}I^-$ Thyroxine by Renal Homogenates.

Lighting	Presence of TCA (420 mM)	Homogenising Buffer			
		HEPES(50mM)	HEPES-EKD	TRIS(50mM)	TRIS-EKD
DARK		19.2	33.1	15.2	27.2
	+	2.0	1.1	1.2	1.3
LIGHT		23.2	31.6	16.5	25.9
	+	1.7	1.6	2.5	1.0

Each value shown is the mean of 5 determinations of $^{125}I^-$ liberated from ^{125}I-T_4 and is expressed as the percentage of the total radioactivity added with correction for $^{125}I^-$ already present. Standard deviations were all <15% of their respective means.

Homogenates were prepared first as a paste by passage of 8 rat kidneys through a Harvard Press. Weighed portions were then mixed with each buffer at 0°C to give a 10% (w/v) homogenate. The buffers were HEPES or TRIS 50mM pH 7.2; EKD is EDTA/KCl/DTT of 15/100/1 mM and pH 7.2.

Incubation was for 2 hours in either the light or in the dark and followed the methods described in Table 2.9.

cannot be ascribed to the non-enzymic deiodination by the buffer components in acid conditions. On the other hand, the homogenates at pH 7.2 show a considerable excess of deiodination over that of their respective TCA-denatured homogenates. The deiodination in homogenates is further increased, by at least half, by the addition of EDTA/KCl/DTT to each buffer.

Light gives a small and statistically non-significant (t-test <80%) increase with either TRIS or HEPES but this difference disappears with the addition of EDTA/KCl/DTT. Overall HEPES is a marginally better buffer than TRIS.

2.7 Conclusion

In conclusion:

(1) Assays for either iodide-125 or ^{125}I- iodothyronine production from ^{125}I- thyroxine have been established.

(2) Buffer compositions have been found which have minimal non-enzymic deiodination rates and which, at the same time, promote enzymic deiodination by employing DTT and EDTA.

(3) The addition of trichloracetic acid to homogenates at zero time provides an adequate control for the measurement of non-enzymic deiodination.

CHAPTER 3

Cell Fractionation and the Intracellular Location of the Deiodinases

3.1 Introduction

In any attempt at purification of an enzyme, both a reasonably concentrated source of enzyme and a reasonably large quantity of starting material is desirable (Dixon and Webb, 1964). In this regard a number of kidneys, from normal Landrace pigs, was made available by Dr K.D. Rainsford of my department so that initially parallel studies on both pig and rat tissues were conducted. As discussed in Chapter 1, kidney tissues were chosen because the kidney has proportionately the highest *in vitro* T_4 deiodinase activity of those tissues tested in the rat (Albright *et. al.*, 1954; Larson *et al.*, 1955) and in man (Albright and Larson, 1959). The high activity in rat kidney was confirmed more recently by Chopra (1977).

Cellular fractionation of kidney and liver homogenates was performed primarily as a step in protein purification and secondly as a procedure to localise the intracellular sites of activity.

As reviewed in Chapter 1, different authors, using RIA to measure the products T_3, rT_3 or 3,3'-T_2, identified different intracellular sites for deiodination. There was general agreement on a microsomal site for both 5- and 5'- deiodinating processes, however there was disagreement between the further possibility of deiodination at either plasma membrane or endoplasmic reticular components of the microsomes. Thus the use of a different and sensitive chromatographic technique to measure deiodination might also provide useful information on the intracellular sites of deiodination. However the prime purpose of fractionation was as a step in the purification of the deiodinases.

3.2 Preparation and Properties of Homogenates

3.2.1 Homogenisation

Before examining the subcellular location of the deiodinases, preliminary studies on homogenates were made. However as the deiodinase activity of homogenates has been extensively investigated by many workers (reviewed in Section 1.2), only that work will be presented which is either relevant to my later studies on purification of the deiodinases or which is necessary to show that my results obtained with the assays in Sections 2.2 and 2.3 are comparable with those obtained by others using RIA.

The available methods of cell breakage prior to cell fractionation have been reviewed by Potter (1955) and more recently by Evans (1978). Initially Teflon in glass Potter-Elvehjem homogenisers having capacities between 25-100 ml were used. The largest had sufficient capacity to hold a whole rat liver or 6 rat kidneys. However when pig kidneys were used, the kidney was cut into segments with a knife and homogenised first in a Waring Blender for 2 min at top speed.

Unless otherwise stated, rat kidney homogenates were prepared from a local strain of male hooded Wistar rats of 180-220 gm. The rats were killed by stunning and cervical dislocation. The kidneys were rapidly removed and chilled in an ice-cold buffer. The kidneys were then homogenised by five passages of the Teflon piston in a Potter-Elvejhem homogeniser.

3.2.2 Homogenising Buffer

The composition of the homogenising medium was examined to see if the deiodinase activity of the homogenate would be improved by using constituents other than those described in section 2.6.4.

As the HEPES or TRIS buffers are not iso-osmotic, the addition of sucrose to maintain cellular organelle integrity was examined for any effect on homogenate deiodinase activity. Experimental details and results are given in Table 3.1 and its caption.

TABLE 3.1 The Effect of Sucrose on Thyroxine 5'-Deiodinase Activity in Rat Kidney Homogenate.

Homogenising buffer[a]	Control	Expt.
Without sucrose	2.3 ± 0.2	12.4 ± 0.7
With sucrose[b]	2.2 ± 0.2	11.7 ± 2.5

[a] HEPES/EDTA 50/15 mM pH 7.4

[b] sucrose 250 mM

Comparable homogenates (10% w/v) were obtained by homogenising one kidney taken from each of two rats in the two buffers. The added substrate gave 122,000 cpm and a final concentration of T_4 of 10^{-7}M. Incubation was for 2 hours at 37°C. Iodide values were measured by the butanol method and are uncorrected for iodide already present in the substrate and are expressed as percent of radioactivity liberated as mean ± S.D. for five determinations.

Table 3.1 shows that the presence of sucrose has no measurable effect on the activity of the renal homogenate. Nevertheless, it was decided to incorporate sucrose into the homogenising buffer or buffers to facilitate the later cell fractionation studies.

Proteins and polymers such as albumin or polyvinyl pyrolidine (PVP) can be added to homogenising buffers to improve yields of enzyme activity in homogenates (reviewed by Crabtree et. al., 1979). To test this possibility, rat kidney homogenates were prepared in

sucrose-HEPES buffer alone or with either 1% w/v of bovine serum albumin (Sigma, St. Louis) or 1% w/v PVP (Sigma, St. Louis) and deiodinase activity measured as before. The effect of both these substances on the non-enzymic deiodination was measured at the same time. Experimental details and results are given in Table 3.2.

TABLE 3.2 The Effect of Albumin or Polyvinyl pyrolidine on the Deiodination of Thyroxine in Buffer or in Kidney Homogenate.

Assay Medium		Addition to Buffer[a]		
		None	Albumin[b]	PVP[b]
Buffer alone[a]	Control	6.3	3.0	3.2
	Expt.	4.9	3.0	3.3
Homogenate	Control	2.9	2.8	3.1
	Expt.	24.6	4.2	16.8

[a] Buffer: Sucrose/HEPES/EDTA/DTT (250/25/3/1 mM)

[b] Final concentration of 1% w/v

The three homogenates were rapidly prepared in the ice-cold buffers from 6 rat kidneys each of which was sliced into three equal portions one each of which was added to each buffer and homogenised as described in section 3.2.1. Incubation conditions and the added concentration of substrate were the same as in Table 3.1. The values for percent iodide released are the means of triplicate determinations.

Table 3.2 shows that both PVP and albumin decreased, rather than increased, the apparent activity of the homogenate. This result was not unexpected as both substances are known to bind thyroid

hormones and thus would reduce the activity in solution of thyroxine relative to its total concentration. Of interest also was the reduced control values in the buffers and in the homogenates. This suggested that there might be a concentration of albumin which inhibited non-enzymic deiodination but not enzymic deiodination. This possibility was examined and the results and methods are given in Figure 3.1.

It is apparent from Figure 3.1 that whilst increasing albumin concentration slowly reduces the non-enzymic deiodination, there is a markedly decreased enzymic activity, probably caused by the binding of the substrate to the albumin. Thus, the use of albumin in homogenates is not warranted.

3.2.3 The Effect of pH on Thyroxine 5'-Deiodinase Activity in Rat Kidney Homogenates

The effect of pH on thyroxine 5'-deiodinating activity in rat kidney was determined and the data and methods are shown and described in Fig. 3.2 There is a broad distribution of enzyme activity which appears to have several maxima at pH 6.0, 7.5 and 9.0.

3.2.4 The Effect of pH on Thyroxine 5'-Deiodinase Activity in Pig Kidney Homogenates

A reasonably large amount of starting material is desirable in any purification attempt. In this regard a number of pig kidneys became available through the courtesy of Dr K.D. Rainsford of this Department.

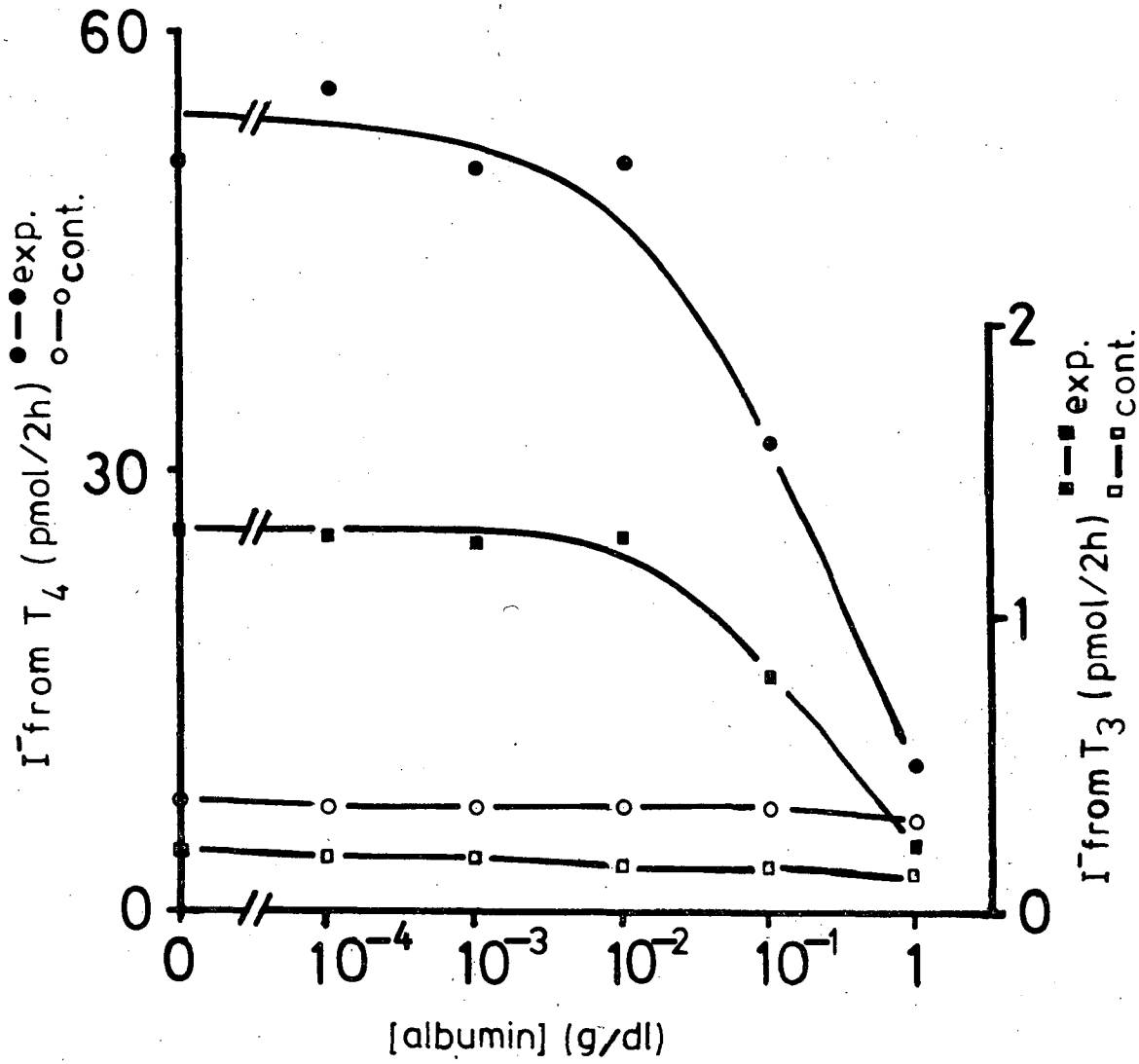


FIGURE 3.1 The Effect of Albumin on 5'-Deiodination of T_4 and T_3 by Rat Kidney Homogenate.

Four rat kidneys were homogenised in buffer consisting of sucrose/HEPES/EDTA/KCl/DTT 250/50/15/100/1 mM pH 7.4 and then aliquots were mixed with an equal volume of one of a range of concentrations of bovine serum albumin (Sigma, St. Louis) in the same buffer to give the final concentrations shown on the abscissa. The mixtures were allowed to stand at 0°C for 30 min before incubation.

Released iodide after 2 hours at 37°C was measured by butanol extraction in duplicate control and experimental samples after addition of substrate which contained either T_4 (144,000 cpm and final concentration 10^{-7}M) or T_3 (292,000 cpm and final concentration 10^{-8}M). Values were converted to p mol iodide released but were not corrected for iodide already present in either substrate.

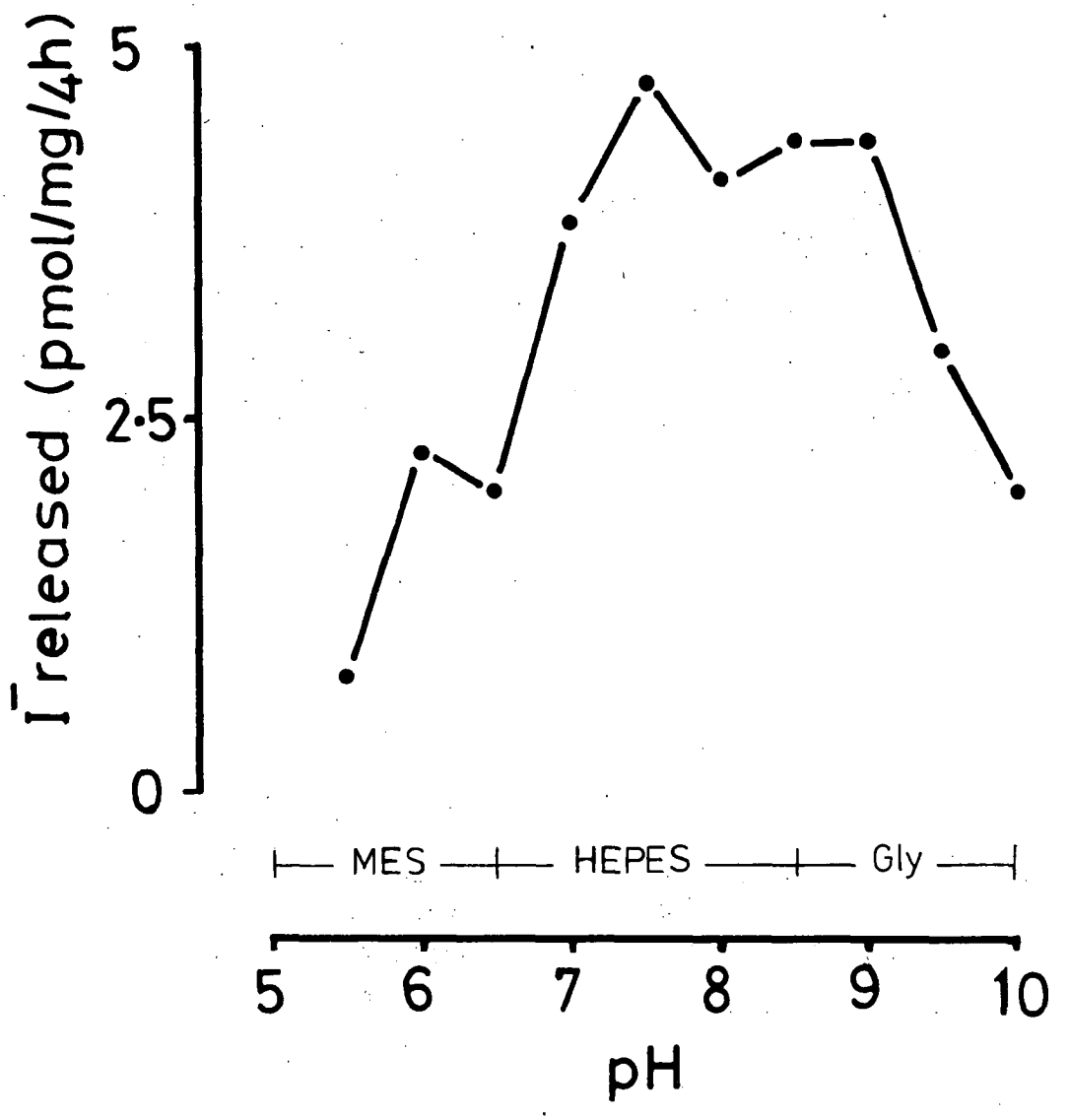


FIGURE 3.2 The Effect of pH on Rat Kidney Homogenate Thyroxine 5'-Deiodinase Activity.

Rat kidneys were homogenised in ice-cold sucrose/EDTA 250/5 mM buffer pH 7.4. Portions of homogenate were then mixed with an equal volume of the same buffer to which had been added one of the following additional buffer ions: Morpholine-ethanesulphonic acid (MES), pKa6.0; HEPES, pKa 7.55; and Glycine, pKa_2 9,6; to give final concentrations of 50 mM. The pH of these latter buffers was adjusted with either HCl or NaOH before mixing to give the pH range shown on the abscissa.

The values shown for iodide production were measured in duplicate by the ion exchange method after incubation for 2 hours at 37°C. The substrate contained 156,000 cpm and gave a final concentration of 10^{-7}M T_4. Control values have been subtracted which provides correction for both iodide already present in substrate but also for any non-enzymic iodide production. Protein was measured using biuret reagent with bovine albumin as standard.

The pigs were killed by the use of a captive bolt pistol, followed by exanguination. The kidneys were rapidly removed, dissected free of connective tissue and sliced once longitudinally and cortex and medulla dissected free and immersed separately in ice-cold buffer. Less than 30 minutes later the kidneys were transferred to fresh cold buffer and homogenised in a Waring Blender to give an approximate concentration of 16 g wet weight/dl. The effect of varied pH on 5'-deiodinase activity was then measured in both cortical and medullary homogenates. The results are shown in Figure 3.3. The cortical homogenate shows a similar plot to that obtained with rat kidney homogenate with a maxima of pH 8, but the presence of other maxima is less obvious. The medullary homogenate however shows a much flatter curve of less specific activity than that of the cortical homogenate. However the amount of medulla present in the kidney when compared to the cortex is very much smaller ($\leq$ 5% wet weight), so that in any homogenate of the whole gland, the contribution of the medulla would be small. At pH 9 the activity would almost be exclusively due to the cortical deiodinase.

3.3 Cell Fractionation of Kidney

Fractionation of either rat or pig kidney was performed as shown in Figure 3.4. Arising out of the data shown in Figures 3.2 and 3.3, the fractions were assayed for T_4 5'-deiodinase activity at pH 7.4 using the same incubation conditions as described in those figures. Results for rat and pig kidney are shown in Tables 3.3 and 3.4 respectively.

The 5'-deiodination of the triiodothyronines by rat kidney cell fractions was also assessed using T_3 and rT_3 and compared to that of

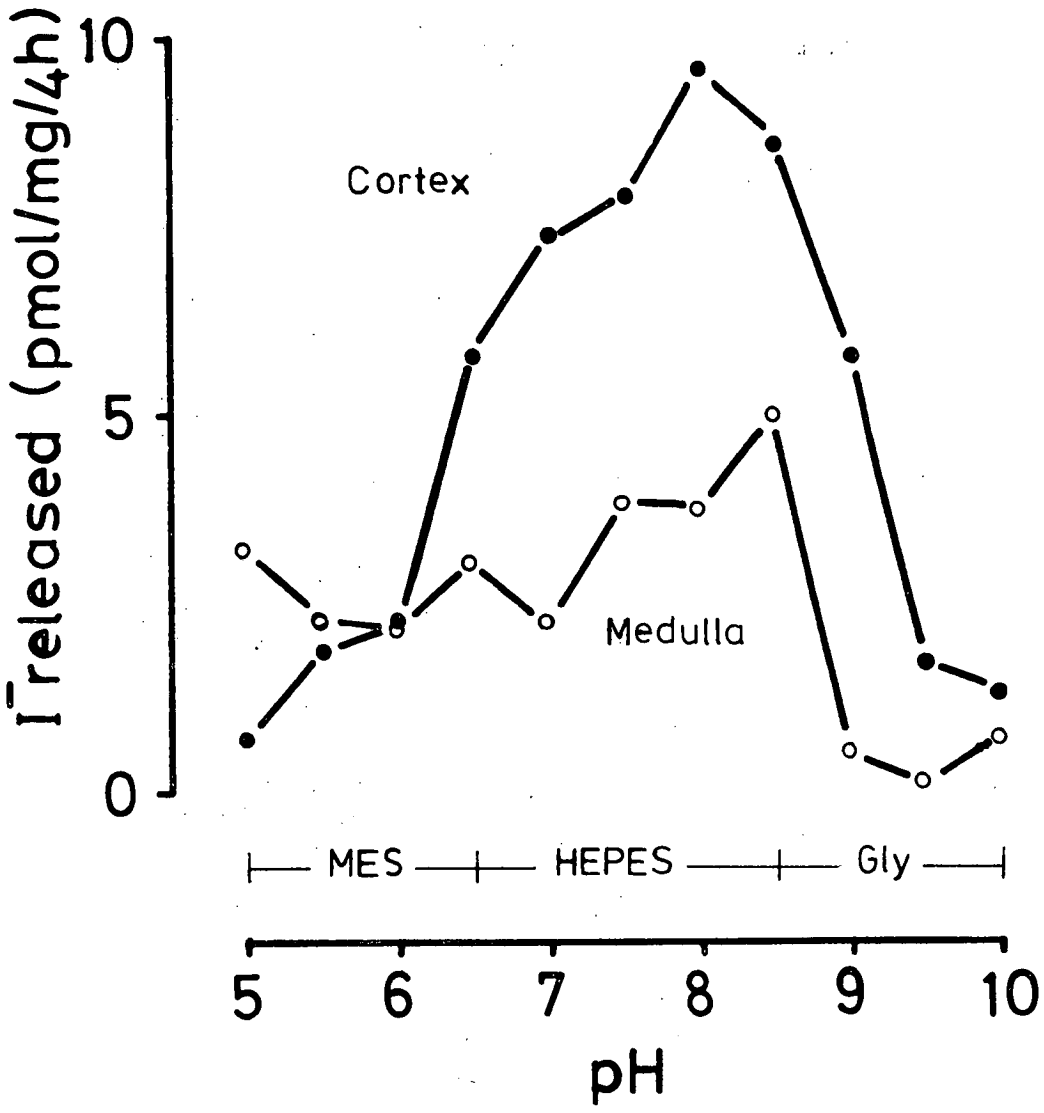


FIGURE 3.3 The Effect of pH on Pig Kidney Homogenate Thyroxine 5'-Deiodinase Activity.

The values shown are the means of duplicate determinations of iodide released over the four hour incubation as measured by the ion-exchange method. Other procedures were the same as described in Figure 3.2.

FIGURE 3.4 Flow Chart of Cell Fractionation by Centrifugation

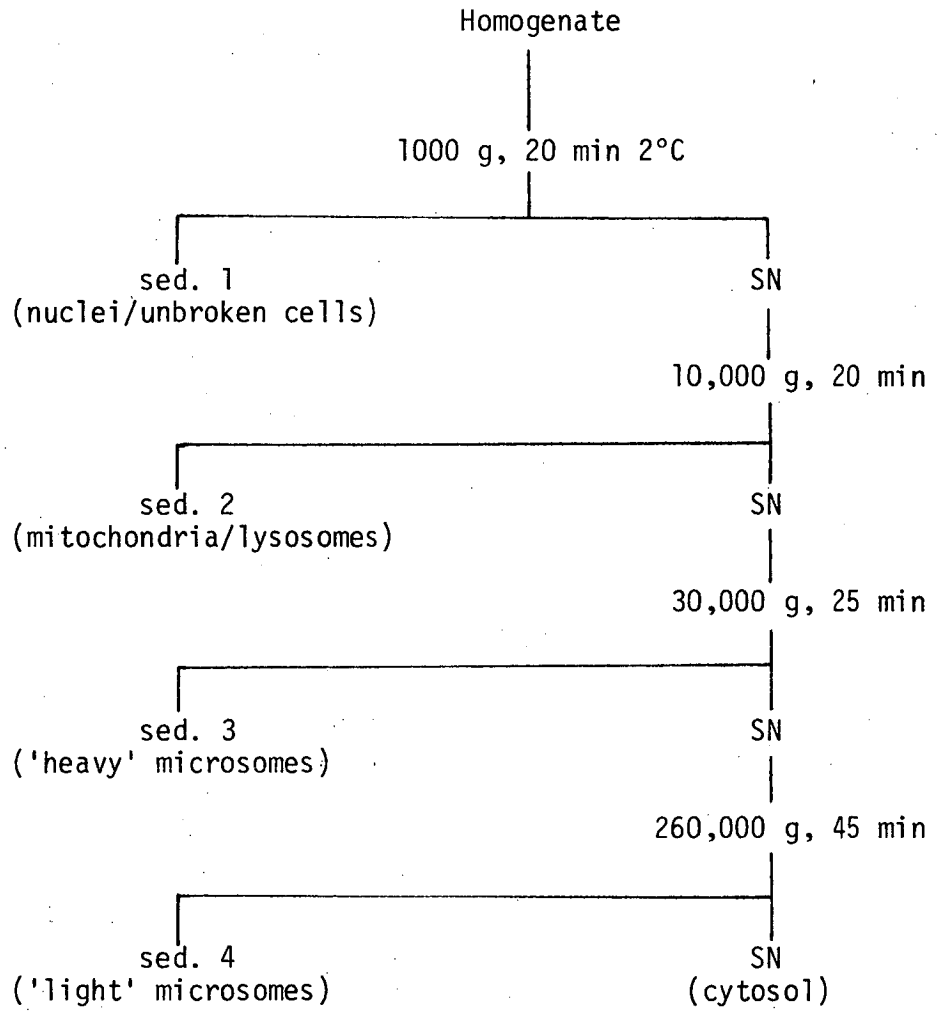


TABLE 3.3 Thyroxine 5'-Deiodinase Activity in Cell Fractions of Rat Kidney

Cell Fraction[a]	Volume (ml)	Total Protein (g)	Protein Conc. (g/l)	5'-Deiodinase Activity	
				Specific Activity (pmol/ 2hr/mg)	Total Activity (nmol/ 2hr)
Homogenate	185	0.80	4.3	6.3	5.0
10,000 g sed. (mitochondria/ lysosomes)	165	0.71	4.3	6.3	4.5
260,000 g sed. (microsomes)	18.5	0.06	3.4	13.3	0.8
260,000 g sn (cytosol)	162	0.62	3.8	0.9	0.6

[a] Preparation of a 30,000 g sediment was omitted in this preparation.

TABLE 3.4 Thyroxine 5'-Deiodinase Activity in Cell Fractions of Pig Kidney Cortex

Cell Fraction	Volume (ml)	Total Protein (g)	Protein Conc. (g/l)	5'-Deiodinase Activity	
				Specific Activity (pmol/ 2hr/mg)	Total Activity (nmol/ 2hr)
Homogenate	333	11.36	34.1	2.3 ± 0.3	26.2 ± 2.3
10,000 g sed. mitochondria/ lysosomes)	233	5.55	23.8	4.1 ± 0.7	22.1 ± 2.1
30,000 g sed. (heavy microsomes)	50	0.86	17.2	6.0 ± 1.4	4.9 ± 1.0
260,000 g sed. (light microsomes)	21	0.41	19.7	7.3 ± 1.0	3.0 ± 0.2
260,000 g sn (cytosol)	184	3.61	19.6	3.8 ± 0.7	13.0 ± 2.2

thyroxine. The method and results are shown in Table 3.5.

TABLE 3.5 Iodothyronine 5'-Deiodinase Activity of Cell fractions

Cell Fraction	T_4		rT_3		T_3	
	Sp.Act.[a]	Tot.Act.[b]	Sp.Act.	Tot.Act.	Sp.Act.	Tot.Act.
Homogenate	61	6.9	15	1.7	3.1	0.4
1,000 g sed	40	1.4	15	0.5	1.1	0.04
10,000 g sed	55	1.5	15	0.4	1.0	0.03
30,000 g sed	37	1.9	17	0.9	1.0	0.05
260,000 g sed	56	2.3	17	0.7	1.9	0.08
260,000 g sn	1.4	0.2	3.2	0.4	0.2	0.03

[a] Specific Activity pmol/2h/mg

[b] Total Activity in mol/2h

Cell fractions were prepared as described in the caption to Figure 3.4 from 4 male rats (180 g) and incubated with T_4 (10^{-7} M), rT_3, T_3 (10^{-8} M) for four hours. The values shown are the means of duplicates.

The concentrations of 10^{-7} M (T_4) and 10^{-8} M (rT_3 and T_3) and the 2 hour incubation period were chosen to indicate physiologically relevant 5'-deiodinase activity and at the same time to obtain sufficient iodide release at this concentration for easy measurement of the reaction.

However the use of these two conditions which provide neither maximal nor initial velocities, as defined for enzyme kinetics, means that maximal enzyme activities are not necessarily obtained and thus the recovery of activity, when all fractions are summed, may exceed that of the homogenate. As well, when the protein concentration is less in a particular fraction, there will be less non-specific binding of the substrate present which then leads to a proportionately higher enzyme activity.

3.4 Discussion

The data obtained for the pH optima of T_4 5'-deiodinase in rat kidney homogenates is consistent with the results obtained by Höffken et al. (1977) who found a maximum conversion of T_4 to T_3 at pH 6.5 and of T_4 to rT_3 at pH 9.5 in rat liver homogenate. A maximum at a higher pH of 7.0 to 7.5 was found by Chopra (1977) and by Cavalieri et al. (1977) for T_4 to T_3 conversion by rat liver homogenates. Hüffner and Grussendorf (1978) found in the same preparation that T_4 to T_3 conversion was maximal at pH 6.5 and that any rT_3 formed from T_4 by homogenates was rapidly further deiodinated to 3,3'-T_2. Both these sequential reactions were found to be maximal at about pH 8.0-8.5. The latter deiodination (rT_3 to 3,3'-T_2) would explain how T_4 labelled only in the outer ring might also indicate inner ring deiodination to form rT_3 from T_4 because the rT_3 formed is then subsequently rapidly deiodinated in the outer ring to form 3,3'-T_2 with liberation of $^{125}I^-$ and thus the activity is detectable in the butanol or ion-exchange assay which depends only on the measurement of $^{125}I^-$ release. Thus in a sense, reverse T_3 synthesis and degradation provide a coupled enzyme assay.

Consideration of the cell fractionation data for both pig and rat kidney suggests that the distribution of the T_4 5'-deiodinase activities over the various fractions is different in each species. For pig, the microsomal fractions have the highest specific activity but the order for total activity is: crude mitochondria > cytosol > microsomes. For rat, the combined microsomal fraction has the highest activity but the order for total activity is: crude mitochondria > microsomes > cytosol.

Confirmation of the rapid deiodination of rT_3 at pH 7.4 in homogenates is given in Table 3.5. This is especially so as the concentration of rT_3 is one tenth that of T_4. The table also shows that rT_3 is deiodinated by all those cell fractions which deiodinate T_4. T_3 is only slowly deiodinated by homogenates and by all cell fractions.

The widespread T_4 5'-deiodinase activity found in the kidney cell differed from the results of others who have found a predominant microsomal localisation of T_4 5'-deiodination (reviewed in Chapter 1) and this made the decision difficult as to which of the cell fractions to further purify for T_4 5'-deiodinase activity. Consequently, cytosol, microsomes and the crude mitochondrial fractions were all chosen for further study.

The cytosol fraction was chosen because the activity was already in a soluble form and the cytosol had a significant total deiodinase activity particularly in the pig kidney. The microsomal fraction was chosen because of its large specific activity, its significant total activity and the work of others (section 3.1) which had localised T_3 production to this fraction. The crude mitochondrial fraction was chosen because of its large total activity and because during the study of the cytosol, a possible link between the activity in the cytosol and lysosomes became apparent.

CHAPTER 4

Purification and Properties of Cytosol Thyroxine 5'-Deiodinase

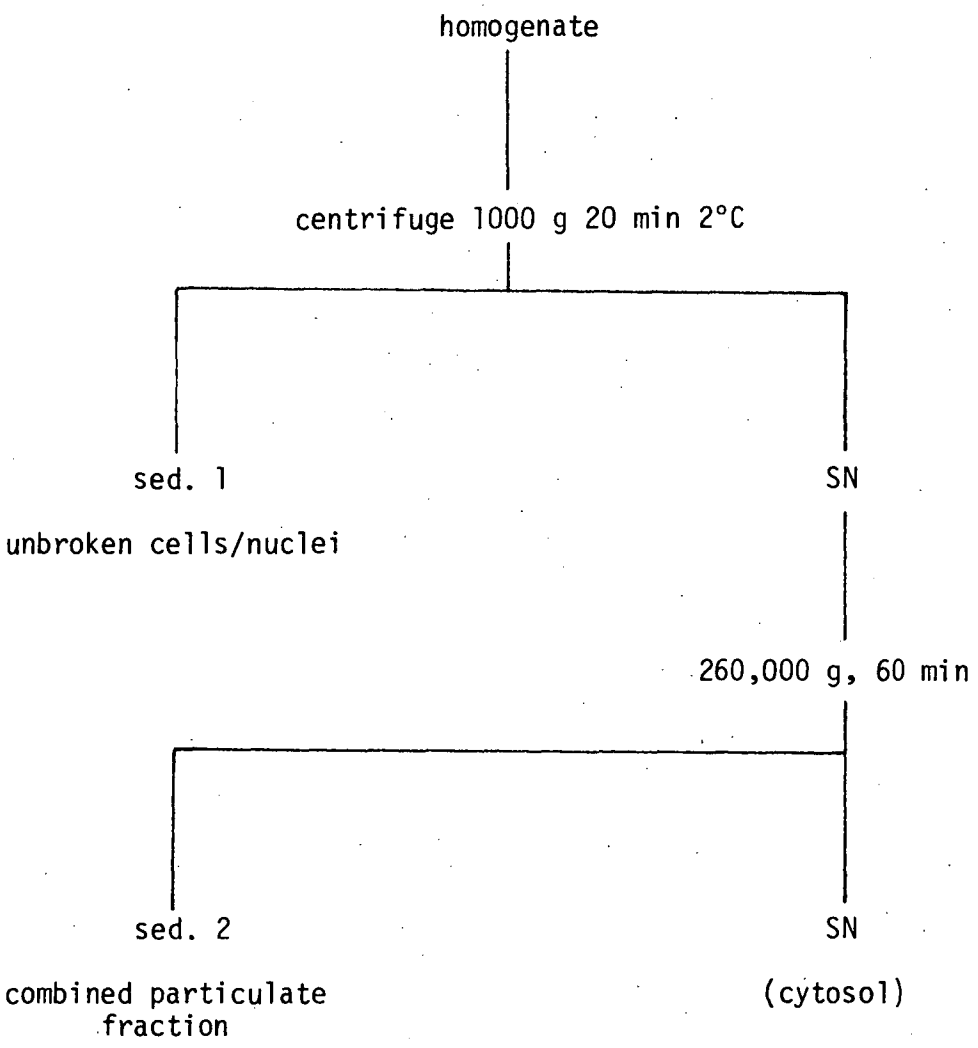
4.1 Preparation and Properties of Thyroxine 5'-Deiodinase from Cytosol

Repeated preparations of cytosol by the method shown in Figure 3.4 were often found to have much lower 5'-deiodinase activity than shown in Table 3.5. Even lower or absent activity was found when the method for preparation of cytosol was abbreviated to that shown in Figure 4.1. This protocol was tried on the rationale that if the 5'-deiodinase was present and in solution in the cytosol, then a single high speed centrifugation step should suffice to remove all the organelles and membranes of the cell and thus prepare the cytosol in a much shorter time.

In view of the reduced activity with the abbreviated preparation, it seemed possible that the 'cytosol' enzyme could be normally associated with either another compartment or membrane of the cell and was thus only found in the cytosol after the cell breakage and the centrifugations involved in the method shown in Figure 3.4.

To test the possibility that the cytosolic 5'-deiodinase activity in the cell was being released by leakage from another cell compartment, cytosol was prepared from rat kidneys which had been homogenised in HEPES-TRIS/EDTA 25/10 mM pH 7.4 with and without 250 mM sucrose in the buffer. The data for the homogenate 5'-deiodinase activity is shown in Table 3.1. Cytosol was prepared in each buffer as shown by the method in Figure 4.1. On assay, both preparations had no detectable T_4-5'-deiodinase activity.

FIGURE 4.1 The Abbreviated Method for Preparing Kidney Cytosol.



Another possibility was that during the manipulations of the multistage centrifugation procedure, the material was not always kept at the same temperature as the ice-bath. Thus elevated temperature might in some fashion increase the enzyme yield in cytosol, for example, by the increased solubilisation of a lipoprotein membrane at the higher temperature or by a cathespin proteolysis liberating a more water soluble protein. Preparation at 4°, and at 20°C, with and without KCl in the homogenising buffer was examined and the method and the data obtained are shown in Table 4.1 and its caption.

Table 4.1 shows that rat cytosol activity is much lower than in other cell fractions, but is slightly greater when prepared at 20°C than at 4°C but that in the presence of 100mM KCl the value at 4°C is greater than at 20°C. Both warmer ambient temperature and KCl caused a decrease in homogenate and microsomal T_4-5'-deiodinase activity.

The possibility that proteolysis was taking place was examined by preparing homogenates at various temperatures and allowing them to stand for an hour before commencing fractionation. The method and data are shown in Table 4.2 and its caption. The table shows that for cytosol there is again low 5'-deiodinase activity which is probably at its highest when prepared at 20°C. For the other fractions, there is little difference between 20° and 4°C, but there is a definite decrease at 37°C. This suggests that autolysis of membrane activity does occur, but there is no subsequent increase in the cytosol activity suggesting that the membrane activity is being destroyed by tissue proteinases rather than being liberated into the cytosol.

TABLE 4.1 The Effect of Temperature and KCl on the Preparation of 5'-Deiodinase Activity from Rat Kidney Cell Fractionations.

Fraction	KCl(100mM) in buffer	Ambient Temperature during Preparation			
		4°C		20°C	
		Spec. Act.[a]	Tot. Act.[b]	Spec. Act.	Tot. Act.
Homogenate		62.6	1253	39.5	790
	+	31.8	635	23.5	469
Microsomes		48.7	584	30.3	364
	+	27.7	332	21.6	259
Cytosol		2.0	39	2.4	45
	+	4.1	78	2.6	49

[a]specific activity of 5'-deiodinase (pmol/mg/4h) as mean of duplicates.

[b]total activity of 5'-deiodinase (pmol/4h) as mean of duplicates.

Rat kidney cytosol was prepared by taking one half of each kidney taken from two male rats of equal weight (200 gm) and homogenising the four groups containing the four quarter kidneys in Sucrose/HEPES-TRIS/EDTA/DTT 205/25/15/1 pH 7.4 with and without KCl 100mM and at either 4°C or 20°C. The four homogenates were fractionated by the method shown in Fig. 3.3 and the 5'-deiodinase activity determined in each fraction using 10^{-7}M T_4 and butanol extraction. The values shown are the means of triplicates.

TABLE 4.2 The Effect of Preincubation at Different Temperatures on the Yield of Thyroxine 5'-Deiodinase Activity from Rat Kidney Cell Fractions.

Cell Fraction	4°C		20°C		37°C	
	Sp. Act.[a]	Tot.Act.[b]	Sp. Act.	Tot.Act.	Sp.Act.	Tot.Act.
Homogenate	9.9	823	8.6	716	7.7	643
Mitochondria (10,000 g sed)	42.8	304	43.1	288	32.8	219
Microsomes (260,000g sed)	43.1	326	41.5	323	30.1	154
Cytosol (260,000 SN)	0.8	31	3.1	87	1.8	61

[a]specific activity of 5'-deiodinase (pmol/mg/2h) as mean of duplicates.

[b]total activity of 5'-deiodinase (pmol/2h) as mean of duplicates.

An homogenate (6 kidneys/30ml) was prepared at 4°C in sucrose/HEPES-TRIS/DTT 250/25/1 mM pH 7.4. It was divided into three aliquots each of which was then allowed to stand at 4°, 20° or 37°C for 1 hour. Each aliquot was then combined with an equal volume of sucrose/HEPES-TRIS/KCl/EDTA/DTT 250/50/100/15/1 mM pH 7.4 and each homogenate was then fractionated by the procedure as shown in Figure 3.3 at 20°C. Before assay, a 2.5 ml sample was taken from each homogenate and diluted to 5 ml, the microsomal and mitochondrial fractions were resuspended in 20 ml and the volume of cytosol was adjusted to 22 ml with the buffer containing DTT.

Another possible reason for the variability was that an essential ion was not present in the preparation, so that various cations and other substances were examined for any effect on either the activity or stability of cytosol thyroxine 5'-deiodinase.

In several experiments it appeared that K^+ ions in high concentration were partially effective in preventing the loss of activity by rat kidney cytosol once prepared (see Figure 4.2), however the effect of KCl as shown in the figure was not always reproducible. Sometimes the 5'-deiodinase was only slightly increased or not increased at all.

The other salts and substances tested for a stabilizing effect were shown to neither stabilise nor inhibit the cytosol 5'-deiodinase in the concentration ranges listed in Table 4.3. The fact that it was KCl and not NaCl which was sometimes effective, suggests that it is the K^+ ion which is the active moiety.

In the light of the evidence that somewhat increased activity of 5'-deiodinase in cytosol could be obtained by using 100 mM KCl and was partially stabilised by 1000 mM KCl and did not inhibit the 5'-deiodinase activity, I was led to use KCl in the homogenising buffer when preparing rat kidney cytosol. I also used 1000 mM KCl when the deiodinase activity could not be assayed immediately, for example after the running of a chromotographic column.

In view of the *in vivo* studies of Oppenheimer *et al.* (1972) and Saberi *et al.* (1975) and the *in vitro* studies of Chopra *et al.* (1978) with rat liver homogenates which have shown that propylthiouracil (PTU), but not methimazole, inhibits the conversion of thyroxine to triiodothyroxine, these substances were tested on cytosol deiodinase activity

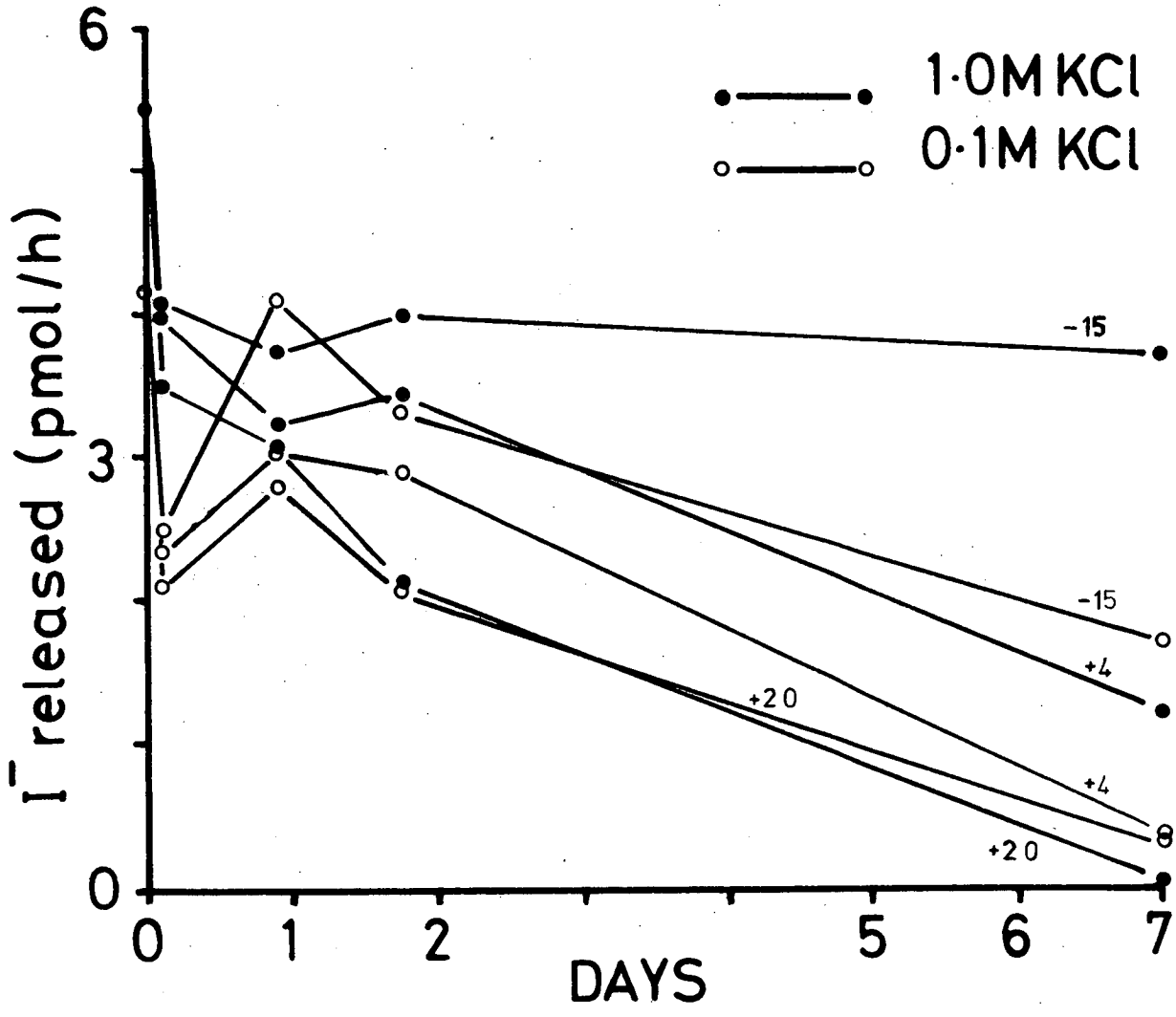


FIGURE 4.2 The Effect of KCl on the Stability of Cytosol Thyroxine 5'-Deiodinase at Different Temperatures.

Cytosol was prepared at 4°C by the method shown in Figures 3.3 using Sucrose/HEPES-TRIS/KCl/EDTA/DTT 250/25/100/15/1 mM pH 7.4. It was divided in two. One half was made to 1000 mM by addition of solid KCl. Aliquots of the two samples were assayed immediately at 37°C and the remainder then divided into 1 ml aliquots which were stood at one of the following temperatures -15°, +4° or +20°C for the times indicated in the figure when they were assayed at 37°C after thawing if necessary.

TABLE 4.3 Compounds Tested for Effect on Rat Cytosol Thyroxine 5'-Deiodinase Activity.

Compound	Concentration Range	Compound	Concentration Range
NaCl	0-1000 mM	Glycerol	0-30%
NH_4Cl	0-1000 mM	Albumin[a]	0-0.01%
$(NH_4)_2SO_4$	0-2,500 mM	Polyvinyl-pyrolidine[a]	0-0.01%
$MgCl_2$	0-5 mM	Heparin	0-1%
$CaCl_2$	0-5 mM	Protamine-sulphate	0-1%

[a]Both albumin and polyvinylpyrolidine will inhibit in higher concentration because of substrate binding.

Compounds were tested as in Figure 4.2 except that EDTA was omitted for cytosol containing $MgCl_2$ and $CaCl_2$ during storage but added for assay.

as shown in Figure 4.3. The figure shows that the data for 5'-deiodinase in cytosol, is consistent with the reported finding that PTU inhibits the deiodinase whilst methimazole has a slight stimulatory or no effect at low concentrations but inhibits at high concentrations.

In order that the optimal pH could be chosen to monitor 5'-deiodinase activity during purification, the effect of pH on the cytosolic thyroxine 5'-deiodinase from pig kidney was determined. The method and data are shown in Figure 4.4 and its caption.

The plot of 5'-deiodinase activity for pig cytosol shows a similar but sharper curve to that for the homogenate in Figure 3.2 and indicates that pH 7.4 is appropriate for monitoring deiodinase activity during purification. There is however significant activity at low pH.

4.2 Salt Fractionation of Cytosol Thyroxine 5'-Deiodinase Activity

Table 4.4 shows the effect of $(NH_4)_2SO_4$ on the precipitation of thyroxine 5'-deiodinase activity from pig cytosol. The method is described in the caption. It is obvious that almost all activity is precipitated in the broad range between 20%-40%. The apparent loss of activity is probably due to losses during dialysis. Visser et al. (1976) found total loss of activity on dialysis of microsomal enzyme but most activity is retained if DTT is added to the dialysis medium. Comparison between the fractions must also take into account that the activity was measured using 10^{-7}M T_4 which is not a saturating substrate concentration.

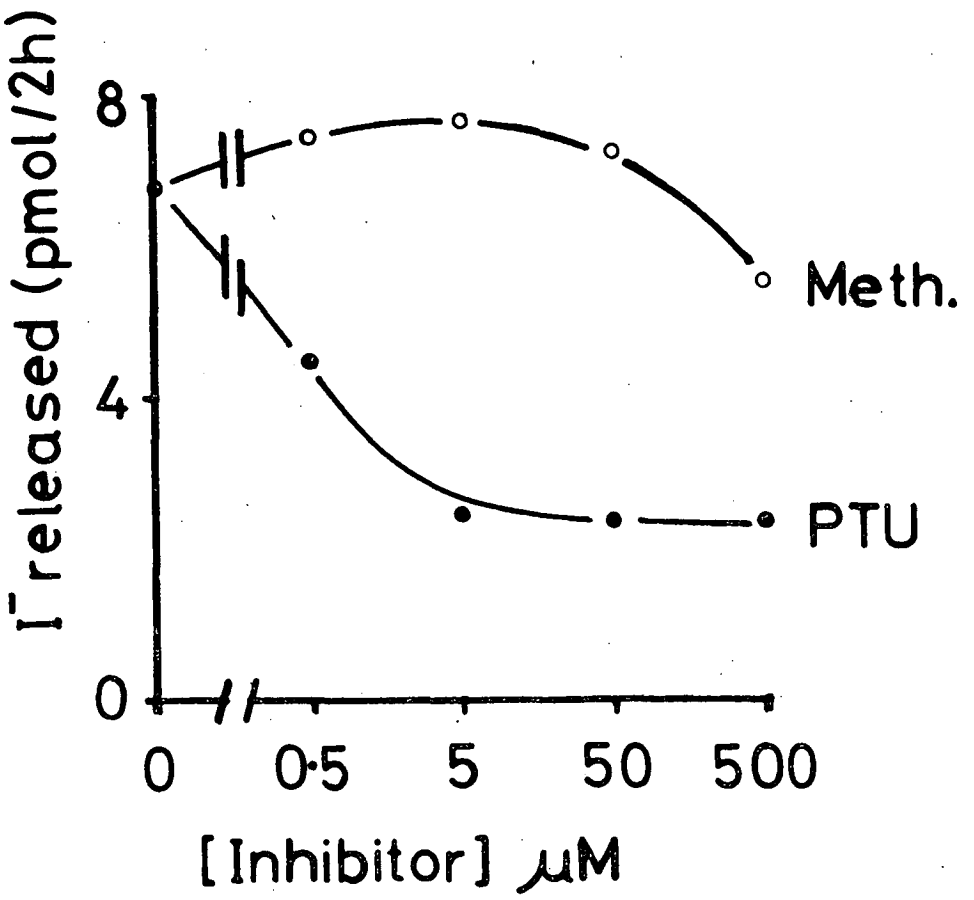


FIGURE 4.3 Effect of Anti-Thyroid Drugs on Rat Kidney Cytosol Thyroxine 5'-Deiodinase.

Cytosol was prepared as shown in Figure 3.4. Each inhibitor was added in a volume of 25 μl, to give final concentrations shown in the figure, 30 minutes before the assay was commenced by the addition of 25 μl of ^{125}I-T_4 to give a final concentration of 10^{-7}M.

Each value was determined in duplicate by butanol extraction after incubation at 37°C for 2 hours using a TCA control.

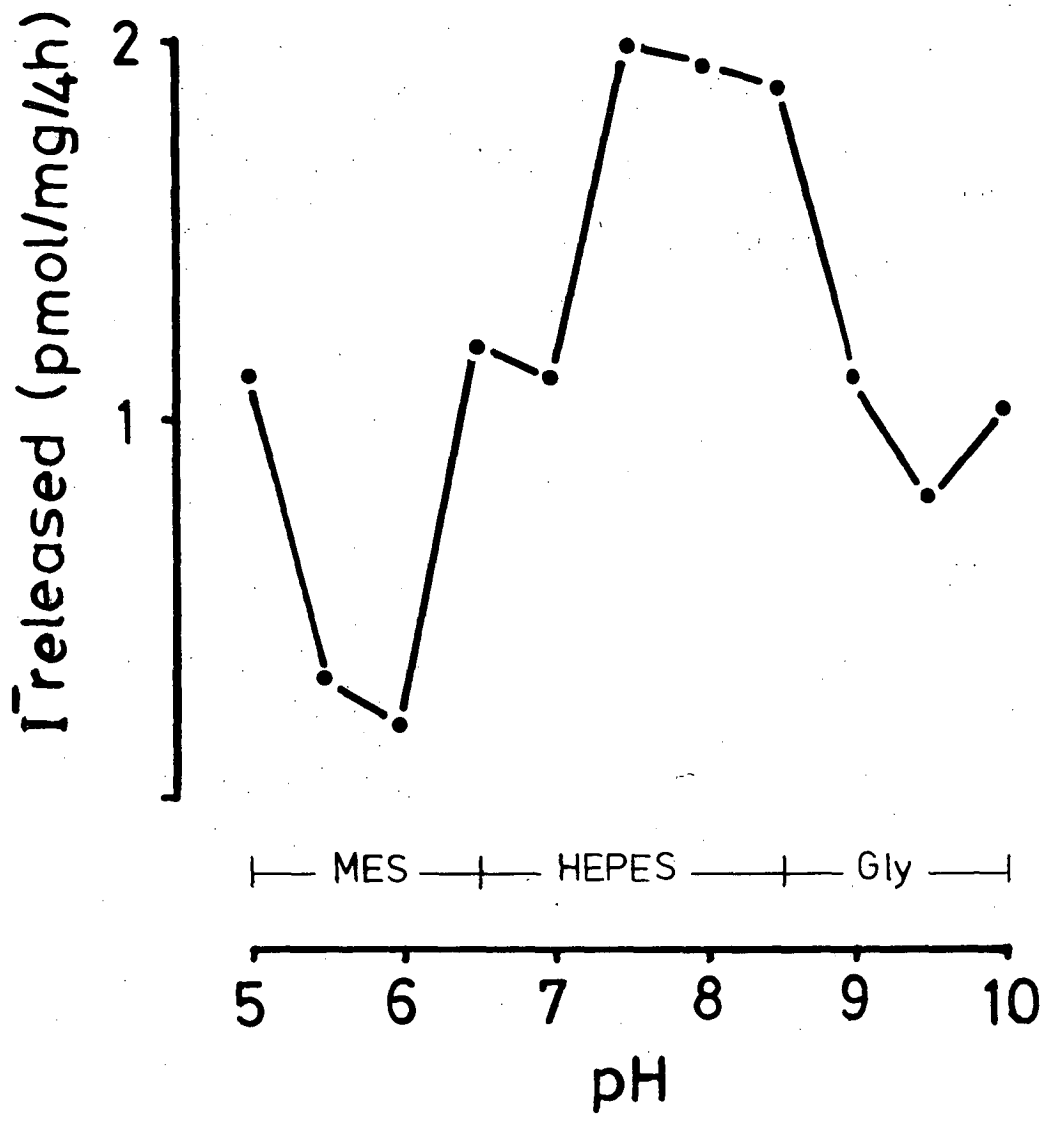


FIGURE 4.4 The Effect of pH on Pig Kidney Cytosol Thyroxine 5'-Deiodinase.

Pig kidney cytosol was prepared as in Section 3.3 using Sucrose/EDTA/DTT 250/15/1 mM pH 7.4 as the homogenising buffer. Aliquots of the cytosol were then mixed with equal volumes of MES or HEPES or Glycine buffers of various pH values and incubated as described in Figure 3.2. The pH was checked after mixing.

TABLE 4.4 Ammonium Sulphate Precipitation of Thyroxine 5'-Deiodinase from Pig Kidney Cytosol.

Fraction	Volume (ml)	Protein Concentration (mg/ml)	Thyroxine 5'-Deiodinase	
			Spec.Act.[a]	Tot.Act.[b]
Cytosol	100	7	1.31	1178
20%[c] sed	7.2	1.1	1.36	11
30% sed	10.8	2.5	4.56	122
40% sed	10.6	5.9	2.42	150
60% sed	11.3	14.9	0.82	92
60% S.N.	23.7	1.35	0.00	0

[a]Specific activity (pmol/mg/4h) as means of duplicates.

[b]Total activity (pmol/4h) as means of duplicates.

[c]Per cent saturation of ammonium sulphate at 0°C.

Pig kidney cytosol (100 ml) was prepared as shown in Section 3.3. Saturated ammonium sulphate, dissolved in 15 mM citrate, 5 mM EDTA pH 6.5, was added at 0°C to make the cytosol 20% saturated with salt. The solution was allowed to mix for 20 min and was then centrifuged at 30,000 g for 20 min and the supernatant decanted and solid salt added to make to 30%, 40% and 60% saturation successively, with removal by centrifugation of sediments at each stage. The sediments and final supernatant were dialysed overnight against 1 mM DTT, 25 mM citric acid adjusted to pH 7.4 with Na_2HPO_4. After dialysis the volume, protein concentration (Pelley *et al.*, 1978) and T_4 5'-deiodinase (ion-exchange method) were determined on each fraction.

A similar pattern of precipitation occurred with rat kidney cytosol as shown in Table 4.5, but in general, a higher concentration of ammonium sulphate was required to precipitate activity even though the cytosol had been made to 1000 mM with KCl to help stabilise activity.

4.3 Affinity Chromatography of Thyroxine 5'-Deiodinase Activity from Kidney Cytosol

4.3.1 Introduction

Affinity chromatography has been used by many workers in recent years as a powerful technique for the purification of proteins. It has often given a major increase in the purity of the protein of interest. Although the idea of affinity chromatography, in which a ligand, fixed to a solid support matrix, binds specifically to the protein of interest has been known for many years, it was not until the use of agarose gel was developed as a support medium, together with methods of attaching the ligands to the agarose that affinity chromatography methods have become widespread (reviewed by Turková, 1978).

Pensky and Marshall (1974) and Kagedal and Kallberg (1977) found that thyroid binding globulin (TBG) from plasma could be purified by attachment to a thyroxine-agarose affinity gel. This work suggested that thyroxine deiodinases might also be purified in a similar manner as they must bind to thyroxine at their active centres during the course of the catalytic deiodination of thyroxine. Therefore, a priori, a thyroxine affinity gel was a potential ligand for these deiodinase enzyme(s)

TABLE 4.5 Salt Precipitation of Thyroxine 5'-Deiodinase from Rat Kidney Cytosol.

Fraction	Volume (ml)	Protein Concentration (mg/ml)	Thyroxine 5'-Deiodinase	
			Spec. Act.[a]	Tot. Act.[b]
Cytosol	37	65	0.19	465
20%[c] sed	20	2.3	2.87	132
30% sed	20	8.1	1.67	270
40% sed	20	18.2	1.38	504
50% sed	20	54.8	0.09	99
60% sed	20	10.5	0.00	1
60% S.N.	33	19.1	0.01	8

[a]Specific activity (pmol/mg/2h) as means of duplicates.

[b]Total activity (pmol/2h) as means of duplicates.

[c]Percent saturation of ammonium sulphate at 0°C.

Rat kidney cytosol was prepared as in Section 3.3 from 8 kidneys and then made to 1000 mM with KCl. Ammonium sulphate was added as in Table 4.4. Deiodinase activity was determined by butanol extraction method without prior dialysis nor removal of the salt.

and hence a means of purification by affinity chromatography. However the binding of thyroxine to the active site raised the possibility that it might also deiodinate the bound thyroxine.

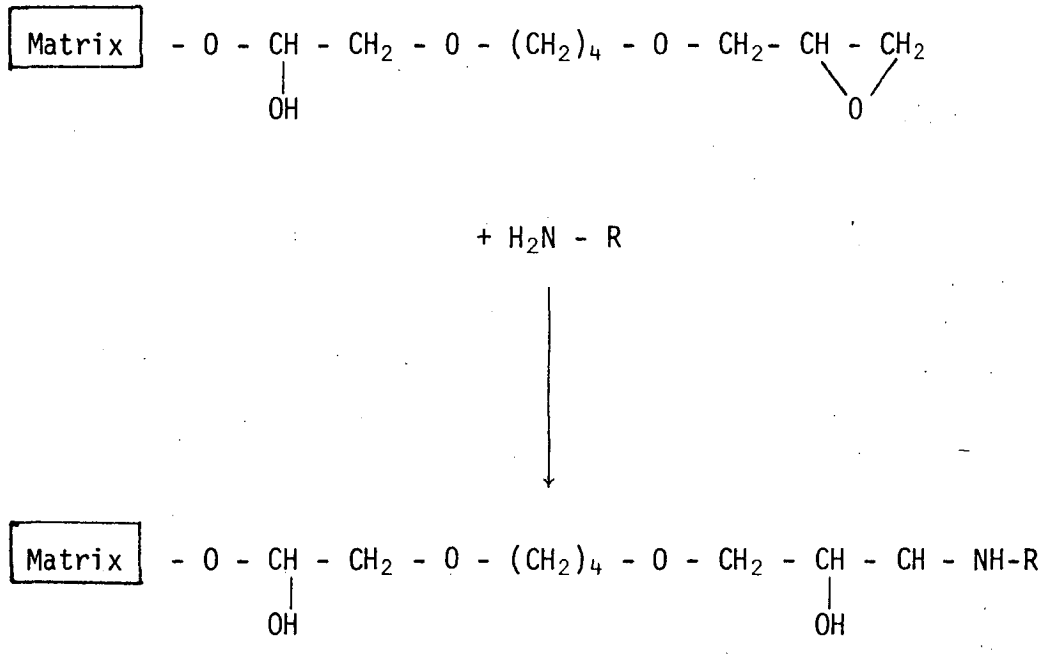
Kagedal and Kallberg (1977) improved the yield of TBG by using as a matrix Epoxy Activated Sepharose (Pharmacia) to covalently bind thyroxine through a moderately hydrophilic spacer arm 13 atoms long (Figure 4.5). They found that it gave a much better purification of TBG than the matrix used by Pensky and Marshall (1974) who employed cyanogen bromide-activation of agarose to couple it to thyroxine. The improved separation with a long hydrophilic spacer arm (as in this Pharmacia product) is thought to allow binding without steric hindrance from the agarose matrix (reviewed by Turková, 1977 and by Lowe, 1979).

4.3.2 Preparation of Thyroxine-Agarose Affinity Gel

Coupling of thyroxine to epoxy activated Sepharose 6B (Pharmacia) was performed essentially as described by Kagedal and Kallberg (1977). In brief: the freeze dried gel was reswollen and washed repeatedly by the addition of distilled water (100 ml/gm gel) to the gel in 100 ml plastic centrifuge tubes. Decanting was performed immediately after centrifugation at 1000 g. The use of plastic implements prevents the gel sticking (as it will to glass). One gm of dry gel gives about 2.5 - 3.0 mls of swollen gel.

Coupling of the gel was performed in the same centrifuge tubes in the dark at 25°C for 24-26 h by the gentle shaking of

FIGURE 4.5 Reactions of Epoxy Activated Agarose with Thyroxine (modified from Porath, 1974).



2 parts of a solution containing 3.6 mg sodium thyroxine (Calbiochem) to which a tracer amount I^{125}-thyroxine had also been added, 15 ml carbonate-bicarbonate buffer (50 mM, pH 9.5) and 5 ml dimethylformamide (Koch-Light) with one part of the swollen gel. The pH of the coupling solution is checked by pH paper to avoid damage to a glass pH electrode.

After coupling, the gel was centrifuged, decanted and then washed 2-3 times (depending on the amount of radioactivity in the washings) with the bicarbonate dimethylformamide solution without thyroxine and the decantings kept. Residual oxirane groups were then blocked by further incubation in the dark for 24 hours at 25°C with 1 M ethanolamine (pH 10). The coupled gel was again decanted and then washed and stored in unbuffered 10 mM Na_2 EDTA at 4°C. An approximate estimate of coupling is determined by measuring the radioactivity in the various solutions. A final and better estimate was made by counting an aliquot of the extensively washed coupled gel. A typical value is 0.35 µmol thyroxine/ml of wet gel.

4.3.3 The Elution of 5'-Deiodinase Activity from the Thyroxine Affinity Gel

Pensky and Marshall (1974) successfully eluted thyroid binding globulin (TBG) from their T_4-gel by using the binding-inhibitor 8-anilino-1-napthalene - sulphonic acid (ANS) or by using weak (2 mM) potassium hydroxide.

Preliminary studies showed that whilst both KOH and ANS would elute cytosol proteins bound to the T_4-gel, no activity

could be measured in the eluant. A lowered pH to 5.0 or 5.5 is suggested as a general procedure (Porath, 1974) for eluting protein from affinity columns. A preliminary study found that 5'-deiodinase activity from pig cytosol could be eluted by changing the pH from 7.4 to 5.5 so that a scaled up study was performed. The method and elution profile are shown in Figure 4.6 and its caption.

From Figure 4.6 a single band of protein was seen to be eluted, however most of the 5'-deiodinase activity was spread in a broad band well beyond the main protein peak. This suggests that proteins other than the 5'-deiodinase are binding to the ligand and are eluted by the change in pH.

Unfortunately, no more pig kidneys were available for further study. Therefore, all further studies were made on rat kidney cytosol even though it had proportionately less 5'-deiodinase activity than the pig cytosol.

As the rat cytosol 5'-deiodinase activity is labile when prepared, 2 mM DTT was maintained in all the solutions containing cytosol proteins which included the mobile phase buffers for affinity chromatography. The presence of DTT did not allow deiodination of the T_4 ligand by the loaded enzyme as shown by the absence of any liberated $^{125}I^-$ after the enzyme preparation had been run on the thyroxine-gel into which a tracer amount of ^{125}I T_4 had been incorporated at the time of its preparation.

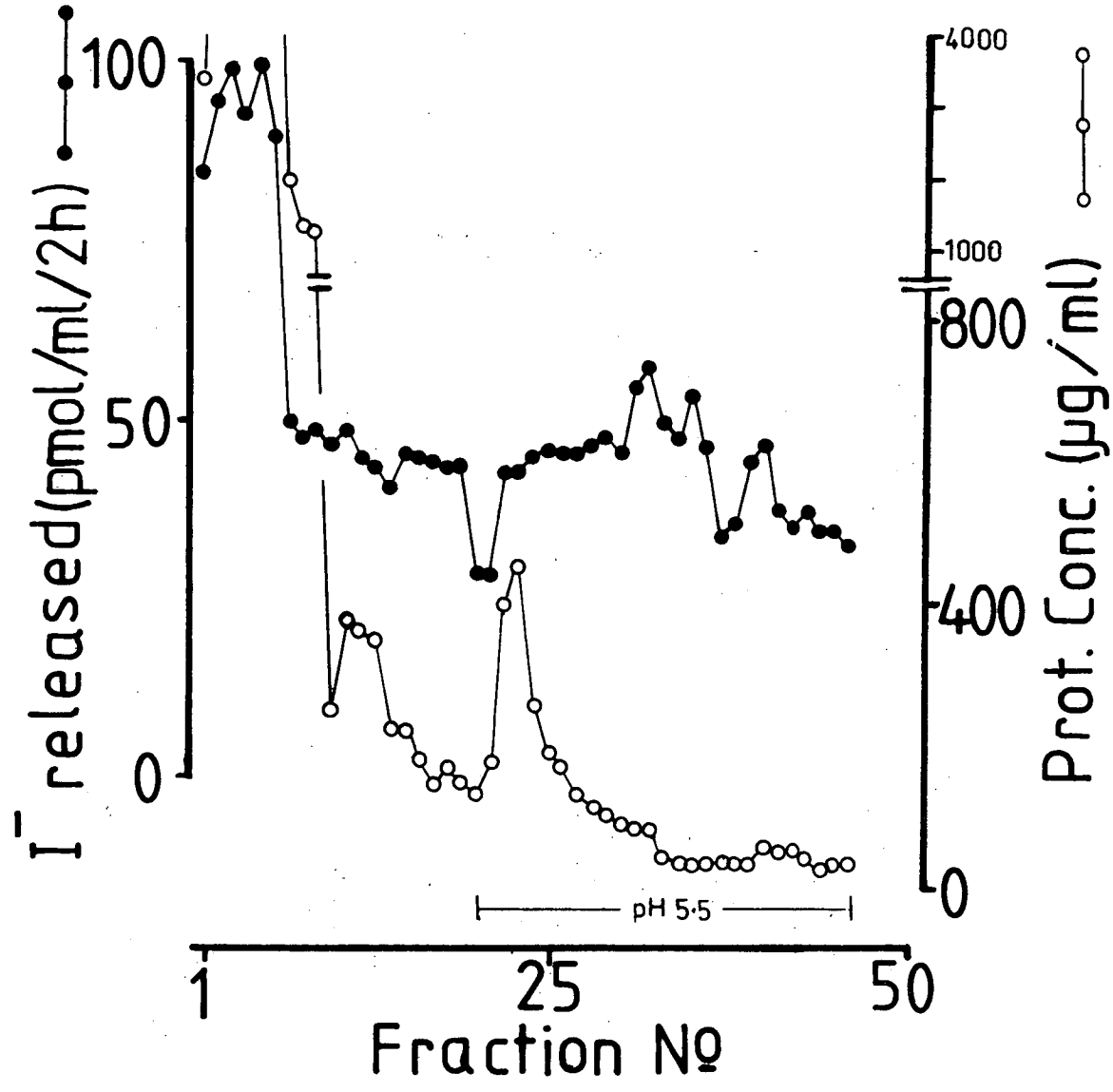


FIGURE 4.6 Thyroxine Affinity Chromatography of Pig Kidney Cytosol.

The load protein consisted of the precipitate formed by making 180 ml of pig kidney cytosol containing 20 mg/ml protein to 40% saturation with ammonium sulphate. The precipitate was collected by centrifugation and dissolved in 40 mls of HEPES/EDTA 25/5 mM buffer pH 7.4. It was then 'desalted' into the same buffer on several small Sephadex G-25 columns. (PD 10, Pharmacia) run in parallel. A final volume of 52.5 ml was collected and loaded by gravity flow onto a 12.0 x 1.5 cm column of T_4-gel and fractions of 10 ml collected. Unbound protein was then washed off with HEPES buffer as the mobile phase until the protein concentration fell to 150 µg/ml by spot test using the method of Sedmark and Grossberg (1977). The mobile phase was then changed to MES/EDTA/DTT 25/5/2 mM pH 5.5 to elute the bound protein. T_4-5'-deiodinase activity was estimated by combining 0.2 ml aliquots from each fraction with 0.8 ml of HEPES/DTT 100/2 mM pH 7.4 buffer. Thyroxine (10^{-7}M) substrate was added as in Chapter 3 and the iodide released during incubation at 37°C for 2 hours was then measured by the ion-exchange method.

The affinity chromatography of rat cytosol was then performed using the change in pH from 7.4 to 5.5 to elute the proteins. The results and method are described in Figure 4.7 and its caption.

Figure 4.7 shows that a relatively large amount of protein is not bound to the gel and is washed from the column and then it is some time before the change in pH leads to elution of the bound protein in a series of small peaks. This is quite a different chromatogram in appearance to that of pig cytosol (Figure 4.6) in which the proteins were eluted as a single band even though the activity was spread out beyond this band.

In view of the fact that the cytosolic 5'-deiodinase enzyme seems to be active and also stabilized in strong KCl solutions, the experiment in Figure 4.7 was repeated using as the mobile phase KCl/HEPES-TRIS/EDTA/DTT 1000/25/15/2 pH 7.4, however no protein was bound to the column so that changing the pH had no effect. This result was not unexpected, although Turková (1978) and Pharmacia Technical Bulletin recommend a trial of 0.5 - 1.0 M salt to prevent non-specific binding of other proteins before more specific elution of the protein of interest. However the elution of deiodinase by 1000 mM KCl suggested that KCl could be used as a desorber. This was examined by using a linear gradient from 0 to 1500 mM KCl. The data and experimental method are given in Figure 4.8 and its caption. The figure shows a single protein peak eluted early (approx. 100-200 mM KCl) with the KCl gradient, but possessing a trailed shoulder. Measurement of 5'-deiodinase activity shows again that there appear to be multiple peaks of activity.

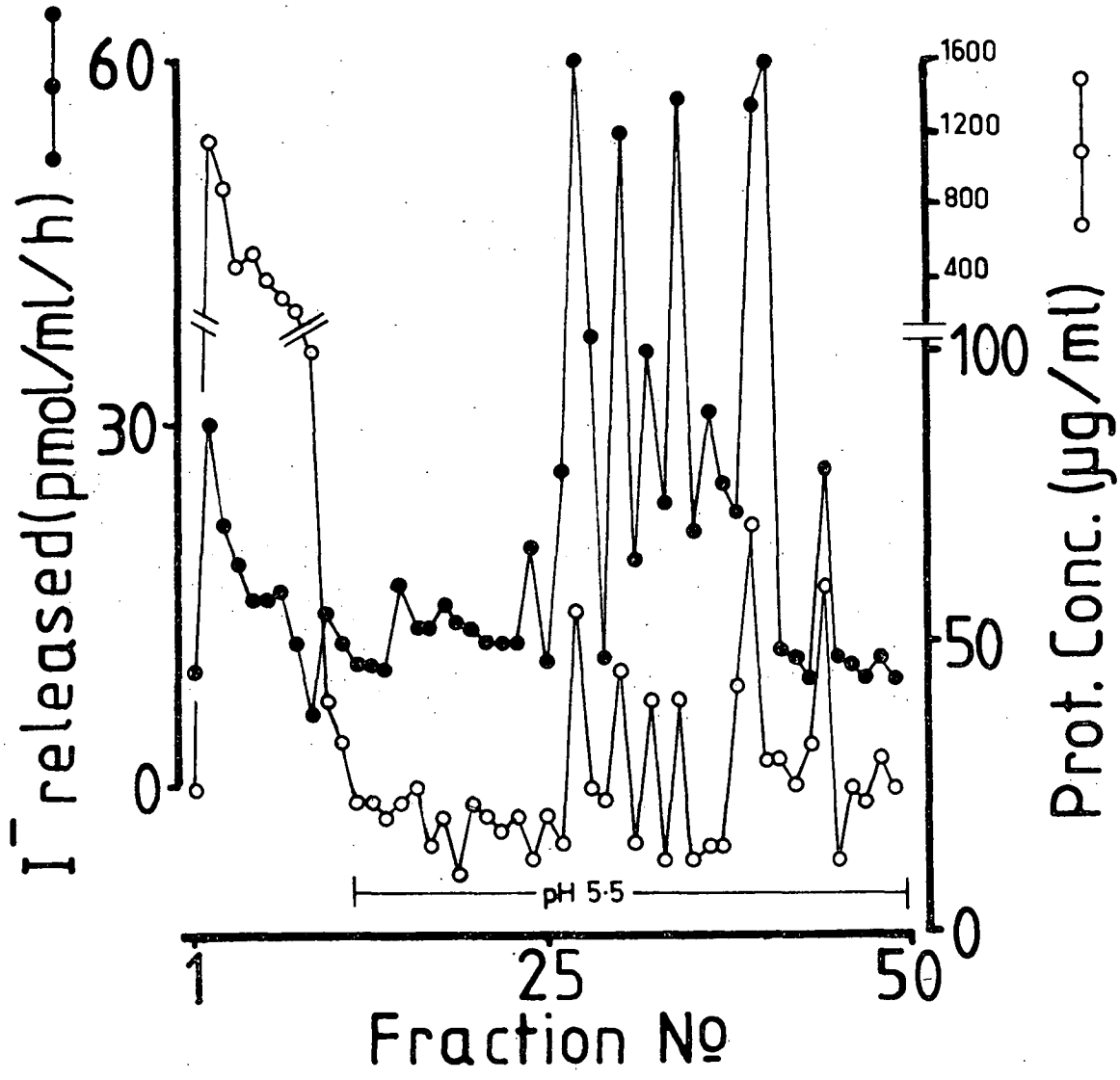


FIGURE 4.7 Thyroxine Affinity Chromatography of Rat Kidney Cytosol with Elution by Decreased pH.

A cytosol fraction was made from two rat kidneys by the method in Figure 3.3 using sucrose/KCl/HEPES/EDTA/DTT 250/100/25/15/2 mM pH 7.4 as the homogenising buffer. The cytosol was then made to 1 M with KCl and then to 50% saturation (2.85 M) with $(NH_4)_2SO_4$ and the precipitate collected and desalted on two small Sephadex G-25 columns (PD 10 Pharmacia) which had been preequilibrated with HEPES-TRIS/EDTA/DTT 25/15/2 mM pH 7.4. The 5 ml of desalted protein solution was then loaded on to a 10 x 1.5 cm column of thyroxine affinity gel prepared as in Section 4.3.2. The same buffer was then run as the mobile phase until the eluted protein concentration fell to less than 50 µg/ml. The mobile phase was then changed to MES/EDTA/DTT 25/15/2 pH 5.5 to elute the 5'-deiodinase activity which was measured in each fraction by taking an 0.5 ml aliquot from each 10 ml fraction and combining it with an equal volume of HEPES-TRIS/KCl/DTT 100/100/2 mM pH 7.4 buffer and then assayed in duplicate using the butanol method with 10^{-7}M thyroxine. Protein concentrations were measured by the method of Sedmark and Grossberg (1977).

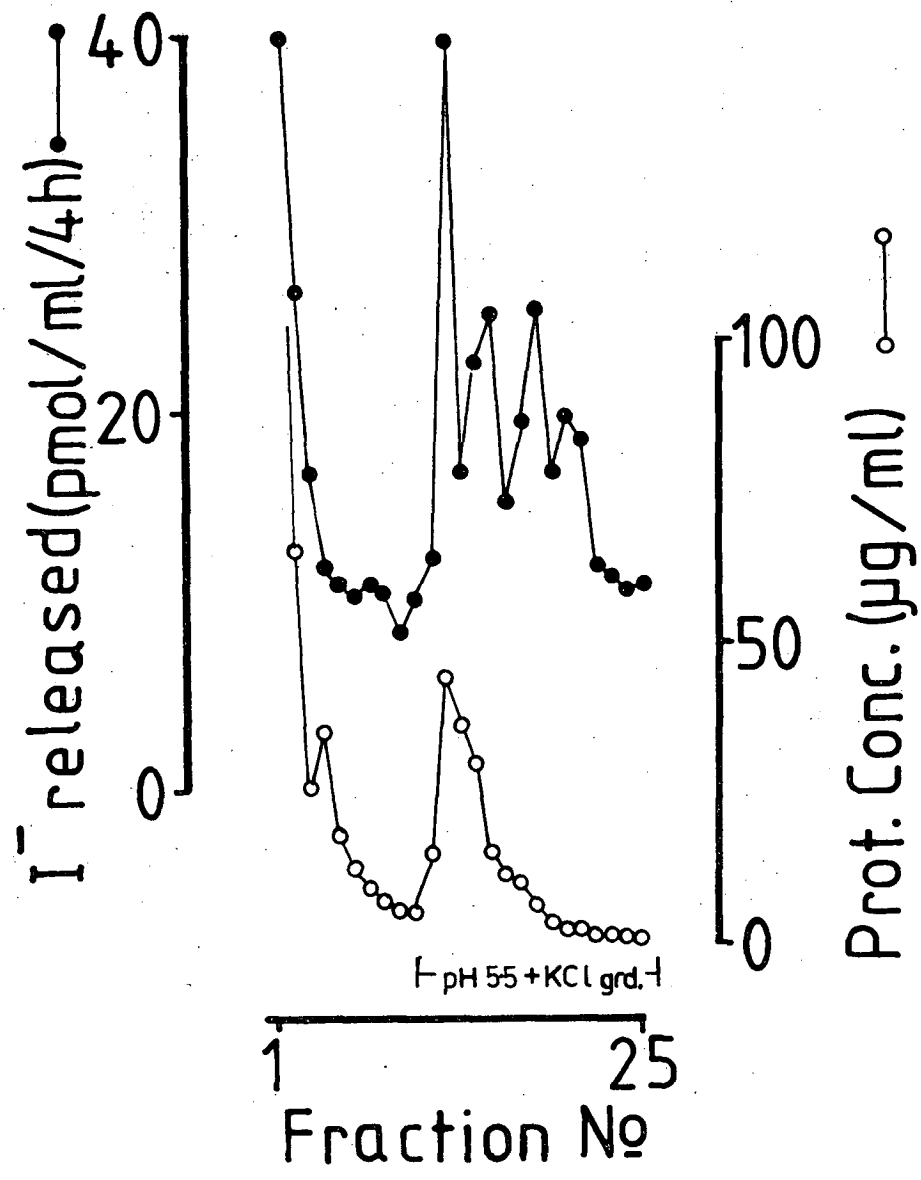


FIGURE 4.8 The Elution of Cytosol 5'-Deiodinase from T_4 Affinity-Gel by a Linear Gradient of KCl.

An ammonium sulphate fraction was prepared from two rat kidneys and desalted and resuspended in homogenising buffer (see Figure 4.7). The desalted protein (10 ml) from the ammonium sulphate precipitate of the cytosol fraction was applied to a 12 x 1.5 cm column of affinity gel consisting of thyroxine linked to epoxy activated Sepharose 6B. After washing with 80 ml of HEPES-TRIS/EDTA/DTT 25/15/2 mM pH 7.4, the bound protein was eluted by changing the mobile phase to MES/EDTA/DTT 25/15/2 mM pH 5.5 and simultaneously starting a linear KCl gradient from 0 to 1500 mM KCl over 500 ml at 50 ml/hour. Each fraction was 10 ml and the whole apparatus was maintained at 10°C.

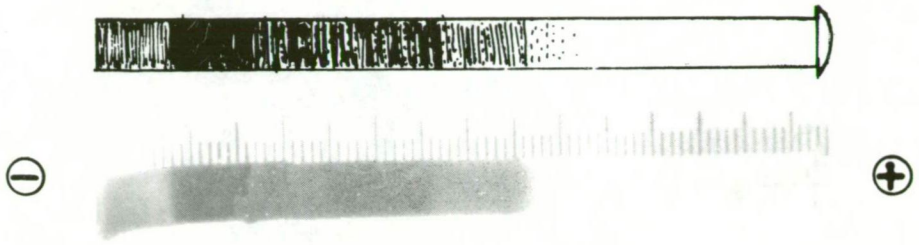
4.3.4 Polyacrylamide Gel Electrophoresis of the Eluted Protein from Affinity Chromatography

For electrophoresis, a further batch of kidney cytosol was prepared from 6 rats, fractionated, and loaded on to the T_4 affinity column and washed free of unbound protein as before. The 5'-deiodinase activity was then eluted by 300 mM KCl in MES/EDTA/DTT 25/15/2 mM pH 7.4 as an isocratic mobile phase rather than using a gradient of KCl as was done previously. The fractions containing the eluted band of proteins were identified by the Coomassie Blue Spot test of Sedmark and Grossberg (1977), pooled, and then concentrated in dialysis tubing against Aquacide I (Sigma) and when reduced to less than 2 ml the protein was further concentrated in an Amicon B_{15} concentrator to approx. 200 µl. Samples (30 µl) of the concentrate were prepared and then run in either 50 mM borate/phosphate buffer pH 8.0 or in 100 mM phosphate pH 7.2 containing 0.2% SDS on 3.3% polyacrylamide gels at 7.5 mA/gel by the method of Weber et al. (1972). Photographs of the gels are shown in Figure 4.9.

The normal disc electrophoresis showed a diffuse broad band from the origin to approximately two thirds along the gel. The stained gels run in SDS show at least 18 bands along their length and correspond to molecular weights ranging from approximately 2×10^7 to 2×10^4 when compared to the migration of thyroglobulin (640,000), ferritin (440,000), albumin (67,000), ovalbumin (43,000), and myoglobin (17,600) as standards. The band migrations distances and their corresponding molecular weights are shown in Table 4.6.

Of particular note is the very large range of molecular weights shown. The presence of very large protein units in SDS

PAGE



PAGE-SDS

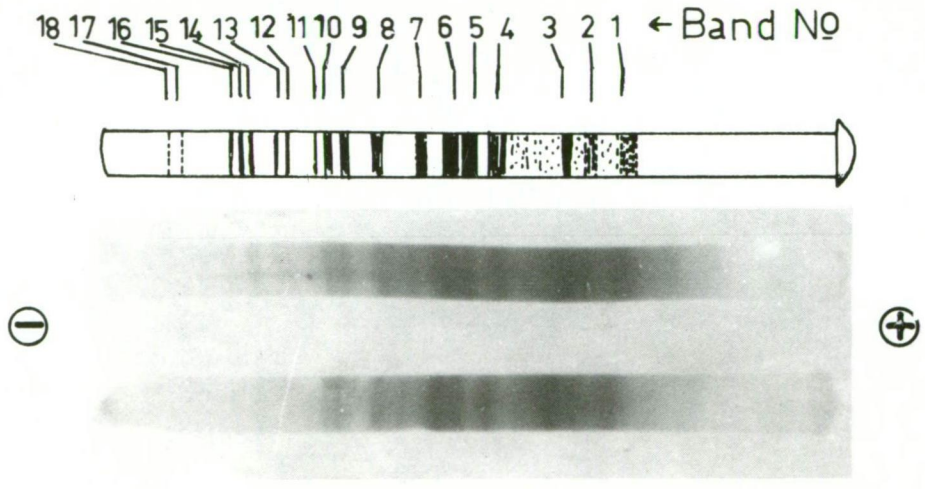


FIGURE 4.9 The Appearance After PAGE-SDS of the T_4-Affinity Chromatographically Purified Cytosol T_4 5'-Deiodinase.

TABLE 4.6 The Molecular Weights on PAGE-SDS of Cytosol Proteins Obtained after T_4-Affinity Chromatography.

Band No.	Band Migration (mm)	Log M_i	Molecular Weight (M_i)
18	7	7.3222	21,000,000
17	8	7.2620	18,280,000
16	14	6.9011	7,964,000
15	15	6.8410	6,934,000
14	16	6.7808	6,037,000
13	19	6.6004	3,985,000
12	20	6.5402	3,469,000
11	23	6.3598	2,290,000
10	24	6.2996	1,940,000
9	26	6.1793	1,511,000
8	30	5.9387	868,400
7	34	5.6981	499,000
6	38	5.4575	286,700
5	40	5.3372	217,400
4	43	5.1568	143,500
3	50	4.7358	54,430
2	53	4.5553	35,910
1	56	4.3749	23,710

suggests that the denaturing conditions of Weber et al. (1972) in which the sample is heated at 100°C in 2% SDS for 5 minutes is insufficient for their full denaturation and combination with SDS. To test this possibility, a further batch of cytosol protein was prepared by affinity chromatography and concentrated as above. Samples (60 µl) of the concentrate were subjected to one of the following treatments in a microreaction vial.

(i) extracted twice with $CHCl_3/CH_3OH$ 50/50 (v/v) and the protein precipitate dissolved in 1% SDS and 1% mercaptoethanol with a further 10 mg of SDS being added to the vial before boiling for 5 min.

(ii) Protein precipitated with 20% (w/v) trichloracetic acid and then dissolved in 1% SDS and 1% mercaptoethanol and boiled for 5 min.

(iii) boiled for 5 min in 1% SDS and 1% mercaptoethanol to which was added 10 mg of SDS.

Samples (30 µl) from each treatment were then separated by electrophoresis on polyacrylamide gels in SDS as described above. Photographs of the gels are shown in Figure 4.10 and the band migration distances together with the calculated molecular weights are shown in Table 4.7.

It is apparent that all of the stronger denaturing conditions reduce both the number and intensity of the large molecular weight bands. This indicates that aggregates are present which can be reduced to three or four main bands of very much lower molecular weight under very strong denaturing conditions as shown by 20% TCA or $CHCl_3/CH_3OH$ combined with SDS. The data in Tables 4.6 and 4.7 is further discussed in Section 4.6.

FIGURE 4.10 The Effect of Various Denaturing Regimes on the Appearance after PAGE-SDS of the T_4-Affinity Chromatographic Purified Cytosol 5'-Deiodinase.

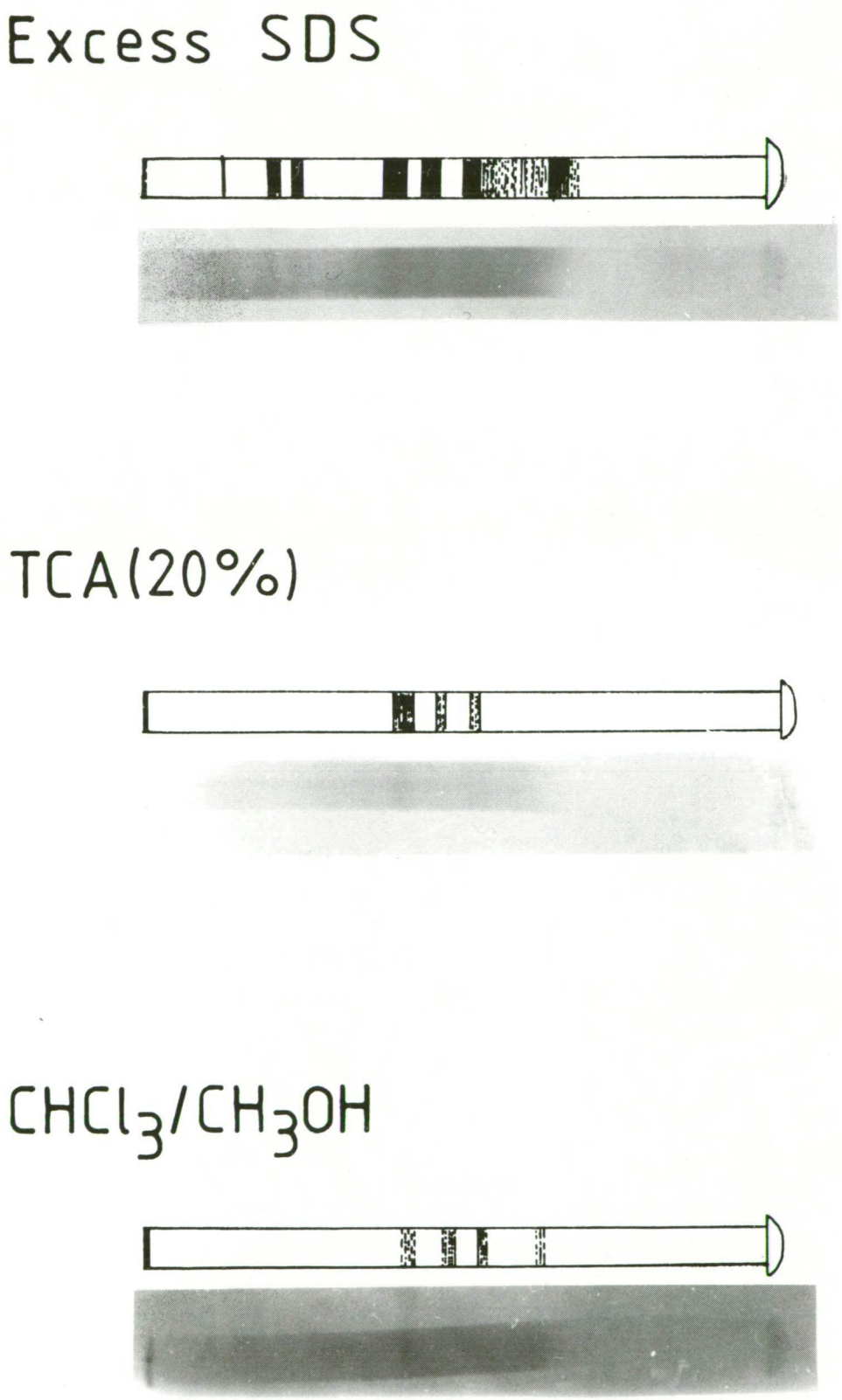


TABLE 4.7 The Molecular Weights on PAGE-SDS after Additional Denaturation of Cytosol Proteins obtained from T_4-Affinity Chromatography.

Denaturing Conditions	Band Migration (mm)	Log M_i	Molecular Weight (M_i)
Excess SDS	0	6.0371	1,089,000
	9.5	5.6437	440,000
	16	5.3746	236,000
	19	5.2503	177,000
	31	4.7534	56,000
	36	4.5464	35,190
	40	4.3807	24,010
*	50	3.9666	9,260
	52	3.8838	7,652
TCA (20%) + SDS	32	4.7120	51,520
	35	4.5878	38,710
	40	4.3807	24,010
$CHCl_3/CH_3OH$ + SDS	32	4.7120	51,520
	36.5	4.5257	33,550
	41	4.3393	21,840
	47	4.0910	12,330

* A broad band between 40 to 52 mm with maximum staining at 50 mm.

4.4 Molecular Exclusion (Gel-Filtration) Chromatography of Cytosol Thyroxine 5'-Deiodinase

The possibility of protein aggregation in a non-denatured preparation of the cytosol T_4 5'deiodinase was examined by molecular exclusion (gel filtration) chromatography using crosslinked agarose (Sepharose CL-6B, Pharmacia) as the stationary phase. The data and method are shown in Figure 4.11 and its caption. Three broad bands of 5'-deiodinase activity are seen at A ($4 - 2 \times 10^6$), B ($5 - 2 \times 10^5$) and C ($19 - 6 \times 10^3$). This range of molecular weights agrees with the range of molecular weights found in a preliminary study published earlier (Colquhoun et al., 1981). They are consistent with aggregates of non-denatured subunits in solution.

The protein bands A, B, C in Figure 4.11 were concentrated as before and run on either 3.3% (A, B) or 5% (C) polyacrylamide gels in SDS by the method of Weber et al. (1972). The band migrations are listed in Table 4.8.

Inspection of Table 4.8 shows that band A is associated with protein of $\geq 3 \times 10^6$ mol. wt. which is consistent with the molecular weight obtained from elution volume on Sepharose CL-6B. Bands B and C after PAGE-SDS show not only protein bands of expected molecular weight but also of greater and lesser molecular weight than would be expected from their elution volume on Sepharose CL-6B. These observations are consistent with aggregation of non-denatured protein before running on Sepharose CL-6B as shown by peaks A and B. The widening of the range of molecular weights on PAGE-SDS suggests that denaturing aggregation can also occur by heating (during sample preparation for PAGE-SDS).

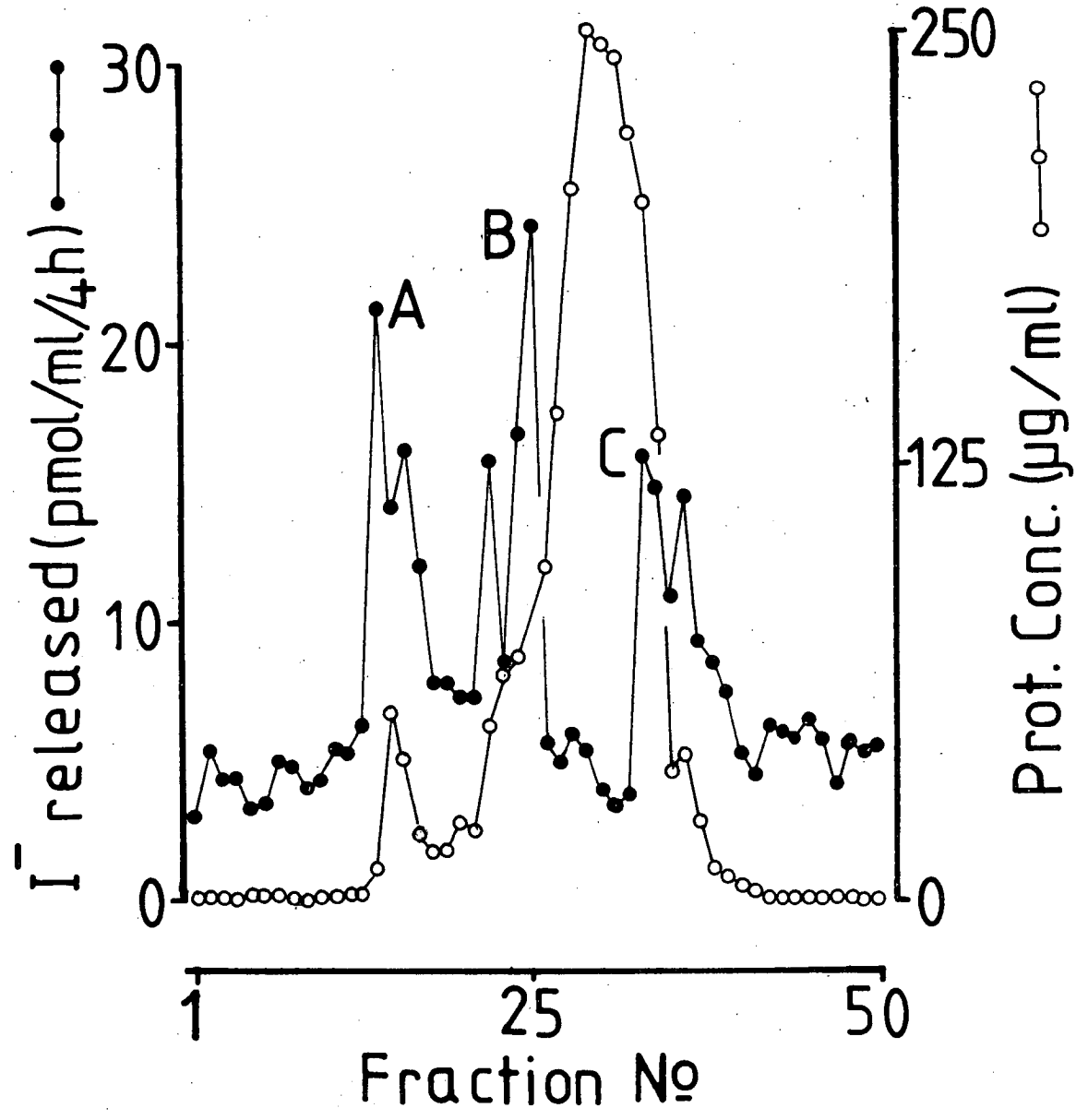


FIGURE 4.11 Chromatography of Cytosol 5'Deiodinase on Sepharose CL-6B.

Desalted protein from ammonium sulphate precipitation of the 260,000 g supernatant (cytosol) fraction of rat kidney was applied in 4 ml of eluant buffer containing 100 mM TRIS, 15 mM EDTA, 1 mM DTT, pH 7.4 to an 81 x 2.5 cm jacketed column of Sepharose CL-6B and eluted in fractions of 10.5 ml over 16 hours. The column and fractions were maintained at 10°C. Before removing 1 ml aliquots for incubation, 500 µl of 2000 mM KCl in eluant buffer was added to each fraction. Calibration of the column under running conditions was performed with thyroglobulin (640,000), ferritin (440,000), albumin (67,000), ovalbumin (43,000), myoglobin (17,600) and cobalamin (1,200) as standards. Protein was assayed by the method of Sedmark and Grossberg (1978).

TABLE 4.8 Migration of Protein Bands on PAGE-SDS from Sepharose CL-6B Chromatography.

Fraction No.	Distance of Migration (mm)	Log M_i	Molecular Weight
13-18 (A) (3.3%)	0	6.5127	$\geq 3.256 \times 10^6$
22-26 (B) (3.3%)	1	6.4779	$\geq 3.00 \times 10^6$
	42	5.5289	1.81×10^5
	50	5.0150	1.03×10^5
	64	4.5971	3.95×10^4
33-40 (C) (5.0%)	0	6.2537	$\geq 1.8 \times 10^6$
	26	5.5427	3.49×10^5
	41	5.1233	1.33×10^5
	60	4.5946	3.93×10^4

The migration of the cytosol T_4 5'-deiodinase was also examined by chromatography on Sephacryl 300 (Pharmacia) which is a cross-linked alkyl dextran. The method and elution profile are shown in Figure 4.12 and its caption.

The migration of the main band of the T_4 5'-deiodinase corresponds to a molecular weight of 45,000. This is markedly retarded when compared to the migration on Sepharose CL-6B. Hydrophobic proteins are often retarded when using Sephacryl as the stationary phase (Pharmacia literature) which implies there may be a hydrophobic site on the cytosol 5'-deiodinase.

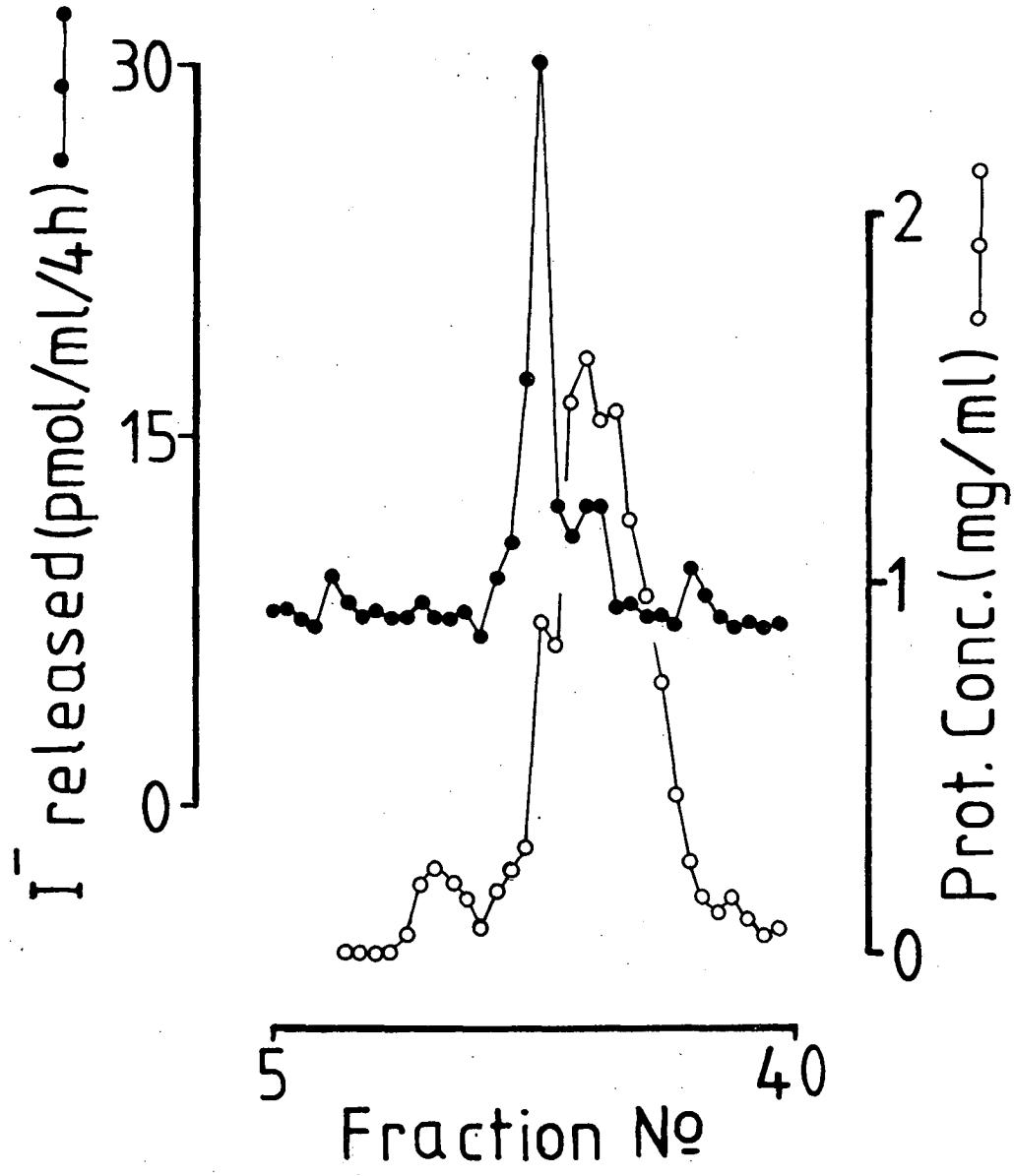


FIGURE 4.12 Chromatography of Cytosol T_4 5'-Deiodinase on Sephacryl 300.

Cytosol protein was prepared as in Chapter 3. The column was calibrated, pre-equilibrated, loaded, and run as described in the caption to Figure 4.11. The column contained 75 x 2.5 cm of Sephacyl 300. Fraction volume was 9.5 ml.

The effect of strong salt on the aggregation of T_4 5'-deiodinase activity was tested by running the same cytosol preparation in the presence and absence of 1M KCl on parallel CL-6B gel columns at the same time to avoid loss of activity. The elution profiles are shown in Figure 4.13 from which it can be seen that the presence of KCl spreads the 5'-deiodinase activity throughout the chromatogram. This is consistent with partial disaggregation to form a large number of smaller aggregates of differing molecular weights. The enzyme also remains active in strong KCl as was expected from the stability studies conducted in Section 4.1.

Confirmatory evidence for normal aggregation in solution of the cytosol 5'-deiodinase was obtained by applying the protein prepared by affinity chromatography to a Sepharose CL-6B gel column and finding that both the 5'-deiodinase activity and protein is spread throughout the elution profile as shown in Figure 4.14.

The 5'-deiodinase activity values are for incubation at pH 7.4, uncorrected for non-enzymic deiodination. That deiodinase activity was indeed present, is shown by the activity levels falling to that of the free iodide already present in the substrate when no protein is present.

Confirmation of considerable purification by either affinity chromatography or molecular exclusion chromatography is given by the specific activities shown in Table 4.9.

4.5 Analysis of the Products Formed by Cytosol 5'-Deiodinase Using HPLC

Cytosol and the cytosol proteins precipitated by ammonium sulphate were incubated separately at pH 7.4 for up to 4 hours with

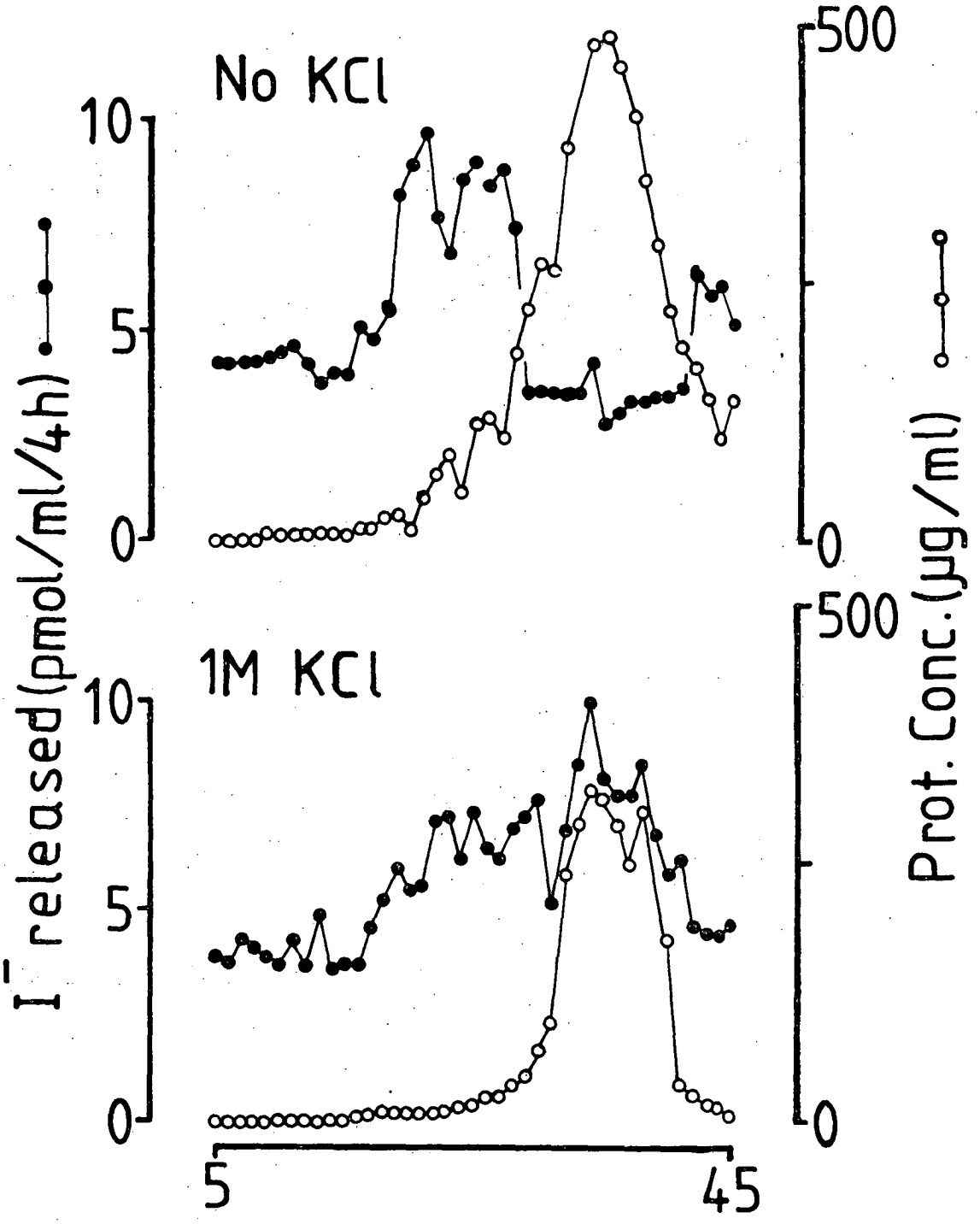


FIGURE 4.13 The Effect of KCl on Aggregation Size of Cytosol T_4 5'-Deiodinase when Chromatographed on Sepharose CL-6B.

The ammonium sulphate precipitate from rat kidney cytosol was halved and each half resuspended in either sucrose/HEPES/EDTA/DTT 250/50/15/0.1 pH 7.4 or with the same buffer including 1000 mM KCl. Then each sample (9 ml) was applied to two 81 cm columns of Sepharose CL-6B pre-equilibrated with the respective buffer and then run overnight. Fractions of 9.1 ml were collected. To the fractions not containing 1 M KCl, 3 ml of 4 M KCl were added to make it 1 M KCl. The activity plotted for zero KCl in the figure has been corrected for the dilution of the fractions.

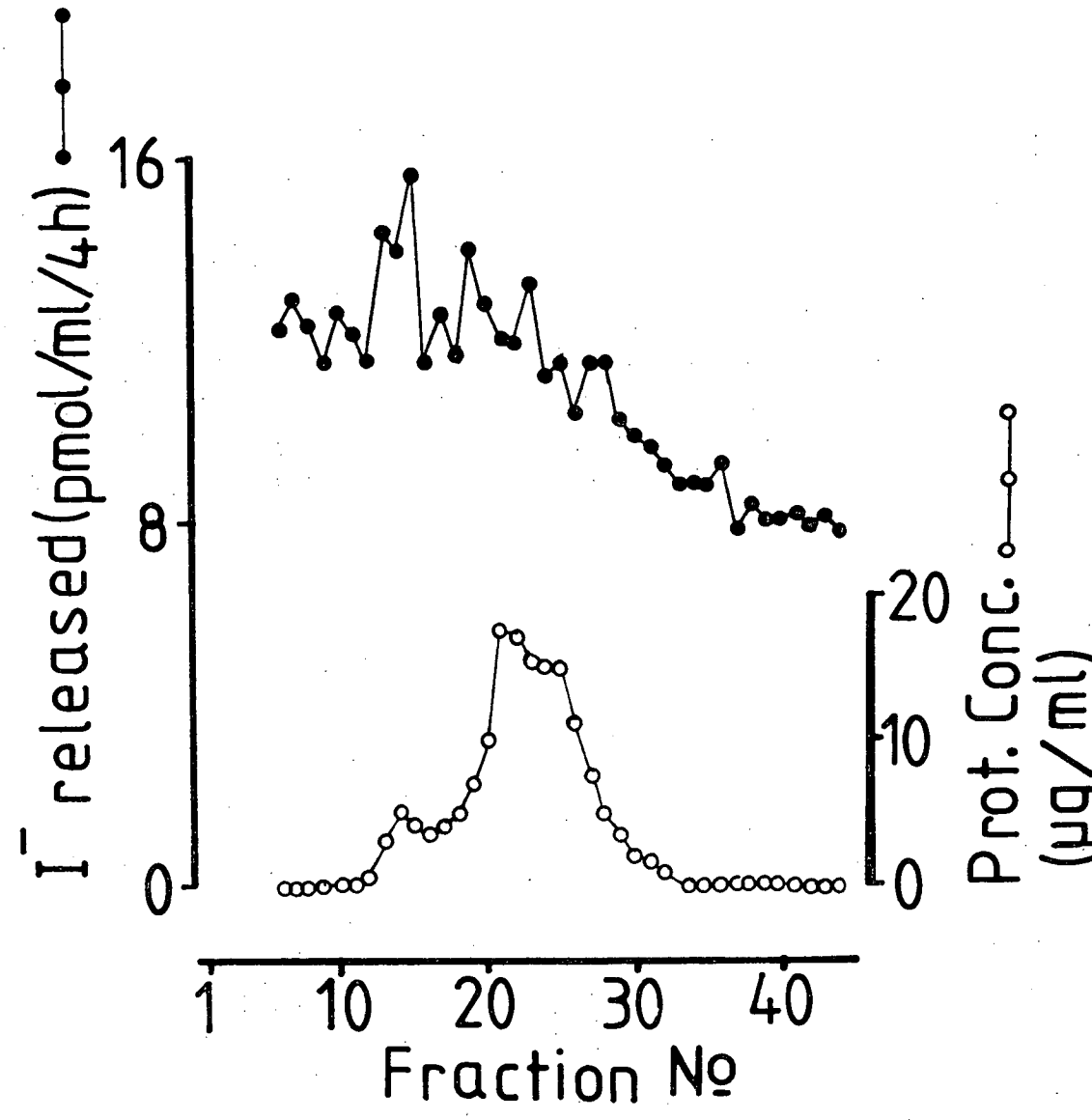


FIGURE 4.14 Chromatography on Sepharose CL-6B of the Cytosol 5'-Deiodinase purified by Thyroxine Affinity Chromatography.

Cytosol was prepared and run on T_4-agarose affinity column as in Section 4.3.4. The eluted protein was concentrated to 10 mls against aquacide and then run on Sepharose CL-6B as described in the caption to Figure 4.11.

TABLE 4.9 Specific Activities of Purified Fractions of Cytosol 5'-Deiodinase.

Fraction	Vol. (ml)	Prot.Conc. (mg/ml)	Tot.Act. (pmol/4h)	Spec.Act. (pmol/mg/4h)	Purifi-cation
Homog.	185	4.32	4958	6.27	1.0
10,000 g sn.	165	4.32	4454	6.25	1.0
260,000 g sn.	162	3.83	558	0.9	0.1
amm.sulp.pt.	32	9.63	4022	1.29	2.0
aff.chr.pk.	95	0.02	320	1900	303
CL-6B pk.A	10.6	0.04	110	2750	440
CL-6B pk.B	10.6	0.10	126	1260	200

[3,'5'-^{125}I] - T_4 (3×10^{-5} M, 120,000 cpm). The iodothyronines were extracted by the ion-exchange system and examined by the HPLC system described in Sections 2.2 and 2.4 using UV detection at 254 nm.

Control extractions of both protein preparations shows strong UV interference obscuring the appearance of thyronine (T_0) and both the monoiodothyronines as well as the iodotyrosines.

After extraction, T_4 was consistently seen on HPLC, but no di- or tri-iodothyronines could be identified at any time during the incubations. This included many samples in which there was greater than 10% deiodination of T_4 as measured by ion-exchange. This result was quite unexpected, as the T_3 was the expected product of 5'-deiodination. T_3 had earlier been shown to be deiodinated only very slowly by rat cytosol (Chapter 3).

Assuming all the iodothyronine product was T_3 (the iodine-125 was only in the outer ring) then 10% deiodination would give 3 nmol/ml of T_3 which is three times the detection limit. If the deiodination were proceeding to either 3,5-T_2 or 3,3'-T_2 then 3nmol would be at least six times the detection limit.

In an effort to resolve this problem of detection, the incubations of cytosol with T_4 were repeated as above (0-2h). The HPLC was however coupled to a 120 tube fraction collector set at one tube per 10 seconds. Each fraction was then transferred to the gamma counter for measurement. The fraction collection however blurs the separation of three pairs of iodothyronines, viz: T_4/rT_3, 3,3'-T_2/3',5'-T_2, and 3'-T_1/3-T_1.

No peaks other than T_4 could be detected until the 1 and 2 hour incubations were examined when small peaks corresponding to 3,3'-T_2/3',5'-T_2,

3,5-T_2, and 3'-T_1 were seen but which in total would account for <20% of the deiodination. 3-T_1 and T_0 would not be seen by this technique as they would have no iodine-125.

4.6 Analysis for Carbohydrate

In an attempt to relate 5'-deiodinase activity with the presence of glycoprotein, T_4 5'-deiodinase enzyme was again prepared by affinity chromatography, concentrated, and then run overnight on a gradient polyacrylamide gel. The gel was then divided into three longitudinal sections as shown in Figure 4.15. The two smaller slices were stained for either protein (Coomassie Blue) or carbohydrate (PAS) and the third larger slice subdivided transversely and T_4 5'-deiodinase estimated in each subdivision. The method and data are shown in Figure 4.15 and its caption. The figure shows that glycoprotein is present, and that 5'-deiodinase activity is in part associated with the glycoprotein bands. It is of interest that some activity was still present in the TCA controls for bands 1 and 3.

The property of the partially purified deiodinase in resisting the denaturing effect of trichloracetic acid when assayed on the gel suggested that it might either be a glycoprotein or be of unusual aminoacid composition. Such proteins manifest these characteristics (Young, 1963). This is especially so when they are not associated with other more easily denatured proteins such as those found in either kidney homogenate or cytosol.

The protein derived from affinity chromatography was therefore examined for neutral carbohydrates and sialic acids as shown in Table 4.10.

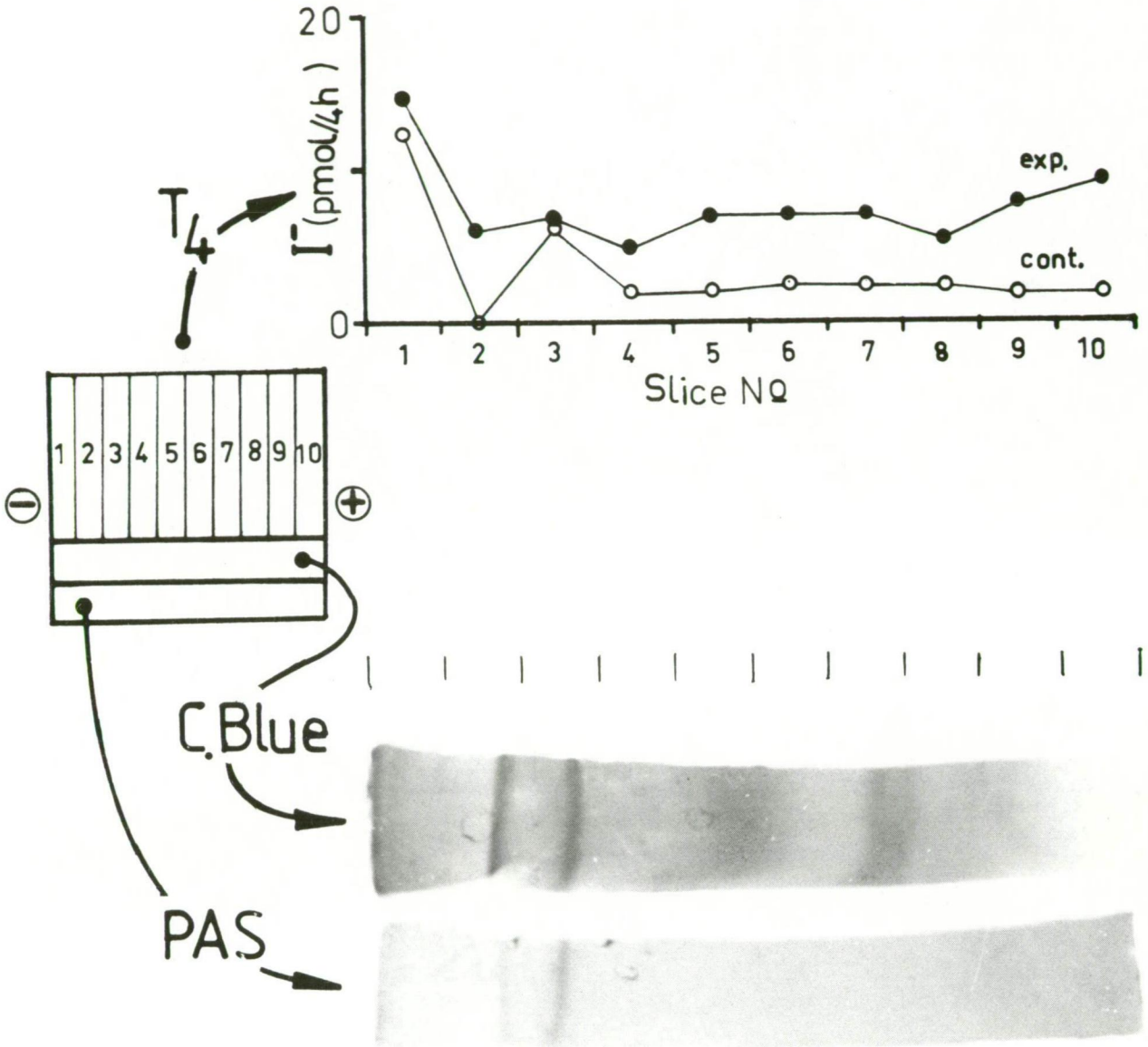


FIGURE 4.15 Pore Gel Electrophoresis of Affinity Chromatography Purified Cytosol 5'-Deiodinase.

Cytosol protein was eluted from T_4-affinity gel as described in Section 4.3.4. The concentrated protein was loaded onto a polyacrylamide gradient gel 2.5-13% (Gradipore, Sydney) and run overnight at 50 V and 10 mA at 0°C. Protein was stained by the method of Weber et al. (1972), and glycoprotein by PAS method of Segrest and Jackson (1972). T_4 5'-deiodinase activity was measured in 8 mm slices after homogenisation of each slice in 4 ml of HEPES/KCl/EDTA/DTT 100/100/15/1 pH 7.4 and incubated for 4 h at 37°C in 10^{-7}M thyroxine.

TABLE 4.10 Carbohydrate Composition of Affinity Chromatography Purified T_4 5'-Deiodinase.

Protein Fraction	Total Hexose (µg/ml)	Fucose (µg/ml)	Sialic Acid (µg/ml)	Protein (µg/ml)
Supernatant	690	100	0	112
Precipitate	28	15	0	136

Protein was determined by the method of Sedmark and Grossberg (1977), total hexose and anthrone by the method of Roe (1955) using galactose as standard as modified by Spiro (1966); sialic acid by the thiobarbituric acid method of Svennerholm (1957) as modified by Spiro (1966); and by the cysteine/sulphuric acid method of Dische and Shettles for fucose as modified by Spiro (1966).

To prevent interference by the dithiothretiol, the protein was dialysed against water containing 0.001% sodium azide overnight at 4°C in a bag made of 6000-8000 molecular weight cut-off dialysis tubing (Spectrophor). It was found that a white fluffy precipitate formed overnight. This was separated by centrifugation from the supernatant and the two fractions analysed separately. No T_4 5'-deiodinase activity in either of these purified fractions remained after dialysis. Activity was absent even in the presence of DTT during dialysis.

Table 4.10 shows that both fractions contain large amounts of carbohydrate relative to the amount of protein. The soluble fraction has proportionately more carbohydrate than the sediment. It is of interest firstly, that the sediment precipitates even though it has so much carbohydrate and secondly, that neither fraction shows enzymic activity after dialysis in the presence of DTT.

4.7 Discussion and Conclusions

The properties of the thyroxine 5'-deiodinase in cytosol prepared from rat kidney can be summarised as follows:

(1) It is prepared from kidney cytosol in variable yield which is in part dependent on the temperature of preparation, and on the KCl concentration but not on the osmolarity of the suspending buffer.

(2) It is labile and the activity is quickly lost on freezing. It is partially stabilized by strong KCl solutions in the cold, and when frozen.

(3) It has a broad pH optimum between 7.4 and 8.5.

(4) Analysis of the deiodination products from T_4 by HPLC reveals only small quantities of di- and mono-iodothyronines.

(5) The rat cytosol activity is precipitated through the broad range of 30-50% saturation by $(NH_4)SO_4$ in the presence of 1 M KCl. Pig cytosol activity is precipitated between 20-40% saturated $(NH_4)SO_4$ without KCl.

(6) Both pig and kidney cytosol are considerably purified by thyroxine affinity chromatography. The rat enzyme so produced is polydisperse in terms of molecular weight when examined by conventional PAGE-SDS. However the number of the bands could be considerably reduced by more vigourously denaturing the proteins prior to PAGE-SDS.

(7) Aggregates of non-denatured protein were shown to exist in solution and migrated in a hydrophobic manner on Sephacryl. In solution the aggregates could be partially dispersed by strong KCl without loss of deiodinase activity.

(8) The more purified preparations of the enzyme (for example after pore-gel electrophoresis), showed resistance to denaturation by trichloracetic acid.

(9) The enzyme preparation after affinity chromatography was fractionated by dialysis into two glycoprotein containing fractions, one soluble and one insoluble in water. The fractions differed greatly in their carbohydrate to protein ratio. The soluble fraction had more carbohydrate.

(10) Part of the thyroxine 5'-deiodinase activity appeared to be associated with PAS staining protein on polyacrylamide pore gel electrophoresis.

Strong evidence for the presence of only a small number of proteins in the aggregates after affinity chromatography can be deduced from the inspection of the log M_i values taken from Tables 4.6 and 4.7 and which, for convenience, have been retabulated in Table 4.11.

Considering first data from Table 4.6 of the logarithm of the molecular weights of any two adjacent bands, it can be seen that the values are integral multiples of a constant value;

$$\text{i.e.} \quad z = \log \frac{M_{i+1}}{M_i} = np \qquad (4.1)$$

where M_i = molecular weight of ith band

$p = 0.0602$

$n = 1, 2, 3, 4, 6, 7$

This data immediately suggests that any band is an aggregate of the preceding smaller bands. Equation 4.1 implies that the molecular weight of any band M_i is given by

$$M_i = A10^{mp} \qquad (4.2)$$

where A is a constant and equal to the molecular weight of band 1

and $m = \sum_{o}^{i} n_i$

It is thus possible to equate the measured molecular weight of each band with equation 4.2.

TABLE 4.11

Denaturing Conditions	Band Number	Log M_i	Log $\frac{M_{i+1}}{M_i} = 3$	$\frac{z}{p}$ (p=0.0601)	$\frac{z}{q}$	$\frac{M_i}{A}$
Table 4.6						
Normal SDS	b1	4.3749	.1804	3.00	9	675.1
	b2	4.5553	.1805	3.00	9	587.8
	b3	4.7358	.4210	7.00	21	337.6
	b4	5.1568	.1804	3.00	9	294.0
	b5	5.3372	.1203	2.00	6	255.9
	b6	5.4575	.2406	4.00	12	168.9
	b7	5.6981	.2406	4.00	12	147.0
	b8	5.9387	.2406	4.00	12	96.96
	b9	6.1793	.1203	2.00	6	84.41
	b10	6.2996	.0602	1.00	3	63.97
	b11	6.3598	.1804	3.00	9	36.75
	b12	6.5402	.0602	1.00	3	21.11
	b13	6.6004	.1804	3.00	9	12.13
	b14	6.7808	.0602	1.00	3	9.187
	b15	6.8410	.0601	1.00	3	6.061
	b16	6.9011	.3609	6.00	18	2.297
	b17	7.2620	.0602	1.00	3	1.515
	b18	7.3222				1.00
Table 4.7				$\frac{z}{p}$, (p'=.0621)	$\frac{z}{q}$, (q'= $\frac{1}{3}$p')	
Excess SDS	b1	3.8838	.0828	1.33	4	
	b2	3.9666	.4141	6.67	20	
	b3	4.3807	.1657	2.67	8	
	b4	4.5464	.2070	3.33	10	
	b5	4.7534	.4969	8.00	24	
	b6	5.2503	.1243	2.00	12	
	b7	5.3746	.2691	4.33	13	
	b8	5.6437	.3934	6.33	19	
	b9	6.0371				
TCA + SDS	b1	4.3807	.2071	3.33	10	
	b2	4.5878	.1242	2.00	6	
	b3	4.7120				
$CHCl_3/CH_3OH$ + SDS	b1	4.0910	.2483	4.00	12	
	b2	4.3393	.1864	3.00	9	
	b3	4.5257	.1863	3.00	9	
	b4	4.7120				

Of great interest is that the values for $\left(\frac{M_i}{A}\right)$ derived from this relationship bear relationships to each other as two dimensional rectangular shapes. These relationships are shown in Figure 4.16 and discussed below.

If for ease of representation we let band 1 (b1) be a square of 4 units of area, then to a very close approximation b2 = 6(6.06) units and b3 = 9(9.19) units → when compared to the calculated values in brackets.

Consideration of these areas suggests that b3 = 9 =(4 + 3 + 2)units which forms another square as shown in Figure 4.16. The model developed in the figure is based on the premise that the aggregation process uses blocks of 9 units (which are the most stable) and occasionally of 6 units to make larger squares or rectangles.

Thus b4 = 24 =(9 + 9 + 6)= (2 x 63 + b2)

b5 = 36 =(4 x 9)= (4 x b3)

b6 = 48 = (b4 + b4)

b7 = 84 = (b5 + b6)

b8 = 147 = (b7 + 7 x 9)

b9 = 255 = (b8 + 12 x 9)

b10 = 336 = (b9 + 9 x 9)

The possibilities for different shaped aggregates are obviously more numerous with increasing molecular weight. The models for b4 - b10 shown in the figure are consistent with the postulate that the aggregates are made from two smaller blocks which join along a common border of the same length as a preceeding block. Although not shown in the figure, a linear end to end aggregate of 9 unit blocks is also quite possible.

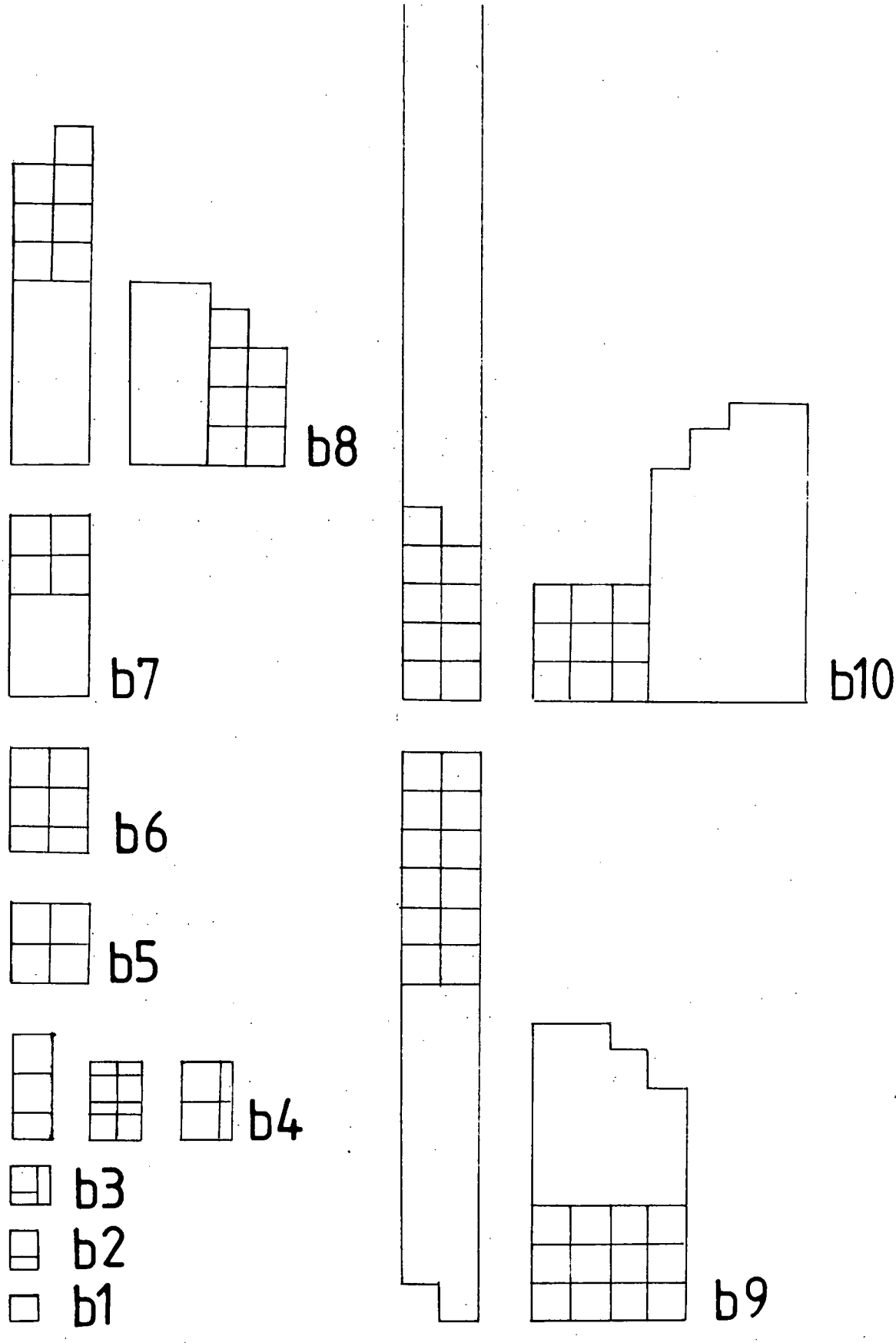


FIGURE 4.16 A Geometric Model for the Structural Relationships Between the Cytosol Bands on PAGE-SDS.

The model does not distinguish between several possibilities regarding subunit composition. They are, firstly, that all bands consist of one subunit so that b1 = 4, b2 = 6, b3 = 9 subunits, etc. and that these are stable and resistant to SDS in blocks of 9, 6, and 4 or secondly, that three separate proteins are present which combine as shown in the figure and have molecular weights in the fairly precise ratio of 4:3:2. A third possibility is that there is a protein of 9 subunits in size and that the other smaller bands (b1 and b2) are constant fragments of this protein.

Confirmatory evidence for a small number of proteins is given by the small number of bands present in PAGE-SDS of the $CHCl_3/CH_3OH$ and of the trichloracetic acid denatured cytosol proteins. Although in this data $p' \simeq 0.0621$ rather than $p = 0.0601$ as in Table 4.6, completely integral values of n and m are obtained by using $z = nq'$ where $q' = \frac{p'}{3}$.

The $CHCl_3/CH_3OH$ denatured protein shows only four distinct bands (apart from origin material) on PAGE-SDS, the largest of which (b4) at 51,520 is within 5% of the molecular weight of band 3 in Table 4.7. This then implies that the other bands consist of 6 (5.86), 4 (3.81), and 2 (2.15) subunits. Similarly for the TCA precipitated protein the bands represent 9, 7 (6.76), and 4 (4.2) subunits. The 7 unit band could consist of (4 + 3) units rather than (4 + 2) units previously encountered.

The multiplicity of bands from the saturated SDS solution on PAGE-SDS confirms that aggregation is possible under the conditions of reaction with SDS. Interestingly, it also shows a band

at 52 mm (7650) which, although larger than the expected 6300 mol. wt., might represent the 1 unit peptide, as the resolution of small peptides on 3.3% PA gels is inaccurate.

On taking into account that the measurement of band migration is at best no better than $\pm$ 0.5 mm the reproducibility of values of $p \simeq p'$, $q \simeq q'$ and of $z \simeq z'$ in both experiments is good and supports the above simple model to explain the aggregating bands on PAGE-SDS and implies a preparation containing only a small number of proteins (perhaps between one and three).

Other experimental data is relevant regarding the number of different protein subunits present. The observed difference in protein to carbohydrate ratio after dialysis (Section 4.5) suggests at least two different proteins, one having a much higher proportion of carbohydrate than the other. Furthermore, it is unlikely that the deiodinase enzyme is of a molecular weight of 6000 as most enzymes are at least double this value (although it could be a proteolytic fragment) and this then implies several peptides to explain all the small bands.

Considering the experimental observations and the deductions made from PAGE-SDS, I believe they are best explained by postulating that the enzyme in cytosol is derived from membranes within the kidney call or less likely, from within an enclosed cell compartment such as lysosomes or mitochondria. Such sites would explain the variable proportion of activity and would be consistent with the presence of glycoprotein. A soluble or 'latent' enzyme within lysosomes or mitochondria which is not part of a membrane is unlikely because there was no increase in 5'-deiodinase activity in the cytosol when the kidney was homogenised in hypotonic medium.

Another and not mutually exclusive explanation for the low and varied cytosol activity comes from the repeated observation that the recovery of activity from the affinity or gel columns often greatly exceeds that of the total activity of the loaded protein. Although this total activity is measured with non-saturating substrate concentrations, it does imply that other proteins in cytosol either strongly bind thyroxine such as the 'cytosol receptors' described by Tata (1975) or that there might be physiological protein inhibitors of the enzyme present. Either kind of protein might be separated from the enzyme during the chromatographic procedures.

The property of aggregation can be explained by hydrophobic patches on the membrane protein which leads to their aggregation to quite large sizes in two dimensions (sheets). Helenius and Simons (1975), in their review on solubilisation of cell membranes, list seven membrane proteins which once solubilised, then remain as water soluble homogenous or dispersed aggregates after removal of detergent and which had molecular weights ranging from 3×10^5 to 3×10^6. Hydrophobic induced aggregation would also explain the relative resistance to dispersion by SDS.

In summary, the thyroxine 5'-deiodinase found in cytosol is characterised by the following properties:

(1) It is unlikely to be found normally in cytosol <u>in vivo</u> but rather on homogenisation is liberated from a membraneous site within the kidney cell.

(2) It is most likely to be a glycoprotein in composition with at least several subunits.

(3) It is aggregated in aqueous solution and partially dispersed by KCl. Part of it is precipitated when dialysed against water.

(4) It can be substantially purified by thyroxine as an affinity chromatographic ligand using agarose as a supporting matrix.

(5) It deiodinates T_4 to T_0. Intermediary iodothyronines have short lifetimes.

CHAPTER 5

Purification and Properties of Thyroxine 5'-Deiodinase from Rat Kidney Microsomes

5.1 Introduction

The purification of a membrane bound protein is complicated by the need to render the protein soluble in water, as most of the available puricifation techniques are dependent on the protein being in aqueous solution. Thus the successful solubilisation of the deiodinase was an essential step in the purification following preparation of the membrane.

Microsomes were prepared from rat kidneys by using the homogenisation and centrifugation protocol shown in Figure 3.4. The 260,000 g particulate sediment was described as 'microsomes'. The measurement of thyroxine 5'-deiodinase activity employed the same substrate and trichloracetic acid control used in Chapter 3.

5.2 The Properties and the Solubilisation of the Microsomal Deiodinase

The pH optimum curve for deiodinase activity of microsomes was measured and is shown in Figure 5.1. The method is described in the caption. Maxima are present at pH 5.5 and at pH 7.5.

The effect of storage, after freezing, on the microsomal 5'-deiodinase activity was examined. The method and results are shown in Figure 5.2 and its caption. The figure shows a linear decrease of activity with time which was not stabilised by 100 mM KCl.

The need for dithiothreitol in the assay for microsomal T_4 5'-deiodinase was measured on washed microsomes and shown in

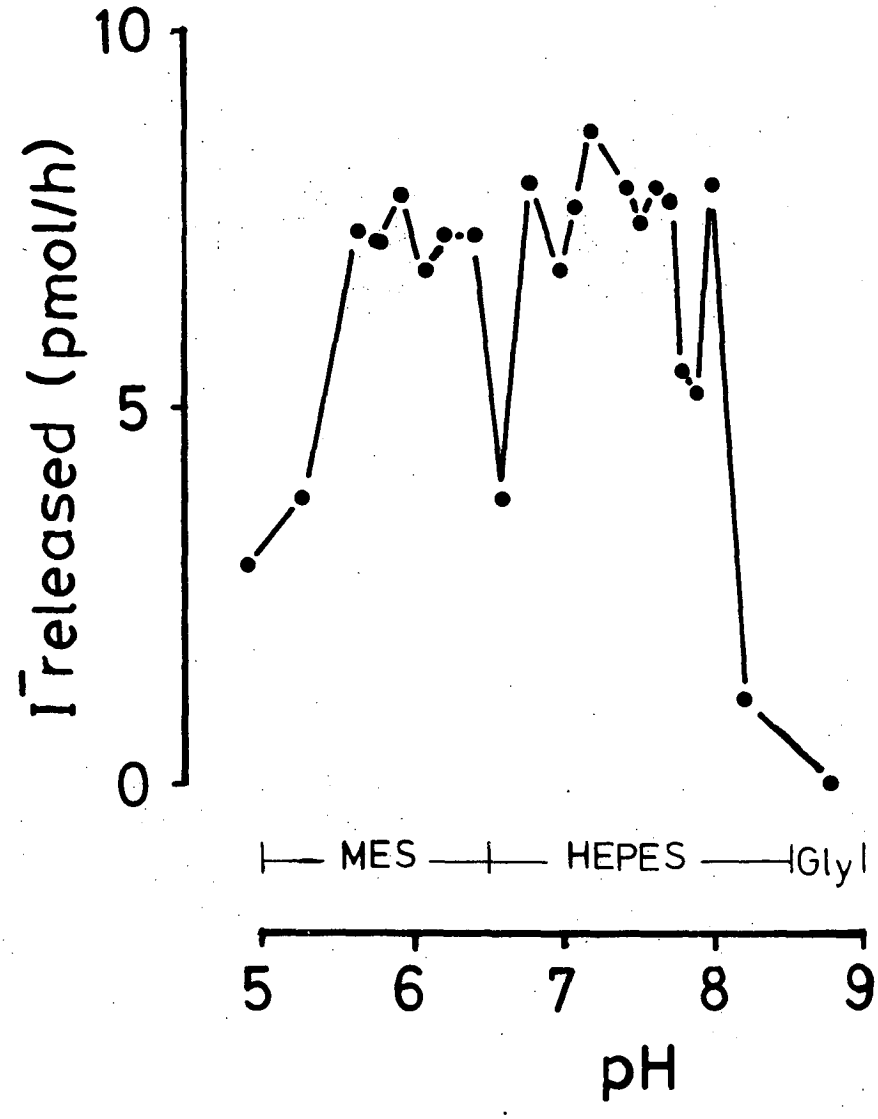


FIGURE 5.1 Effect of pH on Particulate Microsomal T_4 5'-Deiodinase.

Microsomes were prepared as in Figure 3.4 and resuspended in the appropriate buffers as described in Figure 3.3 to give the pH range shown in the figure. The pH values were checked with a pH meter after the addition of protein.

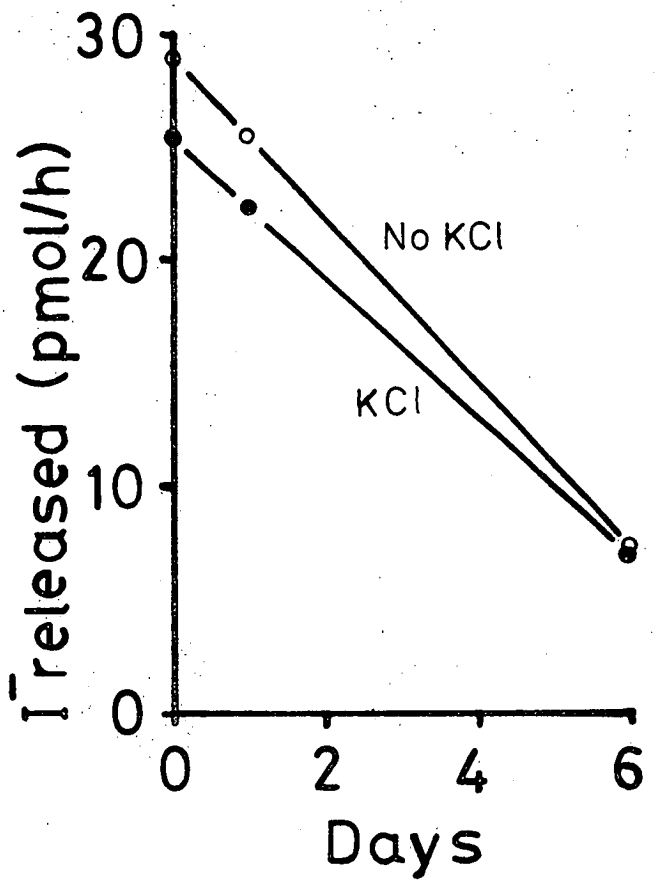


FIGURE 5.2 The Effect of Freezing and Storage on Microsomal 5'-Deiodinase Activity.

Microsomes were prepared in HEPES/EDTA/DTT 50/15/1 mM pH 7.4 as in Figure 3.3. They were resuspended in the same buffer to give 3.5 mg/ml and the solution divided in half. One portion was then made to 100 mM KCl with solid salt, and the T_4 5'-deiodinase activities measured in duplicate for both preparations. Both suspensions were then frozen in liquid nitrogen and stored at -20°C. Thereafter at various times, aliquots of each suspension were thawed quickly in a 37°C shaking water bath and their T_4 5'-deiodinase activities measured in duplicate.

Figure 5.3. The data in the figure generally agree with that of Visser et al. (1976), but maximal activity was obtained with a lower concentration of DTT (0.5 mM).

Preliminary studies found that strong salt solutions would not solubilise the microsomal T_4 5'-deiodinase. This suggested that the enzyme was an integral protein (Coleman, 1973) and would require detergent for its solubilisation.

The effect of Triton X-100 on microsomal thyroxine 5'-deiodinase activity was measured and is shown in Figure 5.4, from which it can be seen that the detergent markedly inhibits the deiodinase at a concentration above 0.01% (v/v). This inhibition was shown to be reversible by dilution.

Thus after the addition of Triton X-100, the assay of 5'-deiodinase was done only after the detergent concentration was either lowered by dilution to 0.01% (v/v) or reduced to values $\leq$ 0.01% (v/v) by the method of Holloway (1973) in which the protein detergent mixture is passed through a small column of Biobeads SM-2 (Bio-Rad, California), a polystyrene-divinyl-benzene polymer. However this method can remove too much detergent and thus allow the protein to reaggregate. So that usually for deiodinase assay, dilution was employed to lower the final detergent concentration.

The use of gel-filtration (molecular exclusion) chromatography was also tried for removal of detergent. With both Sephadex G 25 or Sephadex G 100, the Triton X-100 was not removed from the protein, but rather a protein-detergent micellar complex emerged in the void volume of each gel.

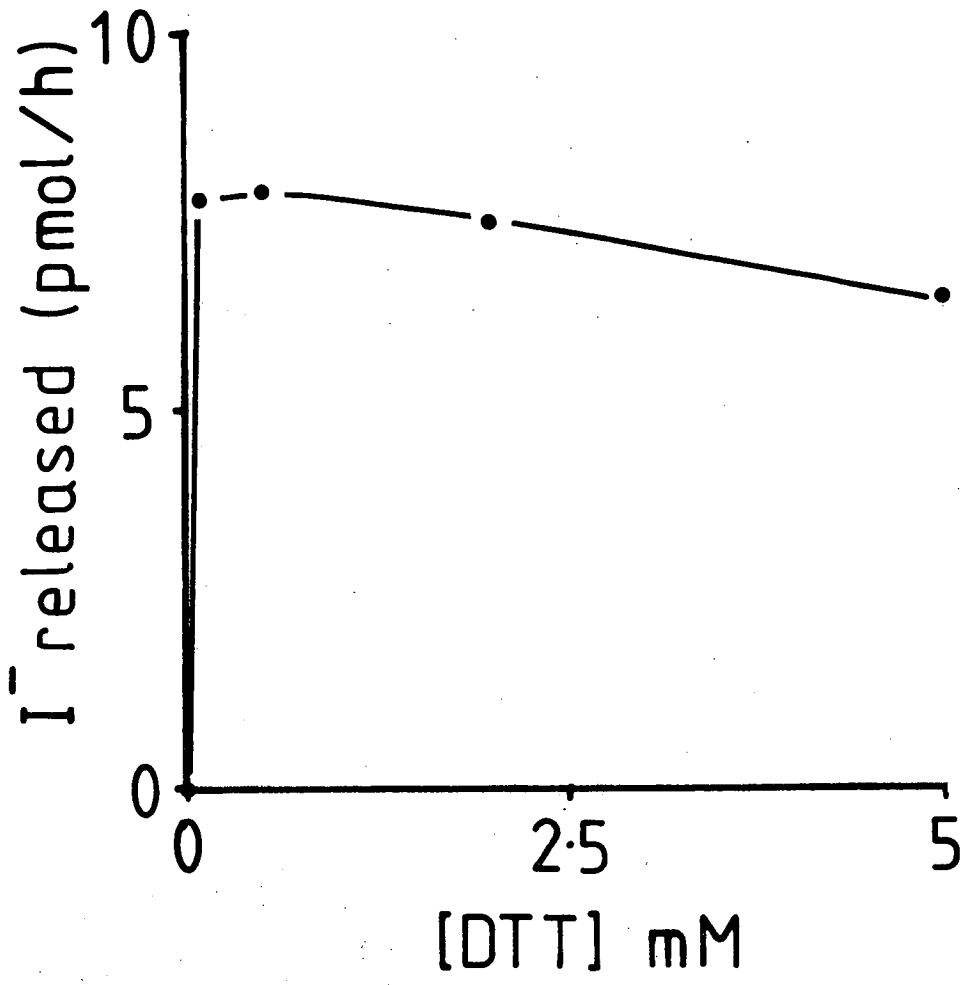


FIGURE 5.3 The Effect of Dithitheitol (DTT) on Microsomal T_4 5'-Deiodinase.

Microsomes were prepared in Sucrose/HEPES/EDTA without DTT pH 7.4 as in Figure 3.3, washed once and then resuspended in the homogenising buffer. Aliquots (1 ml) of the resuspension were taken and DTT added in a constant volume of 25 µl to give the concentrations shown.

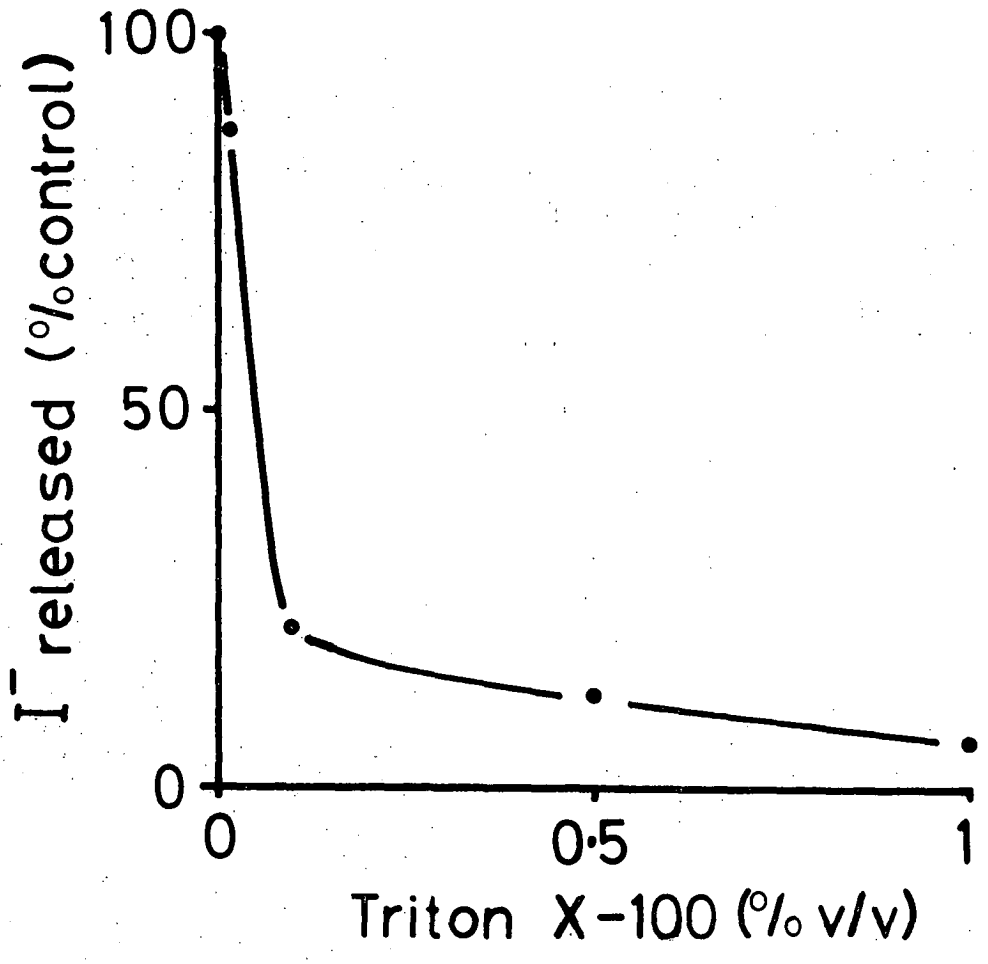


FIGURE 5.4 The Inhibition of Rat Kidney Microsomal 5'-Deiodinase Activity by Triton X-100.

Microsomes were prepared as in Figure 3.3. Aliquots were assayed in duplicate using [3'5'-^{125}I]-thyroxine 10^{-7} M in the presence of Triton X-100.

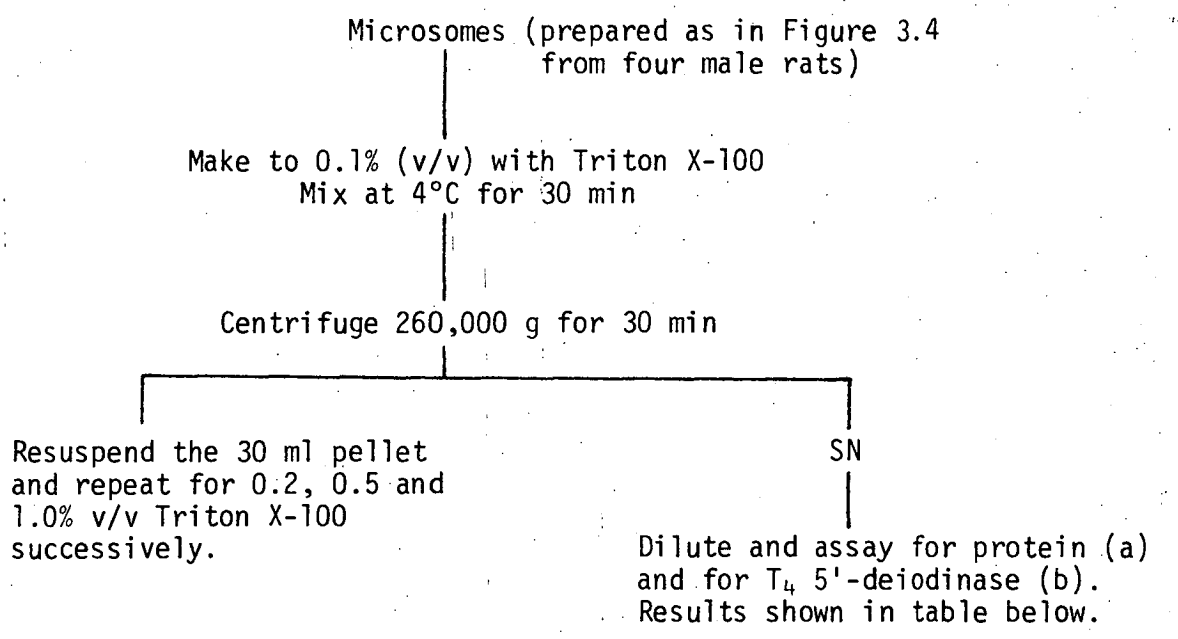
The concentration of Triton X-100 was measured by either UV spectroscopy at 283 nm (Technical Bulletin, Rohm and Haas, Philadelphia, 1967) or by the method of Weber et al. (1964) which employs ammonium cobaltothiocyanate in dichloroethane. The latter method, though less sensitive, can be used after the precipitation of protein which then no longer interferes in the assay, as happens with UV spectroscopy.

The ability of Triton X-100 to solubilise T_4 5'-deiodinase activity is shown in Figure 5.5 which employed the protocol shown in that figure. From Figure 5.5 it is clear that between 0.5 and 1.0% (v/v) Triton X-100 solubilises the T_4 5'-deiodinase activity. The amount solubilised depends not just on the concentration of detergent but also on the detergent to protein ratio (Helenius and Simons, 1975). For this reason an approximate ratio of 5:1 of detergent to protein was always maintained during solubilisation procedures.

The measurement of the pH optimum curve was repeated with the solubilised microsomal activity and is shown in Figure 5.6. The curve is more symetrical with a maximum at approximately pH 7.0, however a smaller maximum is still present at pH 5.5 when compared with Figure 5.1 which has maxima at pH 5.5 and 7.4. Thus the assay of particulate and solubilised microsomal 5'-deiodinase activity was performed at pH 7.4.

The effect of ionic strength was measured on both particulate and solubilised microsomes as shown in Figure 5.7. The results in this figure are not strictly comparable because the solubilised microsomes were diluted to reduce the Triton X-100 concentration after preparation to 0.01% (v/v). This means that there will be an increase in activity of the enzyme because the substrate concentration does not give maximum velocity and the selective solubilisation of the enzyme removes inactive proteins. However it is clear that the activity of the

FIGURE 5.5 Protocol for the Step-Wise Solubilisation of Thyroxine 5'-Deiodinase from Rat Kidney Microsomes.



SOLUBILISATION FRACTION	VOLUME (ml)	PROTEIN (mg)	THYROXINE 5'-DEIODINASE ACTIVITY (pmol/4 hours)
Microsomes in 0.01% Triton X-100	30	96	326
0.1% supernatant	28	83.32	198
0.2% supernatant	31	4.03	79
0.5% supernatant	34	9.52	113
1.0% supernatant	38	1.52	18

Footnotes

(a) Pelley et al. (1976)

(b) butanol extraction.

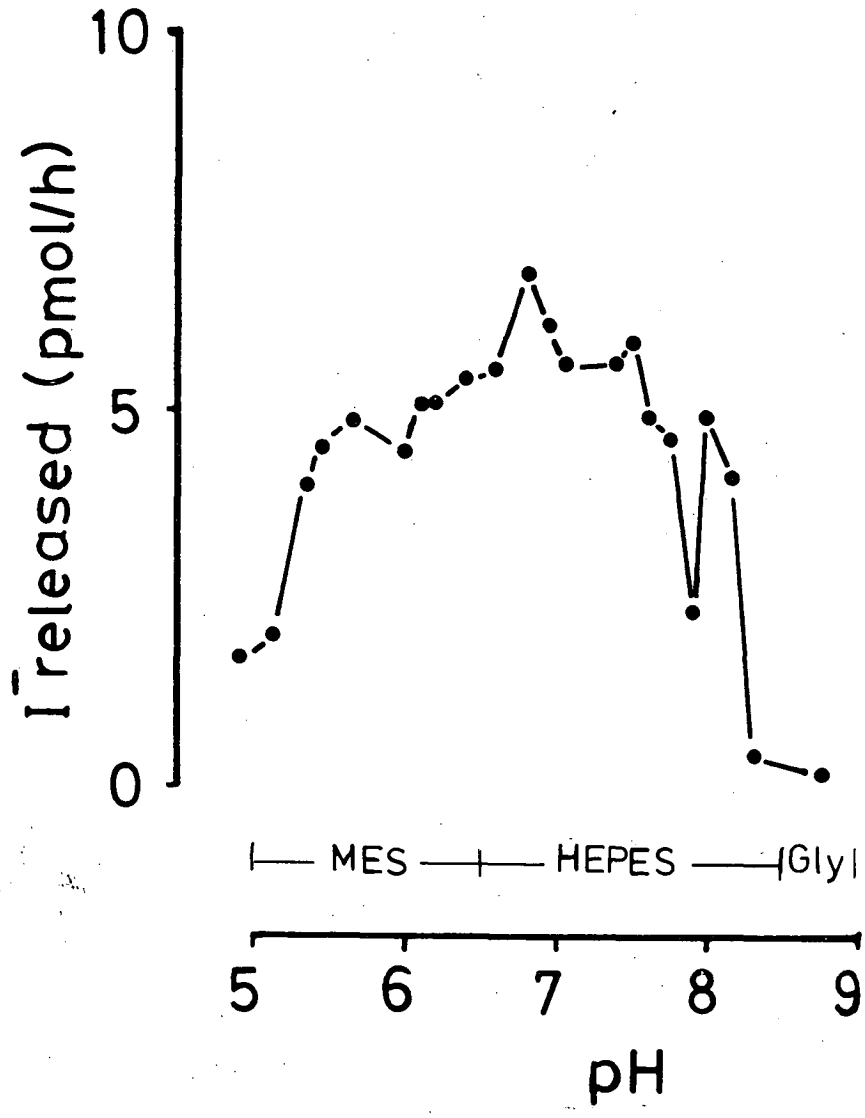


FIGURE 5.6 The Effect of pH on the Solubilised Thyroxine 5'-Deiodinase Activity from Rat Kidney Microsomes.

Solubilised microsomal activity was prepared as described in Section 5.4 using 0.5% Triton X-100 and assayed at different pH after dilution using the protocol in Figure 3.3.

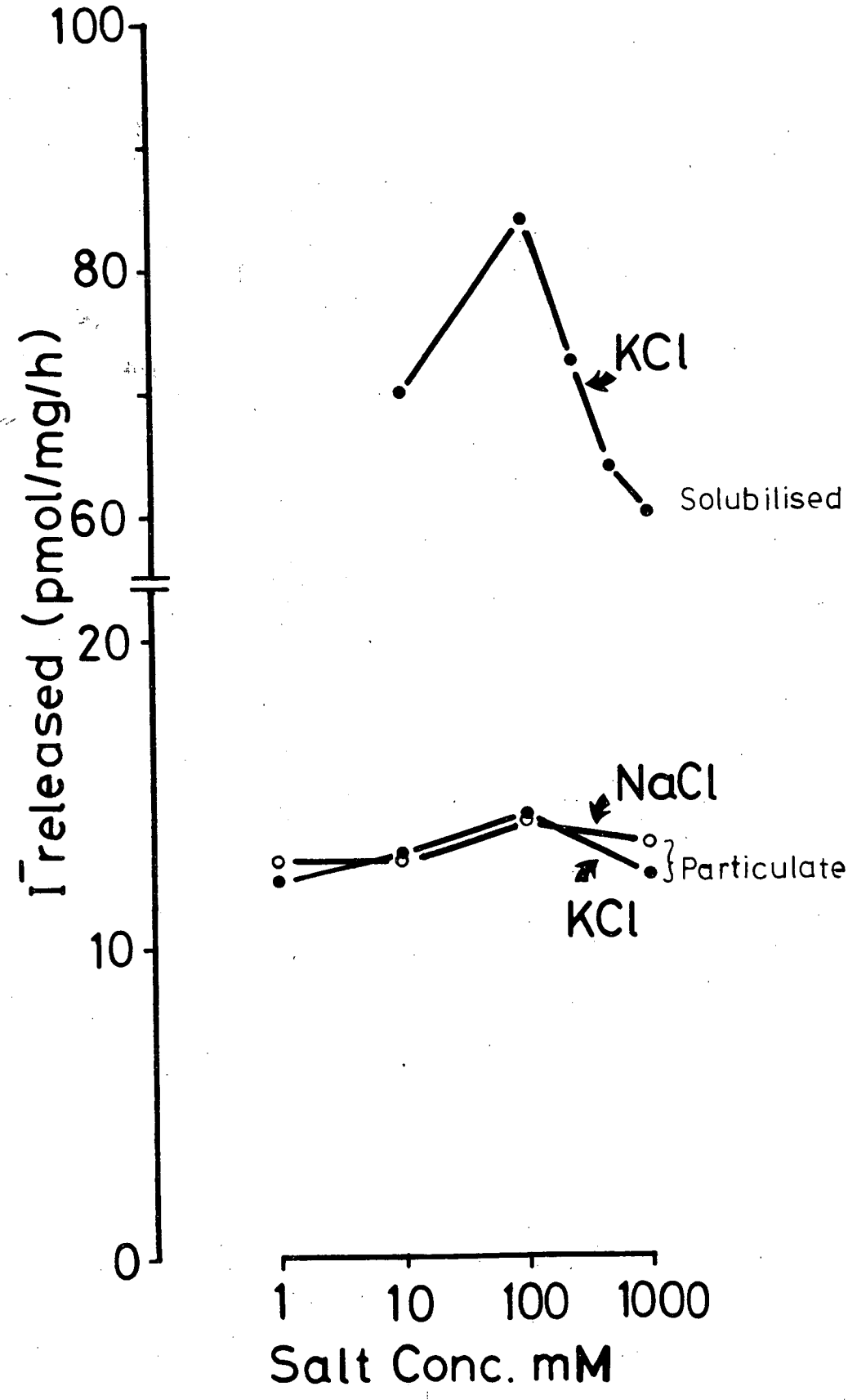


FIGURE 5.7 The Effect of Ionic Strength on the Microsomal T_4 5'-Deiodinase Activity from Rat Kidney.

Microsomes were prepared and incubated with T_4 in the presence of salt. The effect of KCl was also examined on microsomes solubilized in 0.5% (v/v) Triton X-100 and diluted to 0.01% (v/v).

solubilised deiodinase is much more subject to the effects of salt concentration with an optimum of 100 mM.

5.3 Affinity Chromatography of the Thyroxine 5'-Deiodinase from Microsomes

The absorption and desorption of the solubilised microsomal protein and 5'-deiodinase was first investigated under the conditions found suitable for the affinity chromatography of the 5'-deiodinase of cytosol. The elution profile is shown in Figure 5.8. This figure shows that protein and activity were bound to the column at pH 5.5 and both could be eluted by strong KCl solution.

As the increase in specific activity between the load protein and the eluted protein peak was small, other ligands were sought which might give a more specific binding, and hence a higher purification. In this regard various known inhibitors of the 5'-deiodinase were examined for their suitability as ligands on the assumption that their ability to inhibit was dependent on enzyme binding. They were first tested as inhibitors using microsomes and assaying by the butanol method. The results are shown in Figure 5.9. The data in Figure 5.9 agrees closely with that of Chopra et al. (1980) who used RIA to measure the inhibition.

Propylthiouracil and iopanoic acid were selected for further trial because they were the most effective inhibitors and because they have functional amino groups with which to couple to the supporting agarose gel. Their structures and their absorption spectra are shown in Figure 5.10.

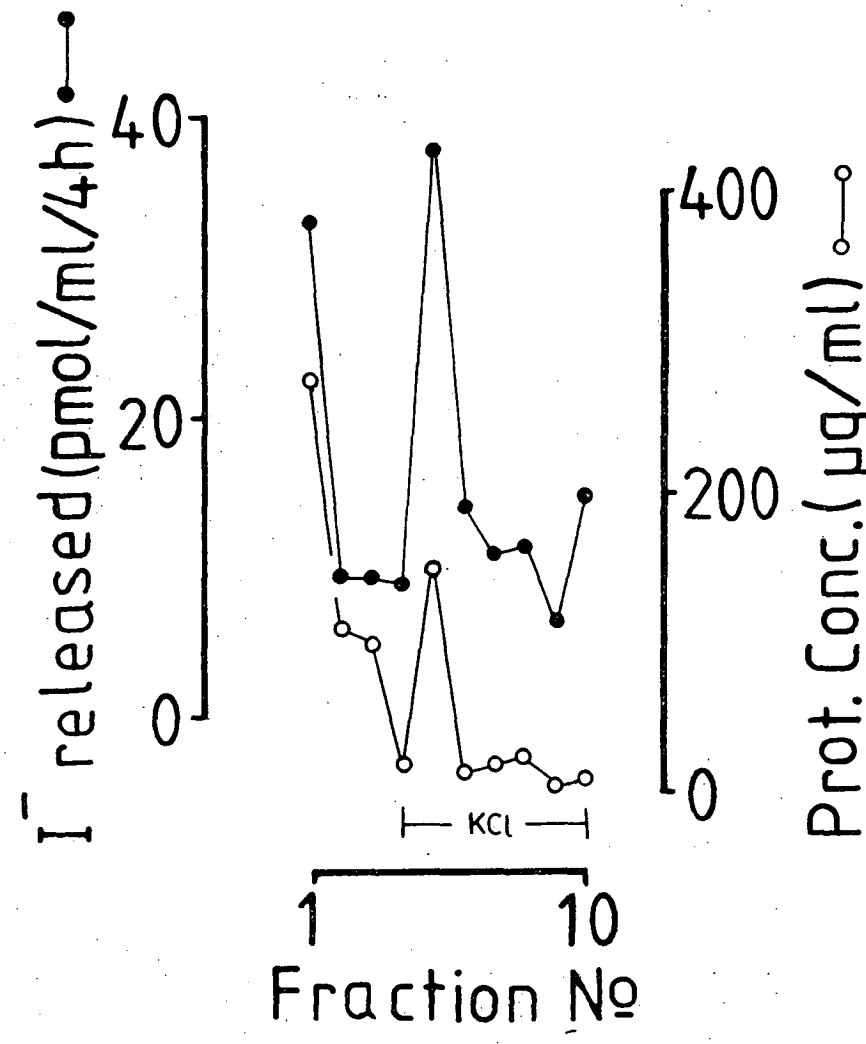


FIGURE 5.8 Affinity Chromatography of Solubilised Microsomes on a Thyroxine-Sepharose 6B Affinity Gel.

Microsomes were prepared and then resuspended in 10 ml of homogenising buffer (Sucrose/KCl/HEPES/EDTA/DTT 250/100/50/15/1 mM at pH 7.4) and made to 0.5% v/v with Triton X-100. After stirring for 1 hour at 4°C the microsomes were centrifuged at 260,000 g for 1 hour and the supernatant was diluted to 0.05% Triton X-100 and applied in a volume of 30 mls to a 1.5 ml T_4-gel affinity column prepared as in Chapter 4 and pre-equilibrated in the same buffer. The column was then washed with MES/EDTA/DTT 25/15/1 mM pH 5.5 containing 0.02% v/v Triton X-100 until no protein could be detected by the 'spot' method of Sedmark and Grossberg (1977) and then eluted by the same MES buffer with the addition of 500 mM KCl. Protein was then measured in duplicate by the method of Sedmark and Grossberg (1977) and T_4 5'-deiodinase activity in duplicate by the butanol method with readjustment of pH to 7.4 and KCl to 100 mM by dilution with HEPES/EDTA/DTT 100/15/1 mM pH 7.4 buffer.

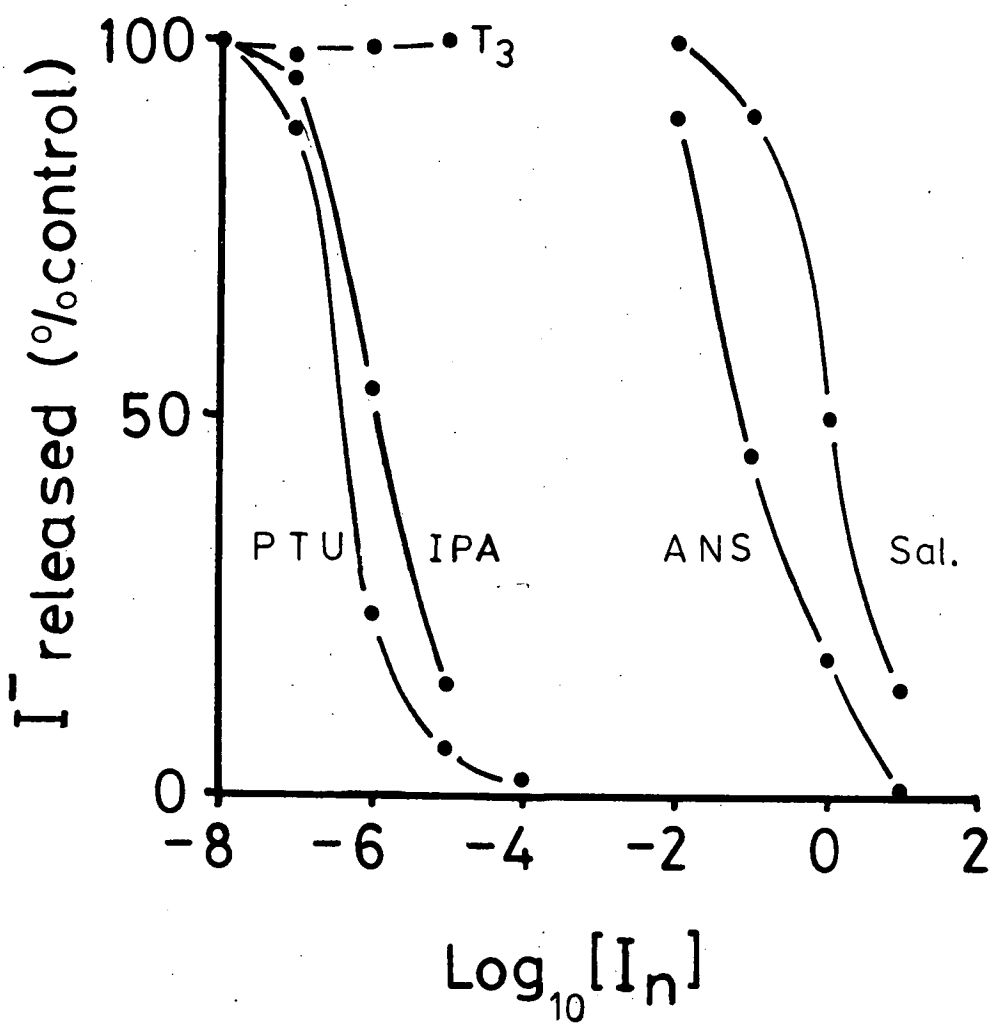


FIGURE 5.9 The Effect on Rat Kidney Microsomal 5'-Deiodinase by Various Inhibitors.

Microsomes were prepared as in Figure 3.4 and assayed in duplicate using 10^{-7} M thyroxine as substrate by the butanol method. Abbreviations: I_n, inhibitor; T_3, 3,5,3' triiodothyroxine; PTU, propylthiouracil; IPA, iopanoic acid (Telepaque-Stirling Winthrop); ANS, 8-anilo-1-napthalene-sulphonic acid, Sal, sodium salicylate.

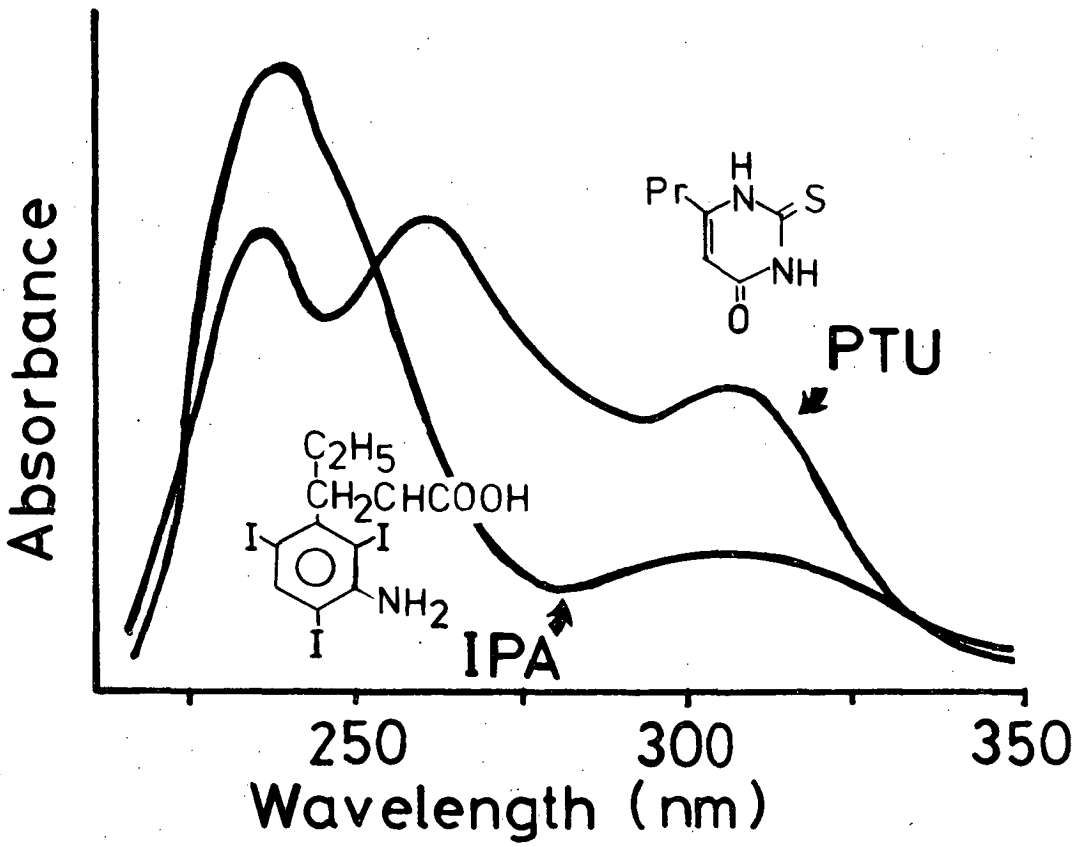


FIGURE 5.10 The Structures and the Absorption Spectra of Iopanoic Acid (IPA) and of Propylthiouracil (PTU).

Spectra were performed on aqueous solutions (7×10^{-7} M) in 1 cm quartz cells using a water blank in a Pye Unicam split beam scanning spectrometer.

The absorption spectra gave several possible wavelengths for spectroscopic assay of PTU and IPA, however it was decided to use the lesser maxima at 315 nm for IPA and 307 nm for PTU in the measurement of coupling to the Sepharose 6B because the other wavelengths could be subject to interference by the absorption maxima of protein (280 nm), or the affinity gel matrix (230 nm). Beer's Law was found to hold in the range 8 to 140 nmol/l.

Iopanoic acid and propylthiouracil were then coupled to epoxy-activated Sepharose 6B (Pharmacia) by the same protocol as used in Chapter 4 but using a reaction time of 42 h at 30°C. Unreacted oxirane groups were again blocked with ethanolamine. A control gel in which all the oxirane groups were reacted with ethanolamine was also made. The amount of ligand bound was determined by suspending a sample of the reaction product in glycerol and was read against standards of IPA or PTU made up in glycerol.

The amounts of each ligand bound were IPA 2.04 µmol/ml gel; PTU 0.55 µmol/ml gel. This compares to T_4 0.39 µmol/ml of gel. Under the reaction conditions it is expected that IPA will bind through its primary amino group, but PTU might bind through either its secondary amino group or through the hydroxyl or sulphydryl groups that a tautomeric isomer of PTU can adopt.

The ability of the four affinity gels: IPA, PTU, T_4 and blank to separate the microsomal T_4 5'-deiodinase was examined in parallel experiments with the same starting material. The data for each column are shown in Table 5.1. The table shows that the IPA-Gel is the best affinity gel as it retains 2-3 fold the activity retained on T_4 or PTU

TABLE 5.1 Comparison of T_4 5'-Deiodinase Purification by Various Affinity Gel Columns.

FRACTION	VOLUME (ml)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (pmol/2 h)	SPECIFIC ACTIVITY (pmol/mg/2 h)
Load Protein	20	4.00	256	64
Ethanolamine (Blank) Gel Eluant	10	1.35	63	47
T_4-Gel Eluant	10	0.65	34	52
PTU-Gel Eluant	10	0.80	38	47
IPA-Gel Eluant	10	0.95	91	96

Microsomes from the kidneys of four rats were prepared and solubilised in 10 ml as before (Sections 5.1, 5.2) and the supernatant prepared by centrifugation at 260,000 g for 1 hour.

The supernatant was then diluted 10 fold, and 20 mls applied to each of the four columns containing 5 ml of their respective affinity gels. The protein was run on, and then the unbound protein eluted by washing with Hepes/EDTA/DTT 25/15/1 mM pH 7.4 buffer containing 0.02% Triton X-100 until the protein in the eluant was undetectable by the spot test of Sedmark and Grossberg (1977). The bound protein was then eluted in a volume of 10 mls by the addition of 500 mM KCl to the elution buffer. Protein and 5'-deiodinase activity was determined as in Figure 5.9.

gels. Interestingly, the ethanolamine gel is superior in its performance to both PTU and T_4 gels but not to the IPA gel.

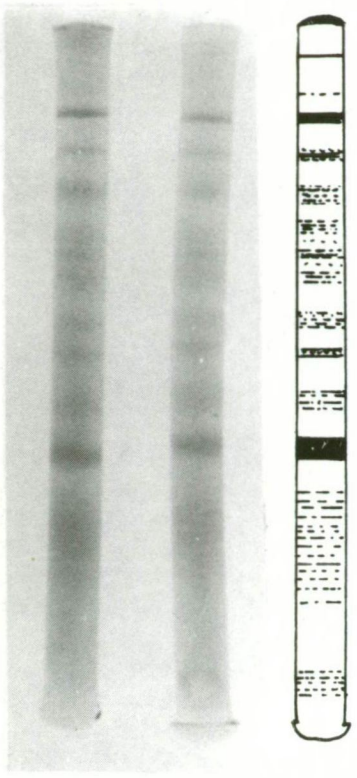
The elution of 5'-deiodinase activity from the IPA-agarose was further examined by the stepped elution with KCl by using 100, 500 and 1000 mM KCl. The method and results are shown in Table 5.2. The table shows that the IPA bound protein is in part further fractionated by elution with differing KCl concentrations. The difference in specific activity suggests that there is still some non-enzymic protein bound to IPA which tends to elute earlier with the lower KCl concentration.

Comparison by PAGE-SDS (Weber et al., 1972) of the proteins from solubilised microsomes and of the proteins in the 100 and 500 mM eluants for the IPA-agarose gel showed a marked reduction in protein bands (see Figure 5.11). Of those proteins found in the eluant, no integral logarithmic relationship was apparent between their molecular weights such as was found with the cytosolic enzyme. The detergent SDS is sufficiently strong to solubilise almost all proteins and displaces Triton X-100.

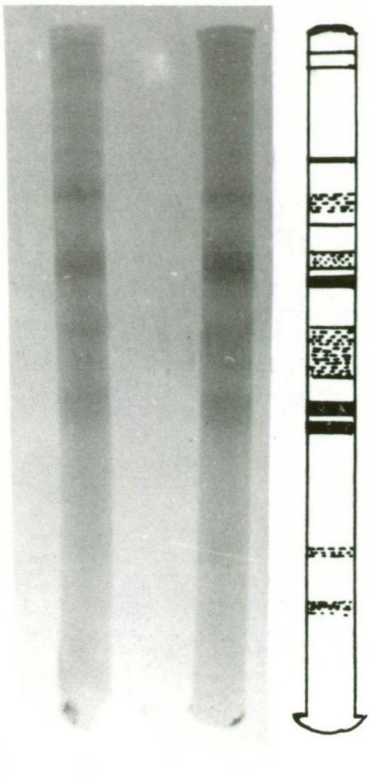
5.4 Discussion and Conclusions

Coleman (1973) has suggested that the membrane proteins can be roughly described as either integral or peripheral. Integral proteins are tightly bound to, or within the membrane and can only be solubilised by disrupting the membrane with detergents or organic solvents. Peripheral proteins are less tightly bound and are solubilised by less drastic measures such as the disruption of homogenisation or by solutions of strong salt.

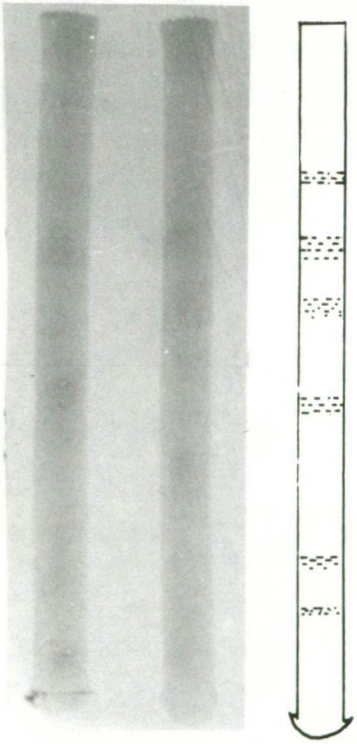
Detergents have been used extensively for the solubilisation of cell membrane components, although a delicate balance often exists



Microsomes(sol.)



100 mM KCl eluant



500 mM KCl eluant

FIGURE 5.11 The Appearance on PAGE-SDS of the Triton X-100 Solubilised Microsomes and of the Protein in the KCl Eluants from the IPA-Affinity Gel.

TABLE 5.2 Elution of Thyroxine 5'-Deiodinase Activity from Iopanoic Acid Affinity Gel.

FRACTION	VOLUME (ml)	PROTEIN (mg)	TOTAL ACTIVITY (pmol/2 h)	SPECIFIC ACTIVITY (pmol/mg/2 h)
Load Protein	50	10.7	704	66
100 mM KCl Eluant	10	500	97	194
500 mM KCl Eluant	10	100	61	610
1000 mM KCl Eluant	10	0	0	0

Microsomes from the kidneys of six rats were prepared as in Sections 5.1 and 5.2. The solubilised microsomal protein (50 ml) was loaded into the column and was treated as in Table 5.1. The bound protein was successively eluted by 100, 500 and 1000 mM KCl in the buffer containing 0.02% Triton X-100.

between solubilisation and denaturation of the protein. In general, Coleman (1973) suggests that the potency for inactivation of proteins by different detergents occurs in the following order:

ionic >> bile salts > non-ionic.

In fact most membrane proteins are denatured by ionic detergents such as sodium dodecyl sulphate (SDS), however the bile salts, the glycoside digitonin and the non-ionic detergents especially, such as Triton X-100 (Rohm and Haas) have often been used successfully to solubilise integral proteins (reviewed by Helenius and Simons, 1975).

Initial studies on solubilising the microsomal 5'-deiodinase were made with Triton X-100 because of: (a) its previous successful use with many other different enzymes and (b) the successful use of affinity chromatography in the presence of Triton X-100 by Dawson and Crane (1974) during the purification of acetylcholine receptor proteins. Triton X-100 is a non-ionic octylphenoxyethanol detergent having a chain length of 9-10 units (Technical Bulletin, Rohm and Haas, Philadelphia).

During solubilisation the detergent binds to membranes at low concentrations resulting in a change in a number of their properties including increases in both membrane area, and in membrane permeability and a reduction in buoyant density (Helenius and Soderlund, 1973). Most membrane bound enzymatic activities are affected in some way or another (Coleman, 1973). As the detergent concentration is further increased, macro-molecules are able to pass through the membrane, a process known as lysis. After lysis of a membrane there is evidence (Deamer, 1973) of increased binding of the detergent to membranes with increasing detergent concentration until a concentration is reached when the membrane begins to disintegrate, i.e. solubilise.

The final step in solubilisation occurs when the Triton X-100 concentration reaches a sufficient value such that separate lipid and protein micelles are formed. These different micelles have been separated by ion exchange chromatography (Jacobs et al., 1966), density gradient centrifugation (Engelman et al., 1967) and also by phase partition separation (Albertson, 1973).

The criteria for solubilisation is operational. It is common for workers to define solubilised material as that which does not form a 100,000 g sediment after 1 h. For the purpose of this thesis, solubility was taken as the inability to form a 260,000 g sediment after 30 min.

The free equilibrium concentration of Triton X-100 has been shown to be below or equal to the CMC* in the presence of membranes (Helenius and Simons, 1974). The extent of solubilisation can best be correlated with the ratio of bound detergent to membrane. As this ratio is often laborious to determine, the effects of the Triton X-100 correlate well with the total detergent/membrane ratio (Helenius and Simons, 1975) if the membrane concentration is relatively high (approximately 2 mg/ml) as was done in this thesis.

Triton X-100, although a useful detergent for membrane solubilisation, is difficult to remove from samples by the usual methods such as dialysis, because of its very low CMC*(0.015, v/v). Triton X-100 has been reported to have been removed from solubilised membranes by gel filtration (Roltem et al., 1968), and Bio-Gel A5m (Loach et al., 1970), but most authors have found that the detergent behaves as a large molecular weight aggregate (approximately 100,000) as was found for the

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* critical micelle concentration.

microsomal T_4 5'-deiodinase and hence these methods are not generally applicable. Bio-beads SM-2 (a neutral, porous styrene-divinylbenzene co-polymer) were used in a small column as a simple, rapid and mild procedure for the removal of Triton X-100 (Holloway, 1973). However in practice it was found that the Bio-beads could remove too much Triton X-100 allowing the protein to come out of solution (Section 5.2).

The solubilised microsomes displayed little change in the pH optimum for T_4 5'deiodinase activity, but they did display a much greater activity in the presence of 100 mM KCl.

Although the purification achieved by affinity chromatography, using iopanoic acid as a ligand, appears modest in terms of the increase in specific activity (3-10 fold) and by the decreased number of bands on PAGE-SDS, it compares quite favourably with the purification achieved by other workers.

Köhrle et al. (1980) solubilised the 5'-deiodinase in deoxycholate followed by either gel filtration or ion-exchange chromatography to separate 5'-deiodinase activity and obtained to give a 1.5 to 2 fold purification. They found no increase in purification using T_4 as an affinity ligand. Fekkes et al. (1980) used polyoxyethylene ether W.1 followed by covalent chromatography using activated thiol-Sepharose to give 3 fold purification. They found no increase in purification using glutathione as an affinity ligand. Both groups found marked variation in the molecular weight of 5'-deiodinase, as judged by molecular exclusion chromatography with different detergents in the mobile phase, indicating variation in the protein-detergent micelle size with the different detergents. Thus affinity chromatography has the advantage of isolating

5'-deiodinase by mechanisms which do not depend on molecular size.

Different workers (see Section 1.2.4.2) have localised the microsomal thyroxine 5'-deiodinase to either or both the plasma membranes and the endoplasmic reticulum of the rat liver and kidney. Therefore it was felt important, to carefully evaluate by the assay methods used in this thesis the plasma membrane and endoplasmic reticulum as sites of the 5'-deiodinase activity because this might allow a better purification prior to affinity chromatography. This work is discussed in Chapter 6.

CHAPTER 6

Further Examination of the Subcellular Sites of 5'-Deiodination

6.1 Introduction

As was discussed in the conclusion to Chapter 5, it was decided to examine both the plasma membrane and endoplasmic reticulum to assess their relative contributions to the microsomal 5'-deiodinase activity using the deiodinase assays established earlier in the thesis employing iodine-125 labelled substrates.

Similarly, the strong likelihood that the soluble 5'-deiodinase found in cytosol after cell fractionation comes from a location other than cytosol implies that it might be coming from an enclosed cell organelle containing soluble enzymes such as the lysosome. Further circumstantial evidence was that the crude mitochondrial fraction had substantial activity (Chapter 3), even though homogenisation in hypotonic medium (Chapter 4) did not increase either the total homogenate or the cytosol activity as is characteristic of the latent soluble enzymes of lysosomes (de Duve, 1965).

Accordingly it was decided to conduct parallel studies both on lysosomes, plasma membranes and endoplasmic reticulum.

The strategy adoped for these studies was firstly, to use highly selective methods for the preparation of cell organelles from the above fractions and secondly, to use marker enzymes which were as unambiguous as possible to identify the cell organelle in each prepared fraction, rather

than depending on centrifugational criteria. On one occasion, mitochondrial and lysosomal fractions were identified by electron microscopy.

6.2 Methods for the Preparation and Identification of Cell Fractions

6.2.1 Preparation of Lysosomes

Lysosomes can be separated from mitochondria and peroxisomes of both liver and kidney cells by utilising the cells' ability to take up and store the detergent Triton WR-1339 in their lysosomes which then leads to a change in lysosomal density (Wattiaux et al., 1963). This observation was employed by Trouet (1964) for the isolation of highly purified lysosomes in high yield from rat liver on a discontinuous sucrose gradient, and by Wattiaux-De Coninck et al., (1965) for the isolation of lysosomes in lower yield but in highly purified form from rat kidney on a continuous sucrose gradient. Such preloaded lysosomes are often called "tritosomes".

The lysosomes prepared by the above methods are easily ruptured by mechanical or osmotic stress so that the method of Trouet (1964) was modified for both liver and kidney lysosomes as follows:

(i) The method of homogenisation was changed from a Potter-Elvehjem device to a tissue press with a plate containing 1 mm holes 2 mm apart followed by 8 passages in a hand held glass Dounce homogeniser.

(ii) The homogenising buffer was Sucrose/TRIS-HCl/KCl/EDTA/DTT 250/100/15/1 pH 7.4 rather than just sucrose 250 mM.

(iii) Chloroquine 2×10^{-5} M was added to all solutions, as it is known to stabilize lysosomal membranes in vitro against rupture (Miller and Smith, 1966).

6.2.2 Preparation of Plasma Membranes and Endoplasmic Reticulum

Plasma membranes were prepared from kidney and liver either by the method of Booth and Kenny (1976) or by the Wisher and Evans (1975) modification of the hypotonic bicarbonate method of Neville (1960). Both methods were modified to use the same homogenisation method and buffer described in Section 6.2.1.

6.2.3 Marker Enzymes

The following enzymes were used as markers for cell components of rat liver and kidney: glucose-6-phosphatase (EC 3.1.3.9) for endoplasmic reticulum was assayed by the method of Swanson (1955) as modified by Evans (1978); 5'-nucleotidase (EC 3.1.3.5) for plasma membrane was assayed by the method of Evans (1978) using AMP as substrate and measuring the liberated phosphate by the method of Le Bel et al. (1978); succinic dehydrogenase (EC 1.3.99.1) for mitochondria (inner membrane) was assayed by the method of Holdsworth (unpublished) in which 0.1 ml of fraction was combined with 0.4 ml of 0.2 M NaH_2PO_4 pH 7.4, 0.3 ml of 0.2 M succinate pH 7.4 and 0.1 ml 1% 3,(4,5 dimethyl thiazole) 2,5 diphenyl tetrazolium bromide (MTT, Sigma), the reaction was stopped after 15 minutes with 1 ml 1% w/v trichloracetic acid and the colour extracted into 4 ml of tetrahydrofuran and read at 550 nm; β galacturonidase (EC 3.2.1.31) and N-acetyl-β-glucosaminidase (EC 3.2.1.30) for lysosomes were assayed by the methods of Gianetto and de Duve (1955) using the p-nitrophenol derivative (Calbiochem) and of Peters et al. (1972) using the 4-methyl umbelliferone derivative (Boehringer) respectively.

N-acetyl-β-glucosaminidase is an almost completely latent lysosomal enzyme, hence its assay has the advantage of allowing assessment of lysosomal integrity (Peters et al., 1975).

6.3 5'-Deiodinase Activity of Lysosomes

Rat liver and kidney homogenates and their lysosomal fractions were prepared after the prior injection of Triton WR-1339. The marker enzymes (section 6.2.3) and the different 5'-deiodinase activities were then determined on these fractions. The methods and the values obtained are shown in Table 6.1 and its caption.

The values in the table show that the yield of lysosomal protein is about 4-5% of homogenate which is slightly greater than normally obtained (Beaufay, 1969). This is probably due to microsomal contamination as shown by the presence of glucose-6-phosphatase and 5'-nucleotidase. However the lysosomal enzymes β galacturonidase and N-acetyl-β-glucosaminidase are selectively increased in this fraction and suggest substantial enrichment of lysosomes. Of great interest is the marked increase in T_4 and rT_3 5'-deiodinase. There is very little increase in T_3 outer-ring deiodination. As the concentration of T_4 is tenfold that of rT_3, the specificity of the lysososomal 5'-deiodinase enzyme appears to be $rT_3 > T_4 > T_3$.

As tritosomes, prepared by the method of Trouet (1964), usually have about 20% contamination of microsomal elements (Beaufay, 1969), it was necessary to ascertain if the rT_3 5'-deiodinase activity was a microsomal contaminant. This was done in the next experiment as shown in the protocol of Figure 6.1 and Table 6.2. At the same time an

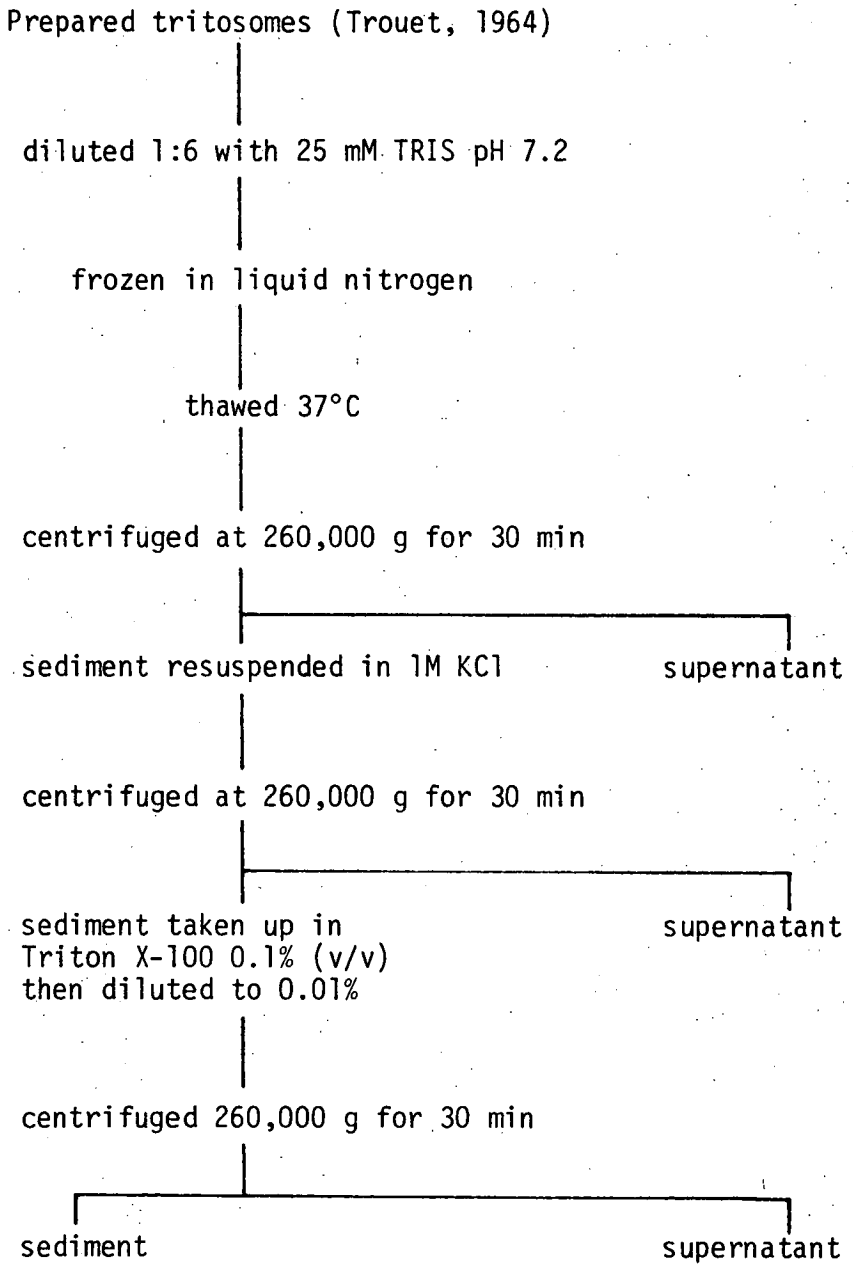
TABLE 6.1 Iodothyronine 5'-Deiodinase and Marker Enzyme Activities of Kidney and of Liver Homogenates and Lysosomes Prepared from Triton WR-1339 Pretreated Rats.

Protein or Enzyme Activity	Liver homogenate	Kidney homogenate	Liver lysosomes[a]	Kidney lysosomes[a]
Protein (mg/ml)	3.1	1.4	0.168	0.058
5'nucleotidase (pmol/mg)	14	28	357	30
Succinic[b] dehydrogenase (units/mg)	10	13	53	0
Glucose-6-phosphatase (pmol/mg)	26	45	173	759
β-galacturonidase (μmol/mg)	27	13	107	35
N-acetyl-β[b] glucosaminidase (units/mg)	2.2	6.1	18	13
T_4-5' deiodinase (pmol/mg)	3.1	11	80	107
rT_3-5' deiodinase (pmol/mg)	3.3	7.3	55	185
T_3-5' deiodinase (pmol/mg)	0.2	0.4	0.8	1.2

[a] lysosomes made to 0.1% then diluted to 0.01% Triton X-100 before assay.
[b] arbitary units.

Two male hooded Wistar rats (180 gm) were pretreated with Triton WR-1339. Homogenates and their lysosomal fractions were prepared from both livers and kidneys as in Section 6.2.1 modified to increase the yields of lysosomes by loading a combined particulate fraction (260,000 g), rather than a heavy mitochondrial fraction, on to the discontinuous sucrose gradient. Marker enzyme activities were determined in duplicate as described in Section 6.2.3 and 5'-deiodinase activities were measured in triplicate by the butanol extraction method using T_4 (10^{-7} M), rT_3 (10^{-8} M) and T_3 (10^{-8} M) as substrates incubated for 1.5 hours. Protein was measured by the method of Sedmark and Grossberg (1977).

FIGURE 6.1 Protocol for Extraction of Lysosomes by KCl and Triton X-100.



Fractions were assayed for both rT_3 5'-deiodinase and N-acetyl-β-glucosaminidase as described in Table 6.2.

TABLE 6.2 Extraction of Lysosomes with KCl and Triton X-100.

Fraction	rT_3 5'-deiodinase[a]		N-acetyl-β-glucosaminidase[a]	
	Liver	Kidney	Liver	Kidney
tritosomes	100	100	100	100
tritosomes after freeze-thaw	135	117	108	128
freeze thaw supernatant	10	7	60	43
KCl supernatant	6	19	23	23
Triton X-100 supernatant	41	84	6	16
Triton X-100 sediment	14	13	2	10

[a] Enzyme activities are the total for each fraction (mean of duplicates) expressed as a percentage of the value for tritosomes. All activities were measured after making each fraction to 0.1% (v/v) Triton X-100 and then diluting to 0.01%.

assessment was made as to whether the enzyme was soluble or membrane-bound.

Table 6.2 shows that most of the N-acetyl-β-glucosaminidase is released by osmotic shock and the single freeze-thaw cycle, although as expected, the kidney lysosomes, because of their smaller size, are more resistant to this process. The lysis is virtually complete after exposure to the strong salt. On the other hand, very little rT_3 5'-deiodinase is released until the membrane fraction is treated with 0.1% Triton X-100. In a parallel experiment, KCl and NaCl (0-500 mM) were found to have no effect on either rT_3 or T_4 5'-deiodinase of tritosomes.

As the use of strong salt removes most of the endoplasmic reticulum contamination from lysosomes (Beaufay, 1969, Thines-Sempoux, 1976), this implies that the rT_3 5'-deiodinase is a membrane bound lysosomal enzyme.

The requirement for dithiothreitol (DTT) as a cofactor for rT_3 5'-deiodinase was examined wtih tritosomes prepared from both liver and kidney in media without DTT. The results in Figure 6.2 show an obligatory requirement with maximum activity for the deiodinase of either tissue at about 10 mM DTT. Consequently subsequent assays were performed at 10 mM DTT rather than at 1 mM DTT.

Since many of the enzymes of lysosomes have pH optima in the acid range, the effect of pH on rT_3 5'-deiodinase was determined. Liver tritosomes were used because of the much higher yield of lysosomal protein from liver. Preliminary experiments showed possible inhibition by various buffer ions such as cacodylate, barbiturate and citrate so that the pH of the incubation media was adjusted using only sodium phosphate buffers which appear not to inhibit the deiodinase. As certain values

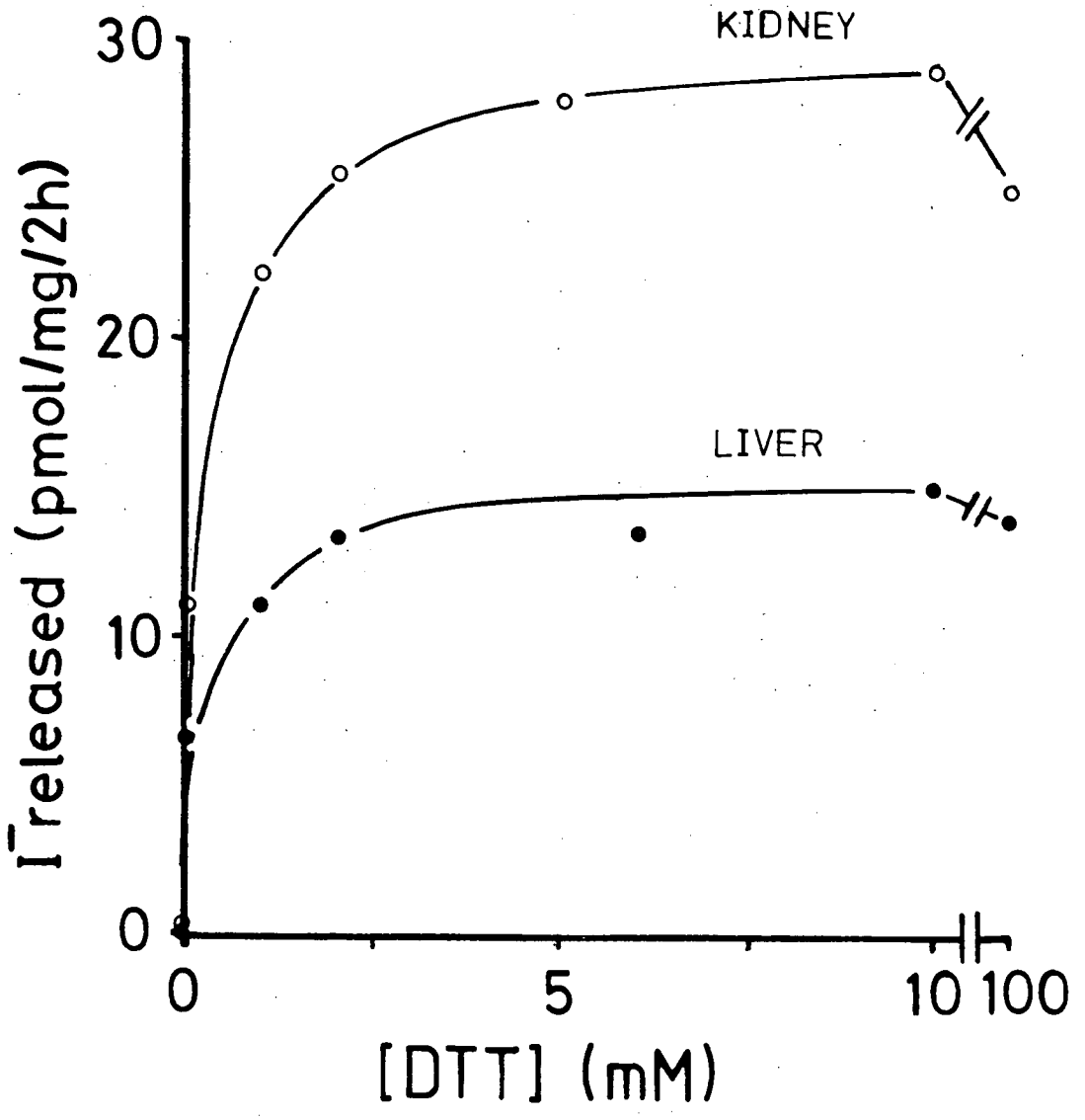


FIGURE 6.2 The Effect of Dithithreitol on Lysosomal 5'-Deiodination of rT_3.

Tritosomes were prepared (section 6.3.2) and made to 0.1% (v/v) Triton X-100 and then aliquots were diluted to 0.01% Triton X-100. At the same time DTT was added to give the final concentrations shown. Reverse T_3 5'-deiodinase was assayed as in Table 6.1.

of pH are more than one pH unit removed from the three pK_a of the phosphate buffers, the pH was measured during the reaction and no change was observed, but as expected, the addition of lysosomal protein did on occasion shift the nominal pH of the buffer. The results and method are shown in Figure 6.3 and its caption.

Figure 6.3 shows a sharp maximum for rT_3 5'-deiodinase at about pH 7.2, with a broader maximum at pH 7.2 for T_4 5'-deiodinase. It also confirms the lack of activity against T_3. Enzyme activities (not shown) were similar with both TRIS and HEPES buffers between pH 7 to 8. Thus subsequent lysosomal 5'-deiodinase activities were performed at pH 7.2 and 37°C.

6.4 The Intracellular Localisation of the Microsomal Thyroxine 5'-Deiodinase

Plasma membranes were prepared by both the modified methods described in Section 6.2.2. From the 7000 g supernatant of the Booth and Kenny method, a 10,000 g (20 min) and then a 260,000 g (30 min) sediment were successively prepared. The latter sediment was taken as the endoplasmic reticulum component of the microsomal fraction. The T_4 5'-deiodinase, 5'-nucleotidase, and glucose-6-phosphatase activities of these fractions were then determined and are shown in Figure 6.4.

Figure 6.4 allows a comparison between the methods of Booth and Kenny (1976) and Wisher and Evans (1975). With the former method, a clear separation of 5'-nucleotidase and glucose-6-phosphatase with the plasma membrane and endoplasmic reticulum fractions was achieved. T_4 5'-deiodinase was present in both fractions but was of higher relative activity in the plasma membrane fraction. The results from the

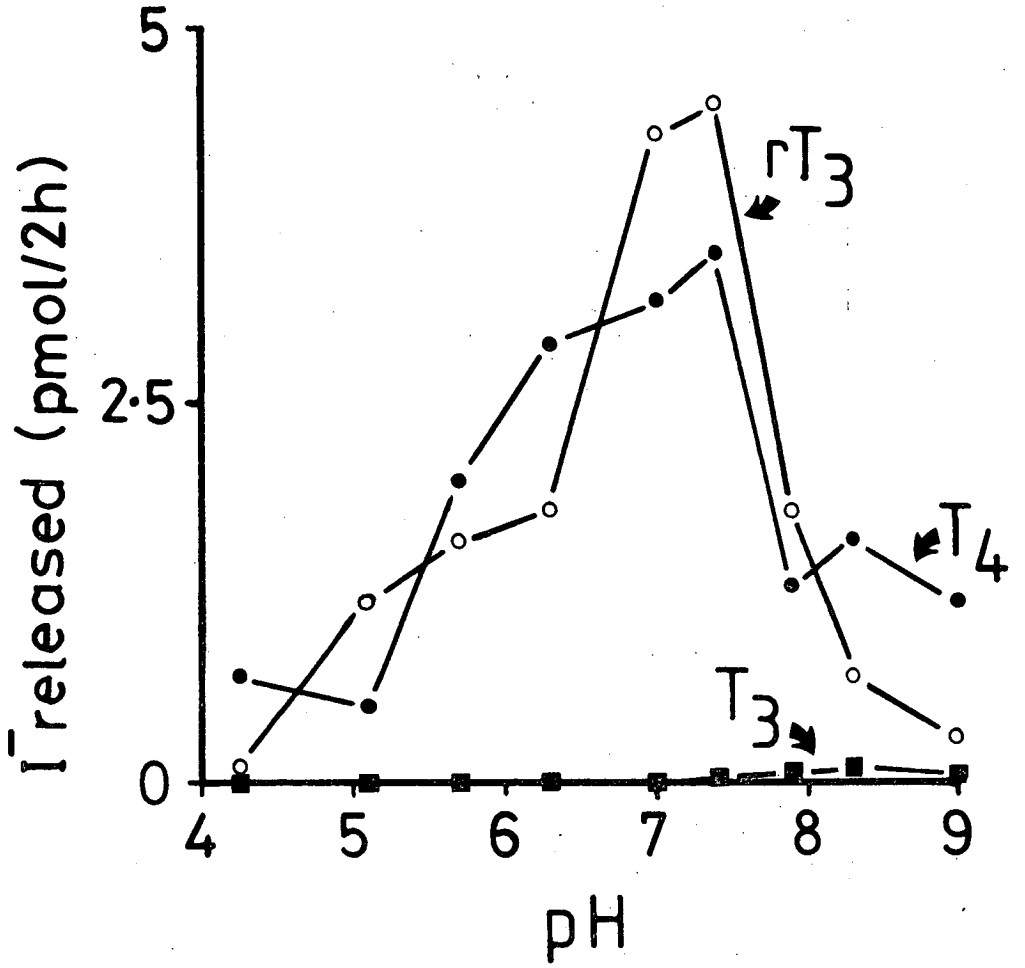


FIGURE 6.3 The Effect of pH on Liver Lysosomal 5'-Deiodination of Iodothyronines.

Tritosomes from liver were prepared as in Section 6.3.1 and assayed for T_4, rT_3 and T_3 5'-deiodinase activity as in Table 6.1. The pH was adjusted by tenfold dilution of tritosomes with the appropriate phosphate buffer (200 mM) and DTT after the addition of 0.1% Triton X-100. The actual pH was measured before and after with a glass pH electrode.

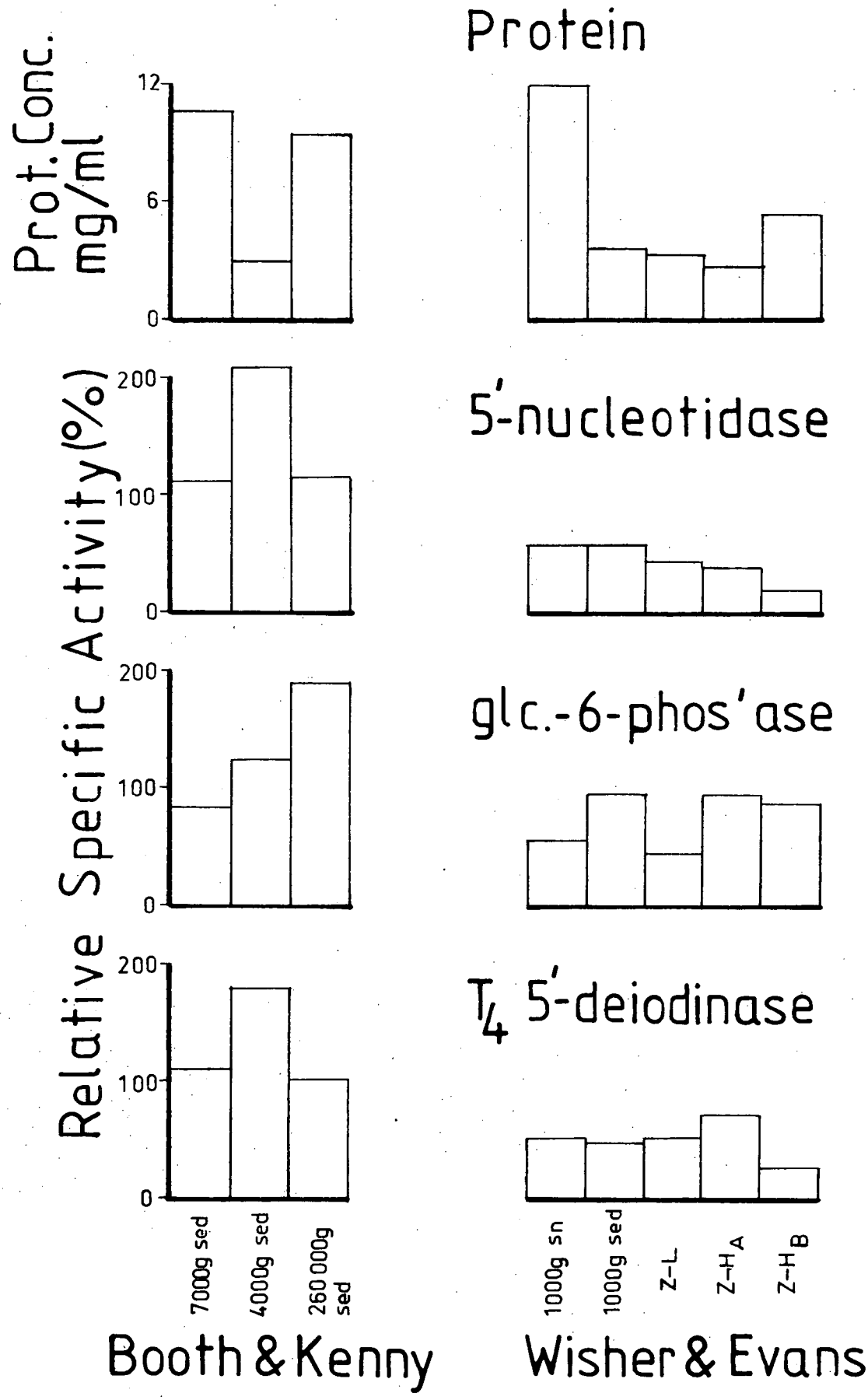


FIGURE 6.4 Enzyme Activities in Microsomal Fractions of Rat Liver. Enzyme activities were measured as described in the text.

fractionation by the Wisher and Evans method were less differentiated and show that T_4 5'-deiodinase was spread into the two poorly separated 5'-nucleotidase and glucose-6-phosphatase fractions.

6.5 Iodothyronine 5'-Deiodinases of Microsomes

In view of the known rapid deiodination of reverse T_3 by microsomes (Chapter 1), the 5'-deiodinase activities of the plasma membrane and endoplasmic reticulum fractions were measured using rT_3, T_3 and T_4. Marker enzyme activities were also determined.

The method and results are shown in Table 6.3 and its caption. It can be seen from the table that both fractions had rT_3 and T_4 5'-deiodinase activities with very little T_3 5'-deiodinase activity. The order of specificity of the 5'-deiodinases for the substrates is $rT_3 > T_4 > T_3$ which is similar to the order for lysosomal 5'-deiodinase activity.

6.6 Kinetic Analysis of the Iodothyronine Deiodinase Activities of Rat Liver

The uncertainty in assessing both the specific activities and the substrate specificities when using non-saturating substrate concentration was brought out after a kinetic analysis in which the K_m^{APP} and V_{max}^{APP} and K_{cat}^{APP} were determined for each substrate and enzyme source. This method and results are shown in Figure 6.5 and its caption. The calculated values for K_m^{APP} and K_{cat}^{APP} are shown in Table 6.4.

Figure 6.5 shows the initial enzyme velocities for each enzyme source. The data for T_3 and T_4 5'-deiodinase is only approximate because

TABLE 6.3 Iodothyronine 5'-Deiodinase and Marker Enzyme Activities of Rat Liver Homogenate and Microsome Fractions.

Enzyme	Homogenate (100 ml)	Plasma Membranes[a] (25 ml)	Endoplasmic Reticulum[b] (25 ml)
Protein (mg/ml)	1.55	1.20	1.60
5'-nucleotidase (pmol/mg)	21	26	18
Succinic dehydrogenase[c] (units/mg)	21	0	42
Glucose-6-phosphatase (pmol/mg)	16	30	65
β-galacturonidase (μmol/mg)	29	24	38
N-acetyl-β-glucosaminidase[c] (units/mg)	26	23	16
T_4 5'-deiodinase (pmol/mg)	17	25	13
rT_3 5'-deiodinase (pmol/mg)	12	14	13
T_3 5'-deiodinase (pmol/mg)	1.2	1.5	1.1

[a] 7,000 g sediment

[b] 260,000 g sediment

[c] arbitary units

Protein and enzyme activities were determined as in Table 6.1. The plasma membrane and endoplasmic reticulum fractions were prepared by the modified method of Booth and Kenny (1976) as described in section 6.4, except that the 7,000 g sediment was not recentrifuged at 4,000 g.

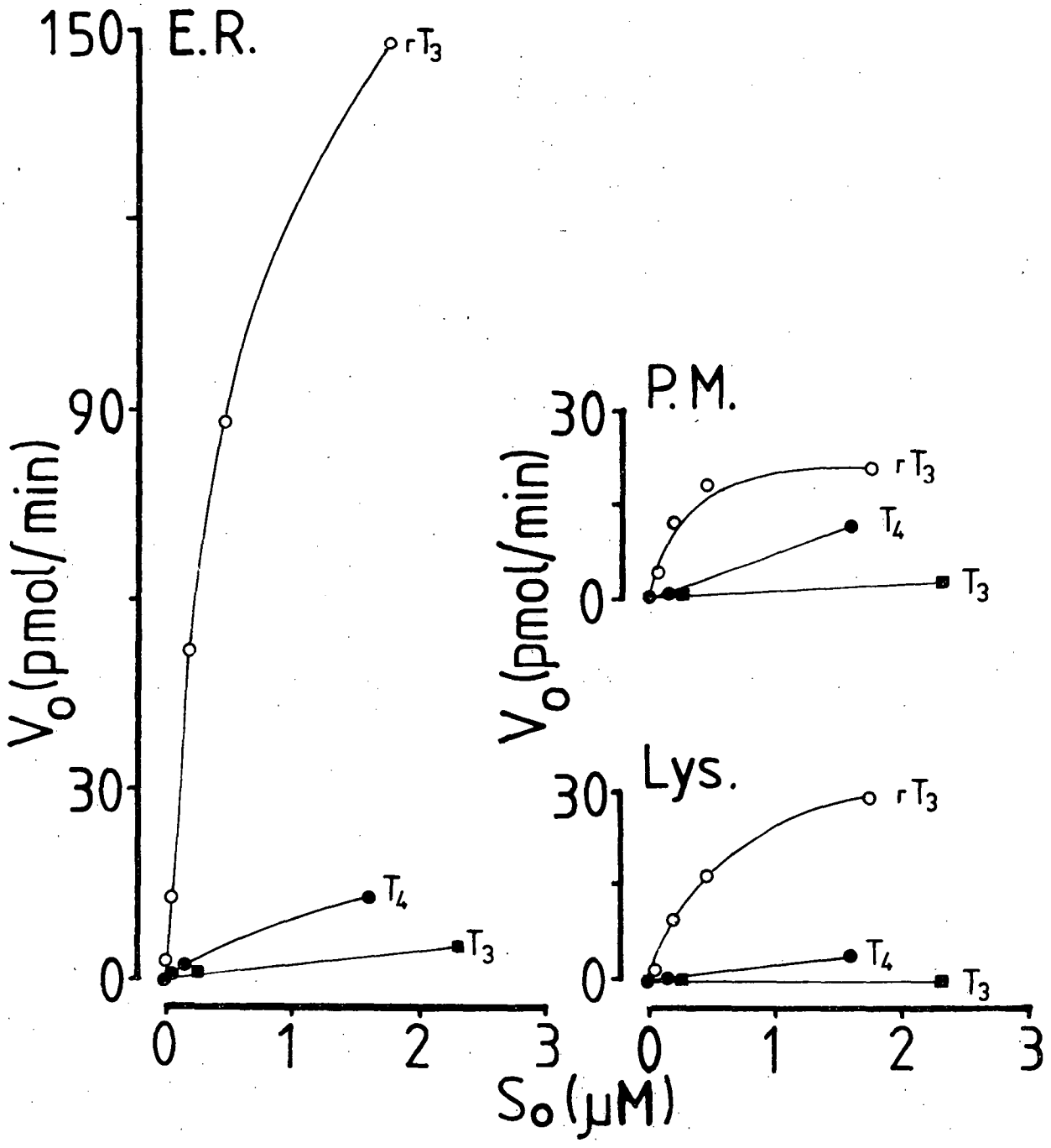


FIGURE 6.5 Initial Velocities of the 5'-Deiodinase Activities Found in Different Cell Fractions of Rat Liver.

Cell fractions (endoplasmic reticulum, plasma membranes from the same homogenate) and tritosomes (from preinjected rats with Triton WR-1339) were prepared from rat liver as in sections 6.2.1 and 6.2.2. Protein were measured by Sedmark and Grossberg (1978). 5'-deiodinase activity was measured in the presence of 0.01% (v/v) Triton X-100 and 10 mM DTT. Samples of enzyme preparation pre-warmed at 37° for 5 min and then added to the radioactive substrate to give the final concentrations shown. For each of the substrates there was approximately 40,000 cpm/ml. At measured intervals (0, 1, 2, 5, 10, 20 mins for rT_3 and 0, 5, 10, 15, 30, 60 mins for T_4, T_3) duplicate 0.5 ml aliquots were removed and added immediately to TCA and the liberated iodide measured by butanol extraction. Initial velocities were determined by drawing tangents to the curve at the origin.

TABLE 6.4 Apparent Kinetic Constants for Iodothyronine 5'-Deiodinases of Rat Liver.

Substrate	Kinetic Parameters	Plasma Membrane	Endoplasmic Reticulum	Lysosomes
	Vol(ml)	200	200	200
rT_3	Protein conc.(mg/ml)	0.3	0.3	0.09
	K_m^{APP} µmol/l	0.22	0.57	0.53
	V_{max}^{APP} pmol/min/ml	12.7	94	18.8
	K_{cat}^{APP} pmol/min/mg	42	313	208
	Vol(ml)	200	200	200
T_4	Protein conc.(mg/ml)	0.3	0.3	0.09
	K_m^{APP} µmol/l	0.24	1.9	0.5
	V_{max}^{APP} pmol/min/ml	2.4	11.5	5
	K_{cat}^{APP} pmol/min/mg	8	38	56

their much lower deiodination rates made the measurement of the initial velocities difficult. As the specific activity was different at each concentration the expected statistical error was also different, therefore K_m^{APP} and V_{max}^{APP} were determined by the direct plot method of Eisenthal and Cornish-Bowden (1974) which is less affected by the variable expected error. The calculated values are tabulated in Table 6.4. Values for T_3 are not shown because of the very low activity and associated large error in the measurements.

6.7 The Use of HPLC in the Assay of Deiodination

As a qualitative study, the experiment in Figure 6.5 was repeated but the timed aliquots were added to four volumes of ice-cold ethanol to which had been added 4 µg of isopropyl diiodothyronine as an internal standard. ^{125}I-labelled tracer was omitted. Iodothyronines were then extracted as a group by the ion-exchange method (Section 2.2) and taken to dryness under air at 56°C. Samples were then redissolved in 120 µl of mobile phase, of which 100 µl was injected into the HPLC system described in Section 2.4.

Two important problems emerged with this qualitative HPLC assay of the iodothyronine products of deiodination. Firstly, the modified method of Tajuddin and Elfbaum (1973) described in Section 2.2 extracts a band of strongly U.V. absorbing material from endoplasmic reticulum, plasma membrane and cytosol which elutes early in the chromatogram and which thus obscures the presence of mono- and di-iodothyronines. With assay of lysosomal deiodinase activity only the mono-iodothyronines were obscured. See Figure 6.6.

Thus with the present methods, the use of HPLC was generally only of use to give a qualitative indication of the deiodination products.

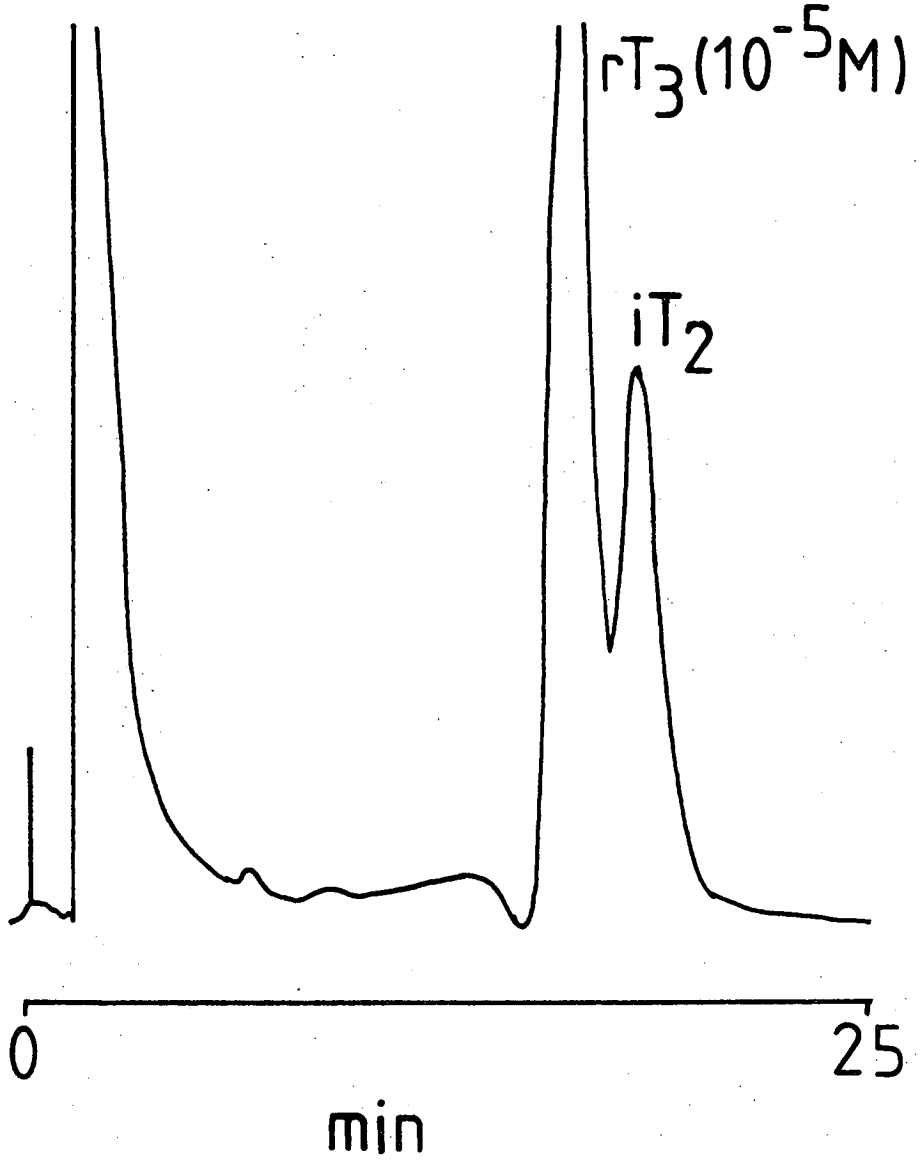


FIGURE 6.6 HPLC appearance of lysosomal extract after incubation with rT_3.

When a combination of highly active deiodinase and substrate could be studied, as with the lysosomal enzyme and rT_3, then quantitative results could be obtained.

Figure 6.7 shows a comparison between the measurement by HPLC of the release of 3,3'-T_2 from rT_3 and of the measurement of the release of I^- from rT_3 by ion-exchange assay in the same experiment. No other iodothyronine was detected by HPLC. As the correspondence between I^- and 3,3'T_2 is very nearly one to one, it indicates that no other product is formed over the 20 minute period of incubation.

Observation and kinetic analysis of both curves in Figure 6.7 suggests that substrate inhibition occurs at higher substrate concentrations. Analysis of both sets of data by the method of Eisenthal and Cornish-Bowden (1974) gives values for K_m^{APP} of 0.54 μM, which agrees closely with the value of 0.53 μM obtained in Table 6.4.

6.8 Discussion and Conclusions

The heterogeneity of the various cell fractions as judged by their marker enzymes activities needs to be recognized, but nevertheless, a substantial separation of endoplasmic reticulum and plasma membranes from the same homogenate was achieved using the method of Booth and Kenny (1976). A very substantial purification of lysosomes (tritosomes) was also achieved. However there was still residual contamination of the lysosomes as judged by the marker enzymes indicative of plasma membrane and endoplasmic reticulum. This is in agreement with Beaufay (1969) and more recently with Waltiaux et al. (1978). These authors prepared highly purified normal lysosomes in a metrizamide gradient and still

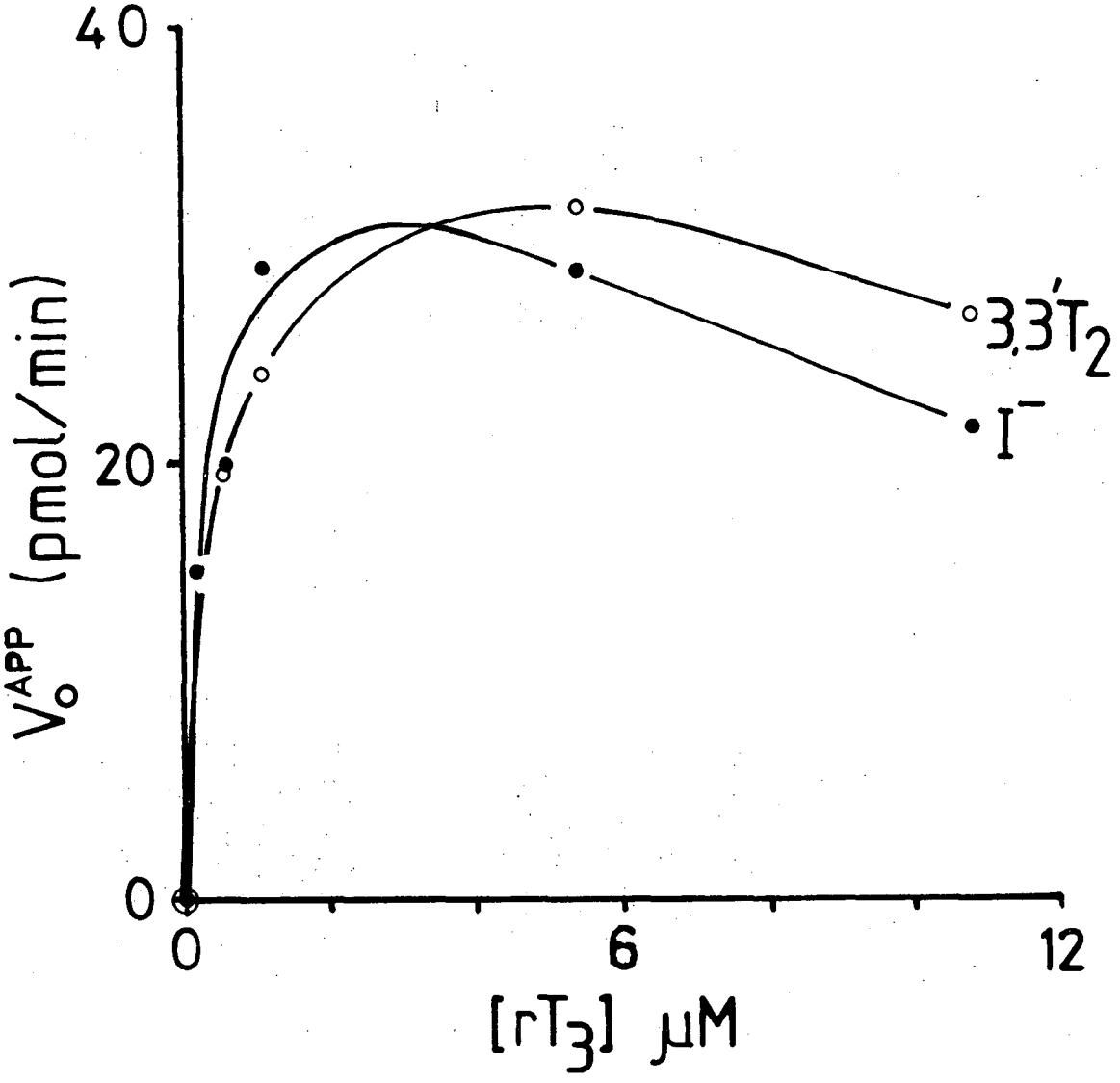


FIGURE 6.7 Comparison of the Measurement of Initial Velocity by Ion Exchange ($^{125}I^-$) and HPLC (3,3'-T_2).

Lysosomal protein was incubated with rT_3 including [3'5'-^{125}I] - rT_3 as tracer by the method described in Figure 6.5. The iodothyronines and iodide were extracted and assayed by ion exchange and HPLC as described in Sections 2.2 and 2.4.

found significant plasma membrane markers (e.g. 5'-nucleotidase) present. This might well be explained by the normal endocytotic transfer of plasma membrane to lysosomes.

In the presence of saturating concentrations of dithiothreitol, the data obtained in Figure 6.3 and Table 6.5 suggests that the 5'-deiodinase activities from each of the three sites in the liver cell deiodinates the substrates tested in the following order: $rT_3 > T_4 > T_3$.

The presence of Triton X-100 alters the relative activities of plasma (PM) membrane and endoplasmic reticulum (ER). Before detergent: PM > ER for T_4 and rT_3, after detergent: ER > PM for T_4 and rT_3. The much lower K_m^{APP} (after detergent) suggests that the PM maybe more active at lower concentrations of T_4 and rT_3 (which may also be more in the physiological range) than the ER.

The activation of ER enzyme activities by detergents is well known (reviewed by Evans, 1978). In this particular case, activation may occur for two reasons: firstly, by solubilising the enzyme and thus allowing free access of substrate from any direction; secondly, by an increase in the activity in solution of the poorly soluble substrates which bind non-specifically to protein and glass.

Thus on the basis of centrifugation and marker enzymes and of the different kinetic parameters, there appear to be at least three different 5'-deiodinase activities in rat liver. Whether the different sites represent the one enzyme at a different site, or different isoenzymes, or quite different enzymes of different protein composition is not obvious and might not be known until each of the activities is purified and their properties and composition determined. The interaction of these enzymic sites is further discussed in Chapter 7.

The activation of enzyme activity by detergent in plasma membrane (PM) and endoplasmic reticulum (ER), suggests that much of the activity is latent. This latency may explain in part the differing conclusions of Leonard and Rosenberg (1978) who found a PM site for T_4 5'-deiodinase in kidney, whereas Maciel et al. (1979) found both a PM and ER site, and Fekkes et al. (1979) and Auf dem Brinke et al. (1979) who found only an ER site in liver.

The identification of a lysosomal 5'-deiodinase has not previously been made. Although Auf dem Brinke et al. (1979), using the Leighton et al. (1968) modification of Trouet (1964) technique to prepare lysosomes by preloading with Triton WR-1339, did suggest that there might be a T_4 5'-deiodinase associated with lysosomes. The definite presence of a highly active rT_3 5'-deiodinase remained undetected as the use of rT_3 as a substrate was not examinedby these authors.

Thus the work in this chapter agrees with that of Maciel et al. (1979) in that there is 5'-deiodinase activity in both endoplasmic reticulum and plasma membranes, but also finds a membranous 5'-deiodinase activity in the lysosomes of the liver cell.

CHAPTER 7

Discussion and Summary

7.1 Introduction

In this chapter, the experimental techniques which were employed in this thesis are critically reviewed. The results obtained from these techniques are then integrated into a model which describes the metabolism of the iodothyronines by the liver and kidney during stress and starvation. The model makes several predictions which could be tested by experiment.

7.2 Review of Analytical Methods

7.2.1 Butanol Extraction

The use of ^{125}I-labelled substrates and their subsequent extraction by the butanol/petroleum spirit mixture has proved to be a reliable and rapid means for the assay of 5'-deiodination by broken cell systems *in vitro*.

The assay does however have two disadvantages. The first is that it measures only iodide which is only one of the reaction products. Thus it is unable to distinguish how many deiodination steps occur. The use of substrate labelled with iodine-125 in one ring reduces the number of possible reactions.

The second disadvantage is that the accuracy of the technique is dependent on the radioactive specific activity. This means that when large concentrations are used, in kinetic analysis, and a fixed amount of radioactive tracer is used for each concentration, the

accuracy of measurement of initial velocity (V_0) decreases. This is compensated in part by the V_0 remaining constant for a much longer period of time so the disadvantage is not overly restrictive.

The need for the addition of a fixed amount of tracer is mostly a problem of the financial restraints imposed by a limited research budget. It does also have an experimental justification, in that the amount of ethanol or propanol solvent for the tracer is kept well below values which will inhibit the deiodinase reactions.

The measurement of the release of $^{125}I^-$ from labelled substrates has the advantage of being more easily applied to study in vitro deiodinase reactions than radio immuno assay (RIA). This is because no antibody is required and the response metameter is linear and not sigmoidal as it is in RIA (Ekins et al., 1967).

By suitable labelling of substrates, most of the deiodinating pathways shown in Figure 1.2 could be monitored by the butanol extraction. At present however, only T_4, rT_3 and T_3 labelled in the outer or phenolic ring are commercially available.

The use of concentrations of substrate (i.e. T_4 10^{-7} M, T_3 and rT_3 10^{-8} M) which were close to physiological values not only indicated physiologically relevant deiodinase activities but ensured the use of high specific activity of tracer. This allowed consistent determinations of deiodination by gamma counting.

The technique also allowed 5'-deiodination of cytosol to be detected by the I^- produced, even though the iodothyronine intermediates were short-lived.

7.2.2 HPLC

The use of HPLC to separate the iodothyronines after their preliminary ethanolic extraction and their isolation as a group on AG50W-X2 sulphonic ion-exchange resin has complemented the butanol technique.

HPLC with detection at 254 nm using a mercury lamp light source, has provided confirmation that the main iodothyronine deiodination product catalysed by lysosomes of rT_3, is 3,3'-T_2 and that further deiodination does not occur within the 20 minute period of the assay. However U.V. detection has not yet been useful in measuring the deiodination products of T_4 because of the much slower deiodination and because of the strongly absorbing band of interference which obscures the mono- and di-iodothyronines when ethanolic extracts are made from the tissue fractions.

As the substances present in the absorbing band migrate very early in the chromatogram they are either more polar or of smaller molecular weight than the iodothyronines. This means that potentially a preliminary group separation on a polar stationary phase such as silica or alumina might eventually prove successful. Preliminary separation by paper chromatography or by thin layer chromatography on cellulose or silica was not successful because of the intrinsic U.V. contaminating substances in these phases. Prolonged washing with solvents removed most, but not all contaminants.

When iodine-125 labelled substrates were used, a fraction collector was used to collect, identify and measure each band. However the collection into tubes blurs the separation of the

difficult pairs viz: T_4/rT_3, 3'5'-T_2/3,3'-T_2 and 3'-T_1/3-T_1. As well, the technique is very slow because of the need to count 120 fractions for each chromatogram. Despite these difficulties, the fraction collection technique was used to show that the deiodination of T_4 by cytosol proceeds via either or both 3,3'-T_2 or 3',5'-T_2 and either or both 3-T_1 and 3'-T_1. Cytosol had been previously thought to completely deiodinate T_4 without producing measurable quantities of any of the lesser iodothyronines (Colquhoun et al., 1980).

Thus as the cytosol deiodinates rT_3 much more rapidly than T_3 (Chapter 3), and Cavalieri et al. (1977) have reported 5-deiodination of T_4 to rT_3 by cytosol, the most likely route for T_4 deiodination by cytosol is firstly 5-deiodination to rT_3 then 5'-deiodination to 3,3'-T_2 with further deiodination to 3 or 3'-T_1.

In conclusion, the separations of iodothyronines achieved by HPLC must be considered to be far superior than the means of their detection yet available.

7.3 The Purification of Deiodinase Enzymes

Several authors (e.g. Köhrle et al., 1980; Fekkes et al., 1980) have experienced difficulty in attempting to purify microsomal deiodinase enzymes. The thiol covalent gel chromatography technique of Fekkes et al. (1980) which gave a three-fold increase in activity appears to be the most successful technique previously published.

Similar difficulties in purifying the microsomal enzymes were encountered during the work described in this thesis. Nevertheless, the successful use of affinity chromatography as a purification step for the deiodinase enzymes has not been reported by other workers (reviewed in Chapter 5).

The use of affinity chromatography in the purification of the deiodinases is therefore a significant experimental advance and suggests that ligands other than those already used might provide better purifications in the future.

The successful use of thyroxine (which is a substrate) for the cytosol 5'-deiodinase suggests that reverse T_3 might be a more successful ligand for the purification of all of the 5'-deiodinases as all of the 5'-deiodinases tested have a greater activity towards rT_3 as a substrate.

Reasoning in a similar manner, the successful use of the 5'-deiodinase inhibitor iopanoic acid, suggests that other inhibitors, e.g. iodipate, may be of use, especially for the purification of the endoplasmic reticulum and plasma membrane enzymes.

Another group of ligands which may be of use in affinity chromatography of the deiodinases, are the thyromimetic analogues, e.g. isopropyl-diiodothyronine, which bind strongly to thyroid hormone receptors and by analogy may well bind to the deiodinase enzymes.

The use of thyroxine as the ligand in affinity chromatography, has shown that its coupling to the support matrix prevents its deiodination by the enzymes in both cytosol and microsomes. This indicates that the normal binding of the amino group near the active centre is an essential prerequisite for deiodination. Furthermore, other parts of the thyroxine molecule must bind to the enzyme for it to be retained on the column. This raises the possibility that coupling of T_4 or other ligand to the matrix via a different group than the amino group used in this thesis, may provide an improved retention of the membrane bound 5'-deiodinases and hence an improved purification.

That binding of different deiodinase enzymes to a ligand can be different, is shown by the substantial purification of the cytosol 5'-deiodinase activity using T_4 as the ligand. This degree of purification was not obtained with the microsomal activity even though a substantial amount of deiodinase activity bound to the ligand.

7.4 The Number, Location and Function of the 5'-Deiodinase Enzymes

The work described in this thesis clearly suggests three of possibly four different subcellular sites in rat liver and kidney cells which have 5'-deiodinase activities. They are the plasma membrane, the endoplasmic reticulum, the lysosomes and perhaps the cytosol.

As discussed in Chapter 4, there is suggestive evidence that *in vivo*, the cytosol activity may normally reside elsewhere in the cell and that it enters the cytosol during the homogenising process.

On the other hand, the properties of the cytosol 5'-deiodinase enzyme are in part different to those of the membrane bound enzymes. Thus it is not yet possible to nominate the putative site of origin for the cytosol enzyme.

Previous research had found deiodinase activity in the cytosol. Cavalieri *et al.* (1977) found a T_4 5-deiodinase which produced rT_3 although its activity was low. Sinsheimer *et al.* (1977) have described a glutathione (GSH) dependent and a GSH independent deiodinating activity in cytosol, which were active against synthetic iodinated compounds.

Recently, Smallridge *et al.* (1981) have described a deiodinating enzyme in cytosol and in microsomes of rat kidney which is specific for 3',5'-T_2 as a substrate. These observations of deiodinase activity in cytosol

raised the possibility that there might be a deiodinase enzyme normally present in cytosol which has different substrate specificities to those deiodinases found elsewhere in the cell.

With respect to the other more definitely established membrane sites of 5'-deiodinase activity, it is difficult to see the biological advantage of having the three sites in kidney and liver cells. The explanation for the number of sites may lie at several levels.

Firstly, the various sites may simply reflect both the site of synthesis of the enzyme on the rough endoplasmic reticulum together with its destinations (after its synthesis and subsequent incorporation) in the plasma membrane or the lysosomes. This explanation is perhaps less likely as Maciel et al. (1978) found no 5'-deiodination activity in the golgi apparatus. The golgi apparatus is thought to be responsible for the incorporation of the carbohydrate into the glycoproteins of plasma membrane and lysosomes.

Transport of protein between cell sites might explain multiple membrane sites for the 5'-deiodinase in another manner. Membrane transfer between plasma membranes (PM) and lysosomes occurs via the processes of pinocytosis, and the fusion of the pinocytotic vesicle with the lysosome would provide a route from plasma membrane to lysosome. This process is thought to explain the normal presence of 5' nucleotidase and other PM maker enzymes in lysosomes (Wattiaux et al., 1978). Reverse transport of membranes also occurs in which there is externalisation of lysosomal enzymes (Hasilik, 1980). This two-way traffic might explain the simultaneous presence of the 5'-deiodinase in both sites. However one would expect similar K_m values for the iodothyronines, which is not the case (see Table 6.4).

In fact, the two 5'-deiodinase activities with similar K_m values are the lysosomal and the endoplasmic reticulum 5'-deiodinase activities. This might suggest that it is the same enzyme in ER and lysosomes. Novikoff (1973) has shown in general that the GERL complex normally gives rise to the lysosomal proteins during the life of the cell.

A second level of explanation may lie in there being a functional necessity for the various sites of the 5'-deiodinase enzyme to achieve normal thyroid hormone homeostasis. This possibility will be discussed in the following section.

Given that T_3 is the active thyroid principle necessary for the elicitation of most, if not all, thyroid hormone effects (Chapter 1), then any cell that has adapted, during the evolution of specialisation, so that T_3 controls part of its metabolism thus has only two options for the supply of T_3. It can either actively synthesise T_3 by deiodinating T_4 in situ, or it can depend on other cells to synthesise T_3 and import it from plasma. The options are not mutually exclusive so that it may do both. The latter combined method would allow local control in response to local factors and systemic control in response to systemic or whole-body factors. There is evidence that pituitary and liver which have active 5'-deiodinases, in fact, do both in different proportions (reviewed by Hesch, 1981).

Regardless of how a given cell type acts in the thyroid hormone economy of the body, it would be difficult to believe that a control mechanism does not operate. Indeed, it would seem unlikely that the feed-back or servoloop which involves the hypothalamus, the adenohypophysis and the thyroid and which so tightly controls the release of T_4 does not in some way control the conversion of T_4 to T_3 and of T_4 to rT_3.

Support for this assertion comes from the studies of Loos et al. (1980) who confirmed that in athyreotic patients receiving a constant infusion of T_4, there was a diurnal fluctuation in rT_3 and T_3. The rise in rT_3 preceeded that of T_3 in time by about 2 hrs. In normal people given intra venous TRH, there is a fall in rT_3 within 45 mins and it preceeds the rise in T_3 which occurs at about 45-60 minutes (Gaitan et al., 1980). These latter workers also showed that the release of prolactin may have an effect on the rT_3/T_3 ratio.

Any hypothesis to explain the different plasma levels of the tri-iodothyronines must take into account the kinetic studies performed in the low T_3 state (see Chapter 1). These studies suggest that in man the rate of production of T_3 falls and the rate of catabolism of rT_3 (which is normally rapid) also falls with little change in its production rate (reviewed by Chopra, 1981).

The observed plasma changes in T_3 and rT_3, together with the in vitro studies showing markedly different pH optimum values for the production of T_3 and rT_3 from T_4, suggests that the production of T_3 and rT_3 is a controlled, non-random process carried out by at least two enzymes (Visser, 1980; Chopra et al., 1978). As yet however, no worker has successfully separated or purified either of the 5- or 5'-deiodinases of thyroxine. Although recently, in human placenta, Roti et al. (1981) have described a T_4 5-deiodinase which deiodinates T_4, T_3 but not rT_3. With the two enzyme hypothesis, the changes in the low T_3 states would be due to an inhibition or decrease in the 5'-deiodinase activity in the cell and thus a decrease in T_3 synthesis and decrease in rT_3 degradation would occur simultaneously (Kaplan and Utiger, 1978).

Several authors have attempted to explain the control of production T_3 and rT_3 using either alterations in other metabolites such as glutathione, NADPH, or pH, which either stimulate or inhibit their production, or by the direct inhibition of the synthesis of T_3 by regulatory iodothyronines such as rT_3 or 3',5'-T_2 (Chopra et al., 1978; Chopra, 1981; Visser, 1980; Visser, 1981; Hesch et al., 1980; Köhrle, 1981).

The discovery by Visser et al. (1976) of the necessity for a reduced thiol compound as a co-factor has lead several workers to try and link changes in T_3 and rT_3 production to either reduced glutathione (GSH) or to oxidized glutathione (GSSG) or to the ratio of GSH to GSSG (e.g. Visser, 1980; and Chopra, 1981).

The model of GSH control of the production of tri-iodothyronines has some deficiencies. Firstly, dithiothreitol (DTT) is much more effective than GSH at any molar concentration in stimulating 5'-deiodinase. Secondly the response curve of liver homogenates to GSH is very much lower than for DTT (Chopra, 1978). Thirdly, the possible physiological changes in the intracellular concentrations of GSH in starvation from 5.3 to 2.7 mM (Vina et al., 1978) would therefore only elicit a small response ($\leq$ 10% using the data of Chopra, 1978) in 5'-deiodinase activity. It would also be necessary to postulate that 5'-deiodinase enzyme was more sensitive to GSH than the 5-deiodinase enzyme to cause decreased T_3 but not rT_3 production. No such evidence has yet been obtained.

Visser (1978; 1981) has argued that the decreased glucose concentration in liver during fasting leads to decreased production of NADPH from the pentose phosphate shunt and hence of GSH. Whilst decreased production of NADPH during starvation is generally accepted, elevated levels of GSSG in liver to account for the fall in GSH have not been found. Rather, the levels

of GSH relate to protein intake and degradation rates which provide sulphydryl aminoacids to the liver (Tateishi and Higashi, 1978).

In the starved rat, GSH values return to fed levels by about day 4 to 5 of the fast, yet 5'-deiodinase activities remain depressed. This is especially so for the rT_3 5'-deiodinase activity. However its activity can be restored with large concentrations (20 mM) of DTT (Chopra, 1980). This study of Chopra provides strong evidence for factors other than the nett intracellular concentration of GSH in the control of deiodination. The study does not exclude a localised low concentration in a cell compartment which requires a high concentration outside to promote the entry of GSH through a relatively impermeable membrane.

The localisation of the rT_3 5'-deiodinase to the lysosomal membrane is the less common site for lysosomal enzymes but still occurs for a significant number of lysosomal enzymes (Lucy, 1969; Thines-Sempoux, 1973; Yamamoto et al., 1980). The membrane site raises the question of whether the active site of the enzyme faces the cytoplasm or the lysosomal matrix, i.e. whether it is on the inner or outer face.

On the basis of protein labelling experiments, most lysosomal membrane proteins have part of their peptide chain exposed to the cytoplasm (Schneider et al., 1978). However no information is available regarding the orientation of the active site of the rT_3 5'-deiodinase. Thus it is unclear whether the concentration of the thiol co-factor GSH in the cytoplasm is relevant or whether it is the intra-lysosomal concentration of GSH.

No figures are available for the intra-lysosomal concentration of GSH, but the lysosomes have a proven reductive system which reduces cystine to cysteine residues (Griffiths, 1979). The measurement of GSH or reducing

power inside lysosomes in different states would be an important step in further assessing the role of lysosomal 5'-deiodinase and might explain the finding of Chopra (1980) of decreased rT_3 5'-deiodinase during starvation, which is restored with high concentrations of DTT.

Thus the requirement for high concentrations of DTT, which is a much stronger reducing agent than GSH, is a serious impediment to believing that *in vitro* activities of deiodinases described in the literature reflect those occurring *in vivo*. When using ruptured lysosomes, 80% of the maximal activation for rT_3 5'-deiodinase was achieved between 0.1 and 0.5 mM DTT, which was much closer in reducing power to equivalent physiological values of GSH. However future studies on intact lysosomes will be important, not only for assessing the access of GSH to the lysosomal deiodinases but also of the access of substrate to the lysosomal enzyme. Goldman (1975) has reviewed the permeation of amino acids into the interior of intact lysosomes. Single aminoacids and other molecules of molecular weight up to 200 are freely permeable but oligopeptides of 2 or 3 aminoacids are retarded. It would be of great interest to measure permeation of iodothyronines into intact lysosomes.

Hesch and co-workers (Hesch *et al.*, 1980; Köhrle, 1981) have argued strongly that the intracellular pH is the main determinant of whether T_4 5 or 5'-deiodinase is most active within the liver cell. Their model couples pH control together with the inhibition of T_4 to T_3 deiodination by rT_3 and 3',5'-T_2 which has been shown *in vitro* using broken cell preparations by Höffken *et al.* (1976) and by Chopra (1977), and using intact renal tubules by Heyma *et al.* (1980b).

Whilst the model of Hesch and co-workers has the potential to explain many of the changes in iodothyronine metabolism seen in illness, it is

severely hampered by its proponents' inability to show either a relation between change in pH and change in rT_3 or T_3 in severely ill patients with either acidosis or alkalosis or to show in the perfused liver changes consistent with the model (Köhrle, 1981).

As no worker has yet separated, nor purified, the T_4 5 or 5'-deiodinases, and as their properties are very similar (Chopra and Teco, 1981), an alternative explanation to the two deiodinase hypothesis would be to postulate a single T_4 deiodinase which produces both T_3 and rT_3. Once produced, the tri-iodothyronines are then deiodinated at very different rates, i.e. T_3 would be slowly deiodinated and rT_3 would be very rapidly deiodinated. Thus, rather than just controlling the enzymic production of the tri-iodothyronines, their catabolism could also be controlled.

Rapid deiodination of rT_3 and very slow deiodination of T_3 were in fact observed for all the renal and hepatic 5'-deiodinase activities described in this thesis, especially with the lysosomal 5'-deiodinase. Thus, using the results of this thesis, it is possible to construct a fairly pragmatic working hypothesis. The model is based on the hypothesis of different rates of metabolism of T_3 and rT_3 and on the inhibition by rT_3 of the production of T_3 (and rT_3). Control of production and catabolism of tri-iodothyronines are not necessarily mutually exclusive.

Before discussing the model, certain chemical properties of the iodothyronines need to be reviewed. Iodothyronines are aminoacids with markedly hydrophobic and bulky iodinated aromatic side chains. They have a phenolic ring in which the pK_a varies depending on the number of iodines present in phenolic ring, i.e. for T_4 (and? rT_3) pK_a = 6.73, for T_3 (and ? 3,3'-T_2 and 3'-T_2) pK_a = 8.47, and for T_0 (and ? 3,5-T_2 and

? 3-T_1) pK_a - 10.00 (reviewed by Cody, 1981). The iodothyronines are poorly soluble in aqueous or saline solutions (Gemmill, 1955). Their solubilities are markedly temperature dependent and also pH dependent when the pH is above that of the pK_a of the phenolic group (Evert, 1960). They form insoluble complexes with many divalent ions (Kendall and Osterberg, 1920). The Mg^{2+} complex has been used by Sterling and Brenner (1966) to precipitate free thyroxine for its assay.

The intra cellular concentration of Mg^{2+} is relatively high at about 10 mM (Gamble, 1954) of which about 1 mM is the free ion (Walser, 1967). This means that any cell will require protein carriers for the iodothyronines to prevent their precipitation as magnesium complexes. This would explain the finding of many binding sites in the cell (Tata, 1975) without the proteins necessarily being receptors for initiating thyroid hormone action.

The model proposed is shown schematically in Figure 7.1. Because of the considerations in the previous paragraph, no binding proteins are shown in order to simplify the diagram. The figure shows a plasma membrane (PM) enzyme which produces both T_3 and rT_3. Both hormones are either imported into, or exported from the liver cell. Some T_4 may also enter the cell. The T_3 which enters the cell is only slowly metabolised and can thus interact with its receptors in the nucleus, the mitochondria and possibly the plasma membrane. The rT_3 which enters the cell is in equilibrium between production at the PM and its degradation by lysosomes and to a lesser extent by endoplasmic reticulum (ER). The rT_3 in the cytosol adjacent to the PM will inhibit or control the production of T_3 Reverse T_3 might also be metabolised by PM, ER and cytosol (not shown). Further degradation di-iodothyronines may occur in either the cytosol or on the ER.

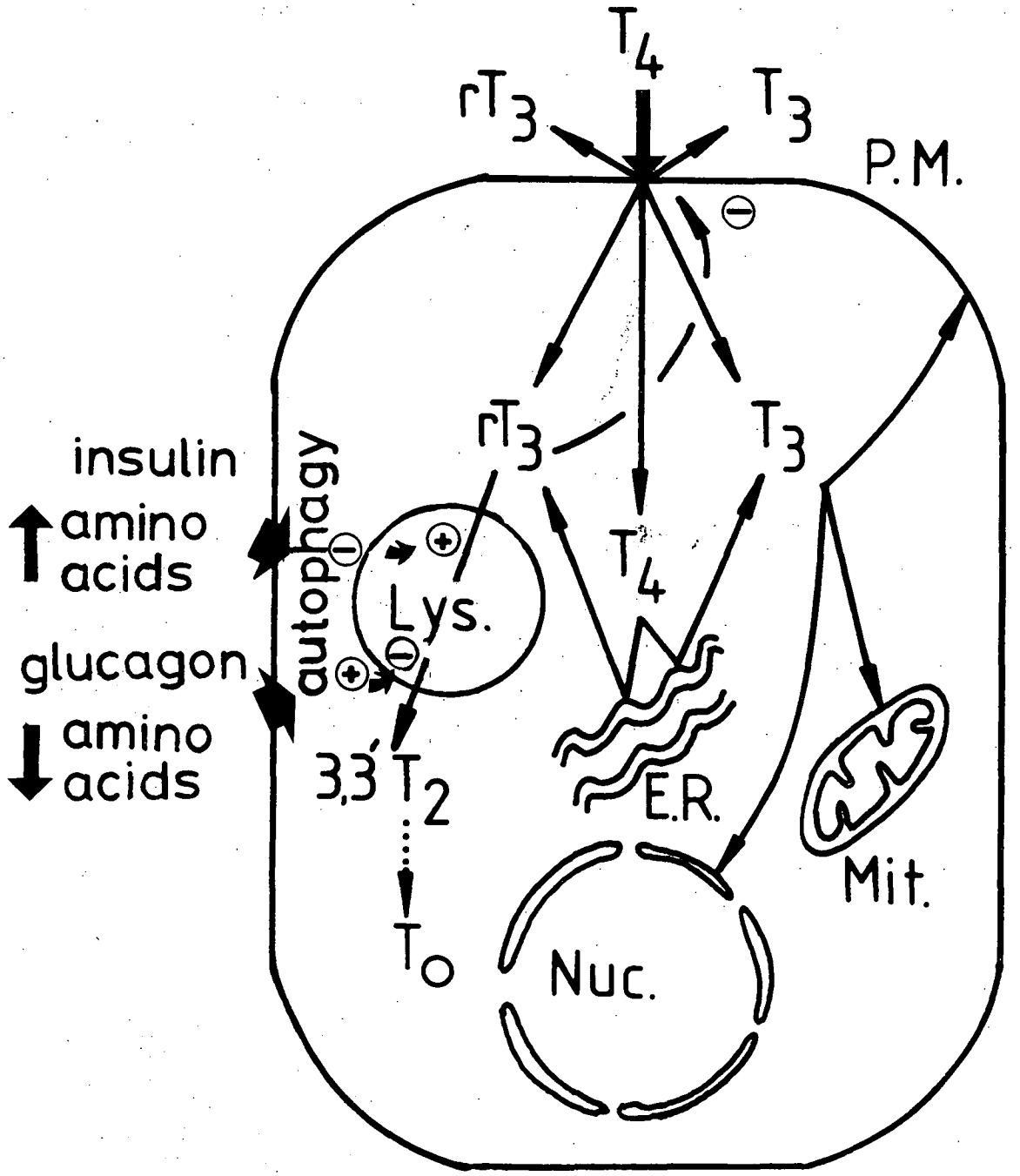


FIGURE 7.1 Speculative Model for the Control of T_3 and rT_3 Function in Rat Liver and Kidney.

Thus in situations giving rise to low T_3 states, the decrease of the lysosomal rT_3 5'-deiodinase activity will cause a rise in cytosol rT_3, which in turn will inhibit T_3 production by the PM. The same chronological sequence of a rise in rT_3 then a fall in T_3 was observed in vivo by Loos et al. (1980) and by Gaitan et al. (1980) in thyroidectomised and normal people respectively.

The evidence for choosing a PM site as being the more important site for T_3 production, rather than an ER site in the model is two-fold but by no means certain. Firstly, the PM site is exposed to the circulating plasma (via extracellular fluid) which would allow export of T_3 without it entering the liver cell and secondly, the data in Table 6.3 suggests that in their particulate forms, the PM is more active than the ER in 5'-deiodination of T_4.

The large latency of the deiodinase of the ER, as revealed when exposed to Triton X-100, together with its similar K_m value and specificities as the lysosomes, suggests that some of the activity is vesicular and may be destined for the lysosomes, i.e. it is synthesised on the RER and transported to the lysosomes where the enzyme becomes active.

Catabolism of rT_3 by its deiodination in the lysosome could have the advantages of compartmentalisation for the cell. Thus it is of interest that many of the low T_3 states are characterised by hormonal changes or are induced by drugs which are known to have marked effects on lysosomal structure and function. For example, propranolol and dexamethasone which are associated with low T_3 and elevated rT_3 (Chapter 1) are known to interact and stabilise the lysosomal membrane (Welman, 1979; Weissman, 1969). Such a structural alteration due to the rearrangement of

membrane phospholipid might well decrease the activity of the 5'-deiodinase enzymes embedded in the lysosomal membrane and thus cause a rise in intracellular rT_3 which would spill over into the plasma. The rise in rT_3 inside the cell would then inhibit T_3 production. In addition these drugs also interact with PM and therefore might have a direct effect on the PM deiodinase.

The model would predict therefore, that lysomotrophic drugs (de Duve et al., 1974) which are known to stabilize lysosomes, e.g. chloroquine and chlorpromazine (Weissman, 1969; Chien et al., 1977), would inhibit rT_3 deiodination and might be associated with elevated rT_3/low T_3 in the plasma and that drugs such as reserpine (Pfeiffer et al., 1980) which labilise lysosomes might lower rT_3 and raise T_3.

Physiological and pathological stress states characterized by low T_3 and elevated rT_3 such as starvation, surgery/anaesthesia, untreated diabetes mellitus, and other acute and chronic illnesses (Chapter 1) are also characterised by a rise in counter regulatory hormones which oppose the action of insulin, i.e. cortisol, glucagon and adrenalin (reviewed by Allison et al., 1976).

Glucocorticoids and glucagon have marked effects on gluconeogenesis in the body by increasing mobilisation of aminoacids from muscle to provide increased aminoacid substrates for gluconeogenesis in the liver.

Both cortisol and glucagon are known to have marked effects on lysosomal function. Philip and Kurup (1978) have shown that chemical adrenalectomy in the rat leads to increased lability in vitro of lysosomes in liver and aorta. Thus corticosterone in the rat may have a background stabilising effect on lysosomes when present in physiological

concentrations, especially when elevated during times of stress and hence may have an effect on the degradation of rT_3 by lysosomes. This effect may well be accentuated in the presence of glucagon because of the effects of glucagon on lysosomal autophagy.

In the presence of glucagon, low amino acid levels (which are required for hepatic gluconeogenesis) markedly stimulate autophagy of intracellular proteins by lysosomes in the perfused rat liver. The process of autophagy begins within a short period of exposure to the combination of low levels of amino acids and glucagon (Neely et al., 1977; Ward et al., 1977; and Schworer and Mortimore, 1979).

Segelen et al. (1980) have found that mixtures of amino acids will inhibit lysosomal autophagy. In particular a mixture of the seven amino acids: leucine, phenylalanine, tyrosine, tryptophane, histidine, asparagine and glutamine are most effective. Of these, asparagine and glutamine were the most effective alone, and were especially effective in inhibition of autophagy when in combination with leucine. The combination of gluconeogenic (Asn, Gln) and anabolic (Leu) amino acids might therefore be a biological signal that there is sufficient amino acid mobilisation or dietary intake for the maintenance of blood glucose and hence there is no need for autophagy.

The inhibition of rT_3 5'-deiodination could occur by an intralysosomal decrease of glutathione or other reducing compounds as discussed earlier. There are, however, a number of other possibilities, viz: competitive inhibition of the 5'-deiodinase by the proteolytically released amino acids; binding of rT_3 to proteins inside the autophagic vacuoles; allosteric control of the 5'-deiodinase by amino acids or peptides and membrane lipid

changes affecting the 5'-deiodinase or the permeation of rT_3 through the lysosomal membrane.

An important possibility not listed above for the control of lysosomal rT_3 deiodination is that of the effect of pH on the rT_3 r'-deiodinase. Examination of Figure 6.3 shows a marked increase of the lysosomal deiodinase activity between pH 5 and 7. This range is in good agreement with many estimates of the prevailing pH within lysosomes (reviewed by Reijingoud and Tager (1977).

The pH inside phagocytic vacuoles of polymorphonuclear leukocytes falls rapidly when proteolysis is occurring (Jensen and Bainton, 1973) so that during autophagy the pH inside liver lysosomes could be expected to fall and thus inhibit the rT_3 5'-deiodinase. Recently Ohkuma and Poole (1978) have measured *in vitro* the intralysosomal pH in living macrophages. They found a resting pH of about 4.5-4.7. When ammonia or weak bases were added, the pH rose immediately up to pH 6.5.

Thus, as the aminoacid levels are raised inside the liver cell, either as a consequence of feeding, or of mobilisation from muscle, increasing quantities of ammonia, of the basic urea-cycle amino acids and of glutamine and asparagine from transamination, will be produced during gluconeogenesis.

A proportion of these bases will be trapped within the lysosomes (de Duve *et al.*, 1974), and raise the intralysosomal pH as shown by Ohkuma and Poole (1978). The rise in pH will then stimulate the rT_3 5'-deiodinase.

Thus the control of the rate of rT_3 deiodination by lysosomes might well be a balance between the fall in intralysosomal pH due to autophagy,

and the rise due to the liberated amino-bases formed during gluconeogenesis.

Autophagy would decrease the amount of endoplasmic reticulum protein within the liver and might therefore reduce both the catabolism of rT_3 and the production of T_3. The greater protein degradation involving endoplasmic reticulum which occurs in liver, when compared to kidney, might also explain the greater changes in T_3 production by the liver which have been shown by Kaplan (1979) to occur in starvation.

Thus the control of lysosomal autophagy by aminoacid levels, which are in turn strongly controlled by insulin, glucagon and the glucocorticoids, may provide the final common pathway for metabolite control of T_3 and rT_3 during starvation. Low intracellular levels of aminoacids in liver, especially when combined with elevated glucagon and low insulin, would stimulate autophagy, whereas elevated levels of aminoacids and insulin would inhibit lysosomal autophagy.

This pathway would explain how a small amount of oral carbohydrate and larger amounts of oral protein but not lipid would reverse the rise in rT_3 and the fall in T_3 in serum, by inhibiting the lysosomal rT_3 5'-deiodinase. The findings of Westgren et al. (1977) of the reversal of dietary changes in the tri-iodothyronines would be consistent with this hypothesis because of the well known effect of increased insulin release by oral glucose and the insulin's subsequent increase of aminoacid transport into liver. Similarly, the findings of Richmand et al. (1980) that septicaemic patients required both intravenous glucose and aminoacids to reverse the tri-iodothyronine changes are consistent with the lack of insulin response and therefore the requirement of both glucose and aminoacids to be given together to raise intracellular aminoacid levels.

The failure of lipid to reverse the changes in tri-iodothyronine levels when given during starvation would be explained on the model by the catabolism of fatty acids since during ß oxidation of fatty acids, the only product is acetyl-CoA which is converted in each cycle of the tricaboxylic acid cycle to carbon dioxide. This means that fatty acids do not normally give rise to significant nett synthesis of glucose or glucogenic ketoacids or aminoacids.

The model allows a further prediction which could be tested. The drug propylamine inhibits the formation of autophagic vacuoles (Segelen et al., 1980) and thus would be predicted to inhibit the changes in rT_3 and T_3 induced by starvation in rats or perfused livers.

7.5 Summary and Conclusions

Assay systems involving iodine-125 labelled iodothyronine substrates have been established which can detect deiodination with high sensitivity. A HPLC separation has also been established which complements the radiochemical assays.

Using these assays, 5'-deiodinase enzyme activities have been localized to the plasma membranes, to the endoplasmic reticulum and to the lysosomes prepared from liver and kidney homogenates. A further activity in the cytosol of these tissues was also found, but evidence suggested, in part, that its presence there may be due to an artefact of the homogenisation procedures.

Purification studies were performed on the cytosol 5'-deiodinase (because of its solubility) and on the combined microsomal 5'-deiodinase activity. Substantial purification (300 fold) of the 5'-deiodinase from

cytosol was achieved using thyroxine as the ligand in affinity chromatography. Gel electrophoresis of this purified product suggested the presence of only a small number of proteins which combine in a regular fashion to produce aggregates of related structures.

The combined plasma membrane and endoplasmic reticulum microsomal activity was significantly purified (3-10 fold) using iopanoic acid (which is a 5'-deiodinase inhibitor) as the ligand in affinity chromatography. The degree of purification compares favourably to that achieved by other workers.

A model for deiodination of T_4 is suggested in which the production of both T_3 and rT_3 occurs predominantly at the plasma membrane. The rT_3 produced is then subject to rapid catabolism by deiodination in the lysosome. The rate of rT_3 deiodination by lysosomes is controlled by the inhibition or stimulation of lysosomal autophagy or changes in the lysosomal membrane or both by drugs, hormones or aminoacids. This leads to alterations in the intracellular levels of rT_3 which in turn feeds-back negatively on the plasma membrane enzyme (and also perhaps the endoplasmic reticulum deiodinase) to inhibit the production of T_3. This then leads to a rise in plasma rT_3 followed soon after by a fall in plasma T_3.

It is further postulated that the final common pathway for dietary changes of rT_3/T_3 levels is via the intracellular levels of aminoacids in the liver, especially those (e.g. Asn, Gln, Leu) which are gluconeogenic or anabolic.

Finally drugs which are known to be lysomotrophic and markedly affect the stability of lysosomes are predicted to have effects on the ratio of rT_3 to T_3 found in plasma.

REFERENCES

Albertson, P.A. (1973) Application of the phase partition method to a hydrophobic membrane protein phospholipase Al from Escharichia coli. Biochemistry 12, 2525.

Albright, E.C. and Larson, F.C. (1959) Metabolism of L-Thyroxine by human tissue slices. J. Clin. Invest. 35, 1899.

Albright, E.C., Larson, F.C. and Tust, R.H. (1954) In vitro conversion of thyroxine to triiodothyronine by kidney slices. Proc. Soc. Exp. Biol. 86, 137.

Allison, S.P., Hinton, P., Woolfson, A. and Heatley, R.V.C. (1976) in Metabolism and the Response to Injury (Wilkinson, A.W. and Cuthbertson, D, eds), Pitman, London.

Aron, M., van Caulaert, C. and Stahl, J. (1931) L'équilibre entre l'hormone préhypophysaire et l'hormone thyroïdienne dans le milieu intérieur, à l'état normal et l'état pathologique. Compt. Rend. Soc. Biol. 107, 64.

Azizi, F. (1978) Effect of dietary composition on fasting-induced changes in serum thyroid hormones and thyrotropin. Metabolism 27, 935.

Backer, E.T., Postmes, T.J. and Wiener, J.D. (1967) Determination of thyroid hormones in serum by means of a cation exchange resin and chloric acid digestion. Clin. Chim. Acta 15, 77.

Balsam, A., Sexton, F. and Ingbar, S.H. (1978) The effect of thyroidectomy, hypophysectomy and hormone replacement on the formation of triiodothyronine from thyroxine in rat liver and kidney. Endocrinology 103, 1759.

Balsam, A., Ingbar S.H. and Sexton, F. (1979) Observations on the factors that control the generation of triiodothyronine from thyroxine in rat liver and the nature of the defect induced by fasting. J. Clin. Invest. 63, 1145.

Baumann, E. (1896a) Uber das normale Vorkommen von Jod im Thierkörper. Z. phys. Chem 21, 319.

Baumann, E. (1896b) Uber das Thyrojodin. Münch. med. Wschr. 43, 309.

Beaufay, H. (1969) in Lysosomes in Biology and Pathology Vol. 2 (Dingle, J.T. and Fell, H.B., eds), North-Holland, Amsterdam.

Becker, D.V. and Prudden, J.F. (1959) The metabolism of I^{131}-labelled thyroxine, triiodothyronine and diiodotyrosine by an isolated perfused rabbit liver. Endocrinology 64, 136.

Le Bel, D., Poirier, G.G. and Beaudoin, A.R. (1978) A convenient method for the ATPase Assay. Anal. Biochem. 85, 86.

Bermudez, F., Serks, M.I. and Oppenheimer, J.H. (1975) High incidence of decreased serum triiodothyronine concentration in patients with nonthyroidal disease. J. Clin. End. Metab. 41, 27.

Bollet, C. and Caude, M. (1976) Sépération par chromatographie en phase liquid rapide des dérivés phenylthiohydantoine des amino-acids rencontres lors de la dégradation d'Edman. J.Chromatog. 121, 323.

Booth, A.G. and Kenny, A.J. (1976) A morphometric and biochemical investigation of the vesiculation of kidney microvilli. J. Cell. Sci. 21, 449.

Braverman, L.E, Ingbar, S.H. and Sterling, K. (1970) Conversion of thyroxine (T_4) to triiodothyronine (T_3) in athyreotic human subjects. J. Clin. Invest. 49, 855.

Auf dem Brinke, D., Hesch, R.D. and Köhrle, J. (1979) Re-examination of the subcellular localization of thyroxine 5' deiodination in rat liver. Biochem. J. 180, 273.

Brown, B.L., Ekins, R.P., Ellis, S.M. and Reith, W.S. (1970) Specific antibodies to triiodothyronine hormone. Nature 226, 359.

Brown, P.R. (1973) High Pressure Liquid Chromatography: Biochemical and Biomedical Applications. Academic Press, New York.

Brown, P.R. and Krstulovic, A.M. (1979) Practical aspects of reversed-phase liquid chromatography applied to biochemical and biomedical research. Anal. Biochem. 99, 1.

Burger, A.G. (1980) in Endocrinology 1980 (Cunningham, I.A., Funder, J.W. and Mendlesohn, F.A.O., eds), p. 223. Australian Academy of Science, Canberra.

Burke, C.W. and Eastman, C.J. (1974) Thyroid hormones. Brit. Med. Bull. 30, 93.

Burman, K., Dimond, R.C., Harrey, G.S., O'Brian, J.T., Georges, L.P., Bruton, J., Wright, F.D. and Wartofsky, L. (1979) Glucose modulation of alterations in serum iodothyronine concentrations induced by fasting. Metabolism 28, 291.

Burr, W.A., Black, E.G., Griffiths, R.S., Hoffenberg, R., Meinhold, K. and Wenzel, K.W. Serum triiodothyronine and reverse triiodothyronine concentrations after surgical operation. Lancet 2, 1277.

Cahnmann, H.J. (1972) in The Thyroid and Biogenic Amines (Rall, J.E. and Kopin, I.J., eds), p. 27. North-Holland, Amsterdam.

Carter, J.N., Eastman, C.E., Carcoran, J.M. and Lazarus, L. (1974) Effect of severe, chronic illness on thyroid function. Lancet 2, 971.

Cavalieri, R.R., Gavin, L.A., Bui, F., McMahon, F. and Hammond, M. (1977) Conversion of thyroxine to 3,3',5'-triiodothyronine (reverse-T_3) by a soluble enzyme system of rat liver. Biochem. Biophys. Res. Com. 79, 897.

Chien, K., Abrams, J., Serroni, A., Martin, J.T. and Farber, J. (1978) Accelerated phospholipid degradation and associated membrane dysfunction in irreversible, ischemic liver cell injury. J. Biol. Chem. 253, 4809.

Chiraseveenuprapund, P., Buergi, U., Goswami, A. and Rosenberg, I.N. (1975) in Thyroid Research (Robbins, J. and Braverman, L.E., eds) Excerpta Medica, Amsterdam.

Chiraseveenuprapund, P., Buergi, U., Goswami, A. and Rosenberg, I.N. (1978) Conversion of L-thyroxine to triiodothyronine in rat kidney homogenates. Endocrinology 102, 612.

Chopra, I.J. (1974) A radioimmunoassay for measurement of 3,3',5'-triiodothyronine (rT_3). J. Clin. Invest. 54, 583.

Chopra, I.J. (1976) An assessment of daily production and significance of thyroidal secretion of 3,3',5'-triiodothyronine (reverse T_3) in man. J. Clin. Invest. 58, 32.

Chopra, I.J. (1977) A study of extra thyroidal conversion of thyroxine (T_4) to 3,3'5-triiodothyronine (T_3) in vitro. Endocrinology 101, 453.

Chopra, I.J. (1978) Sulphydryl groups and the monodeiodination of thyroxine to triiodothyronine. Science 199, 904.

Chopra, I.J. (1980) Alterations in monodeiodination of iodothyronines in the fasting rat. Effects of reduced non-protein sulphydryl groups and hypothyroidism. Metabolism 29, 161.

Chopra, I.J. (1981) Triiodothyronines in Health and Disease. Springer-Verlag, Berlin.

Chopra, I.J. and Solomon, D.H. (1976) Thyroid function tests and their alterations by drugs. Pharmacol. Therap 1, 367.

Chopra, I.J. and Solomon, D.H. (1980) in Endocrinology 1980 (Cumming, I.A., Funder, J.W. and Mendelsohn, F.A.O., eds). Australian Academy of Science, Canberra.

Chopra, I.J. and Teco, G.N.C. (1982) Characteristics of inner ring (3 or 5) monodeiodination of 3,5 diiodothyronine in rat liver: evidence suggesting marked similarities of inner and outer ring deiodinases for iodothyronines. Endocrinology 110, 89.

Chopra, I.J., Fisher, D.A. and Teco, C. (1973) Thyroxine: just a prohormone or a hormone too ? J. Clin. End. Metab. 36, 1050.

Chopra, I.J., Sack, J. and Fisher, D.A. (1975a) 3,3',5'-triiodothyronine (reverse T_3) and 3,3',5-triiodothyronine (T_3) in fetal and adult sheep: studies of metabolic clearance rates, production rates, serum binding, and thyroidal content relative to thyroxine. Endocrinology 97, 1080.

Chopra, I.J., Sack, J. and Fisher, D.A. (1975b) Circulating 3,3',5'-triiodothyronine (reverse T_3) in the human newborn. J. Clin. Invest. 55, 1137.

Chopra, I.J., Chopra, U., Smith, S.R., Reza, M. and Solomon, D.H. (1975) Reciprocal changes in serum concentrations of 3,3',5'-triiodothyronines (reverse T_3) and 3,3'5-triiodothyronine (T_3) in systemic illnesses. J. Clin. Endocrinol. Metab. 41, 1043.

Chopra, I.J., Solomon, D.H., Chopra, U., Wu, S.Y., Fisher, D.A. and Nakamura, Y. (1978) Pathways of metabolism of thyroid hormones. Rec. Prog. Horm. Res. 34, 521.

Cody, V. (1981) in Triiodothyronines in Health and Disease (Chopra, I.J. ed.) p. 15. Springer-Verlag, Berlin.

Coiro, V., Harris, A., Goodman, H.M., Vagenakis, A. and Braverman, L. (1980) Effect of pharmacological quantities of infused 3,3',5'-triiodothyronine on thyroxine monodeiodination to 3,5,3'-triiodothyronine. Endocrinology 106, 68.

Coleman, R. (1973) Membrane-bound enzymes and membrane ultrastructure. Biochem. Biophys. Acta 300, 1.

Crabtree, B.R., Leech, A.R. and Newsholme, E.A. (1979) in Techniques in Life Sciences B2/1 (B211)
Elsevier/North Holland, Amsterdam.

Cruchaud, S., Vannotti, A., Mahaim, C. Deckelman, J. (1955) The in vitro effect of methylthiouracil and oestradiol monophosphate on the conversion of thyroxine to triiodothyronine by kidney slices. Lancet 2, 906.

Danforth, E.Jr., Horton, E.S., O'Connell, M., Sims, E.A., Burger, A.G., Ingbar, S.H., Braverman, L. and Vagenakis, A.G. (1979) Dietary-induced alterations in thyroid hormone metabolism during overnutrition. J. Clin. Invest. 64, 1336.

de Duve, C. (1965) The separation and characterization of subcellular particles. Harvey Lect. 59, 49.

de Duve, C., Pressman, B.C., Bianetto, R., Wattiaux, R. and Applemans, F. (1955) Tissue fractionation studies. Biochem. J. 60, 604.

de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P. and van Hoof, F. (1974) Lysomotrophic agents. Biochem Pharmacol. 23, 2495.

de Vries, J.X., Frank, R. and Birr, C. (1975) The separation and quantitative identification of PTH-aminoacids by high pressure liquid chromatography. FEBS Lett 55, 65.

Denning, S.N. and Turoff, M.L.H. (1978) Optimisation of reverse-phase liquid chromatographic separation of weak organic acids. Anal.Chem. 50, 546.

Di Stefano, J.J. and Fisher, D.A. (1976) Peripheral distribution and metabolism of the thyroid hormones: a primarily quantitative assessemnt. Pharmac. Ther. B. 2, 539.

Dixon, M. and Webb, E.C. (1964) Enzymes, 2nd ed. Longmans, London.

Done, J.N., Knox, J.H. and Loheac, J. (1974) Applications of High Speed Liquid Chromatography. John Wiley and Sons, London.

Duick, D.S., Warren, D.W., Nicoloff, J.T., Otis, C.L. and Croxson, M.S. (1974) Effect of single dose dexamethasone on the concentration of serum triiodothyronine in man. J. Clin. Endocrinol. Metab. 39, 1151.

Eisenthal, R. and Cornish-Bowden, A. (1974) The Direct Linear Plot. Biochem. J. 139, 715.

Ekins, R.P., Newman, G.B. and J.L.H. O'Riordan (1967) in Radioisotopes in Medicine: In Vitro Studies (Hayes, R.L., Goswitz, F.A. and Pearson-Murphy, P.E., eds), U.S. Atomic Energy Commission, Washington.

El-Zaheri, M.M., Braverman, L.E. and Vagenakis, A.G. (1980) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S., eds) Australian Academy of Science, Canberra.

Engelman, D.M., Terry, T.M. and Morowitz, H.J. (1967) Characterisation of the Plasma Membrane of Mycoplasma lailawii Sodium Dodecyl Sulphate Solubilisation. Biochem. Biophys. Acta 135, 381.

Erenberg, A.E., Phelps, D.L., Lam, R. and Fisher, D.A. (1974) Total and free thyroid hormone concentrations in the neonatal period. Pediatrics 53, 211.

Erickson, V.J., Cavalieri, R.R. and Rosenberg, L. (1980) A thyroxine-5'-deiodinase from rat thyroid gland. Thyroid Research 8, 355.

Etkin, W. (1978) The thyroid - A gland in search of a function. Pers. Biol. Med. 19, 19.

Etling, N. and Barker, S.B. (1959) Metabolism of thyroxine during prolonged kidney catex incubation. Endocrinology 64, 753.

Evans, W.H. (1978) Preparation and Characterisation of Mammalian Plasma Membranes. North Holland, Amsterdam.

Evert, H.E. (1960) The solubility of L-thyroxine (Na) in the presence of phosphate buffer and of neutral salts. J. Phys. Chem. 64, 478.

Fekkes, D., van Overmeeren, E., Hennemann, G. and Visser, T.J. (1980) Solubilization and partial characterisation of rat liver iodothyronine deiodinases. Biochim. Biophys. Acta 613, 41.

Fekkes, D., van Overmeeren, E., Docter, R., Hennemann, G. and Visser, T.J. (1979) Location of rat liver iodothyronine deiodinating enzymes in the endoplasmic reticulum. Biochim. Biophys. Acta 587, 12.

Fisher, D.A. and Dussault, J.H. (1971) Contribution of methodological artifacts to the measurement of T_3 concentration in serum. J. Clin. End. Metab. 32, 675.

Fisher, D.A. and Oddie, T.H. (1964) Whole body counting of 131-I labelled triiodothyronine. J. Clin. End. Metab. 24, 733.

Flock, E.V. and Bollman, J.L. (1955) The metabolism of thyroxine and triiodothyronine in the eviscerated rat. J. Biol. Chem. 214, 709.

Flock, E.V., BOllman, J.L., Grindlay, J.H. and Orvis, A.L. (1956) Metabolism of L-thyroxine and L-triiodothyronine in the absence of the liver. Am. J. Physiol. 187, 407.

Ford, D.H., Carey, K.R. and Gross, J. (1957) The localization of thyroid hormones in the organs and tissues of the guinea pig: an autoradiographic and chromatographic study. Endocrinology 61, 426.

Gaitan, E., Siegel, A.L., Pittman, C.S., Dino, J.A., Roddam, R.F. and Cooksey, R.C. (1980) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S., eds) p. 424, Australian Academy of Science, Canberra.

Galton, V.A. (1968) in Recent Advances in Endocrinology (James, V.H.T., ed.) 8th ed., Churchill, London.

Galton, V.A. (1975) in Thyroid Research (Robbins, J. and Braverman, L.E., eds) p. 251, Excerpta Medica, Amsterdam.

Galton, V.H. and Ingbar, S.H. (1961a) The influence of reserpine, serotonin and metabolites of tryptophane on the degradation of thyroxine and its derivatives. Endocrinology, 68, 435.

Galton, V.A. and Ingbar, S.H. (1961b) Mechanism of protein iodination during metabolism of thyroid hormones by peripheral tissues. Endocrinology, 69, 30.

Galton, V.A. and Ingbar, S.H. (1962) A photoactivated flavin-induced degradation of thyroxine and related phenols. Endocrinology, 70, 210.

Galton, V.A. and Ingbar, S.H. (1963) Role of peroxidase and catalase in the physiological deiodination of thyroxine. Endocrinology, 73, 596.

Galton, V.A. and Ingbar, S.H. (1966) Observations on the nature of the heat resistant thyroxine deiodinating system of rat liver. Endocrinology, 78, 855.

Gamble, J.L. (1954) Chemical Anatomy, Physiology and Extracellular Fluid. Harvard University Press, Cambridge, Mass.

Geffner, D.L., Azukikawa, M. and Hershman, J. (1975) Propylthiouracil blocks extrathyroidal conversion of thyroxine to triiodothyronine and augments thyrotropin secretion in man. J. Clin. Invest. 61, 1276.

Gemmill, C.L. (1955) The apparent ionization constants of the phenolic hydroxyl groups of thyroxine and related compounds. Arch. Biochem. Biophys. 54, 359.

Giddings, J.C. (1965) Dynamics of Chromatography. Marcel Dekker, New York.

Glitzer, M.S., Symchowicz, S. and Gross, J. (1956) Metabolism of labelled thyroxine in perfused rabbit kidney in vitro. Fed. Proc. 15, 76.

Goldfine, I.D., Smith, G.J. and Simons, C.G. (1976) Activities of thyroid hormones and related compounds in an in vitro thymocyte assay. J. Biol. Chem. 251, 4233.

Goldman, R. (1975) in Lysosomes in Biology and Pathology (Dingle, J.T. and Dean, R.T., eds) Vol. 5, p. 309, North Holland, Amsterdam.

Goldstein, A. (1949) The interactions of drugs and plasma proteins. J. Pharmacol. & Exper. Therap. Part II 95, 102.

Green, J.D. and Harris, G.W. (1947) The neurovascular link between the neurohypophysis and the adenohyphonysis. J. Endocrinol. 5, 136.

Greer, M.A. (1951) Evidence of hypothalamic control of pituitary release of thyrotrophin. Proc. Soc. Exper. Biol. Med. 77, 603.

Greer, M.A. (1955) Demonstration of thyroidal response to exogenous thyrotropin in rats with anterior hypothalamic lesions. Endocrinology 57, 755.

Griffiths, P.A. (1979) Evidence for lysosomal reduction of cystine residues. Biochem. Biophys. Res. Com. 89, 428.

Grinberg, R., Volpert, E.M. and Werner, S.C. (1963) In vivo deiodination of labelled L-thyroxine to L-c,5,3'-triiodothyronine in mouse and human pituitaries. J. Clin. End. Metab. 23, 140.

Gross, J. and Lebland, C.P. (1951) Metabolites of thyroxine. Proc. Soc. Exp. Biol. Med. 76, 686.

Gross, J. and Pitt-Rivers, R. (1951) Unidentified iodine compounds in human plasma in addition to thyroxine and iodide. Lancet 2, 766.

Gross, J. and Pitt-Rivers, R. (1952a) The identification of 3,5,3'-L-triiodothyronine in human plasma. Lancet 1, 439.

Gross, J. and Pitt-Rivers, R. (1952b) Physiological Activity of 3,5,3'-L-triiodothyronine. Lancet 1, 593.

Gross, J. and Pitt-Rivers, R. (1953a) 3,5,3'-triodothyronine. (1) Isolation from thyroid gland and synthesis. Biochem. J. 53, 645.

Gross, J. and Pitt-Rivers, R. (1953b) 3,5,3'-triiodothyronine (2) Physiological activity. Biochem. J. 53, 652.

Gross, J., Pitt-Rivers, R. and Trotter, W.R. (1952) Effect of 3,5,3'-L-triiodothyronine in myxoedema. Lancet 1, 1044.

Grushka, E. and Kikta, E.J.(Jr.) (1977) Chemically Bonded Stationary Phases in Chromatography. Anal. Chem. 49, 1004A.

Haibach, H. (1971) Evidence for thyroxine deiodinating mechanism in the rat thyroid different from iodotyrosine deiodinase. Endocrinology 88, 918.

Hamilton, P.B. (1966) Ion exchange chromatography of amino-acids: Recent advances in analytical determinations. Advan. Chromatography 2, 3.

Hancock, W.S., Bishop, C.A. and Hearn, W.T.H. (1976) High pressure liquid chromatography in the analysis of underivatised peptides using a sensitive and rapid procedure. FEBS Lett 72, 139.

Hancock, W.S., Bishop, .CA., Prestidge, R.L., Harding, D.R.K. and Hearn, M.T.W. (1978) The use of phosphoric acid in the analysis of underivatised peptides by reversed-phase high-pressure liquid chromatography. J. Chromatog. 153, 391.

Harrington, C.R. (1926a) Chemistry of thyroxine. I. Isolation of thyroxine from the thyroid gland. Biochem. J. 20, 293.

Harrington, C.R. (1926b) Chemistry of thyroxine. II. Constitution and synthesis of desiodothyroxine. Biochem. J. 20, 300.

Harrington, C.R. (1927) Chemistry of thyroxine. III. Constitution and synthesis of thyroxine. Biochem. J. 21, 169.

Harris, A.R.C., Vagenakis, A.G. and Braverman, L.E. (1979a) Sex related differences in outer ring monodeiodination of thyroxine with 3,3',5'-triiodothyronine by rat liver homogenates. Endocrinology 104, 645.

Harris, A.R.C., Fang, S.L., Hinerfeld, L., Braverman, L.E. and Vagenakis, A.G. (1979b) The role of sulphydryl groups on the impaired hepatic 3',3,5-triiodothyronine generation from thyroxine in the hypothyroid, stored fetal and neonatal rodent. J. Clin. Invest. 63, 516.

Harrower, A.D.B., Fyffe, J.A. and Horn, D.B. (1977) Thyroxine and triiodothyronine levels in hyperthyroid patients during treatment with propranalol. Clin. Endocrinol. 7, 41.

Hasilik, A. (1980) Biosynthesis of Lysosomal Enzymes. Trends Biochem. Sci. 5, 237.

Hearn, M.T.W. and Hancock, W.S. (1979) Ion pair partition reversed phase HPLC. Trends Biochem. Sci. 4, N58.

Hearn, M.T.W., Hancock, W.S. and Bishop, C.A. (1978) High-pressure liquid chromatography of aminoacids, peptides and proteins V. J. Chromatog. 157, 337.

Helenius, A. and Simons, K. (1975) Solubilization of Membranes by Detergents. Biochim. Biophys. Acta 415, 29.

Helenius, A. and Soderlund, H. (1973) Stepwise Dissociation of the Semliki Forest virus membrane with Triton X-100. Biochim. Biophys. Acta 307, 287.

Hershman, J.M. and van Middlesworth, L. (1962) Effect of antithyroid compounds on the deiodination of thyroxine in the rat. Endocrinology 71, 94.

Hesch, R.D. (1981) in The "Low T_3 Syndrome" (Hesch, R.D., ed) p. 1, Academic Press, London.

Hesch, R.D., Brunner, G. and Söling, H.D. (1975) Conversion of thyroxine and T_3 and the subcellular localization of the converting enzyme. Clin. Chim. Acta 59, 209.

Hesch, R.D., Höffken, B., Ködding, R. and Köhrle, J. (1980) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S., eds) p. 400, Australian Academy of Science, Canberra.

Heyma, P. Larkins, R.G. and Campbell, D.G. (1980a) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S., eds) Australian Academy of Science, Canberra.

Heyma, P., Larkins, R.G. and Campbell, D.G. (1980b) The effect of iodothyronines on the conversion of thyroxine into 3,3',5-triiodothyronine in isolated rat renal tubules. Biochem. J. 190, 239.

Höffken, B., Ködding, R. and Hesch, R.D. (1977) Conversion of T_4 to T_3 and rT_3 and their cytoplasmic binding: pH dependency. Clin. Chim. Acta 78, 261.

Höffken, B., Ködding, R., Hehrmann, R., von zur Mühlen, A., Jüppner, F. and Hesch, R.D. (1976) in Biochemical Basis of Thyroid Stimulation and Thyroid Hormone Action (von zur Mühlen, A. and Schleusner, H., eds) p. 204, G. Thieme Verlag, Stuttgart.

Hogness, J.R., Berg, M., Van Arsdell, P.P., Jr and Williams, R.H. (1955) Tissue conversion of thyroxine to triiodothyronine. Proc. Soc. Exp. Biol. Med. 90, 93.

Holloway, P.N. (1973) A simple procedure for removal of Triton X-100 from protein samples. Anal. Biochem. 53, 304.

Hoskins, R.G. (1949) The thyroid-pituitary apparatus as a servo (feed-back) mechanism. J. Clin. Endocrinol. Metab. 9, 1429.

Hüfner, M. and Grussendorf, M. (1978) Investigations on the deiodination of thyroxine (T_4) to 3,3'-diiodothyronine (3,3'-T_2) in rat liver homogenate. Clin. Chim. Acta 85, 243.

Hüfner, M., Grussendorf, M. and Ntokalou, M. (1977) Properties of the thyroxine (T_4) monodeiodinating system in rat liver homogenate. Clin. Chim. Acta 78, 251.

Imai, Y., Kataoka, K. and Nishikimi, M. (1980) A possible function of thiols, including glutathione, as cofactors in the conversion of thyroxine to 3,3'5-triiodothyronine in rat liver microsomes. Endocrinol. Japon 27, 201.

Ingbar, S.H. and Galton, V.A. (1963) Thyroid. Ann. Rev. Physiol. 25, 361.

Jacobs, E.E., Kirkpatrick, F.H., Andrews, B.C., Cunningham, W. and Crane, F.L. (1966) Lipid-free soluble cytochrome oxidase, purification, properties and reaction characteristics. Biochem. Biophys. Res. Com. 25, 96.

Jensen, M.S. and Bainton, D.F. (1973) Temporal changes in pH within the phagocytic vacuole of the polymorphonuclear leukocyte. J. Cell. Biol. 56, 379.

Johnson, E.L. and Stevenson, R. (1978) Basic Liquid Chromatography. Varian Assoc. Inc., Palo Alto.

Kagedal, B. and Kallberg, M. (1977) An improved procedure for isolation of thyroxine-binding globulin from human pregnancy serum. Clin. Chim. Acta 78, 103.

Kalant, H., Lee, R. and Sellers, E.A. (1955) Metabolic fate of radioactive thyroid hormones in normal and propylthiouracil-treated rats. Endocrinology 56, 127.

Kaplan, M.M. (1979) Subcellular alterations causing reduced hepatic thyroxine-5'-monodeiodinase activity in fasted rats. Endocrinology 104, 58.

Kaplan, M.M. (1980) Thyroxine 5'-monodeiodination in rat anterior pituitary homogenates. Endocrinology 106, 567.

Kaplan, M.M. and Utiger, R.D. (1978) Iodothyronine metabolism in rat liver homogenates. J. Clin. Invest. 61, 459.

Kaplan, M.M. and Utiger, R.D. (1978) Iodothyronine metabolism in liver and kidney homogenates from hyperthyroid and hypothyroid rats. Endocrinology 103, 156.

Kaplan, M.M. and Yaskoski, K.A. (1980) Phenolic and tyrosyl ring deiodination of iodothyronines in rat brain homogenates. J. Clin. Invest. 66, 551.

Kaplan, M.M., McCann, U.D., Yaskoski, K.A., Larsen, P.R. and Leonard, J.L. (1981) Anatomical distribution of phenolic and tyrosyl ring iodothyronine deiodinases in the nervous system of normal and hypothyroid rats. Endocrinology 109, 397.

Karger, B.L. and Giese, R.W. (1978) Reversed phase liquid chromatography and its application to biochemistry. Analyt. Chem. 50, 1049A.

Karger, B.L., Snyder, L.R. and Horvath, C. (1973) An Introduction to Separation Science. John Wiley & Sons, New York.

Karger, B.L., Su, S.C., Marchese, S. and Persson, B.A. (1974) High performance ion pair partition chromatography; the separation of thyroid hormones and sulfur drugs. J. Chromatogr. Sci. 12, 678.

Kelly, F.C. (1961) Iodine in medicine and pharmacy since its discovery 1811-1961. Proc. Roy. Soc. Med. 54, 831.

Kendall, E.C. (1915) The isolation in crystaline form of the compound containing iodine which occurs in the thyroid. J. Amer. Med. Ass. 64, 2042.

Kendall, E.C. (1919) Isolation of the iodine compound which occurs in thyroid. J. Biol. Chem. 39, 125.

Kendall, E.C. and Osterberg, A.E. (1920) The chemical identification of thyroxin. J. Biol. Chem. 192, 265.

Kirkland, J.J. (ed) (1971) Modern Practice of Liquid Chromatography. Wiley-Interscience, New York.

Kobayashi, I., Yamada, T. and Shichijo, K. (1966) Nonenzymatic deiodination of thyroxine in vitro. Metabolism 15(12), 1140.

Köhrle, J. (1981) in The "Low T_3 Syndrome" (Hesch, R.D., ed.) p. 27, Academic Press, London.

Köhrle, J., Kodding, R. and Hesch, R.D. (1980) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S. eds), Australian Academy of Science, Canberra.

Laidlaw, J.C. (1949) Nature of the circulating thyroid hormone. Nature 164, 927.

Larsen, P.R. (1971) Technical aspects of the estimation of triiodothyronine in human serum: evidence of conversion of thyroxine to triiodothyronine during assay. Metabolism 20, 609.

Larsen, P.R. (1972a) Tri-iodothyronine: review of recent studies of its physiology and pathophysiology in man. Metabolism 21, 1073.

Larsen, P.R. (1972b) Direct immunoassay of triiodothyronine in human serum. J. Clin. Invest. 51, 1939.

Larsen, P.R., Castonguar, S. and Jove, R. (1980) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S. eds), Australian Academy of Science, Canberra.

Larson, F.C., Tomita, K. and Albright, E.C. (1955) The deiodination of thyroxine to triiodothyronine by kidney slices of rats with varying thyroid function. Endocrinology 57, 338.

Lassiter, W.E. and Stanbury, J.B. (1958) The in vivo conversion of thyroxine to 3:5:3' triiodothyronine. J. Clin. End. Metab. 18, 903.

Laub, R.J. and Purnell, J.H. (1975) Criteria for the use of mixed solvents in gas-liquid chromatography. J. Chromatog. 112, 71.

Laub, R.J. and Purnell, J.H. (1976) Optimization of gas ghrmoatographic analysis of complex mixtures of unknown composition. Anal. Chem. 48, 1720.

Leonard, J.L. and Rosenberg, I.N. (1978a) Subcellular distribution of thyroxine 5'-deiodinase in the rat kidney: a plasma membrane location. Endocrinology 103, 274.

Leonard, J.L. and Rosenberg, I.N. (1978b) Thyroxine 5'-deiodinase activity of rat kidney: observations on activation by thiols and inhibition by propyl thiouracil. Endocrinology 103, 2137.

Leonard, J.L. and Rosenberg, I.N.(1980) Characterisation of essential enzyme sulfhydryl groups of thyroxine 5'-deiodinase from rat kidney. Endocrinology 106, 444.

Lerman, J. and Slater, W.T. (1940) The relief of myxoedema with proteins of extrathyroidal origin. J. Clin. Invest. 19, 555.

Lewis, M., Bourke, J.R., Conn, P. and Evered, D.C. (1980) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S. eds), p. 445, Australian Academy of Science, Canberra.

Lissitzky, S., Benevent, M.-T. and Roques, M. (1961) Désiodination photochimique des dérivés iodés de la thyronine et de la tyrosine en présence de flavin mononucléotide. Biochim. Biophys. Acta 51, 407.

Lissitzky, S., Michel, R., Roche, J. and Roques, M. (1956) Sur la désiodination des hormones thyroidiennes par des extraits d'organes. Bull. Soc. Chim. Biol. Paris 38, 1413.

Lissitzky, S., Torresani, J., Pinchera, A. and Andreoli, M. (1962) Methods for the measurement of thyroxine deiodinase activity of human muscle tissue. Clin. Chim. Acta 7, 13.

Loach, P.A., Sekura, D.L., Hadsell, R.M. and Stemer, A. (1970) Quantitative dissolution of the membrane and preparation of photoreceptor subunits from Rhodopseudomonas spheroides. Biochemistry 9, 724.

Loos, U., Winter, H., Duntas, L., Etzrodt, H., Grau, R., Rosenthal, J. and Pfeiffer, E.F. (1980) in Thryoid Research VIII (Stockigt, J.R. and Nagataki, S. eds) p. 428, Australian Academy of Science, Canberra.

Lowe, C.R. (1979) An Introduction to Affinity Chromatography. North Holland, Amsterdam.

Lucy, J. (1969) in Lysosomes in Biology and Pathology, Vol. 2 (Dingle, J.T. and Fell, H. eds) p. 313, North Holland, Amsterdam.

MacLagan, N.F. and Sprott, W.E. (1954) The in vitro deiodination of thyroxine and triiodothyronine. Lancet 2, 368.

MacLagan, N.F. and Wilkinson, J.H. (1954) Some differences in metabolism of thyroxine and triiodothyronine in the rat. J. Physiol. 125, 405.

MacLagan, N.F., Bowden, C.H. and Wilkinson, J.H. (1957) Metabolism of thyroid hormones. 2. Detection of thyroxine and triiodothyronine in human plasma. Biochem. J. 67, 5.

MacLagan, N.F., Sprott, W.E. and Wilkinson, J.H. (1952) Effect of 3,5,3'-L-triiodothyronine and certain antithyroxine substances on the oxygen consumption of mice. Lancet 2, 915.

McLarty, D.G., Ratcliffe, J.G., Sammon, D.J., Ratcliffe, W.A., McColl, K.E.L., Meinhold, H. and Wenzel, K.W. (1976) Letter. Lancet 2, 1070.

McLean, F.C. and Hastings, A.B. (1935) The state of calcium in the fluids of the body. I. The conditions affecting the ionization of calcium. J. Biol. Chem. 108, 285.

Maciel, R.M.B., Ozawa, Y. and Chopra, I.J. (1979) Subcellular localization of thyroxine and reverse triiodothyronine outer ring monodeiodinating activities. Endocrinology 104, 365.

Martin, A.J.P. and Synge, R.L.M. (1941) A new form of chromatogram employing two liquid phases. Biochem. J. 35, 1358.

Miller, W.S. and Smith, J.G. (1966) Effect of acetylsalicylic acid on lysosomes. Proc. Soc. Exptl. Biol. Med. 122, 634.

Money, W.L., Kumaoka, S., Rawson, R.W. and Kroc, R.L. (1960) Comparative effects of thyroxine analogues in experimental animals. Ann. N.Y. Acad. Sci. 86, 512.

Morreale de Escobar, G., Escobar del Rey, F. and Rodriguez, P.L. (1962) Activation by inert proteins of the flavin-induced photodependent deiodination of thyroxine. J. Biol. Chem. 237, PC2041.

Morreale de Escobar, G., Llorente, P., Jolin, T., Escobar del Rey, F. (1963b) The 'Transient Instability' of Thyroxine and its Biochemical Applications. Biochem. J. 88, 526.

Morreale de Escobar, G., Rodriguez, P.L., Jolin, T. and Escobar del Ray, F. (1963b) Activation of the flavin photodeiodination of thyroxine by "Thyroxine Deiodinase" and other proteins. J. Biol. Chem. 238(10), 3508.

Nachtmann, F., Knapp, G. and Spitzy, H. (1978) Catalytic detection principle for high performance liquid chromatography. J. Chromatog. 149, 693.

Naeije, R., Clumeck, N., Somers, G., Vanhaelst, L. and Goldstine, J. (1976) Letter. Lancet 1, 1070.

Nakagawa, S. and Ruegamer, W.R. (1967) Properties of rat tissue iodothyronine deiodinase and its natural inhibitor. Biochem. 6(5), 1249.

Nauman, J.A., Mauman, A. and Werner, S.C. (1967) Total and free triiodothyronine in human serum. J. Clin. Invest. 46, 1346.

Neely, A.N., Cox, J.R., Fortney, J.A., Schworer, C.M. and Mortimore, G.E. (1977) Alteration of lysosomal size and density during rat liver perfusion. J. Biol. Chem. 252, 6948.

Neville, D.M. (1960) The isolation of a cell membrane fraction from rat liver. J. Biophys. Biochem. Cytol. 8, 413.

Neville, D.M. (1968) Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. Biochim. Biophys. Acta 154, 540.

Nicod, P., Burger, A., Staeheli, V. and Vallotton, M.B. (1976) A radioimmunoassay for 3,3',5'-triiodo-L-thyronine in unextracted serum: method and clinical results. J. Clin. End. Metab. 42, 823.

Nomura, S., Pittman, C.S. and Chambers, J.B. (1975) Reduced peripheral conversion of thyroxine to triiodothyronine in patients with hepatic cirrhosis. J. Clin. Invest. 56, 643.

Novikoff, A.B. (1973) in Lysosomes and Storage Diseases (Hers, H.G. and van Hoof, F., eds) p. 1, Academic Press, New York.

Obregón, M.J., Rogriguez, A., Mallol, J., Pascual, A., Bernal, J., Escobar del Ray, F. and Morreale de Escobar, G. (1980) in Thyroid Research VIII (Stockigt, J.R. and Nagataki, S. eds) p. 441, Australian Academy of Science, Canberra.

O'Connor, M. and Woodford, F.P. (1975) Writing Scientific Papers in English. Elsevier, Amsterdam.

Ohkuma, S. and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Soc. Acad. Sci. U.S.A. 75, 3327.

Oppenheimer, J.H., Schwartz, H.L. and Surks, M.I. (1972) Propylthiouracil inhibits the conversion of L-thyroxine to L-triiodothyronine. J. Clin. Invest. 55, 218.

Osborn, R.H. and Simpson, T.H. (1972) The characterisation of iodoaminoacids and their derivatives by thin layer chromatography. J. Chromatog. 40, 219.

Otten, M.H., Hennemann, G. Docter, R. and Visser, T.J. (1980) The role of dietary fat in peripheral thyroid hormone metabolism. Metabolism 29, 930.

Peiffer, C.J., Cho, C.H., Cheema, A. and Saltman, D. (1980) Reserpine induced gastric ulcers: protection by lysosomal stabilization due to zinc. Eur. J. Pharmacol. 61, 347.

Pelley, J.W., Garner, C.W. and Little, G.H. (1978) A simple rapid biuret method for the estimation of protein in samples containing thiols. Anal. Biochem. 86, 341.

Pensky, J. and Marshall, J.S. (1974) in Methods in Enzymology Vol. XXXIV Part B (Jakoby, W.B. and Wilchek, M. eds), Academic Press, New York.

Perry, S., Amos, R. and Brewer, P. (1972) Practical Liquid Chromatography. Plenum, New York.

Peters, T.J., Müller, M. and de Duve, C. (1972) Lysosomes of the arterial wall. J. Exp. Med. 136, 1117.

Peters, T.J., Heath, J.R., Wansbrough-Jones, M.H. and Doe, W.F. (1975) Enzyme activities and properties of lysosomes and brush borders from control subjects and patients with coeliac disease. Clin. Sci. Mol. Med. 48, 259.

Philip, B. and Kurup, P.A. (1978) Adrenocortical suppression and lysosomal stability. Ind. J. Biochem. and Biophys. 15, 193.

Pind, K. (1957) Paper chromatographic determination of thyroid hormone (3,5,3'-triiodothyronine) in serum without radio-iodine. Acta Endocrinol. 26, 263.

Piroska, E. and Földes, J. (1976) Letter. Lancet 1, 1071.

Pittman, C.S., Chambers, J.B. and Read, V.H. (1971) The extrathyroidal conversion rate of thyroxine to triiodothyronine in normal man. J. Clin. Invest. 50, 1187.

Pitt-Rivers, R. (1978) in Hormonal Proteins and Peptides VI (Li, C.H. ed.) p. 391, Academic Press, New York.

Pitt-Rivers, R. and Tata, J.R. (1959) The Thyroid Hormones. Pergamon Press, London.

Pitt-Rivers, R., Stanbury, J.B. and Rapp, B. (1955) Conversion of thyroxine to 3,5,3'-triiodothyronine in vivo. J. Clin. Endocrinol. Metab. 15, 616.

Plaskett, L.G. (1961) Studies on the degradation of thyroid hormones in vitro with compounds labelled in either ring. Biochem. J. 78, 652.

Plaskett, L.G. (1964) Paper chromatography of iodoaminoacids and related compounds. Chromatography Reviews 6, 91.

Pochin, E.E. (1950) Investigation of thyroid function and disease with radioactive iodine. Lancet 2, 41.

Porath, J. (1974) in Methods in Enzymology Vol. XXXIV Part B (Jakoby, W.B. and Wilchek, M. eds), Academic Press, New York.

Portnay, G.I., O'Brian, J.T., Bush, J., Vagenakis, A.G., Azizi, F., Arky, R.A., Ingbar, S.H. and Braverman, L.E. (1974) The effect of starvation on the concentration and binding of thyroxine and triiodothyronine in serum and on the response to TRH. J. Clin. Endocrinol. Metab. 39, 191.

Rabinowitz, J.L. and Hercker, E.S. (1971) Thyroxine: conversion to triiodothyronine by isolated perfused rat heart. Science 173, 1242.

Recant, L. and Riggs, D.S. (1952) Thyroid function in nephrosis. J. Clin. Invest. 31, 789.

Refetoff, S. and Matalou, R. (1972) Conversion of L-thyroxine to triiodothyronine by human fibroblasts in tissue culture. J. Clin. Invest. 49, 78a.

Reijingoud, D.J. and Tager, J.M. (1977) The permeability properties of the lysosomal membrane. Biochim. Biophys. Acta 472, 419.

Reinwein, D. and Rall, J.E. (1966a) Non-enzymatic deiodination of thyroid hormones by FMN and light. J. Biol. Chem. 241, 1636.

Reinwein, D. and Rall, J.E. (1966b) Non-enzymatic deiodination of thyroxine by hydrogen peroxide. Endocrinology 78, 1248.

Richards, A.H. and Mason, W.B. (1966) Gas chromatographic separation of some iodine compounds of serum. Anal. Chem. 38, 1751.

Richmand, D.A., Molitch, M.E. and O'Connell, T.F. (1980) Altered thyroid hormone levels in bacterial sepsis: the role of nutritional adequacy. Metabolism 29, 936.

Riggs, D.S. (1952) Quantitative aspects of iodine metabolism in man. Pharmacol. Reviews 4, 284.

Robbins, J. and Rall, J.E. (1957) The interaction of thyroid hormones and protein in biological fluids. Rec. Prog. Horm. Res. 13, 161.

Robbins, J. and Rall, J.E. (1960) Proteins Associated with the thyroid hormones. Physiol. Review 40, 415.

Robbins, J. and Rall, J.E. (1967) in Hormones in Blood, 2nd ed. (Gray, C.H. and Bacharach, A.L. eds), Academic Press, New York.

Roche, J. and Michel, R. (1954) Thyroid hormones and iodine metabolism. Ann. Rev. Biochem. 23, 481.

Roche, J., Lissitzky, S. and Michel, R. (1952a) Caractérisation des iodohistidines dans les protéines iodées (thyroglobuline et iodoglobine). Biochim. Biophys. Acta 8, 339.

Roche, J., Lissitzky, S. and Michel, R. (1952b) Sur la triiodothyronine, produit intermédiarie de la transformation de la diiodothyronine en thyroxine. C.R. Hebd. Seances Acad. Sci. 234, 997.

Roche, J., Michel, R. and Lissitzky, S. (1964) in Methods of Biochemical Analysis Vol. 12, (Glick, D. ed.), p. 143, Interscience, New York.

Roche, J., Michel, R. and Nunez, J. (1956) Sur la presence de la 3:3:5'-triiodothyronine dans le sang de rat. C.R. Seances Soc. Biol. Fil. 150, 20.

Roche, J., Michel, R., Wolf, W. and Nunez, J. (1956) Sur deux nouveaux constituants hormonaux du corps thyroïde, la 3:3' diiodothyronine et la 3:3':5'-triiodothyronine. Biochim. Biophys. Acta 19, 308.

Roe, J.H. (1955) The determination of sugar in blood and spinal fluid with anthrone reagent. J. Biol. Chem. 212, 335.

Rosenberg, I.N. (1951) The nature of the circulating thyroid hormone in Graves' Disease. J. Clin. Invest. 30, 1.

Roti, E., Fang, S.L., Green, K., Emerson, C.H. and Braverman, L.E. (1981) Human placenta is an active site of thyroxine and 3,3',5-triiodothyronine tyrosyl ring deiodination. J. Clin. Endocrinol. Metab. 53, 498.

Saberi, M., Sterling, F.H. and Utiger, R.D. (1975) Reduction in extrathyroidal triiodothyronine production by propylthiouracil in man. J. Clin. Invest. 55, 218.

Schneider, D.L., Burnside, J., Gorga, F.R. and Nettleton, C.J. (1978) Properties of the membrane proteins of rat liver lysosomes. Biochem. J., 176, 75.

Schwartz, H.L., Surks, M.I. and Oppenheimer, J.H. (1971) Quantitation of extrathyroidal conversion of L-thyroxine to 3,5,3'-triiodo-L-thyronine in the rat. J. Clin. Invest. 50, 1124.

Schworer, C.M. and Mortimore, G.E. (1979) Glucagon-induced autophagy and proteolysis in rat liver: mediation by selective deprivation of intracellular aminoacids. Proc. Natl. Acad. Sci. 76, 3169.

Sedmark, J.J. and Grossberg, S.E. (1977) A rapid sensitive and versatile assay for protein using Coomassie Blue G250. Anal. Biochem. 79, 554.

Seglen, P.O., Gordon, P.B. and Poli, A. (1980) Aminoacid inhibition of the autophagic/lysosomal pathway of protein degradation in isolated rat hepatocytes. Biochim. Biophys. Acta 630, 103.

Segrest, J.P. and Jackson, R.L. (1972) in Methods in Enzymology Vol. 28 (Cobwick, S.P. and Kaplan, N.O. eds) p. 54, Academic Press, New York.

Selye, H. (1946) The general adaption syndrome and diseases of adaption. J. Clin. Endocrinol. 6, 117.

Shimaoka, K. and Jasani, B.M. (1965) The application of two dimensional paper chromatography and low-level counting to the study of triiodothyronine in plasma. J. Endocrinol. 32, 59.

Siebert, W.J. and Smith, R.S. (1930) Effect of various anterior pituitary preparations on basal metabolism in guinea pigs. Proc. Soc. Exptl. Biol. Med. 27, 622.

Silva, J.E. and Larsen, P.R. (1977) Pituitary nuclear 3,5,3'-triiodothyronine and thyrotropin secretion: an explanation for the effect of thyroxine. Science 198, 617.

Silva, J.E. and Larsen, P.R. (1978) Contributions of plasma T_3 and local T_4 to T_3 monodeiodination to nuclear T_3 receptor saturation in pituitary, liver and kidney of hypothyroid rats: further evidence relating saturation of pituitary nuclear T_3 receptors and the acute inhibition of TSH release. J. Clin. Invest. 61, 1247.

Silva, J.E., Dick, T.E. and Larsen, P.R. (1978) The contribution of local tissue thyroxine monodeiodination to the nuclear 3,5,3'-triiodothyronine in pituitary liver and kidney of euthyroid rats. Endocrinology 103, 1196.

Simpson, C.F. (ed) (1976) Practical High Performance Liquid Chromatography. Heyden & Son Ltd., London.

Sinsheimer, J.E., Wong, T., Röder, S. and Shum, Y.Y. (1978) Mechanisms for the biodehalogenation of iodocompounds. Biochem. Biophys. Res. Com. 83, 281.

Smallridge, R.C., Burman, K.D., Ward, K.E., Wartofsky, L., Dimond, R.C., Wright, F.D. and Latham, K.R. (1981) 3',5'-Diiodothyronine to 3'-monoiodothyronine conversion in the fed and fasted rat: enzyme characteristics and evidence for two distinct 5'-deiodinases. Endocrinology 108, 2336.

Smith, P.E. (1930) Hypophysectomy and a replacement therapy in the rat. Amer. J. Anat. 45, 205.

Snyder, L.R. and Kirkland, J.J. (1974) Introduction to Modern Liquid Chromatography. John Wiley and Sons, New York.

Sorimachi, K. and Ui, N. (1975) Ion-exchange chromatographic analysis of iodothyronines. Anal. Biochem. 67, 157.

Spaulding, S.W., Chopra, I.J., Sherwin, R.S. and Lyall, S.S. (1976) Effect of caloric restriction and dietary composition on serum T_3 and reverse T_3 in man. J. Clin. Endocrinol. Metab. 42, 197.

Spiro, R.G. (1966) in Methods in Enzymology Vol. 8 (Neufeld, E.F. and Ginsburg, V. eds), Academic Press, New York.

Sprott, W.E. and MacLagan, N.F. (1955) Metabolism of thyroid hormones: the deiodination of thyroxine and triiodothyronine in vitro. Biochem. J., 59, 288.

Stanbury, J.B., Morris, M.L., Corrigan, H.J. and Lassiter, W.E. (1960) Thyroxine deiodination by a microsomal preparation requiring Fe^{++}, oxygen and cysteine or glutathione. Endocrinology 67, 353.

Stasilli, N.R., Kroc, R.L. and Meltzer, R.A. (1959) Antigoitrogenic and calorigenic activities of thyroxine analogues in rats. Endocrinology 64, 62.

Sterling, K. (1970) The significance of circulating triiodothyronine. Rec. Prog. Horm. Res. 26, 249.

Sterling, K. and Brenner, M.A. (1966) Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J. Clin. Invest. 45, 153.

Sterling, K. and Hegedus, A. (1961) Measurement of free thyroxine concentration in human serum. J. Clin. Invest. 41, 1031.

Sterling, K., Brenner, M.A. and Newman, E.S. Conversion of thyroxine to triiodothyronine in normal human subjects. Science 169, 1099.

Sterling, K., Brenner, M.A. and Saldanha, V.F. (1972) Conversion of thyroxine to triiodothyronine by cultured human cells. Science 179, 1000.

Sterling, K., Rosen, P. and Tabachnick, M. (1962) Equilibrium dialysis studies of the binding of thyroxine by human serum albumin. J. Clin. Invest. 41, 1021.

Sterling, K., Bellabarba, D., Newman, E.S. and BRenner, M.A. (1969) Determination of triiodothyronine concentration in human serum. J. Clin. Invest. 48, 1150.

Stevenson, G.F. (1963) in Evaluation of Thyroid and Parathyroid Functions (Sunderland, F.W. and Sunderland, F.W. Jr. (eds), p. 3, J.B. Lippincott, Philadelphia.

Suda, A., Chambers, J. Jr., Thurston, C. and Pittman, C. (1977) Thyroid hormone kinetics in the fasting and diabetic subjects. Clin. Res. 25, 516A.

Sullivan, P.R.C., Bollinger, J.A. and Reichlin, S. (1973) Selective difficiency of tissue triiodothyronine: a proposed mechanism of elevated free thyroxine in the euthyroid sick. J. Clin. Invest. 52, 83a.

Surks, M.I. and Oppenheimer, J.H. (1971) Metabolism of phenolic- and tyrosyl-ring labeled L-thyroxine in human being and rats. J. Clin. Endocrinol. Metab. 33(4), 612.

Svennerholm, L. (1957) Quantitative estimation of sialic acids. Biochim. Biophys. Acta 24, 604.

Tajuddin, M. and Elfbaum, S.G. (1973) Evaluation of "Dexsil 300 GC" as stationary phase in gas chromatography of triiodothyronine and thyroxine in human serum. Clin. Chem. 19, 109.

Takaishi, M., Shimizu, T. and Shishiba, Y. (1979) Solubilization of thyroxine 5'-deiodinase activity from rat liver microsome fraction. Acta Endocrinol. 92, 694.

Tata, J.R. (1957) Metabolism of l-thyroxine and l 3:5:3'-triiodothyronine by homogenates of rat skeletal muscle. Proc. Soc. Exp. Biol. Med. 95, 362.

Tata, J.R. (1959) Activation of thyroxine deiodinase by ferrous ions and flavin. Biochim. Biophys. Acta 35, 567.

Tata, J.R. (1960a) The partial purification and properties of thyroxine dehalogenase. Biochem. J. 77, 214.

Tata, J.R. (1960b) Transiodination of proteins during enzymic de-iodination of thyroxine. Nature 187, 1025.

Tata, J.R. (1961) The physiological significance of thyroxine dehalogenase. Acta Endocrinol. 37, 125.

Tata, J.R. (1962) Intracellular and extracellular mechanisms for the utilization and action of thyroid hormones. Rec. Prog. Horm. Res. 18, 221.

Tata, J.R. (1975) How specific are nuclear 'receptors' for thyroid hormones ? Nature 257, 18.

Tata, J.R., Rall, J.E. and Rawson, R.W. (1957) Metabolism of L-thyroxine and L-3:5:3' triiodothyronine by brain tissue preparations. Endocrinology 60, 83.

Tateishi, N. and Higashi, T. (1978) in Functions of Glutathione in Liver and Kidney (Sies, H. and Wendel, A., eds) p. 3, Springer-Verlag, Berlin.

Taurog, A. (1963a) Spontaneous deiodination of I-131-labelled thyroxine and related iodophenols on filter paper. Endocrinology 73, 45.

Taurog, A. (1963b) Spontaneous deiodination of I-131-labelled thyroid extracts on filter paper. Endocrinology 73, 57.

Taurog, A. and Chaikoff, I.L. (1948) The nature of the circulating thyroid hormone. J. Biol. Chem. 176, 639.

Thines-Sempoux, D. (1976) in Lysosomes in Biology and Pathology Vol. 3, (Dingle, J.T. ed.), p. 278, North Holland, Amsterdam.

Trotter, W.R. (1964) in Thyroid Gland (Pitt-Rivers, R. and Trotter, W.R. eds) p. 1, Butterworths, London.

Trouet, A. (1964) Arch. Int. Physiol. Biochem. 72, 698. Quoted by Beaufay (1969) in Lysosomes in Biology and Pathology Vol. 2 (Dingle, J.T. and Fell, H.B., eds), North Holland, Amsterdam.

Turková, J. (1978) Affinity Chromatography. Elsevier, Amsterdam.

Vagenakis, A.G., Burger, A., Portnay, G.I., Rudolph, M., O'Brian, J.T., Azizi, F., Arky, R.A., Nicod, P., Ingbar, S.H. and Braverman, L.E. (1975) Diversion of peripheral thyroxine metabolism from activating to enactivating pathways during complete fasting. J. Clin. End. Metab. 41, 191.

Viña, J., Hems, R. and Krebs, H.A. (1978) Maintenance of glutathione content in isolated hepatocytes. Biochem. J. 170, 627.

Visser, T.J. (1978) A tentative review of recent in vitro observations of the enzymatic deiodination of iodothyronines and its possible physiological implications. Molec. Cell. Endocrinol. 10, 241.

Visser, T.J. (1980) Deiodination of thyroid hormone and the role of glutathione. Trends in Biochem. Sci. 5, 222.

Visser, T.J. (1981) in The "Low T_3 Syndrome" (Hesch, R.D. ed.) p. 13, Academic Press, London.

Visser, T.J., van der Does-Tobé, I., Docter,R.and Hennemann, G. (1975) Conversion of thyroxine into triiodothyronine by rat liver homogenate. Biochem. J. 150, 489.

Visser, T.J., van der Does-Tobé, I. Docter, R., Hennemann, G. (1976) Subcellular localization of a rat liver enzyme converting thyroxine into triiodothyronine and possible involvement of essential thiol groups. Biochem. J. 157, 479.

Visser, T.J., Fekkes, D., Docter, R. and Hennemann, G. (1978) Sequential deiodination of thyroxine in rat liver honogenate. Biochem. J. 174, 221.

Visser, T.J., Fekkes, D., Docter, R. and Hennemann, G. (1979) Kinetics of enzymic reductive deiodination of iodothyronines. Effect of pH. Biochem. J. 179, 489.

Visser, T.J., Lamberts, S.W.J., Wilson, J.H.P., Docter, R. and Hennemann, G. (1978) Serum thyroid hormone concentrations during prolonged reduction of dietary intake. Metabolism 27, 405.

Volpert, E.M., Tierney, J. and Werner, S.C. (1966) Studies with mouse pituitary thyrotropic tumors. X. Formation in vitro of 3,5,3'-triiodothyronine from thryoxine by dependent but not autonomous strains. Endocrinology 79, 43.

Walser, M. (1967) Magnesium metabolism. Ergeb. Physiol. 59, 185.

Ward, W.F., Cox, J.R. and Mortimore, G.E. (1977) Lysosomal sequestration of intracellular protein as a regulatory step in hepatic proteolysis. J. Biol. Chem. 252, 6955.

Wattiaux, R., Wibo, M. and Baudhuin, A. (1963) in Ciba Found Symp. Lysosomes (de Reuck, A.U.S. and Cameron, M.P., eds) p. 176, J. and A. Churchill, London.

Wattiaux, R., Wattiaux-de Coninck, S., Ronveaux-Dupal, M.F. and Dubois, F. (1978) Isolation of rat liver lysosomes by isopycnic centrifugation in a metrizamide gradient. J. Cell. Biol. 78, 349.

Wattiaux-De Coninck, S., Rutgeerts, M.J. and Wattiaux, R. (1965) Lysosomes in rat kidney tissue. Biochim. Biophys. Acta 105, 446.

Weber, J.R., Degner, E.F. and Bahjet, K.S. (1964) Determination of nonionic ethylene oxide adduct in some commercial products. Anal. Chem. 36, 378.

Weber, R., Hagenbüchle, O. and Ryffel, G. (1974) Thyroid hormones and RNA metabolism in anuran metamorphosis. Fortsch. Zool. 20, 419.

Weber, K., Pringle, J.R. and Osborne, M. (1972) in Methods in Enzymology Vol. 26 (Colowick, I.P. and Kaplan, N.O., eds), Academic Press, New York.

Weissman, (1969) in Lysosomes in Biology and Pathology Vol. 1, (Dingle, J.T. and Fell, H. eds) p. 277, North Holland, Amsterdam.

Welman, E. (1979) Stabilization of lysosomes in anoxic myocardium by propranolol. Br. J. Pharmacol. 65, 479.

Wenzel, K.W. and Horn, W.R. (1976) in Thyroid Research (Robbins, J. and Braverman, L.E. eds), p. 270, Excerpta Medica, Amsterdam.

West, C.D., Wayne, A.W. and Chavré, V.J. (1965) Thin-layer chromatography for thyroid hormones. Anal. Biochem. 12, 41.

Westgran, U., Ahrén, B. and Burger, A. (1977) Stimulation of peripheral T_3 formation by oral but not intravenous glucose administration in fasted subjects. Acta Endocrinol. 85, 526.

Wisher, M.H. and Evans, W.H. (1975) Functional polarity of the rat hepatocyte surface membrane. Biochem. J. 146, 375.

Woeber, K.A., Doherty, G.F. and Ingbar, S.H. (1972) Stimulation by phagocytosis of the deiodination of L-thyroxine in human leukocytes. Science 176, 1039.

Wynn, J., Gibbs, R. and Royster, B. (1962) Thyroxine degradation. I. Study of optimal reaction conditions of a rat liver thyroxine-degrading system. J. Biol. Chem. 237, 1892.

Yamamoto, K., Ikehara, Y., Kawamoto, S., and Kato, K. (1980). Characterization of enzymes and glycoproteins in rat liver lysomal membranes. J. Biochem. (Tokyo) 87(1), 237.

Yamazaki, E. and Slingerland, D.W. (1959) The in vitro metabolism of thyroxine, triiodothyronine and their acetic and proprionic acid analogues. Endocrinology 64, 126.

Young, E.G. (1963) in Comprehensive Biochemistry (Florkin, M. and Stolz, E.H. eds), Elsevier, Amsterdam.