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Some Effects of Thyroid Hormones on Renal and Hepatic Ultrastructure in the Mouse

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by

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SUMMARY.

The effects of thyroid hormones upon cellular ultrastructure have been investigated in both mouse liver and kidney. The tissues were examined after various prepation procedures, and as well as subjective visual examination of ultrastructure, attempts were made where possible, to gain quantitative information of variations.

Alterations in the morphology of cells were investigated and established that thyroid hormone associated changes in protein synthetic activity in cells is reflected in cellular ultrastructure. Particular attention was paid to the effects of thyroid hormones upon nuclear morphology but was unable to demonstrate any significant variation in the appearance of nuclear components which might be associated with a changed functional state.

Investigation of the morphology of the nuclear envelope revealed the existence of small vesicles associated with the outer membrane of the nuclear envelope of both liver and kidney cells, and large vesicle structures developed from the nuclear envelope of cells of the kidney. Thyroid hormone administration increased the numbers of both types of vesicle, and the accumulation of obscuring material associated with the nuclear envelope of both the liver and the kidney. It was suggested that the changes in the nuclear envelope morphology were connected with nucleo-cytoplasmic transfer mechanisms.

An observed tendency for nucleoli to migrate towards the nuclear envelope with thyroid hormone influence, and the hormone associated variations in the morphology of the nuclear envelope were thought to reflect the increased turnover of material within the nucleus and its transfer to the cytoplasm, known to take place under the influence of thyroid hormones.

Investigation of liver and kidney ultrastructure after

administration of a single dose of triiodothyronine revealed the time course of development of ultrastructural changes. Variations in the morphology of the nuclear envelope were shown to precede cytoplasmic changes associated with increased protein synthetic activity.

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INTRODUCTION

The biochemical effects of thyroid hormones upon mammalian tissues, particularly the liver, have been well established, (Tata 1967a; Tata 1967b; Tata 1970). However, little attention has been paid to the parallel effects which might be expected upon the ultrastructure. Tata (1967a) was able to demonstrate changes in the arrangement of cellular organelles which paralleled biochemical changes in induced amphibian metamorphosis, and Tata et al (1963) reported no alteration of mitochondrial structure or size with physiological doses of triiodothyronine, but there has been no thorough investigation of the ultrastructural effects of thyroid hormones reported in the literature.

There are several points in the established pattern of thyroid hormone action where an investigation of thyroid hormone influence upon ultrastructure might lead to an understanding of the hormonal effects. For this reason investigations of cellular structure, the appearance of nuclei and alterations in the nuclear envelopes under the influence of thyroid hormones were initiated.

It was hoped that an investigation of cellular structure would demonstrate changes in the relative distribution of organelles reflecting the overall increase in anabolic activity produced by thyroid hormone action. Investigation of nuclear structure was directed towards elucidating whether a changed functional state was reflected in the ultrastructural appearance. An investigation of the ultrastructure of the nuclear envelope was undertaken in an attempt to establish the effects of increased turnover of nuclear RNA upon nucleo-cytoplasmic transfer mechanisms, as visualised in the electron microscope.

The appearance of mouse liver and kidney were investigated at various times after the administration of a single dose of triiodothyronine, in an attempt to establish the chronological order of observed ultrastructural changes.

General Survey of the Literature Concerning the Effects of Thyroid Hormones at the Cellular Level.

Many attempts have been made to explain the various biological effects of thyroid hormones. Early work was directed towards the mitochondria, in an attempt to explain the calorigenic effects of thyroid hormone administration. Lardy and Feldott (1951) and Niemeyer et al (1951) demonstrated that administration of thyroxine in vitro produced an uncoupling of oxidative phosphorylation and Tapley et al (1955), Beyer et al (1956), Emmelot and Bos (1958) and Lehninger et al (1959) showed that thyroid hormones had a direct action upon mitochondrial permeability. However, most of the in vitro effects of thyroid hormones upon mitochondria could only be produced by pharm-acological doses (Gustafsson et al 1965). Direct action at the level of the mitochondrion cannot explain the multiple effects upon protein metabolism. (see Pitt-Rivers and Tata, 1959).

Gustafsson et al (1965) showed that thyroid hormones exerted a selective control upon mitochondrial composition, without affecting oxidative phosphorylation, in doses close to the physiological, and that mitochondria under went growth and structural change in vivo, that was distinct from the mitochondrial swelling produced in vitro with high doses of the hormone. Roodyn et al (1965) demonstrated that mitochondria from thyroidectomised animals had a reduced capacity to incorporate amino acids into protein than did those of normal animals, and Sokoloff and Kaufmann (1961) noted that although mitochondria were required for an increased incorporation of amino acids into protein by rat liver homogenates, microsomes were responsible for the greater proportion of amino acid incorporation. Michel et al (1963) demonstrated that although thyroxine administration in vivo produced an increased uptake of amino acids into protein by the various tissues, administration in vitro had no effect. Thus protein synthesis was

implicated as a site for the early action of thyroid hormones.

Since Tata et al (1963) correlated the effects of thyroid hormone administration upon mitochondrial function and basal metabolic rate, and demonstrated alterations in amino acid incorporating activity of cell free liver homogenates, and ribonucleic acid/protein ratios with time after thyroid hormone administration, the involvement of protein synthesis in the early action of thyroid hormones has been conclusively demonostrated.

The events taking place during the latent period before stimulation of basal metabolic rate have been the subject of extensive investigations. The earliest reported effect of triiodothyronine upon the liver of the rat was an accelerated synthesis of rapidly labelled nuclear RNA which was demonstrated at 6hrs. after hormone administration (Widnell and Tata 1966). An increase in the specific activity of nuclear RNA polymerase was detected at 16hrs. after administration of a single dose of triiodothyronine to thyroidectomised rats. There was no detectable accumulation of ribonucleic acid in the nucleus, as a result of the rapid rate of turnover of newly synthesised material (Tata 1964, Tata 1965, Tata 1967.)

A net accumulation of newly synthesised ribosomes in the cytoplasm was detected by Widnell and Tata (1966) 24hrs. after hormone administration. Tata (1970) demonstrated that the appearance of newly formed ribosomes was coordinated with an increased synthesis of phospholipids of both smooth and rough microsomal membranes. Increased incorporation of choline into membrane phospholipids was detectable 20hrs after administration of thyroid hormones.

Increased amino acid incorporating activity of microsomes was demonstrable at 26hrs. after hormone administration, reaching a peak at 36hrs. (Tata et al 1963). Increased amino acid incorporating activity by mitochondria was also detectable 36hrs, after hormone

administration (Roodyn et al 1965). An increased RNA content of ribosomes was detected at 45hrs and an increase in the number of ribosomes recovered as polysomal aggregates from a tissue homogenate was detectable at 42hrs. The peak of amino acid incorporating activity of microsomes was reached at 40-50hrs after triiodothyronine administration (Tata et al 1963; Roodyn et al 1965; Widnell and Tata 1966.). The raised amino acid incorporating activity of microsomes was maintained 300hrs after hormone administration, and specific activities of lactic dehydrogenase and glucose-6-phosphate dehydrogenase were still elevated 6 days later (Tata et al 1963).

Widnell and Tata (1963, 1966) showed that the increase in specific activity of nuclear RNA polymerase was not due to a non-specific stimulation of nuclear activity but due to some interaction with a specific site. The newly synthesised nuclear RNA was mainly ribosomal in character, although there was an increase in the synthesis of messenger-RNA which was masked by the disproportionately greater increase in ribosomal RNA production (Tata 1967). There was no alteration in the base composition of newly synthesised RNA (Widnell and Tata 1966) but there was a threefold increase in the specific activity of nuclear RNA from triiodothyronine treated animals compared to thyroidectomised controls (Tata 1964; Widnell and Tata 1966).

There was a hormone induced net increase in the cytoplasmic RNA content of animals given a single replacement injection of triiodothyronine (Tata 1967). The hormone induced RNA of increased RNA of increased specific activity was found to associate with a lipid rich cellular fraction (Widnell and Tata 1966) and there was found to be twice as much membrane bound RNA in triiodothyronine treated rats compared to thyroidectomised controls. Tata (1967) found that the newly formed microsomes had an increased capacity to incorporate amino acids into protein. As was previously noted, there was a marked increase in the number of ribosomes recovered as polysomal aggregates

after thyroid hormone treatment. Widnell and Tata (1966) and Tata (1970) noted that ribosomes formed under the influence of the hormone action were more firmly attatched to membrane elements. Tata (1970) was also able to demonstrate that the newly formed ribosomes were associated with newly formed membrane elements, and that the membrane bound ribosomes were responsible for the greated proportion of increased protein synthetic activity.

Thus an early action of thyroid hormones upon the cell is to induce an increased specific activity of nuclear RNA polymerase resulting in a production of new messenger-RNA and a disproportionately greater amount of ribosomal-RNA. The newly synthesised RNA subsequently appears in the cytoplasm as ribosomes with an increased capacity to incorporate amino acids into protein. The appearance of newly formed ribosomes in the cytoplasm is accompanied by an increased synthesis of membrane phospholioids. Ribosomes become associated with membrane elements in a tighter binding than that previously present in the cell, and the newly formed ribosomes are responsible for the increased protein synthetic activity.

The accumulation in the cytoplasm, of ribosomes of increased protein synthesising capacity is coordinated with changes in mitochondrial activity. Tata et al (1963) have shown no alteration in the tightness of coupling of the electron transfer system or phosphorylating systems in mitochondria from animals treated with triiodothyronine, but there was an overall increase in mitochondrial activity which was not localised at any one stage in either the respiratory chain or phosphorylarion. Roodyn et al (1965) demonstrated that mitochondria from thyroidectomised rats were less active at incorporating amino acids into protein than those from normal rats, and that administration of triiodo-thyronine to thyroidectomised rats increased the overall amino acid incorporating activity of the mitochondria. This increased amino acid incorporating activity was not dependent upon intramitochondrial pools

of amino acids. It has been suggested that the increased amino acid incorporating activity was a result of an increased rate of oxidative phosphorylation, but the rate of energy production and amino acid incorporating activity were stimulated at the same time and the increased energy production continued long after the amino acid incorporating activity had returned to normal (Roodyn et al 1965). Thus the increased respiratory activity and increased amino acid incorporating activity seen to result from some separate stimulation.

It has been shown that the thyroid hormone administration affects levels of mitochondrial RNA, causing an overall increase detectable 16hrs. after administration of a single dose of triiodothyronine to thyroidectomised animals (Tata 1964; Widnell and Tata 1966). However the significance of this is not fully understood since although stimulation of mitochondrial amino acid incorporating activity occurs somes 20hrs. after the first detectable changes in mitochondrial RNA levels, there is no apparent relationship between mitochondrial RNA and amino acid incorporating activity (Roodyn et al 1965).

Thus it would seem that the main effects of thyroid hormones, on rat liver at least, is to produce an overall stimulation of the normal anabolic processes taking place within the tissue, as is summarised in Diagram 1. on the following page. The hormones may exert their effects at one or more points in the process. Production of new ribosomes of increased amino acid incorporating capacity may be a result of hormone action at some stage in either the production of the ribosome or in its localisation upon microsomal membranes. The increased turnover of nuclear RNA may be a result of direct stimulation of nucleo-cytoplasmic transfer mechanisms by hormone action, or as a result of the increased synthesis of the RNA. Again the increased production of membrane phospholipids may result from direct hormone action, or be a result of increased ribosomal RNA production. Stimulation of mitochondrial activity might be a result of direct hormone action, or as a result of

increased demand for energy from other cellular sites.

Possible sites of action for thyroid hormones in the cell.

Explanation:-

1. Induction of new nuclear RNA polymerase of increased specific activity.

2. Increased turnover of nuclear RNA.

3. Localisation of ribosomes on microsomal membranes.

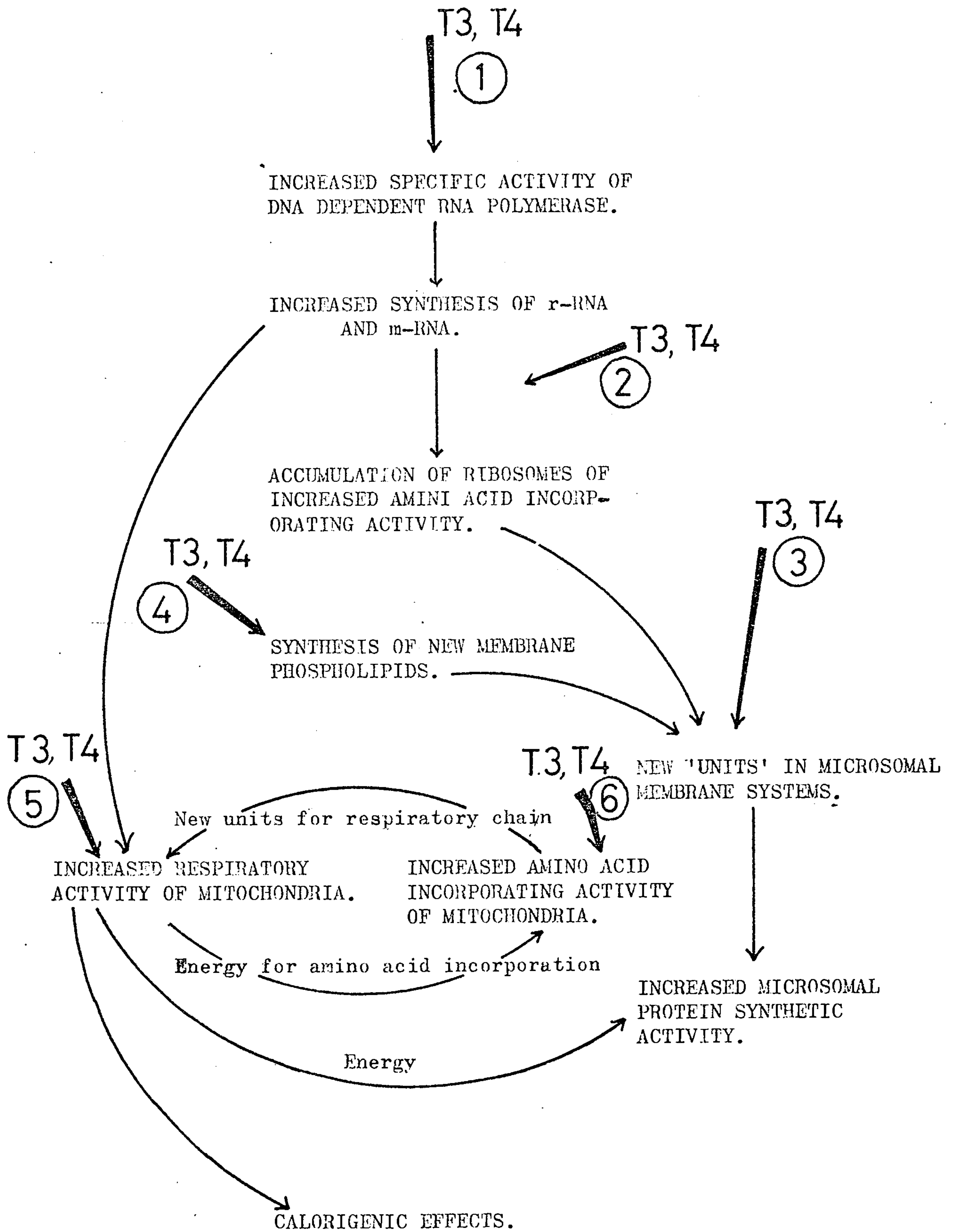
4. Induction of new membrane phospholipids.

5. Increased respiratory activity of mitochondria.

6. Increased amino acid incorporating activity of mitochondria.

Figure 1.

Possible sites of action of thyroid hormones in the cell.



Critical Review of Techniques used in the Preparation of Material for Electron - Microscopy.

Since the resolution obtained with the electron microscope is so much better than that obtained by the use of light microscopy the preparation techniques involved are much more critical. The final appearance of material under examination depends to a great extent, both upon the chemicals used in the preparation and upon the conditions under which they are used. Since much of the detailed cellular structure can be seen only at magnifications within the range of the electron microscope it is necessary to ensure that the results obtained are comparable to those obtained by other techniques and are not dependent upon the chemicals or conditions used.

The chemical nature and the physical properties of the fixative, and the vehicle used with it, the structure of the embedding medium and the contrasting agents used to "stain" the sections can all exert a profound effect upon the appearance of a tissue under electron microscopic examination.

Of the many fixatives used in light microscopy few are used in the preparation of material for electron microscopy. Those that are used are of relatively simple chemical composition and include, osmium tetroxide, various aldehydes, permanganate and chromium salts of which osmium tetroxide and the aldehydes,acrolein and glutaraldehyde, have been of the most use. The chemical composition of these fixatives is, to some extent, responsible for the appearance of the material they are used to prepare.

Fixation. Osmium does not appear to react with polysaccharides or with proteins to any marked extent. According to Hayes et al (1963) osmium reacts with proteins only after prolonged treatment of up to four days. Apparently osmium does cross-link with certain proteins, but the complexes so produced are not as stable as those produced by aldehyde fixation and

gradually breakdown.(Porter and Kallmann,1953). Dallam (1957) showed that after fixation in 1% osmium tetroxide in veronal acetate 11.7 - 16.9% of total nitrogen was lost from kidney slices and 29.2 - 36.5% from isolated liver mitochondria. Aldehydes, however, react strongly with proteins to form stable cross-linked complexes.(Pearse,1960; Fraenkel-Conrat,and Meecham, 1949; and French and Edsall, 1945.) Although aldehydes tend not to react with polysaccharides glycogen is usually retained, probably as a result of cross-linking with some associated protein (Pearse,1960). Aldehydes do not react with lipids and some extraction appears to take place even in aqueous solutions, whereas lipids are the main constituent retained after osmium fixation.

Sabatini, Bensch and Barnett (1963) studied a number of aldehydes for use as fixatives in electron microscopy, including formaldehyde, glutaraldehyde, hydroxy-adipaldehyde, glyoxal, crotonaldehyde and acrolein. They found glutaraldehyde gave the best preservation of cellular fine structure and enzymatic activity, and acrolein was a good preservative of cellular fine structure but was too reactive to preserve much enzymatic activity.

In general the most tissue components are retained after post-osmication of aldehyde fixed tissues. Aldehyde fixation stabilises the protein components and osmium treatment tends to retain the lipids and to protect them from extraction during the subsequent dehydration and embedding proceedures.

The properties of the buffering vehicle used with a fixative may also exert a profound effect upon the ultrastructure of tissues as viewed in the electron microscope. This effect is more marked after osmium fixation than after aldehyde fixation. This is not really surprising in view of the fact that proteins and polysaccharides are not well stabilised by osmium fixation. The degree to which these constituents are extracted differs with the different buffering media used, since protein solubility depends on such factors as pH, total ionic strength,

dialectric composition, and specific ion concentration.

Palade (1952; 1956) found considerable variation in the ultrastructure of cells after fixation in osmium tetroxide in the pH range of 5.0 - 9.0. He observed precipitation of the nucleoplasm and cytoplasmic ground substance at acid pH values and mitochondrial swelling in more alkaline conditions. Rhodin (1954) observed separation of intercellular membranes at acid pH values in his study of fixation in pH ranges 4.5 - 7.9, whereas Claude (1962) found better preservation of certain nuclear components and cytoplasmic fibrils at lower pH's.

There is evidence from light microscopy that the osmolality of the fixative solution is not of a great deal of importance in the preservation of cellular structure and relationships(Rhodin, 1954; Baker, 1958.) However, Caulfield (1957) suggests that there may be alterations in the appearance of mitochondria associated with changes in tonicity, and Holt and Hicks (1961) found that rat liver was sensitve to the tonicity of formalin/veronal fixatives. Various attempts have been made to improve the quality of fixation by the addition of salts to the buffering medium in an attempt to render it more nearly physiological, but without a great deal of success. Rhodin (1954) varied the osmolality of his veronal acetate buffer from 0.28 to 0.42 by the addition of more or less sodium chloride. Both he, and Palade in a similar experiment (Palade, 1952; 1956) found little change except in the degree of separation of the mitochondrial cristal membranes. Trump and Ericsson (1965) found that the quality of fixation of rat kidney proximal convoluted tubules fixed in 1% osmium in distilled water did not differ markedly from that fixed in osmium buffered with either s-collidine, veronal acetate,cacodylate, bicarbonate phosphate or Robinson's balanced salt solution (Robinson 1945). Caulfield (1957) adjusted the tonicity of his veronal acetate buffer by the addition of a non-ionic substance which would readily penetrate tissues, sucrose. Tissues fixed in his veronal acetate/sucrose solution did not differ markedly from those fixed in osmium in veronal acetate alone,

but the amount of well preserved materialper block was greater and the degree of preservation was more consistent. Holt and Hicks (1961) found that the best results were obtained with a formalin/veronal fixative with sucrose concentration of 7.5%. Although changes attributable to tonicity only become patent in extremely hypo- or hypertonic media it may often be advantageous to use a fixative and buffering system as close to physiological conditions as possible, but since direct measurement of the tonicity of cells has shown that their tonicity may be extremely variable (Opie, 1954) selection of a buffering medium, must of necessity, be made on arbitrary grounds, such as the quality of fixation as seen in the electron microscope. Robinson (1960) noted that tissues tend to swell after the inhibition of respiration, which may suggest that it may be advantageous to err on the side of hypertonicity , since no compensatory shrinkage takes place during subsequent embedding in epoxy resins.

Trump and Ericsson (1965) suggested that other properties of the buffering medium , possibly total ionic strength, dialectric composition, specific ion concentration and buffering capacity may effect the quality of fixation of material. Material fixed in osmium tetroxide buffered with either bicarbonate, cacodylate or phosphate was of relatively high density with the cytoplasmic organelles standing out in negative contrast, as pale areas, whereas the cytoplasmic ground substance of material fixed in osmium buffered with either s-collidine, veronal acetate, Robinson's solution or distilled water appeared pale with the cytoplasmic organelles standing out in positive contrast. There were also variations in the appearance of nuclei. After fixation with s-collidine, veronal acetate, cacodylate or phosphate the chromatin was evenly dispersed throughout the nucleoplasm, whereas after bicarbonate or Robinson's solution had been used to buffer the osmium the chromatin was aggregated around the nucleolus and against the nuclear membranes, which would suggest either a differential extraction of some component, probably protein, in the different salt solutions or a differential binding of the osmium

under the different conditions. The first explanation seems the more likely in view of the poor stabilisation of proteins by osmium. Those not adequately stabilised would be expected to be washed out of the sections at different rates during the fixation and subsequent proceedures.

Variation of the buffering medium used with aldehyde fixation did not exert such a profound effect, although Holt and Hicks (1961) noted variations in the results obtained with different buffering media used with formaldehyde. The preservation of fine structure of rat liver fixed with a formaldehyde/calcium medium was not good, but the preservation was considerable improved using veronal acetate or phosphate as buffering systems. No variation was observed after fixation with glutaraldehyde buffered with either phosphate, s-collidine or cacodylate which would be expected in view of the greater stabilising effect of aldehydes upon proteins.

Post osmication of material primarily fixed with an aldehyde tends to resemble the results obtained by primary osmium fixation. The cytoplasmic ground substance is pale with the cytoplasmic organelles appearing in positive contrast. The membranes appear in positive contrast instead of the negative contrast seen after the use of aldehyde fixation alone. Nuclear structure resembles that seen after the use of aldehyde fixation used alone, with the chromatin aggregated against the nucleolus and the nuclear envelope. The variation in contrast and tissue relationships with different buffer systems used with the osmium are not observed if the material has been previously fixed in an aldehyde, since the proteins appear to have been stabilised by the primary fixation.

Sabatini, Bensch and Barrnett (1963) found that it was necessary to introduce an intermediary buffer wash between the primary fixation and osmication proceedures to remove excess aldehyde from the tissue. Immediate postfixation of aldehyde fixed tissue resulted in very low contrast with the cytoplasmic membranes hardly apparent , even if the osmium treatment was prolonged for up to four days, or carried out

at twice the usual concentration. Immediate washing in sucrose buffer for up to three days produced a progressive increase in the contrast obtained after osmication Trump and Ericsson (1965) showed that the contrast gradually disappeared if the sucrose buffer wash was prolonged after.

Sabatini et al (1963) noted that tissues could be stored in this intermediate sucrose buffer wash at 4°C for up to three months without significant deterioration. De Thé (1963) recorded a similar proceedure for storing tissues under dry ice for three months to a year without noting deterioration of ultrastructural preservation or the localisation of ATPase activity. However, I found that glutaraldehyde or acrolein fixed material stored in sucrose phosphate buffer for three months showed a marked deterioration of preservation. There was a marked extraction of material from the glycogen areas of the mouse liver, flocculation of the cytoplasmic matrix material, especially within the microvilli of the proximal convoluted tubules of the kidney, and of the mitochondrial matrix material. There was some extraction of granules from nuclei and of ribosomes, and an overall reduction of contrast of the material which was subsequently post-fixed in osmium tetroxide.

Factors such as temperature, delay in fixation, duration of fixation, concentration of the fixative agent, mode of application of the fixative solution and the size of the block of material may all affect the quality of fixation of the tissue.

The effect of temperature of fixation has not been fully investigated. Rhodin (1954) investigated the effect of fixation at 0, 18, 37 and 48°C noting a tendency toward granularity of the cytoplasmic ground substance and mitochondrial shrinkage at the higher temperatures. It appears that the temperature affects the rate of cytolysis rather more than it does the penetration of the fixative solution, and Rhodin suggested that the changes he observed were early cytolytic changes rather than changes due directly to the temperature of fixation. It may be

that if early cytolytic changes can be prevented by the rapid application of the fixative solution, or by fixation in vivo that the temperature will be found to have little effect.

Onset of cytolysis appears not to be as rapid as was originally thought so that the speed with which the tissue and fixative can be brought together may not be as critical, particularly if low temperatures are used. (Trump, Goldblatt and Stowell, 1962; Ito, 1962;) However, as Trump and Ericsson (1965) suggested, it is probable that the breakdown of organelles is preceeded by a stage of increased susceptibility to mechanical dammage. As might be expected cellular components show dammage at different rates after the onset of anoxia. Trump et al showed that mitochondrial matrix granules showed alterations after 15mins. at 37°C. Smooth endoplasmic reticulum was less stable than rough, and formed chains of vesicles within 30mins. Thus it would appear justifiable to reduce the rate of cytolysis as much as possible by the reduction of the temperature of fixation or by reducing the delay in fixation.

A number of proceedures have been developed to overcome the delay in applying the fixative to the tissue. Immersion fixation of small pieces of tissue has given adequate results for many tissues, but as was noted by Palade (1952; 1956) with osmium fixation, the blocks of tissue must be very small for adequate penetration of the fixative, and even then the preservation varies considerably in different regions of the block. Blocks of tissue fixed by immersion usually show an outer region of mechanical dammage and an inner zone of poorly fixed material showing mitochondrial swelling, expansion of the endoplasmic reticulum, and course precipitation of the nucleoplasm and cytoplasmic ground substance. The intermediate zone shows good preservation of cellular structure. Sabatini et al found adequate penetration of tissue with aldehydes and good preservation as long as the blocks were no bigger than $2mm^3$. Liver shows relatively good preservation by immersion fixation, but the kidney presents more problems. Preservation of cellular structure may be adequate but tubule lumen are almost always collapsed and not obvious. A number of

attempts have been made to improve the fixation by applying the fixative in vivo to an anaesthetised or freshly killed animal. Leeson and Kalant (1961) perfused liver with osmium via the hepatic artery, subsequently completing the fixation process by immersion. Preservation was found not to differ markedly from that of tissue fixed wholly by immersion. Rease (1955; 1955b) attempted vascular perfusion of the kidney with osmium and aldehydes finding reasonable preservation of the cortex but poor results with the medulla. The only technique found to give reliable results in preventing the collapse of kidney tubules was that investigated by Maunsbach, Madden and Latta (1962) which involved dripping the fixative onto the surface of the kidney before excision and final fixation of the small blocks of tissue by immersion. The surface layers showed excellent preservation with open tubule lumena, whereas the deeper layers showed considerable changes, including swelling of the cytoplasm and mitochondria, collapsed tubule lumena and congestion of the vascular bed. The layer of good preservation was found to be deeper if the perirenal capsule was broken before the fixative was applied. In contrast material fixed wholly by perfusion showed collapsed lumena and some degree of cytoplasmic swelling. It seems that such changes occur as a result of severing the blood supply resulting in the rapid onset of anoxia. Physiological studies of Swann (1960) showed that the kidney lost up to 25% of its tissue volume as extracellular fluid and blood within a few seconds of the severing of the hilus, suggesting that the kidney is normally in a state of functional distension. Thus it would be expected that there would be drastic changes in the structure of the kidney with immersion fixation. In an attempt to overcome some of the major changes taking place with immersion fixation I adopted the practice of flooding the body cavity with the fixative solution before excising tissue for fixation by immersion. With this proceedure there was excellent preservation of the cellular structure but the lumena were rarely

found to be open.

Most investigators have selected arbitrary fixation times of 1 - 4hrs for osmium fixation and ½ - 4hrs with aldehyde fixation. The time taken to achieve fixation could be expected to vary with the different tissues and fixative systems, depending on the permeability of the tissue and the rate of penetration of the fixative solution. However, little investigation has been carried out on the subject of minimum duration times for the completion of the fixation process. Although it seems that in some cases at least very short times are adequate, Koshiba, Smetana and Busch(1970) found adequate preservation of structure in Novikoff hepatomas after 10 - 15mins. fixation in 2% glutaraldehyde. The cytologic effects of overfixation are also little understood, although extraction or leaching effects might be expected. PaLade (1952,1956) found extraction of material from the Z and I bands of muscle fixed for up to 48hrs in osmium, whereas there was excellent preservation of structure with up to 4hrs fixation. Similar experiments with pancreatic acinar cells showed almost complete extraction of material from the zymogen granules with 24hrs fixation. This phenomenon seems to be linked to the effects of osmium upon protein stabilisation mentioned above, since protein and polysaccharide components disappear more rapidly than lipid material during prolonged osmium fixation. Holt and Hicks (1961) noted, interestingly, that the preservation of fine structure in rat liver was better after 24hrs. of formalin fixation than 1hr, and better after 1hr of osmium fixation than after 4 hrs. However, little work has been carried out to investigate the effects of different buffer systems upon extraction during prolonged fixation with osmium, and no evidence seems to exist of the processes taking place during prolonged aldehyde fixation.

The effect of the concentration of the fixative solution upon cellular preservation is also little understood. Most investigators appear to have selected arbitrary concentrations of 1 to 2% for osmium

tetroxide and 2 to 10% for the various aldehydes. In view of the reactivity of the solutions used as fixatives it would seen adviseable to use the lowest concentrations reconcilable with good preservation of cellular structure and enzymatic activity, which latter might prove as good an indicator as any of the amount of dammage inflicted upon a tissue during fixation.

Dehydration. The processes of dehydration and embedding can also affect the final appearance of material in the electron microscope. Most investigators have used a graded series of alchohols for the dehydration of material, although other organic solvents such as acetone and dioxan have been used (Pease 1964) without improving the results. Pease emphasized the necessity of completing the early stages of dehydration with rapidity in order to minimise the extraction of poorly stabilised material from the tissue blocks. It is usual to complete the initial stages to absolute alchohol within 15mins. Once most of the water has been removed from the tissue there is little advantage to be found in curtailing the steps in absolute alchohol since the tissue will subsequently be exposed to a prolonged treatment in un-polymerised plastic embedding media which may act as an effective organic solvent.

Embedding. Because of the viscosity of un-polymerised epoxy resins it has been necessary to employ a transition solvent between the alchohol and the resin. Epoxy propane appears to be the best solvent for this purpose although others, such as xylene, toluene and styrene have been used (Birbeck and Mercer, 1957; Kurtz,1967; Pease, 1964.) Epoxy propane has the advantage of miscibility with water, of being sufficiently fluid to reduce the viscosity of the embedding media used to infiltrate the blocks, and of being sufficiently volatile that excess will evaporate out of the tissue. According to Pease, it is also sufficiently reactive with the embedding medium that excess will be incorporated into the final polymer, without causing any detrimental effects. According to

Merillees et al (1963) it may also act as a fixative combining with reactive groups in the tissue and inhibiting certain histochemical or staining reactions.

Epon 812 (as described by Luft,1961) was routinely used throughout this investigation and proved satisfactory.

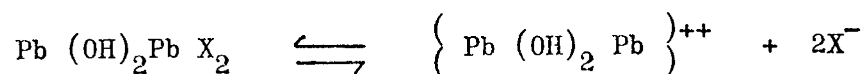
Section staining. Although Sabatini et al (1963) noted that aldehyde fixed tissues post-fixed with osmium tetroxide had enough intrinsic contrast when embedded in Epon 812, to be viewed without subsequent section staining, examination of the tissues was much easier after the use of a contrasting agent.

Various solutions of uranyl acetate have been used as contrasting agents, from saturated methanolic solutionsto 2% ethanolic and 2 - 5% aqueous solutions. Although Bernhard (1968) suggested that only very short staining times were necessary to produce adequate results with any of these formulations, up to an hour is used by most people. Solutions based on organic solvents tend to give better results than aqueous solutions, and tend to require shorter staining times. This is probably a result of the lower surface tension and greater solvent properties of the organic solvents with Epon when compared to water. Proteins stain fairly intensely, but the cytomembranes do not appear very distinctly, so that the overall contrast, although an improvement upon that of unstained material is not very good.

The use of lead salts as contrasting agents yield better results, although they are more difficult to handle. Watson's original lead hydroxide stain (Watson, 1958) produced vast quantities of lead carbonate upon exposure to the air which heavily contaminated sections. Numerous techniques were developed to overcome this problem, of which the most notable are those which combine the lead with a chelating agent such as the lead citrate stain of Reynolds(1963) and the lead tartrate of Millonig (1961). These two staining solutions are said to produce minimal contamination of sections. The results are reliable from section to

section, although they do have the disadvantage of destaining over prolonged periods, so that prolonging the treatment of a section in the staining solution tends to reduce the final contrast rather than to increase it.

Reynolds (1963) discussed the mechanisms of alkaline lead staining and suggested that all the different formulations might act in the same way, the citrate of his stain and the tartrate of Millonig's stain acting as chelating agents sequestering excess lead from the staining solutions and preventing contamination of the sections with a fine precipitate. Reynolds stated that the tissue binding sites sequestered lead more strongly than the citrate so that the citrate did not interfere with the staining reaction. It is probable that the tartrate of Millonig's solution competes more strongly with the tissue bound lead, which would account for the reduction in contrast with the length of time in the staining solution. Reynolds suggested that divalent lead salts in alkaline solutions ionise according to the following equation:-



$$Pb\ (OH)_2Pb\ X_2 \rightleftharpoons \left(Pb\ (OH)_2\ Pb \right)^{++} + 2X^-$$

which would lead to the attachment of the lead atoms at each tissue binding site and account for the advanced contrast observed with lead staining using highly alkaline solutions.

Lead salts in general stain diffuse proteins such as the mitochondrial matrix and cytoplasmic ground substance but poorly but render the cytoplasmic membranes in sharp contrast.

Double staining of sections using uranyl acetate and lead salts gives better results in terms of contrast than the use of either agent on its own. Diffuse and particulate proteins stand out against the cytoplasmic ground substance and the cytoplasmic membranes are sharply contrasted.

Marinozzi and Gautier (Marinozzi and Gautier, 1962; Marinozzi, 1963) studied the role of fixation in the staining of material for

electron microscopy and noted that various stains, previously used as general contrasting agents yielded different results with different fixatives. Neither uranyl acetate nor lead gave preferential staining after primary osmium fixation, whereas there was preferential staining of the chromatin after formalin fixation either with or without post osmication. Uranium stained chromatin under various experimental conditions; without previous fixation (Huxley and Zubay. 1961), after formaldehyde fixation or after formol/osmium treatment, whereas lead stained chromatin after postosmication but not after primary formaldehyde fixation alone. It was suggested that either the fixative bound to the tissue reacted with the staining solution, or in so far as it was chemically active it modified the reactivity of some of the components to which it was bound. It seems that osmium denatures nucleoproteins thus preventing the reaction with reaction with the staining solutions to give preferential results. Aldehyde fixation seems to protect the nucleoprotein from the effects of the osmium tetroxide.

In the absence of osmium treatment lead staining resulted in the selective staining of ribonucleoproteins, after aldehyde fixation uranyl acetate stained the deoxy-ribonucleoproteins , the strongest diferentiation occuring after post-osmication.

It was suggested that the staining of some components of osmium treated tissues, such as the cytoplasmic membranes depended on a reaction with tissue bound osmium, which could be prevented by a prior oxidation. Staining of the nucleoprotein components with lead depended upon some other mechanism, since it was not affected by an oxidation process. It seems that reduced osmium is less important in the staining of tissue components with uranyl acetate although uranium staining of cytoplasmic membranes was affected to some extent by the presence of reduced osmium in the tissues..

Some interaction of the staining solution and the embedding medium may also affect the result of electron staining. Thus

better results may be obtained by using aqueous solutions on water soluble embedding media such as methacrylate, Durcupan or Aquon, and organic solvents systems might give better results on Araldite or Epon. It was found, however, that good results could be obtained using either solvent system on Epon but the staining times had to be prolonged compared to those used to obtain good results with water soluble embedding media.

Greater difficulties were experienced with regard to the penetrability of Epon to staining solutions when staining of thick sections with toluidine blue was attempted for light microscopy, but rigorous 'boiling' of the stain on the sections produced adequate if unreliable results.

Histochemical techniques. Although a number of techniques, based upon the production of an electron - dense reaction product, from tissue bound enzymes have been developed, which have enabled the cellular localisation of various enzymes to be determined, techniques for localising other cellular compenents are as yet in their early stages. Those investigated up to date consist of preferential, non-specific staining by heavy metal complexes, extraction of various components by solution in water or dilute acids or alkalis, reduction in the contrast of certain constituents by chelating agents, or their removal by enzymic digestion.

As Marinozzi pointed out, only those stains which make it possible to increase, in a selective manner, the density of a particular component, have a genuine histochemical value. Unfortunately, most of the available staining techniques are only able to increase the contrast of certain components in a 'selective' manner, few if any are available which are specific for a particular component.

Most of the available selective staining techniques rely upon the variation of the result of contrasting agents with different fixatives. As was pointed out above, lead staining in the absence of osmium results in preferential of ribonucleoproteins, and that after aldehyde fixation

uranium salts stain deoxyribonucleoproteins more intensely than ribonucleoproteins. Huxley and Zubay (1961) noted that deoxyribonucleoprotein took up almost its equivalent weight in uranyl acetate after minimal osmium fixation, whereas ribonucleoprotein had a greater affinity for lead than did deoxyribonucleoprotein structures. Huxley and Zubay pointed out that this uranyl acetate staining of deoxyribonucleoprotein structures gave results comparable to those obtained with the Feulgen reaction in light microscopy.

Differentiation of components can be obtained in osmium fixed tissue by the comparison of oxidised and non-oxidised sections. However, although this allows one to determine whether or not the staining of a component was due to the presence of reduced osmium it does not give much hint as to the identity of the component to which the osmium was bound, since as yet little is known ot the affinity of osmium to different tissue components.

The action of acrolein and silver upon a tissue is more fully understood. Marinozzi (1963) noted that silver impregnation following acrolein gave results comparable to those obtained by the Schiff reaction. According to Van Duijn (1960) acrolein 'labels' sulphydril groups with an aldehyde group which is then available for reaction with the silver nitrate of Marinozzi's staining solution. However, since the silver is deposited as fine granules upon the surface of the section the results are rather difficult to interpret with any accuracy and the technique is, at best, only preferential since it labels proteins fairly indescrimminately (including nucleoproteins, collagen and elastin.) However, greater densities of deposits occur over atructures composed of the more basic proteins. This means that little differentiation can be made between deoxy- and ribonucleoproteins since the associated histones are equally rich in basic amino acids, and produce equally dense deposits of granules.

Bernhard and Leduc (1960) have been able to produce some

differentiation between nucleoproteins by differential extraction with slightly acid or slightly alkaline water. Their technique relied upon the use of a water soluble embedding medium, glycol methacrylate. Treatment of sections with dilute hyperchloric or perchloric acids over prolonged periods (up to 15hrs. with perchloric acid) extracted what were said to be ribonucleoprotein particles, but the treatment lead to severe dammage to the sections and the results were not comparable to those obtained by ribonuclease digestions. Similarly, Marinozzi (1963) was able to differentiate between nucleoproteins by floating acrolein fixed mathacrylate sections on water before staining with silver and/of uranyl acetate. This technique seemed to depend upon the length of the washing steps as to its results.

Bernhard (1968) developed a technique which was able to differentiate between nucleoproteins by a regressive staining proceedure. He was able to remove uranium from certain components of aldehyde fixed tissue by the use of chelating agents such as EDTA. This gave a reduction in the contrast of the deoxyribonucleoprotein components whilst leaving the ribonucleoprotein components heavily stained. The EDTA appears to remove the less tightly bound uranium as a soluble complex which is then washed out of the tissue. Bernhard, stressed however, that there were limmitations to this technique, that it was at best preferential and by no means specific for either of the ribonucleoprotein components.

The one, apparently infallible test for the nature of a component is that of enzymic digestion, involving the selective removal of components by relatively specific enzymes. However this technique is also problematic. There are two approaches in use, the treatment of the fixed block with the enzyme before final embedding, or the digestion of a section of material embedded in a water soluble embedding medium. In both cases the tissue is likely to differ markedly from that occuring in life, in the first case because it has been subjected to a fixation.

process which will have had gross effects upon the chemical composition and/or the molecular structure of the components and in the second place because the tissue is completely surrounded by a plastic embedding medium as well as having been subjected to fixation and dehydration proceedures.

Marinozzi (1962), Marinozzi and Bernhard (1963), Bernhard and Leduc (1960) and Leduc and Bernhard (1962) have successfully used ribonuclease, deoxyribonuclease and pepsin digestion to selectively remove nucleoproteins and proteins from sections, but found that this was only possible if water soluble embedding media are used. Treatment of tissue blocks suffers from many of the problems encountered during fixation, such as inadequate penetration of the tissue block by the enzyme in use. It is possible that the degree of penetration might vary with the size of the enzyme and the vehicle in use with the enzyme, so that only a limmited zone of the block is of any use. Since it is necessary to fix the tissue before treating it with the enzyme inorder to preserve the basic ultrastructure, factors such as the osmolality of the vehicle or its pH are unlikely to affect the result except in so far as they affect the enzyme itself.

There are likely to be problems in the interpretation of pictures obtained from enzyme digested material appart from those inherent upon a technique reliant upon the comparison of control and treated material for results. For instance the lack of digestion of a component need not mean that it is not digestible by the enzyme in use. It is possible that the component is protected by some non-digestible component. Koshiba, Smetana and Busch (1970) noted that certain components of the nucleo-pore complex behaved as ribonucleoproteins with heavy metal staining proceedures but were not ribonuclease digestible, They concluded that these elements were protected by a protein coat. Lack of removal by an enzyme may also mean that the component is so altered by prior fixation and/or dehydration proceedures that it can no longer act

as a substrate for the enzyme, however, if a combination of enzyme digestion techniques and heavy metal staining techniques are critically compared it is possible to come to some valid conclusion as to the identity of certain cellular components.

Inspite of the processes a tissue must be subjected to before it can be examined in the electron microscope, all of which can profoundly alter the ultrastructure, excellent preservation can be obtained if the preservation techniques employed are carefully selected and the results are compared with those obtainable by other techniques. So, although the impressiohs of ultrastructure observed in the electron microscope are artefactual in that they depend upon the quality of the preparation proceedure, it is also possible to ascertain wheter or not they are 'life like artefacts' by comparison of material and by the use of histochemical techniques some attempt can be made to identify the chemical composition of the cellular components.

METHODS.

Treatment of experimental animals.

Experimental treatments were selected which would parallel, as nearly as possible, those used by other workers investigating the effects of thyroid hormones. Tata and co-workers used of dosesof triiodothyronine in the range of 20 - 25μgms/100gms body weight, administered subcutaneously to either normal or thyroidectomised rats when investigating phenomena associated with hyperthyroidism, or in studying the events in the latent period of action (Tata st al, 1963; Roodyn et al, 1965; Widnell and Tata, 1966; Tata and Widnell, 1966.) In studying the effects of hyperthyroidism induced by thyroxine administration Roodyn et al (1966) used doses of thyroxine in the range of 25 - 35μgms/100gms body weight. Sokoloff and Kaufmann (1959, 1961) used doses in the range of 100μgms per rat. In this invsetigation doses of 25μgm/100gm body weight of triiodothyronine, and 100μgm/100gms body weight of L-thyroxine, administered intraperitoneally, were used throughout.

Surgically thyroidectomised mice were obtained from the Scientific Products Farm, and were maintained upon a solution of 1% calcium lactate in the drinking water, to alleviate the effects of the accompanying parathyroidectomy. Animals were thyroidectomised when weighing in the range of 15 - 20gms., and were maintained in the laboratory for three to four weeks before use.

Mice were selected for study, in preference to rats, as they are more suitable for handling in the preparation of material for electron microscopical examination. Male, T.O. strain mice, weighing between 20 and 25gms. were used throughouth this programme of work. Animals were fed and watered ad libitum. Administration of hormones was carried out as nearly as possible the same time of day,

between 11am and noon, to avoid any possible effects of diurnal variations, although this was impracticable in the case of the time course experiments. Animals were killed at approximately the same time of day, although again this was not possible in the case of the time course experiments. Animals were killed by breaking the neck, and small pieces of liver and kidney were removed as rapidly as possible for preparation for electron microscopy.

Induced hypo- or hyperthyroidism.

(a) Hyperthyroidism.

Normal mice were injected with either 25μgm/100gms. body weight of triiodothyronine, or 100μgm/100gms. body weight of thyroxine in 0.2ml. saline daily for seven days, via an intraperitoneal injection.

(b) Hypothyroidism.

Surgically thyroidectomised mice were injected daily for seven days, with saline vehicle.

(c) Normal animals were also injected daily for seven days, with saline vehicle blanks as a control group.

Chronic effects of triiodothyronine.

Mice were given a single intraperitoneal injection of 25μgms/100gms body weight of triiodothyronine and were killed 15, 30, 45 or 60 hrs. later. A group of control animals was killed at the beginning of the experiment, and another group of animals, which had received an injection of saline vehicle 60hrs. before, were killed at the end of the experiment.

Unfortunately, due to the unavailability of surgically thyroidectomised mice at this time, this experiment had to be carried out using normal animals.

Preparation of material for electron microscopy.

For routine examination material was fixed with glutaraldehyde and osmium tetroxide and sections were stained with either uranyl acetate or uranyl acetate and lead tartrate. In order to check the origin of certain of the observations and to eliminate their possible artefactual origin, material which had been fixed in either glutaraldehyde or osmium tetroxide has been examined. In which case the osmium material was either unstained or uranium was used as a contrasting agent. Uranyl acetate and lead tartrate double staining was used after glutaraldehyde fixation.

Acrolein fixation followed by silver nitrate staining (Marinozzi, 1963.) was investigated as a preferential staining technique for nucleoproteins. Pepsin and ribonuclease digestion after glutaraldehyde fixation has been used in attempt to differentiate between the different nucleo-protein components of the nucleus.

Storage of material in cold buffer solutions was investigated as a means to curtail the numbers of animals used and to reduce the time in preparation of material for the electron microscope.

All material was embedded in Epon 812 after routine ethanol dehydration.

Application of the fixative.

All animals were killed by breaking the neck. The abdominal cavity was opened immediately and flooded with the fixative solution in use. Where osmium tetroxide, or acrolein was used as the primary fixative the abdominal cavity was flooded with the buffer vehicle rather than the fixative solution, but the tissues were removed and placed in vials of the fixative solution as rapidly as possible after being removed. The frontal lobe of the liver and the left kidney were

used, ahd were placed in a bath of the fixative solution where they were cut into small blocks(approximately 1 - 2mm.^{3}) using a fresh scalpel blade. The fixation proceedure was completed by immersing the tissue blocks in vials of fresh fixative. To minimise dammage the blocks were not then removed from the vials until they were embedded in resin. Subsequent solutions were changed by careful removal with a Pasteur pipette.

Fixation times.

Arbitrary periods of time were originally selected for fixation of tissue, based upon those recommended for the fixative concerned in the literature. However, much shorter times were often found to be adequate. For instance, two to four hours are in general useage for glutaraldehyde fixation but fifteen to thirty minutes have been found to produce adequate results. Similarly, up to two hours is in general use for osmium tetroxide fixation, fifteen minutes has been found adequate, although in this case, only the outer layers of the tissue blocks were shown to be fixed. Blackening only extended to a a depth of about 0.5 - 1.0mm. Layers deeper in the blocks were unaffected by the fixative and often showed considerable extraction of material.

Temperature of fixation.

Although it has been observed that tissues fixed at higher temperatures tend to show early cytolytic changes (Rhodin, 1954; Trump et al, 1962; Ito, 1962.) fixation at room temperature was found to be adequate particularly if the tissues were exposed to the fixative as soon as possible after the opening of the abdominal cavity.

Variation of the buffer vehicle.

Little difference has been observed with the variation of the buffer vehicle used with the fixative in the case of aldehyde fixation.

Both cacodylate and phosphate buffers have been used, giving satisfactory results, with no difference in the appearance or the relative contrast of the tissues. However, it was upon osmium fixation that the variation of the buffer vehicle was shown to have the most marked effect (Trump and Ericsson, 1965.) To avoid any unnecessary variation, Caulfield's 2% buffered osmium tetroxide was used throughout (Caulfield, 1957.)

The pH of the buffers used in the course of this investigation, have varied from pH 6.4 to 7.6 without affecting the final quality of fixation of either the liver or the kidney.

Osmium fixation.

Small blocks of tissue were fixed with Caulfield's 2% osmium tetroxide for 2hrs. at room temperature, followed by a 5min, buffer wash and routine ethanol dehydration and Epon embedding.

Fixation of tissues with osmium tetroxide, buffered with veronal acetate, yielded material with relatively low contrast when examined in the electron microscope. The cytoplasmic ground substance was pale and somewhat granular in appearance, as is shown in Plate 1 . Cytoplasmic organelles appeared as darker bodies against a lighter background, but the differences in electron density were not sufficient to make detailed examination easy. In particular the cytoplasmic membranes were very similar in electron density to the background substances, making them not readily apparent.

Nuclei appeared paler than the surrounding cytoplasmic ground substance. The chromatin was dispersed throughout the nucleoplasm and not aggregated against the nuclear membranes as after primary aldehyde fixation. Nucleoli appeared as relatively dense, discrete bodies with little associated chromatin. As with the cytoplasmic membranes the nuclear membranes were difficult to distinguish.

Their relative density was so similar to that of the surrounding material and the individual elements appeared to be so closely associated that they were often difficult to resolve even at magnifications of up to x20,000.

Glutaraldehyde fixation.

Material was fixed in 5% glutaraldehyde in cacodylate buffer at pH 7.4 for 1 - 2hrs. at room temperature before washing in the buffer for 1hr. followed by routine ethanol dehydration and embedding in Epon 812.

Material fixed in glutaraldehyde and stained with uranyl acetate and lead tartrate did not differ markedly in appearance from similarly prepared material described by Sabatini et al, (1962). The general contrast of the tissue was low but was adequate for examination, particularly if the sections were double stained with lead and uranium salts rather than if they were stained with uranium alone.

The general cytoplasm was of relatively high electron density with a somewhat homogeneous appearance. Mitochondria stood out as pale bodies of indistinct structure against a darker background. The cristae could usually be seen as darker striations but the outer mitochondrial membranes could not be seen. The endoplasmic reticulum resembled that of the material fixed in osmium but on close observation the membranes could not be discerned. The endoplasmic reticulum was visualised as a result of the attached ribosomes. Glycogen deposits resembled those seen after osmium fixation, appearing as groups of electron dense granules.

Nuclei differed from those seen after osmium fixation. The nuclear membranes could not be distinguished although their presence was indicatied by the cessation of material against the cytoplasmic interface. Chromatinic material was not dispersed as sfter osmium

fixation but was aggregated into compact granular masses which surrounded the nucleolus and were applied to the inner margin of the nuclear envelope. The interchromatinic material consisted of a fine network of fibrils and granules of low electron density compared to that of the surrounding cytoplasm. Plate 2 shows the characteristic appearance of glutaraldehyde fixed material.

Glutaraldehyde and osmium double fixation.

For the initial examination of liver and kidney, small pieces of liver and kidney were fixed in 5% glutaraldehyde in cacodylate buffer at pH 7.4 at room temperature for 1 - 2 hrs, This was followed by a 24hr. wash with a sucrose/phosphate buffer solution. Post-fixation in Caulfield's 2% osmium tetroxide was followed by a short wash in veronal acetate buffer and routine ethanol dehydration and embedding in Epon.

The general appearance of the tissue, as is shown in Plate 3 , resembled that of material fixed in aldehyde alone, with the exception that the mitochondria appeared somewhat darker than the cytoplasmic ground substance. The osmium post-fixation restored the appearance of the membrane elements so that both the outer mitochondrial membranes and the cristae were clearly visible.

The endoplasmic reticulum was prominent and consisted of a double membrane with attached ribosomes, rather than the apparently membranous structure visualised by attached ribosomes, seen after aldehyde fixation alone.

Nuclei were paler than the surrounding cytoplasmic ground substance. Their appearance resembled that of material fixed with aldehyde alone, with the chromatin aggregated against the periphery and around the nucleoli, and not dispersed throughout the nucleoplasm. The nuclear membranes were clear and composed of two elements, the inner being slightly more prominent than the outer.

As is shown in Plate 4 , the appearance of material post-fixed with osmium tetroxide, from the preparation of which the sucrose/phosphate washing stage had been ommitted, resembled similarly prepared material described by Sabatini et al (1962). Some factor, possibly excess aldehyde remaining in the blocks, interfered with the normal action of the osmium so that the tissue resembled that prepared by primary aldehyde fixation, with low general contrast and a lack of obvious visible membrane structures.

Section staining of glutaraldehyde fixed, and glutaraldehyde and osmium double fixed material.

Although, as was pointed out by Sabatini et al (1962), the contrast of aldehyde fixed tissues was sufficient for them to be visualised in the electron microscope without section staining, it was not sufficient to make detailed observations possible. The use of uranyl acetate or lead tartrate as section stains considerably improved the appearance of such tissues and made detailed examination considerably easier.

Uranyl acetate as a contrasting agent: Sections were stained with either 2% aqueous uranyl acetate or saturated methanolic uranyl acetate. Methanolic solutions were found to produce better results than aqueous, possibly because of its lower surface tension which allowed better penetration of the Epon. Inspite of the observation of Bernhard (1968) that the staining times in general use with uranium were much longer than were necessary to produce adequate improvement of contrast, fairly prolonged times were found to be necessary. Bernhard found that as little as five minutes treatment in aqueous uranyl acetate produced a significant improvement in contrast. However, up to 1½hrs was necessary to produce good results with the aqueous solutions and the material in use here. Better results were obtained with methanolic solutions, 30mins. staining times producing adequate results, and the

methanolic solution was used in most of the experiments. This difference was possibly due to the differences in embedding media in use, although Bernhard was also using Epon. The differences in staining times required to produce a significant improvement in contrast between aqueous and methanolic solutions seem more likely to be a result of the different solvent properties rather than the concentrations in use, since a dilute methanolic solution required 40 - 50 mins. to produce equivalent improvement in contrast.

The results of using uranyl acetate as a contrasting agent are illustrated in Plate 3 . It's use produced sections with a much better contrast when compared to unstained material, and even improved the contrast of material that had been post-fixed with osmium. There was a general improvement of the contrast as a result of the heavy metal binding to protein components. The electron density of nucleoprotein components was markedly increased and that of the cytoplasmic ground substance to a lesser extent. There seemed to be little difference between the cytomembranes of the stained and the unstained material.

Uranyl acetate and lead tartrate double staining: The result of uranium salts, used alone, gave a marked improvement of the contrast but detailed structure was often difficult to interpret since the membrane structures and fine particles are not rendered in sharp contrast. For this reason double staining using lead salts, after the uranium, was attempted. Lead stains appear to have a greater affinity for the membrane components.

Sections were stained with Millonig's lead tartrate solution (Millonig, 1961.) for 15 mins. and washed briefly with N/100 NaOH and distilled water, following staining with methanolic uranyl acetate, described above. A marked improvement in the clarity of the sections, as observed in the electron microscope, was obtained. However, it must

be noted that prolonged staining in the lead solution tended to reduce the final contrast, rather than to increase it. This was possibly due to the chelating action of the tartrate removing bound lead from the sections.

The appearance of double stained material is shown in Plate 5 . Sections were very clear and easy to examine. The cytoplasmic membranes and the particulate components, such as ribosomes were rendered in very sharp contrast, whilst the more diffuse components were stained by the uranyl acetate and tended to be somewhat less clear.

Digestion of blocks with pepsin and ribonuclease.

Small pieces of tissue were fixed in 2% glutaraldehyde in phosphate buffer at pH 7.4 for 15mins. at room temperature, and subsequently washed in sucrose/phosphate buffer for 24hrs. before being subjected to one of the following treatments:-

(a) Phosphate buffer, pH 7.4, for 20 mins.

(b) 0.1N HCl for 20mins.

(c) 0.1N HCl with pepsin (0.5gm./100ml.) for 20 mins.

(d) Phosphate buffer pH 6.4, for 180 mins.

(e) Phosphate buffer pH 6.4, with ribonuclease (1mgm./100ml.) for 180 mins.

All the above treatments were carried out at 37°C. The digestion was terminated by a 60 minute post-fixation in Caulfield's osmium tetroxide, and was followed by routine dehydration and embedding. Sections were stained using uranium and lead salts.

Control incubations: A preliminary investigation established that a period of 15 minutes fixation in 2% gluataldehyde was adequate , although the inner depths of the blocks of tissue did show dammage attributable to poor fixation, suggesting that the fixative had not

penetrated to the centre of the blocks,

Material which had been incubated in phosphate buffer at either pH 7.4 for 20 mins., or at pH 6.4 for 180 mins. or in 0.1N HCl for 20 mins. before osmication resembled the routinely prepared material previously described. This would suggest that the fixation proceedure used was sufficient to stabilise the tissues, for them to withstand the adverse treatment to which they were subjected.

There were slight variations in the appearance of the incubated material which could be attributed to the processing proceedures. The general contrast of the material which had been incubated in the phosphate buffer was slightly reduced, but this reduction was not sufficient to cause any difficulty in the examination of the material, and was compensated for by the double staining proceedure There was some degree of dammage to cytoplasmic structures after incubation with 0.1N HCl. The cytoplasmic matrix material tended to be granulated, especially in the liver, and there was some vacuolation of the cytoplasm, but this was restructed to a narrow outer zone of the blocks, where the effects of the hydrochloric acid might be expected to be the most severe. There was some loss of glycogen deposits from the liver, but this was very slight and again restricted to a narrow outer zone of the blocks.

Thus, although the incubation medium had slight effects upon the appearance of the treated material, these were slight and the more severe effects were restricted to a narrow outer zone.

Ribonuclease digestion: Again the appearance of the liver and kidney which had been subjected to ribonuclease digestion resembled routinely prepared material, however, there were detailed differences which could be attributed to the action of the enzyme.

Plate 6 shows the characteristic appearance of ribonuclease digested liver and Plate 7 shows the characteristic appearance of kidney

which had been similarly treated. As examination of these plates shows, ribonuclease digestion produced gross loss of contrast in many ribonucleoprotein structures, for instance in the liver there was little evidence of ribosomes attached attached to the endoplasmic reticulum, so that all the endoplasmic reticulum of treated material appeared to be of the smooth type. There was no evidence of ribosomal structures attached to the outer element of the envelope, where ribosomes are in normal preparations, commonly found, and there was no evidence of polysomal aggregates within the cytoplasm of the kidney, these again were commonly observed after routine preparation .

There were significant effects upon the appearance of the nucleus. There was a general reduction in the contrast of the pars fibrosa region of the nucleolus in almost all of the nuclei examined. There was little apparent effect upon the appearance of the pars granulosa, or upon the chromatin, but detailed observation revealed a loss of the underlying fibrillar structure of the nucleus supporting the suggestions of Swift (1963), Marinozzi (1964), Bernhard and Granboulan (1968) that this is an underlying ribonucleoprotein network.

<u>Pepsin digestion:</u> Pepsin digestion had a less marked effect upon detailed cellular morphology of the liver or the kidney, and the general appearance of pepsin digested material resembled that of normally prepared material. There was however, a slight reduction in the general quality of fixation, probably as a result of the exposure to 0.1N HCl.

Plate 8 shows the characteristic appearance of pepsin digested liver, and Plate 9 shows the characteristic appearance of similarly prepared kidney. The most obvious result of the pepsin digestion was a gross reduction of contrast of the interlamellar spaces of all the cytoplasmic membrane systems, the golgi apparatus and the nuclear

envelope, but especially of the endoplasmic reticulum. There was a reduction in the contrast, and apparently of the size of ribosomal structures. Although the endoplasmic reticulum still possessed a granulated appearance due to the presence of attached ribosomes, the ribosomes were not as distinct as those seen after routine preparation. Ribosomes were not obvious attached to the outer element of the nuclear envelope, and polysomal aggregates in the cytoplasm of the kidney were difficult to visualise and were often not discernible. This would suggest the prexence of some protein component of the ribosome which is susceptible to digestion by the enzymes pepsin.

Within the nucleus, there was a slight reduction in the contrast of the pars fibrosa of the nucleolus, which was less marked than the reduction in contrast produced by the action of ribonuclease, which would again suggest the presence of a proteinaceous component. There was also a reduction in the contrast of chromatin components, which resulted in a loss of the apparently granular structure. The chromatin, after pepsin digestion, generally had the appearance of a relatively homogeneous mass of electron dense material.

Acrolein fixation and silver nitrate staining.

Material was fixed in a solution of 10% acrolein in cacodylate buffer, pH 7.4, for 1 hr. and washed over night in a cacodylate buffer before routine dehydration and embedding. Thin sections were cut and stained in a 0.1% silver nitrate solution, pH 8.5, for 30 - 45 mins. at 70°C before washing in a solution of sodium thiosulphate and mounting on the copper grids. It was found to be necessary to stain the sections before mounting since the staining solution reacted with the copper, causing severe dammage to the grids and interfering with the staining reaction.

Acrolein was not an ideal fixative for use in electron microscopy, for a number of reasons, including its high toxicity and

noxiousness, even in a 10% solution. It had a tendency to polymerise in the blocks before infiltration of the embedding medium, making the final blocks brittle and 'chippy' and difficult to section. Tissue preservation, though adequate, was not as good as that produced by glutaraldehyde.

Some difficulties were also experienced, due to the nature of the silver stain. The silver is deposited as fine granules of metallic silver on the surface of the section, rather than being bound to tissue components, as are more conventional heavy metal stains. Observation of the sections also required some experience since it was necessary to focus upon the silver granules and upon the underlying section in order to identify the tissue component that gave rise to the deposit in question. Focussing of the electron image, for photographic records was often very difficult sonce the silver deposits and the underlying section were rarely in focus in the same plane.

The general background contrast was poor but was sufficient to distinguish most cellular structures, if it was not sufficient to enable detailed examination. The general appearance of material resembled that of other aldehyde fixed material. The silver granules were mainly deposited over structures that might be expected to be nucleoprotein in composition.

Plate 10 shows the appearance of liver prepared in this way. Sections tended to be vacuolated, and much of the glycogen removed, suggesting inadequate fixation, however the endoplasmic reticulum was well preserved and was very clear due to the presence of silver granules over attached ribosomes. Mitochodria were more difficult to identify, usually apppearing as homogeneous pale gray bodies, with no discernible structure and no apparent silver deposits. There was some deposition of silver over the cytoplasm, granules arranged in distinct rosettes and chains were distinct from a general deposition of

fine granules forming a background contamination, which sometines occurred.

Nuclei resembled those usually found after aldehyde fixation, with silver deposits over the chromatin and the nucleoli. The nuclear membranes were not usually apparent, but their position was indicated by the presence of silver granules over ribosomes attached to the outer element and by the abrupt cessation of deposits against the inner element. The pars fibrosa region of the nucleolus frequently appeared as a dense black body, denser than any other tissue component and frequently did not appear to be granular although it was possible to distinguish the granular nature of the deposits over this region, in good photographic prints. This may have been due to exceptionally dense deposition of granules over this region or to a deposition of granules over a structure with a high inherent electron density. Chromatinic material and pars granulosa stain up similarly with dense deposits of granules giving a fairly homogeneous appearance, usually less dense than the pars fibrosa but still in strong contrast to the rest of the components. Small groups and chains of granules were deposited over the nucleoplasmic ground substance giving the impression of a background stain which was restricted to the nucleus. The underlying structure of the nucleoproteinic network can be distinguished in poor contrast as an ill-defined background.

In the kidney, there was some vacuolation of the ground substance and removal of matrix material again suggesting poor fixation. There was little evidence of granules over free ribosomes or over ffagments of endoplasmic reticulum. Mitochondria resembled those of the liver in general appearance.

Again nuclei resembled those described in the liver, although there was frequently a greater degree of reticulation of the nuclear ground substance, with some extraction of the matrix leaving a courser network than that usually found in liver nuclei. This may

have been due to the lower intrinsic contrast of the fibrils or to some extraction of matrix material.

None of the other cytoplasmic structures of the kidney such as the basement membranes or the brush borders, gave rise to any deposition of silver, although they were apparent due to their high intrinsic electron density.

Storage of material in cold buffer solutions.

Material which had been fixed in glutaraldehyde (5% in cacodylate buffer at pH 7.4 for 2 hrs.) was stored in a sucrose/ phosphate buffer at pH 7.4 for three months at 4°C before post-fixation in osmium and routine dehydration and embedding, and material that had been fixed in acrolein (10% in cacodylate buffer at pH 7.4 for 1 hr.) was similarly stored before dehydration and embedding.

Glutaraldehyde fixed material: There was a general tendency towards vacuolation of the cytoplasm, the cytoplasmic ground substance appeared to be clumped and granular in appearance. There was often almost complete removal of glycogen deposits from the liver. In the liver the endoplasmic reticulum was well preserved, together with attached ribosomes. Mitochondria tended to show considerable extraction of matrix material, the outer membranes were often indistinct although the cristae were crisp and clear, often being more obvious than in freshly embedded material.

Nuclei appeared to be unchanged although there was some extraction of dark-staining material in some cases, and a greater degree of reticulation of the nucleoplasmic ground substance as is shown in Plate 11 .

Again there was considerable granulation of the cytoplasmic ground substance in the kidney. Fragments of endoplasmic reticulum were readily observed and free polysomes were often easily distinguished,

suggesting that the background contrast had been reduced in some way. Mitochondria appeared as indistinct structures with reduced electron density, their cristae were not often apparent. This was particularly true of mitochondria occuring in the upper halves of tubule cells, in the nucleus surrounding the cytoplasm. Those occuring in the basal half of the tubule cells resembled those in the liver, in that the electron density of the matrix was reduced and the cristae stood out clearly.

Nuclei were normal in appearance although there was often a reduction in the amount of dark staining material and a greater reticulation of the back ground fibrils.

The basal cell structure of the tubule cells appeared to be relatively unaffected by the prolonged storage, although there was frequently some breakdown of the basement membranes.

Acrolein fixed material: The appearance of material that had been stored in cold buffer after acrolein fixation varied considerably from that of freshly embedded material. There was a general reduction in the electron density of the nucleoli and the ribosomes suggesting that the nucleoproteins, and the ribonuceoproteins in particular, were poorly stabilised by the acrolein fixation and leached out of the blocks during the period of storage. Marinozzi (1963) noted a similar phenomenon in acrolein fixed material embedded in methacrylate and floated on water for several hours prior to staining. In general the material resembled the similarly treated glutaraldehyde fixed material as regards cytoplasmic preservation although the dammage tended to be more severe. Plate 12 shows the general appearance of acrolein fixed material which had been subjected to the cold storage period.

Marinozzi (1963) suggested that the flotation of acrolein fixed, methacrylate embedded material upon water could be used to

differentiate between the nucleoprotein components. Although, as he pointed out, the technique tended to give rather variable results. Similar differentiation of the nucleoprotein components appeared to take place during the prolonged storage of acrolein fixed material in cold buffer. The use of the stored material for the differentiation between nucleoprotein components had several disadvantages, however. The degree of extraction of material from the sections was somewhat variable, the sections cut from the outer zones of the blocks tended to show a greater degree of extraction than those cut from deeper layers even though the quality of fixation was comparable. In the outer zones of the blocks the extraction of nuclear dark staining material was often almost complete, even much of the contrast of the chromatin having been removed, whereas in the inner zones only those areas with an apparently high concentration of ribonucleoproteins, such as the nucleolus showed reduced contrast.

Routine dehydration and embedding.

Tissue blocks were routinely dehydrated through a graded series of alchohols, concentrations of ethanol of 50%, 70% and 90%, within a period of 15 mins. Material was then subjected to two 30 min. changes of absolute alchohol, although the later period has been prolonged up to two hours without impairing the final quality of the blocks.

Dehydration with ethanol was followed by a 30min. treatment with epoxy propane, which was used as a transition solvent between the ethanol and the un-polymerised embedding medium. This was again followed by a 30 min. treatment with a 50% solution of epoxy-resin in epoxy propane, and two changes of unpolymerised resin plus accelerator within an hour. Tissues were blocked out in fresh resin in gelatin capsules. The plastic was cured at 60 C for two days.

Epon 812 was used as an embedding medium, throughout this

investigation, and produced reliable results. Penetration of the tissue blocks was found to be adequate if the above proceedure was followed, and no advantage was found in prolonging the stages in unpolymerised resin. Even polymerisation of the blocks was obtained if the resin and accelerator were adequately mixed, and for this reason resin was always made up in 10 ml. sample bottles and inverted for an hour before use on a standard blood mixer.

Preparation of sections.

Sections were prepared using a Reichert OM U2 ultramicrotome. Pale gold or silver sections were cut with glass knives and floated off on a bath of water or 1% ethanol, which had a lower surface tension and tended to eliminate the compression of some sections. Where compression was found to take place as the section floated off an attempt was made to eliminate this by exposing them to trichloro-ethylene vapour, but this was not particularly effective with Epon sections. Where Epon sections did become compressed in cutting little was found that would correct it except careful sectioning.

The sections were mounted on 200mesh copper grids. Sections were usually picked up from below, on to the dull surface of the grids since the sections were found to adhere better to this side, and were more easily detected by examination with the naked eye. During early work sections were mounted on carbon support films, but during later work the preparation of support films was abandoned as they were found to be unnecessary. The improved contrast obtained by ommitting the carbon layer made up for the lack of stability in the electron beam, It was found that if grids were mounted in the microscope 'upside down' the sections were less susceptible to dammage .

Examination of the material in the Electron Microscope.

Most sections were examined in an AEI EM6B microscope, but, use was made of an EM6G instrument during periods when the departmental instrument was out of operation.

The microscope was usually operated at 60kv., but accelerating voltages of 40 and 50kv were used in examining material of particularly low contrast, or where sections seemed to be particularly susceptible to beam dammage. Changing the size of the objective apperture in use was also found to improve the contrast of the image in the case of very low contrast material. The normal apperture in use was a 50 diameter and a 25 diameter apperture was used as a substitute.

Routine photographic processing.

Photographs were taken using contrasty plates obtained from Ilford Ltd., either N 50 plates or in later work EM 5's. These were developed using a high contrast hydroquinone developer according to a standard proceedure. (Ref. Appendix III.)

For routine analysis, plates were printed onto 16 x 16cm. prints. Photographic paper of varying contrast grades (Ilford grades 1 - 5) were used as appropriate to the quality of the negatives, but in certain cases use of even the hardest papers were not adequate to show up the details on the plates. In such cases prolonged exposures at reduced light intensities often produced adequate results.

Measurement of cellular structures.

A quantitisation of estimation of cytoplasmic structures was acheived by the use of a method described by Loud (1962).

Selection of areas of tissue for quantitisation was carried out at x3,000 in order to avoid prejudice. At this magnification it

TABLE I

NEGATIVE NO. = 712/71 TISSUE = LIVER (M) = 9,300 EXPERIMENTAL TREATMENT. = CONTROL							
	ROW NO.	LENGTH OF SAMPLE LINE	INTER-CELLULAR SPACE	NUCLEUS	MITOCHONDRIA	GLYCOGEN	ER CROSSINGS
	1	152	0	79	11	33	8
	2	152	0	88	11	16	9
	3	152	5	87	16	0	9
	4	152	7	74	12	13	8
	5	152	13	0	4	68	17
	6	152	0	0	29	31	14
TOTAL MITOCHONDRIA = 55	7	152	0	0	38	45	12
	8	152	0	0	35	20	25
TOTALS		1,216	25	328	185	226	102

was difficult to introduce bias since sufficient cellular detail was not discernible. To ensure examination of comparable areas of cytoplasm photographs were taken of areas including a nuclear profile.

Micrographs for estimation were taken at magnifications of x5,000 and were enlarged by two diameters. Sample lines, 2 cms. appart were superimposed upon the micrographs. The length of sample line covering a cytoplasmic structure was measured by placing a millimeter scale against the line covering each structure , and total counts of the length of line over each structure were recorded, as shown on the previous page. The number times profiles of endoplasmic reticulum crossed the sample lines was also counted and recorded.

The percentage area of cytoplasm occupied by a structure was calculated as the length of line over-lying it as a percentage of the total sample line length, as follows:-

% area of micrograph occupied by a structure

$$= \frac{100}{Z} \times A$$

where Z = total length of sample line overlying the micrograph.

and A = length of sample line overlying structure.

Thus % area of cytoplasm occupied by structure

$$= \frac{100}{L} \times A$$

where L = total length of sample line covering cytoplasm (exclusive of intercellular space and the nucleus.)

Example; The percentage area of cytoplasm occupied by glycogen deposits, and by mitochondrial profiles in the micrograph 712/71 are calculated on the following page. The data for this micrograph is shown in Table I on page

% area of cytoplasm occupied by mitochondrial profiles

$$= \frac{100 \times 185}{863}$$

$$= 21.43\%$$

% area of cytoplasm occupied by glycogen deposits

$$= \frac{100 \times 226}{863}$$

$$= 26.18\%$$

<u>An estimation of the endoplasmic reticulum profiles</u> was obtained from a count of the number of times profiles crossed the sample line, as follows:-

mms. of profile per mm^2 of cytoplasm in a micrograph.

$$= \frac{\pi C}{2 L}$$

where C = total number of crossings

average number of ER profile per μ^2 of cytoplasm of un-magnified cell

$$= \frac{\pi C M}{2{,}000L}$$

where M = total magnification of print.

In this example:-

$$= \frac{\pi (102)(9{,}300)}{2{,}000(863)}$$

$$= 1.72 \mu/\mu^2 \text{ cytoplasm.}$$

<u>The numbers of mitochondrial profiles</u> were estimated as total numbers of mitochondria per micrograph, using the micrographs as a sample area of constant size. Since all the micrographs measured $(152 \text{ mm})^2$ at a magnification of x9,300, counts were of numbers of mitochondrial profiles per $232.12 \mu^2$.

<u>The average area of mitochondrial profiles</u> from each micrograph were calculated as follows:-

L was calculated as a percentage of Z in order to determine the proportion of the micrograph occupied by cytoplasm exclusive of nuclei

and intercellular space.

$$\text{which} = \frac{100 \times L}{Z}$$

which in this example

$$L = \frac{100 \times 863}{1,216}$$

$$= 70.97\%$$

Since Z represents an area of $232.12\mu^2$ L can also be calculated as an area:-

$$L = \frac{70.97 \times 232.12}{100} \mu^2$$

$$= 164.74\mu^2$$

and similarly the area of mitochondrial profiles in the micrograph can be calculated from the area represented by L ..

In this example

$$= \frac{164.74 \times 21.43}{100} \mu^2$$

$$= 34.71\mu^2$$

The areas of nuclear profiles were estimated by superimposing a grid of centimeter squares over micrographs. The number of squares covering the nuclear profiles was counted. In the case of incomplete squares, where than half the .half the square lay over the profile it was counted as a complete square, where less than half a aquare overlay nuclear profile it was discounted.

Area of micrograph occupied by nuclear profile

$$= N$$

$$= 56 \text{ cm}^2$$

$$= 5,600\text{mm}^2$$

The area of the nuclear profile was calculated from this by correction for magnification as follows:-

$$= \frac{N}{\text{area equivalent to } 1\mu^2 \text{ at M} \quad \text{mm}^2}$$

$$= \frac{5,600}{9.3 \times 9.3} \mu^2$$

$$= 64.37 \mu^2$$

All results of quantitative estimations were calculated using an 'Olivetti' desk computer.

CHAPTER 1.

The Effects of Thyroid Hormones on Cellular Structure.

A general impression of the patterns of change taking place under the influence of thyroid hormone action, was gained by visual assessment. Three blocks of tissue, from as widely separated parts as possible, were prepared from the liver and the kidney of each of the six mice in each experimental group. Two sections were cut from each block for visual assessment. Photographs were obtained from these sections for quantitative assessment. Areas for photographs were selected on arbitrary grounds at magnifications of x3,000, where it was only possible to select areas on the grounds of gross technical quality. Areas photographed always contained a nucleus in order that roughly comparable areas of cytoplasm were being examined. Twenty four photographs were taken from each of the four experimental treatments, at magnifications of x5,000. The final magnifications of the prints used for analysis was x9,300. Prints were scored with the aid of a x10 hand lens, according to the method of Loud (1962) described in the Methods section.

Cellular effects on the Liver.

General appearance of the tissue: Variations in the appearance of the liver from the different experimental groups, were observed that could be attributed to the influence of the administered hormones.

The nuclei of liver cells from normal animals appeared rounded with little dark-staining material present. That which was present was restricted to around the nucleolus and to a narrow sub-peripheral zone. The nuclear membranes were generally easily resolved as even, double structures at magnifications of x10,000, although some of the nuclear envelopes were obscured by some deposit.

Approximately half of the cytoplasmic areas of the cells were occupied by endoplasmic reticulum and mitochondria and half by glycogen deposits. The glycogen was characteristically granular in appearance. The endoplasmic reticulum was arranged in loose, paralell lamellae and whorls, interspersed with mitochondria. Groups of lamellae were interspersed with small patches of glycogen deposits. Groups and rosettes of isolated polysomes and scraps of endoplasmic reticulum were observable within the glycogen areas. The general appearance of the tissue is shown in the diagrammatic representation shown in Fig. 2 and in Plate 13 .

The amount of endoplasmic reticulum present in the cells was reduced in the livers of thyroidectomised animals and the quantity of glycogen present was increased. The lamellae of endoplasmic reticulum were much less regularly organised and were dispersed among the glycogen deposits, whereas in the hormone treated animals there was a marked increase in the amount of endoplasmic reticulum with a corresponding decrease in the amount of glycogen present in the cells. In hormone treated livers the endoplasmic reticulum was organised into complex whorls and lamellae, interspersed with numerous mitochondria, and was not interspersed with glycogen granules. Glycogen deposits were isolated, without mitochondria or scraps of endoplasmic reticulum as is shown in Fig. 3 and Plate 14 .

Mitochondria appeared to vary in number with the different hormone treatments, being apparently more frequent in the livers of hormone treated animals than in the livers of thyroidectomised animals. Their distribution also appeared to vary. They almost always occured in close association with lamellae of the endoplasmic reticulum, but whereas in the thyroidectomised animals profiles of lamellae were short and were dispersed throughout the cytoplasm, in the livers of hormone treated animals the mitochondria usually occurred in closely packed groups completely surrounded by lamellae of the endoplasmic reticulum.

Figure 2.

APPEARANCE OF LIVER CELLS FROM A NORMAL ANIMAL.

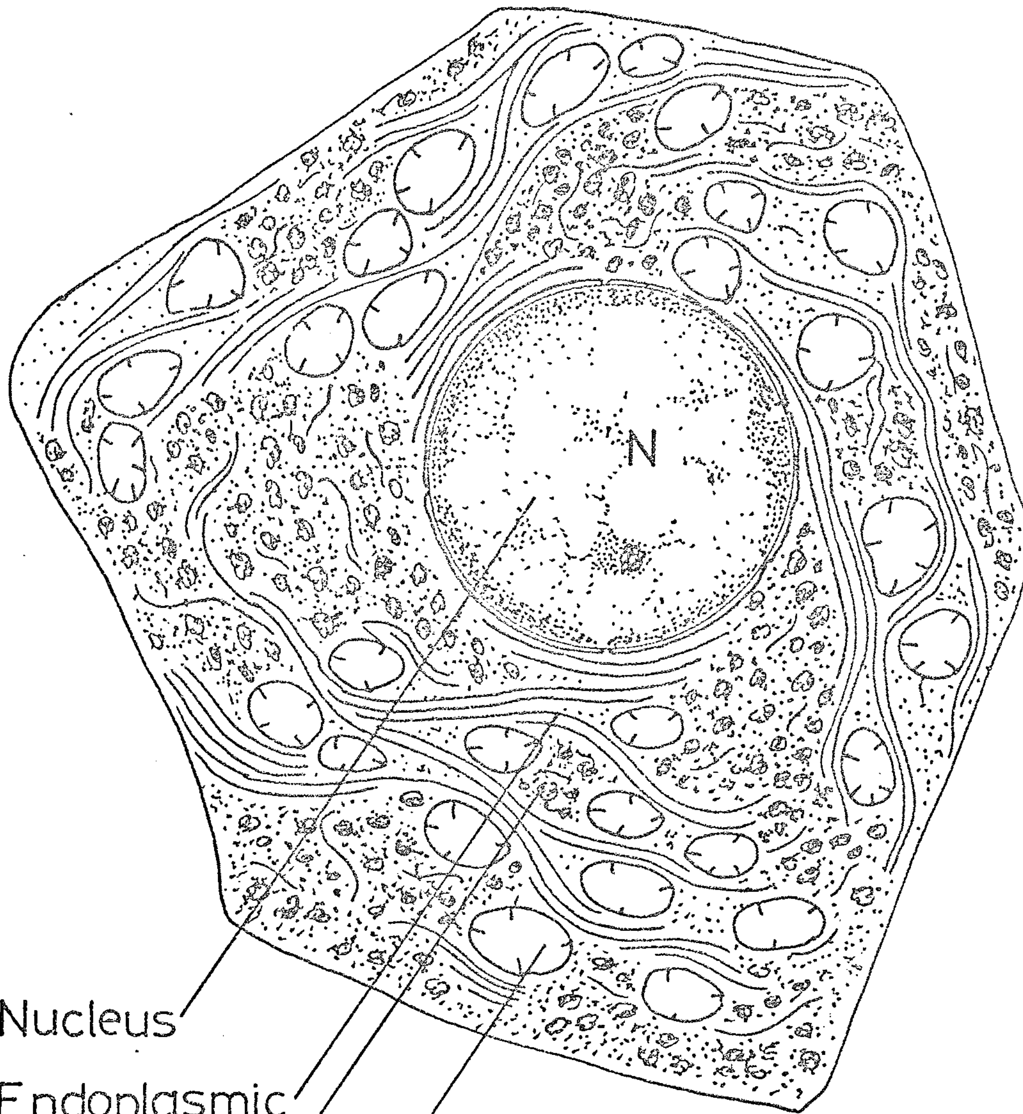
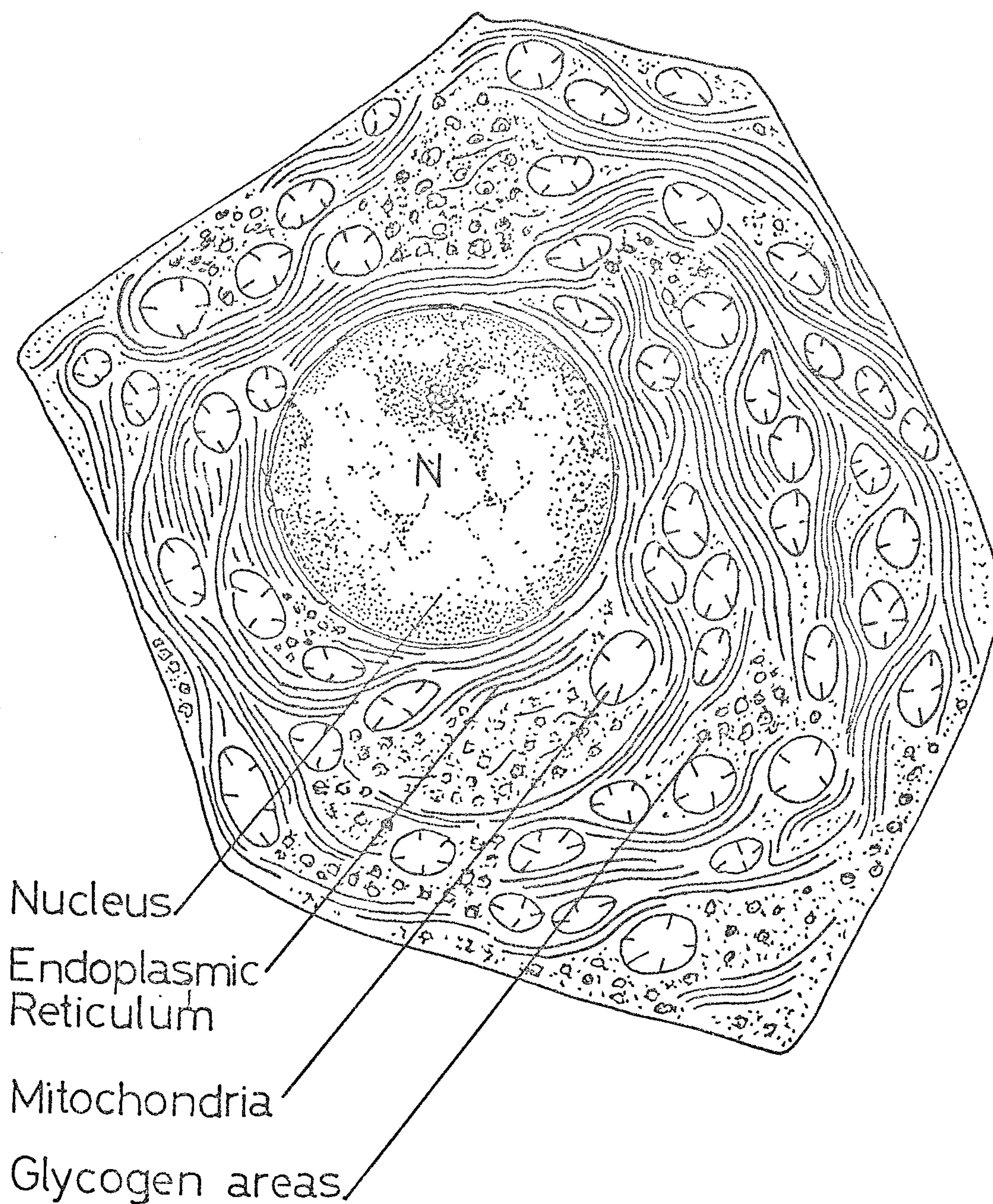


Figure 3.

APPEARANCE OF LIVER CELLS FROM A HORMONE-TREATED ANIMAL.



Isolated mitochondria were often observed among the glycogen of the livers of hypothyroid animals but almost never in the livers of hormone treated animals.

There was no initial impression gained of hormone induced mitochondrial swelling, but quantitative estimations of the mitochondrial sixe were obtained in order to substantiate this.

The liver mitochondria were simple in structure, with rounded or oval profiles and few, randomly orientated cristae, which rarely reached to the middle of the profiles. There appeared to be alterations in the organisation of the cristae, and numbers of cristae present, with the hormone treatments. Thus an attempt was made to gain some quantitative estimations of the variations associated with the hormone influence as is shown below.

Table 2: Mitochondrial shape.

TX		C		T3		T4	
Round	Oval						
24	6	82	18	89	11	86	14

* figures % of total mitochondria counted.

Table 3: Orientation of cristae.

TX			C			T3			T4		
Absent	Radial	Parallel									
34*	57	9	19	74	3	16	74	9	20	53	16

* figures % of total mitochondria counted.

Table 4: Length of cristae.

TX		C		T3		T4	
Short*	Long**						
85+	15	50	50	100	0	76	24

* cristae donot reach to middle of profile

** cristae reach the middle of the profile or beyond the middle.

+ figures % of total mitochondria counted.

Table 5: Numbers of cristae per mitochondrial profile

TX	C	T3	T4
11*	14.5	10	9

* average number over 20 mitochondria.

There would appear to be no overall pattern of change in mitochondrial structure associated with the hormone treatments.

Quantitative analysis of hormone associated changes in the liver.

The method of quantitative analysis described bu Loud (1962) was used to gain an estimate of the percentage cytoplasm occupied by mitochondrial profiles, and by glycogen deposits. The numbers of mitochondrial profiles per sample area was counted, and a figure representing the average cross-sectional area of mitochondrial profiles was calculated. The concentration of endoplasmic reticulum profiles per μ^2 of cytoplasm was calculated, and the cross-sectional areas of nuclear profiles were estimated as described earlier in the Methods section.

The results are tabulated in Table 6, on the following page. On the whole there was little difference between the material from the thyroidectomised and normal animals, as regards the parameters investigated. There was however, a difference in the effects of thyroxine and triiodothyronine upon the liver.

There was no apparent difference between the percentage area of the cytoplasm occupied by mitochondria in the liver of thyroid-ectomised or normal animals treated with thyroxine. However,therewas a significantly greater percentage of cytoplasm occupied by mitochondrial profiles after triiodothyronine treatment. There was no significant difference in the numbers of mitochondrial profiles per sample area from the different treatments,so that the increased cytoplasmic area occupied by mitochondrial profiles is a result of an increase in profile area, reflecting mitochondrial size, rather than in number.

There was no difference in the amount of cytoplasm occupied by glycogen deposits in the livers from thyroidectomised animals and normal animals, although there was a difference in the distribution of the deposits within the cytoplasm. The glycogen deposits of thyroidectomised livers tended to be dispersed throughout the cytoplasm, in small patches and aggregations of granules, whereas the glycogen deposits of normal animals tended to occur in extensive aggregations devoid of other structures.

There was a slight but significant reduction in the amount of glycogen present after prolonged thyroxine treatment, but after triiodothyronine treatment there was a vast reduction in the amount of glycogen present, almost all having been removed. However, it must be pointed out that there was considerable variability in the percentage area occupied by glycogen deposits in the thyroxine treated animals, half the sample areas counted showed less than 10% of the cytoplasm occupied by glycogen, and two out of the twenty two sample areas showed nearly 50% of the cytoplasm to be occupied by glycogen.

Table 6.

Cytoplasmic changes in the liver with hormone treatment.

	TX	N	T3	T4
% area of cytoplasm occupied by mitochondria.	15.64 ±1.04	15.61 ±1.11	23.99 ±1.32	15.85 ±0.70
Nos. of mitochondria	63.78 ±3.65	57.05 ±2.41	63.50 ±3.64	69.55 ±6.48
Areas of mitochondrial profiles in μ^2	0.48 ±0.03	0.55 ±0.04	0.71 ±0.04	0.45 ±0.04
% area of cytoplasm occupied by glycogen deposits.	10.58 ±2.23	22.78 ±1.59	1.72 ± 1.06	16.14 ±2.60
μER / μ^2 cytoplasm.	1.84 ±0.08	2.02 ±0.10	2.30 ±0.16	2.23 ±0.10
Areas of nuclear profiles in μ^2	38.50 ±4.99	38.02 ±2.82	34.59 ±2.49	30.87 ±3.97

VARIATIONS IN THE PROPORTIONS OF CYTOPLASMIC ORGANELLES WITH HORMONE VARIATIONS IN THE LIVER

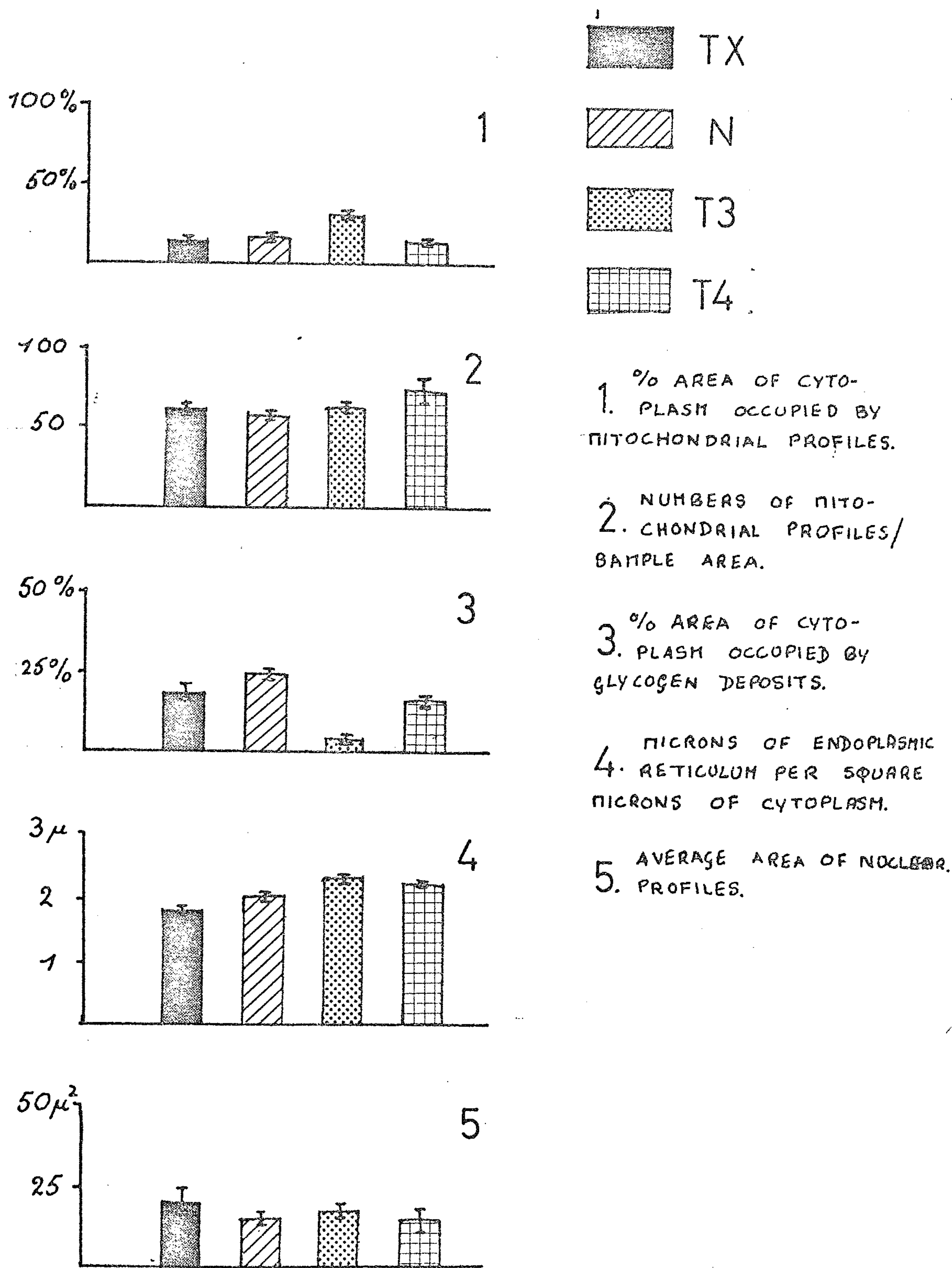


Table 7.

Statistical significance of the changes in cytoplasmic organelles in the liver with hormone treatment.

%area of cytoplasm occupied by mitochondrial profiles.

	TX	N	T3	T4
TX				
N	P > 0.1			
T3	P < 0.001	P < 0.001		
T4	P < 0.1	P < 0.1	P < 0.001	

Numbers of mitochondria

No significant differences.

Areas of mitochondrial profiles in μ^2.

	TX	N	T3	T4
TX				
N	P > 0.1			
T3	P < 0.001	P < 0.02		
T4	P > 0.1	P < 0.1	P < 0.001	

% area of cytoplasm occupied by glycogen deposits.

	TX	N	T3	T4
TX				
N	P > 0.1			
T3	P < 0.001	P < 0.001		
T4	P > 0.1	P < 0.001	P < 0.001	

μER / μ^2 of cytoplasm.

	TX	N	T3	T4
TX				
N	P > 0.1			
T3	P < 0.01	P > 0.1		
T4	P < 0.01	P > 0.1	P > 0.1	

Areas of nuclear profiles in μ^2.

No signifiaant differences.

The difference between the effects of triiodothyronine and thyroxine noted above, were not seen in the case of the concentration of endoplasmic reticulum profiles. There was a gradual trend towards a greater abundance of endoplasmic reticulum from thyroidectomised animals through normal to the hormone treated animals. The differences between the experimental groups were not great, only those between the thyroidectomised animals and the hormone treated animals being significant. There was no significant difference between either thyroidectomised animals and normal animals or between the two hormone treatments as is shown in Table 7.

Again there was no significant difference between the size of the nuclei from the different experimental groups, although there was a tendency towards a reduction in size after hormone treatment, compared to those measured from thyroidectomised animals.

Cellular effects on the kidney.

General appearance of the tissue: Similar nuclear changes as those noted in the liver were observed in the kidney. Changes in nuclear membrane morphology, particularly in the proximal convoluted tubules were marked, as is described in Chapter 4.

General cellular changes were difficult to assess, and could often be attributed to errors in identification of the different, closely intermingled cellular types, and thus to normal functional differences between the proximal and distal convoluted tubules. Generally the differences were more subtle than those seen in the liver and it seemed that, to some extent at least, the kidney could be described as a 'Control' tissue as regards hormone action. However, that thyroid hormone treatment had some effect was obvious from the marked changes in the morphology of the nuclear envelope. This lack of apparent change was possibly attributable to normal differences in

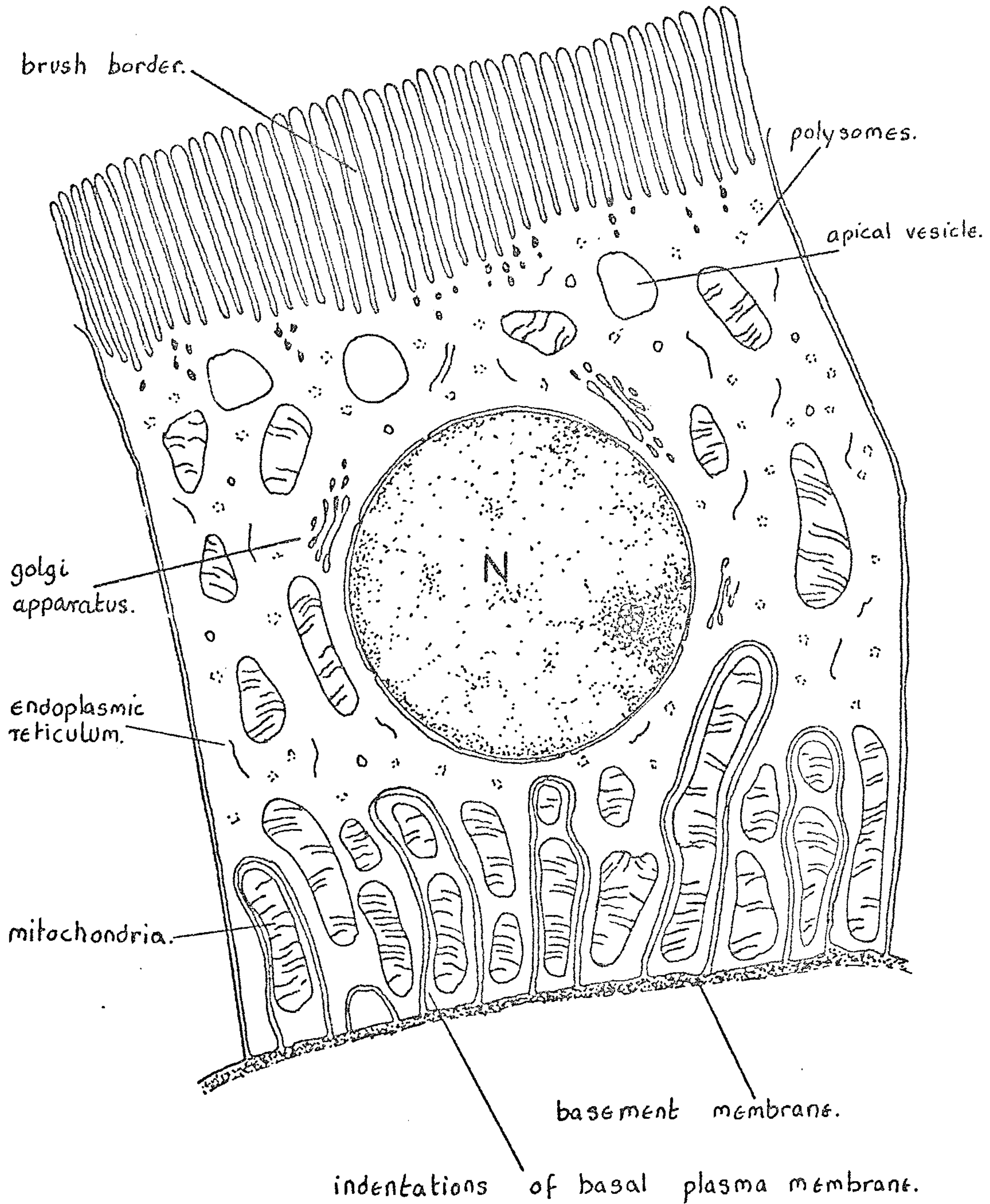
functional response to external influences between the two tissues, the liver being a more 'dynamic' structure reacting to changes in environment by gross changes in activity which are reflected in gross morphological changes, Whereas the kidney, being a more organised structure capable of reacting to gross rapid changes in internal environment without changes in morphological appearance. That changes in the kidney, induced by the influence of the thyroid hormone administration, are probably at the biochemical level rather than the morphological resulting in alterations enzymes concerned with the urea cycle and other aspects of excretion and ion control would account for the apparent lack of structural variation between the experimental groups.

Generally the morphology of the kidney observed in all the experimental groups conformed to the generally accepted patterns of renal morphology, and resembled the pattern shown in Figure 5. and Plate 15.

Quantitative analysis of hormone associated changes in the kidney. Some difficulty was experienced in obtaining quantitative estimates of the amount of cytoplasmic structures present in the kidney as a result of several factors. Firstly, unlike the liver, the kidney is not a relatively homogeneous tissue. Numerous functionally distinct cell types occur in close proximity and are closely intermingled which makes it essential that measurements made from each cell type are obtained independently and not from a pool of all cell types. In this study, attention was confined to the cells of the proximal convoluted tubule, since preliminary observations, particularly of the nucleus, suggested that this was the most susceptible of the cellular components to the effects of thyroid hormones. Secondly, the individual cells of each of the cell types in the kidney exhibit a polarity which is not shown by the cells of the liver, which might affect the randomness of the sample lines used in this method of obtaining quantitative results.

Figure 5.

REPRESENTATION OF THE APPEARANCE OF PROXIMAL TUBULE CELLS FROM ALL HORMONE TREATMENTS



For this reason, sample lines were always drawn as near parallel as possible to the basement membrane, rather than with random orientation to the cell.

The percentage area of cytoplasm occupied by mitochondrial profiles and apical vesicle profiles, the concentration of endoplasmic reticulum profiles, and the average areas of nuclear profiles were calculated from sample areas from each hormone treatment.

There was no change in the percentage area of cytoplasm occupied by apical vesicles, with the hormone treatments. There was a slight decrease in the percentage area of cytoplasm occupied by mitochondrial profiles with the hormone treatment. Approximately 28% of the cytoplasm was occupied by mitochondrial profiles in kidney cells from thyroidectomised animals while only 22% consisted of mitochondrial profiles in cells of thyroxine treated animals. Only the differences between thyroidectomised and hormone treated animals were statistically significant as is shown in Table 9.

As is shown in Figure 6, there was also a reduction in the concentration of endoplasmic reticulum profiles with the hormone treatment, which was statistically significant if the results of the thyroidectomised animals were compared with the hormone treated animals. There were approximately 0.7 μ of endoplasmic reticulum profiles per μ^2 of cytoplasm in cells from thyroidectomised animals compared to 0.44 μ from cells from animals treated with triiodothyronine and 0.39 μ from animals treated with thyroxine.

There was no variation in the sizes of nuclei from cells after each of the different hormone treatments.

Table 8.

Cytoplasmic changes in the kidney with hormone treatments.

	TX	N	T3	T4
% area of cytoplasm occupied by apical vacuoles.	2.37 $\pm$0.34	2.59 $\pm$0.52	2.38 $\pm$0.48	3.21 $\pm$0.63
% area of cytoplasm occupied by mitochondria.	28.49 $\pm$1.06	25.56 $\pm$1.60	24.58 $\pm$1.05	22.78 $\pm$1.05
μER / μ^2 cytoplasm.	0.71 $\pm$0.07	0.56 $\pm$0.07	0.44 $\pm$0.05	0.40 $\pm$0.04
Areas of nuclear profiles in μ^2	24.05 $\pm$2.69	25.02 $\pm$2.00	21.83 $\pm$1.60	19.85 $\pm$2.01

Figure 6.

VARIATIONS IN THE PROPORTIONS OF CYTOPLASMIC ORGANELLES WITH HORMONE VARIATIONS IN THE KIDNEY

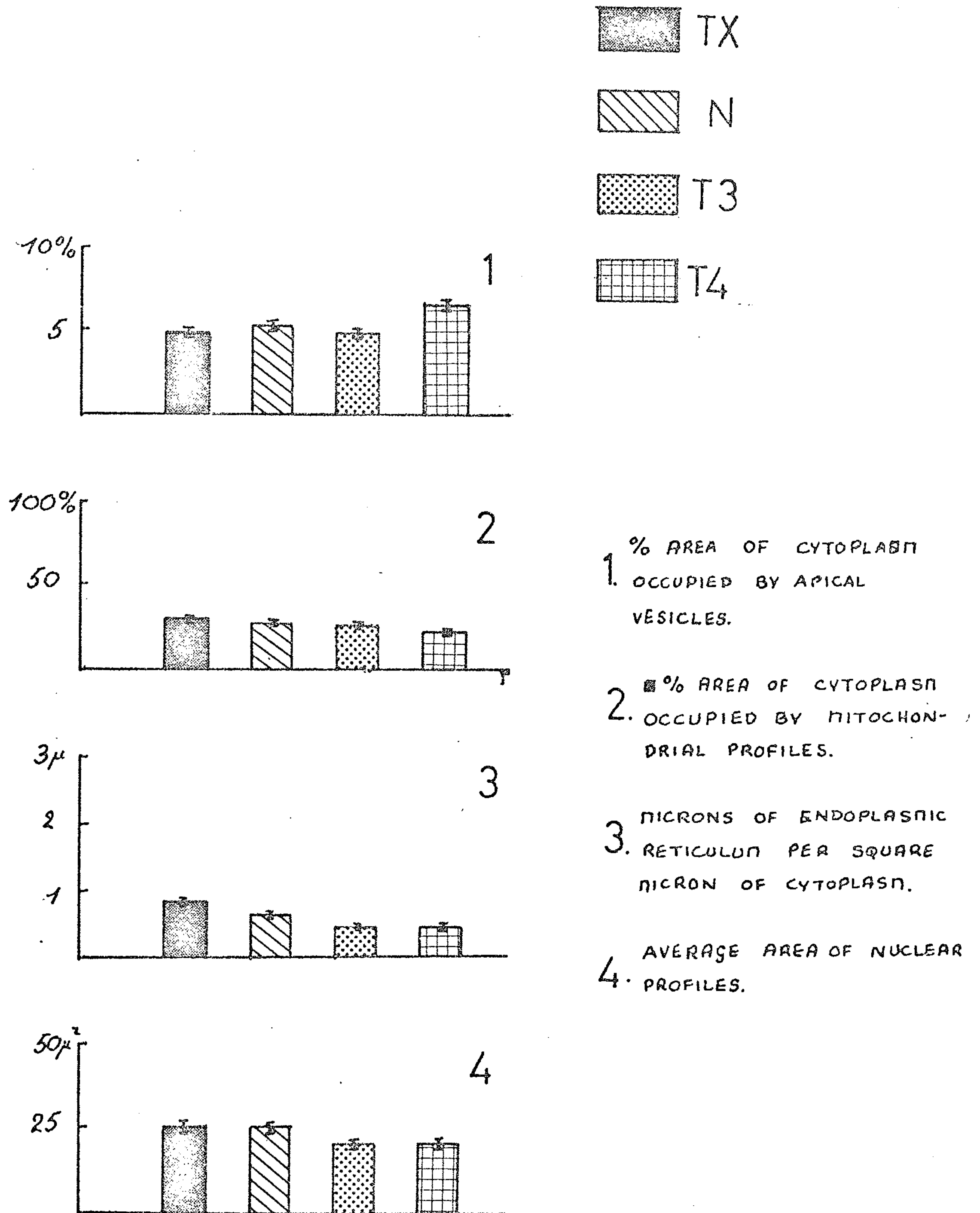


Table 9.

Statistical significance of the changes in cytoplasmic organelles in the kidney with hormone treatment.

% area of cytoplasm occupied by apical vesicles.

No significant differences.

% area of cytoplasm occupied by mitochondria.

	TX	N	T3	T4
TX				
N	$P > 0.1$			
T3	$P < 0.02$	$P > 0.1$		
T4	$P < 0.001$	$P > 0.1$	$P > 0.1$	

μ ER / μ^2 cytoplasm

	TX	N	T3	T4
TX				
N	$P > 0.1$			
T3	$P < 0.01$	$P > 0.1$		
T4	$P < 0.001$	$P > 0.1$	$P > 0.1$	

Areas of nuclear profiles in μ^2.

No significant differences.

Discussion: The effects of thyroid hormones on cellular structure.

There were significant differences in the effects of thyroid hormones upon the distribution of cytoplasmic structures between liver and the proximal convoluted tubule of the kidney, and there was a significant difference in the reaction of the two tissues to thyroxine and triiodothyronine. Triiodothyronine had a significantly greater effect upon the liver than did thyroxine, whereas there was no difference in the effects of the two hormones upon the kidney.

The most obvious change in cellular morphology brought about by the hormone influence was the gross depletion of liver glycogen produced by the action of triiodothyronine. Glycogen deposits were reduced to 1% of the sample area after triiodothyronine treatment compared with 22% and 19% in normal and thyroidectomised animals respectively. This was in accord with the findings of Tata et al (1963) who noted a 50% reduction in liver glycogen of thyroidectomised rats given a single injection of triiodothyronine. The significantly smaller reduction in percentage area of sample area occupied by glycogen after thyroxine treatment was more difficult to explain in view of the observations of other workers. Burton et al (1957) noted a significant depletion of liver glycogen under the influence of thyroxine and Fletcher and Myant (1961) reported a 90% reduction of glycogen content of livers of rats which received a daily dose of 50μ of thyroxine. However, as was pointed out, there was a considerable variation in the amount of liver glycogen present after thyroxine treatment, and a considerable amount of reorganisation of deposits had taken place under the influence of the hormone.

Hormone induced glycogen depletion was accompanied by changes in mitochondrial profile areas, reflecting changes in mitochondrial size, where again there was a significant difference between the effects of thyroxine and triiodothyronine treatment. There

was no significant difference in the number of mitochondria per sample area from any treatment, although there tended to be slightly more after treatment with either of the hormones. There was no change in the percentage area of mitochondrial profiles after thyroxine, but a significant increase after triiodothyronine treatment. Although there appeared to be no change in the number of cristae per mitochondrial profile, the increase in size was probably attributable to growth and addition of new units, rather than swelling due to inbibition of water under the hormonal influence. According to Freeman et al (1963) in vitro swelling of mitochondria under the influence of thyroxine was produced at doses well above the physiological range, whereas the doses used in this investigation were close to the physiological range.

Roodyn et al (1965) and Freeman et al (1963) have demonstrated an increase in amino acid incorporating activity under the influence of triiodothyronine and thyroxine, and various workers have demonstrated an increase in the content of various respiratory enzymes under thyroid hormone influence. (Drabkin 1950; Rugamer et al 1965; Lardy et al 1960; Lee and Lardy 1965;) However the lack of response of liver mitochondria in this investigation to the influence of thyroxine is difficult to understand. Freeman et al (1963) noted no difference in the response of rat liver mitochondria to either hormone, although Lardy et al (1960) found that triiodothyronine had a greater effect than thyroxine upon the stimulation of mitochondrial glycero-phosphate dehydrogenase content.

Again the slight but not significant reduction in the percentage area of kidney cytoplasm occupied by mitochondrial profiles under the influence of either hormone is difficult to explain. Drabkin (1950) found a 20% increase in the cytochrome-c content of rat kidneys from animals rendered hyperthyroid with thyroxine, compared to thyroidectomised animals, and Lardy et al (1960) found gross increases in the glycero- phosphate dehydrogenase content of kidney mitochondria

after treatment with either triiodothyronine or thyroxine. It seems possible that kidney mitochondria undergo reorganisation to accomodate new enzyme units, rather than an increase in size. Although the changes recorded by Drabkin (1950) and Lardy et al (1960) from whole kidneys might reflect changes in mitochondria from other tissue components than the proximal convoluted tubules.

The differential effects of triiodothyronine and thyroxine upon the liver was not as marked unpon the proliferation of endoplasmic reticulum as upon other structures, and measurements made of profiles of endoplasmic reticulum from livers of animals treated with triiodothyronine or thyroxine were not statistically different. This is in accord with the observations of Tata (1967) that there was no difference in the effects of either hormone upon proliferation of rough endoplasmic reticulum. It is now well established by Tata and other workers that the early stimulation by thyroid hormones of ribonucleic acid synthesis is followed by an increase in the cytoplasmic ribosome populations coordinated with an increase in the synthesis of membrane phospholipids, and that increased cytoplasmic protein synthesis, is a result of the increased activity of newly formed ribosomes that are tightly bound to new elements of the endoplasmic reticulum. (Widnell and Tata 1966; Tata 1967; Tata 1970). That the increase in endoplasmic reticulum seen after thyroid hormone treatment of mice, was so slight was surprising but can probably be explained on the basis that the hormones were administered to normal young mice, where protein synthesis was still rapid, and, as was noted by Roodyn et al (1965), normal animals do not make as great a response to thyroid hormone administration as do thyroidectomised animals.

The reduction in the endoplasmic reticulum seen in the kidney after administration of either triiodothyronine or thyroxine was difficult to explain. Almost all the work carried out on proliferation of endoplasmic reticulum or increased cytoplasmic protein synthesis

which is recorded in the literature, has been carried out using rat liver as a model. There are few references to the kidney. Although Michel et al (1963) have recorded a significant increase in the uptake of labelled amino acid precursers into protein by rat kidney they do not state which subcellular fractions were involved, and it is possible that the increased amino acid incorporating activity of mitochondrial fractions was responsible for their observation. It is possible that the endoplasmic reticulum components of the kidney cells undergo some reorganisation after thyroid hormone treatment so that fewer ribosomes are associated with membrane components. This is inconsistent with the view that the greatest protein synthetic activity takes place in membrane bound ribosomes. However, in view of the observations that the mitochondria appear to be responsible for the increased amino acid uptake this explanation could be feasible.

Chapter 2.

An Investigation into the Sequence of Ultrastructural Changes Produced under Thyroid Hormone Influence.

In order to place the ultrastructural changes, described in Chapter 1, into some sequence related to the time course of action of thyroid hormone activity, the ultrastructure of mouse liver and kidney was examined at various times after a single injection of triiodothyronine. The sampling times were selected in order to fit, as nearly as possible, the sequence of biochemical events in the early action of triiodothyronine described by Tata et al (1963), Roodyn et al (1965) and Widnell and Tata (1966), which can be summarised as is shown in Table 10. Mice were thus killed at intervals of 15, 30, 45 and 60hrs after the administration of a single dose of triiodothyronine, and samples of liver and kidney from each were subjected to routine fixation proceedures for the preparation of material for electron microscopy using glutaraldehyde and osmium.

As well as direct visual assessment, material was subjected to quantitative analysis using the method of Loud (1962), used in Chapter 1, and described in the Methods section.

Variations of cytoplasmic organelles after a single injection of triiodothyronine.

The general appearance of the liver.

The appearance of material from animals killed at the beginning of the experiment and that from animals killed at 60hrs after an injection of saline vehicle resembled that of normal animals described in Chapter 1. Approximately half the cytoplasmic area was occupied by glycogen deposits which tended to occurr as extensive areas of granules of characteristic appearance, devoid of other structures, although small groups of granules

Table 10.

Time Scale of Action of Thyroid Hormones.

(Response of rat liver from thyroidectomised animals to a single injection of 16 - 20μgms triiodothyronine.)

Time after T3	Biochemical Response.	Reference
16hrs.	Increased specific activity of nuclear RNA polymerase	Widnell & Tata (1966)
16hrs.	25% reduction in liver glycogen content,	Tata et al (1963)
20hrs.	Increased synthesis of membrane phospholipids.	Tata (1970)
24hrs.	Net accumulation of ribosomes in the cytoplasm.	Widnell & Tata (1966)
36hrs.	Peak amino acid incorporating activity of microsomes.	Tata et al (1963)
36hrs..	Peak amino acid incorporating activity of mitochondria.	Roodyn et al (1965)
50hrs.	Peak stimulation of mitochondrial QO_2	Tata et al (1963)
60-88hrs	Stimulation of microsomal respiratory chain enzymes.	Tata et al (1963)

occurred throughout the cytoplasm. Mitochondria were small, rounded or oval in profile, with relatively few cristae, and were fairly evenly distributed throughout the cytoplasm. Profiles of endoplasmic reticulum tended to be loosely arranged in groups of parallel lamellae, through most of the cytoplasmic area. Plate 16 shows the appearance of material from animals killed at the beginning of the experiment. Plate 17 shows the characteristic appearance of material from animals killed 60hrs after a saline injection. As comparison of the two Plates shows, there was a considerable variation in the distribution of glycogen deposits through the cytoplasm. Certain cells were predominately occupied by glycogen deposits, whilst others showed less extensive deposits of glycogen. Nuclei tended to be rounded, with slightly irregular profiles and relatively little dark staining material.

Material from animals killed 15hrs after a single injection of triiodothyronine resembled the control material described above in its general appearance. Glycogen deposits were again extensive, occupying approximately half of the cytoplasmic area. Most of the deposits were organised into isolated deposits, there was a reduction in the amount of small deposits scattered among other organelles. There was no apparent difference in the amount or distribution of mitochondrial profiles or of lamellae of endoplasmic reticulum, as is shown in Plate 18. Nuclei again resembled those from normal animals, although they tended to be more regular in profile.

There was a marked reduction in the amount of glycogen present 30hrs after a single dose of triiodothyronine. Glycogen deposits were reduced to small aggregates of granules scattered throughout the cytoplasm. There was but a slight increase in the number of endoplasmic reticulum profiles after 30hrs. Mitochondria profiles appeared to be increased in area, and were still distributed evenly throughout the cytoplasm, as is shown in Plate 19. Nuclei were regular in profile,

rounded, with an increase in the amount of dark-staining material present.

After 45hrs there was an apparent increase in the amount of glycogen deposits present. Small, dense masses of granules occurrying throughout the cytoplasm, which were quite extensive in some instances. as is shown in Plate 20. Endoplasmic reticulum profiles were more abundant after 45 hrs. They were usually long, and arranged in groups of parallel lamellae, although the complexity of arrangement after prolonged thyroid hormone administration, described in Chapter 1, was not usually developed. Nuclei again appeared regular in profile with extinsive, diffused deposits of dark-staining material.

After 60hrs of thyroid hormone treatment glycogen deposits again occupied upto half of the cytoplasmic area, althou h the deposits tended to be more dispersed than those observed in normal animals, as is shown in Plate 21. Mitochondria were small, rounded or oval in profile and distributed evenly throughout the cytoplasm. Profiles of endoplasmic reticulum were particularly obvious in all the material from animals killed 60hrs after the administration of a single dose of triiodothyronine. Extensive arrays of parallel lamellae occurred often in association with mitochöndrial profiles. Nuclei were rounded and regular in profile but there was a redu tion in the amount of dark-staining material present, so that nuclei tended to resemble those of normal material.

Quantitative assessment of t e changes in the liver.

Measurement of the cytoplasmic changes taking place under the influence of thyroid hormones did nor reveal such marked changes as were expected from the initial visual assessment. This can probably be attributed to the small numbers of photographs used to obtain quantitative information. It is probable that, due to the small number of areas examined were not fully representative of the tissue from each experimental treatment.

Although there was no overall change in nuclear size detected after treatment with either thyroid hormone for seven days,

Table 11.

Variations in the proportions of cytoplasmic organelles in the liver after a single injection of triiodothyronine.

	Hours after administration of T3					Control (60hrs saline)
	0	15	30	45	60	
Area of nuclear profiles in μ^2	49.24 ±3.54	41.77 ±2.67	50.71 ±3.36	34.91 ±2.27	42.69 ±3.57	47.98 ±7.37
% area occupied by mitochondria.	17.12 ±1.12	16.79 ±1.06	19.89 ±1.43	15.24 ±0.91	13.80 ±0.38	14.93 ±1.49
Numbers of mitochondria	87.42 ±9.30	71.25 ±6.42	91.00 ±14.7	84.36 ±12.5	102.25 ±4.49	69.16 ±7.22
% area occupied by glycogen deposits	14.66 ±1.78	31.04 ±2.75	10.67 ±2.13	25.65 ±4.71	17.43 ±2.63	29.25 ±7.78
μER / μ^2 cytoplasm	1.38 ±0.11	1.20 ±0.17	1.95 ±0.13	1.48 ±0.08	1.62 ±0.09	1.54 ±0.16

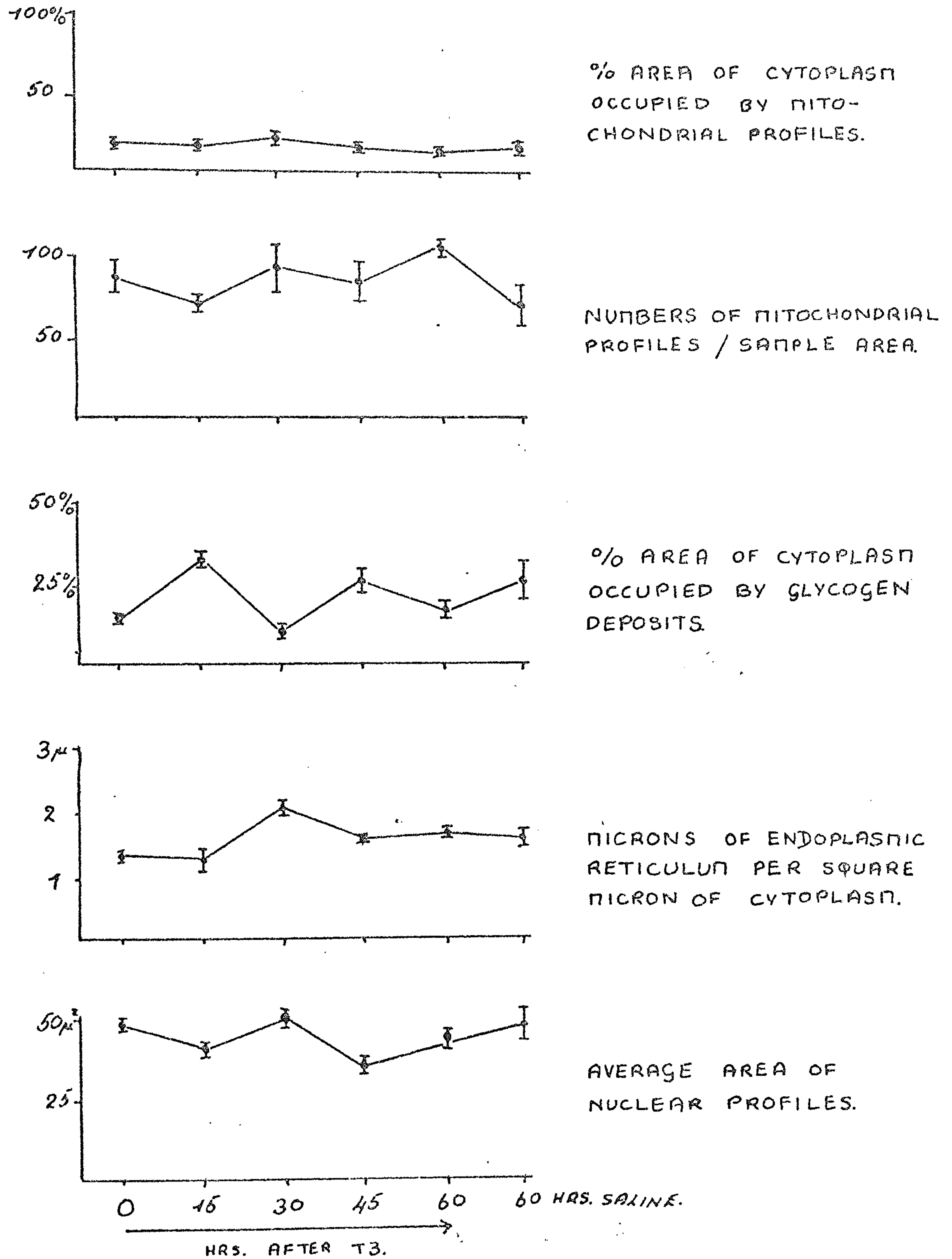
Table 12.

Statistical significance of the variations in cytoplasmic organelles after a single dose of triiodothyronine.

	0 x 15	0 x 30	0 x 45	0 x 60	0 x C
Area of nuclear profiles	$P > 0.1$	$P > 0.1$	$P < 0.01$	$P > 0.1$	$P > 0.1$
% area occupied by mitochondria	$P > 0.1$	$P > 0.1$	$P > 0.1$	$P < 0.01$	$P > 0.1$
Numbers of mitochondria	$P > 0.1$	$P > 0.1$	$P > 0.1$	$P > 0.1$	$P > 0.1$
% area occupied by glycogen deposits	$P < 0.001$	$P > 0.1$	$P < 0.1$	$P > 0.1$	$P > 0.1$
μER/μ^2 cytoplasm	$P > 0.1$	$P < 0.001$	$P > 0.1$	$P > 0.1$	$P > 0.1$

Figure 7.

VARIATIONS IN THE PROPORTIONS OF CYTOPLASMIC ORGANELLES IN THE LIVER AFTER A SINGLE INJECTION OF TRI-IODOTHYRONINE.



there were variations in the size of nuclear profiles during the first 60hrs after the administration of a single dose of triiodothyronine. There were significant fluctuations in the areas of nuclear profiles during the first 30hrs, followed by a significant decrease at 45hrs. The average area of nuclear profiles had returned to normal at 60hrs after triiodothyronine administration.

There was no significant variation in the numbers of mitochondrial profiles counted from each sample area, but there were variations in the percentage area of cytoplasm occupied by mitochondrial profiles. There was no variation in mitochondrial size during the first 15hrs of hormone action, but there was a slight but not significant increase after 30hrs. Thereafter there was a decrease in the area of the cytoplasm occupied by mitochondrial profiles, which continued to 60hrs after hormone administration. The area occupied by mitochondria at 60hrs. was significantly less than at the beginning of the experiment.

The area of the cytoplasm occupied by glycogen deposits varied considerably throughout the period of 60hrs following thyroid hormone administration, with a significant increase in glycogen content at 15hrs. followed by a decrease at 30hrs. and another rise at 45hrs and decrease at 60hrs. These latter fluctuations were not significant.

The fluctuations in the concentrations of endoplasmic reticulum profiles showed a more apparent pattern. There was no significant change during the first 15 hrs but there was a significant increase 30hrs after thyroid hormone administration. At 45hrs the concentration of endoplasmic reticulum profiles had returned more or less to normal.

The results of the quantitative assessment of the changes in the liver are shown in Tables 11 and 12 and in Figure 7.

The general appearance of the kidney.

As in Chapter 1, the investigation of the cellular effects of thyroid hormones upon the kidney was restricted to an examination of the cells of the proximal convoluted tubule. Cellular changes produced by the early action of thyroid hormones were not immediately obvious. The appearance of the cells of the proximal convoluted tubule of animals killed at the beginning of the experiment. and of those killed 60hrs. after an injection of the saline vehicle resembled that of Plate 15, as is shown by the appearance of Plates 22 and 23. The appearance of cells of animals killed 15hrs after a single injection of triiodothyronine also resembled those of the control material as is shown by Plate 24. As is shown by Plates 25 and 26, there appeared to be a reduction in the size of the apical vacuoles after 30hrs of thyroid hormone treatment. There was a reduction in the amount of endoplasmic reticulum after 45hrs of thyroid hormone treatment. Plate 27 shows the appearance of cells of the proximal convoluted tubule animals killed 60hrs after the administration of a single dose of triiodothyronine, this tends to resemble the general appearance of the control material although there did appear to be a reduction in the size of the nuclei.

Quantitative assessment of the changes in the kidney.

There were significant variations in several of the cytoplasmic organelles present in the cells of the proximal convoluted tubule during the first 60hrs following a single injection of triiodothyronine. There was a marked reduction in the areas of nuclear profiles which was significant at 45 and 60hrs.

There were significant variations in the amount of cytoplasm occupied by apical vacuoles during the period of 60hrs following thyroid hormone administration under examination. Examination of material suggested that the variation was due to fluctuations in the

Table 13.

Variations in the proportions of cytoplasmic organelles in the kidney after a single injection of triiodothyronine.

	Hours after administration of T3					Control (60hrs saline)
	0	15	30	45	60	
Area of nuclear profiles in μ^2	37.48 ±2.65	39.42 ±2.76	34.42 ±2.59	31.42 ±1.38	27.38 ±1.14	37.29 ±2.80
% area occupied by apical vacuoles	3.26 ±0.61	3.30 ±1.08	1.74 ±0.53	3.23 ±0.71	3.63 ±0.67	4.92 ±2.49
% area occupied by mitochondria	17.87 ±1.59	24.54 ±2.97	22.61 ±2.15	22.26 ±0.93	22.44 ±1.13	21.40 ±0.91
μ ER/μ^2 cytoplasm	1.15 ±0.06	1.31 ±0.15	1.47 ±0.15	0.74 ±0.07	0.91 ±0.11	0.85 ±0.08

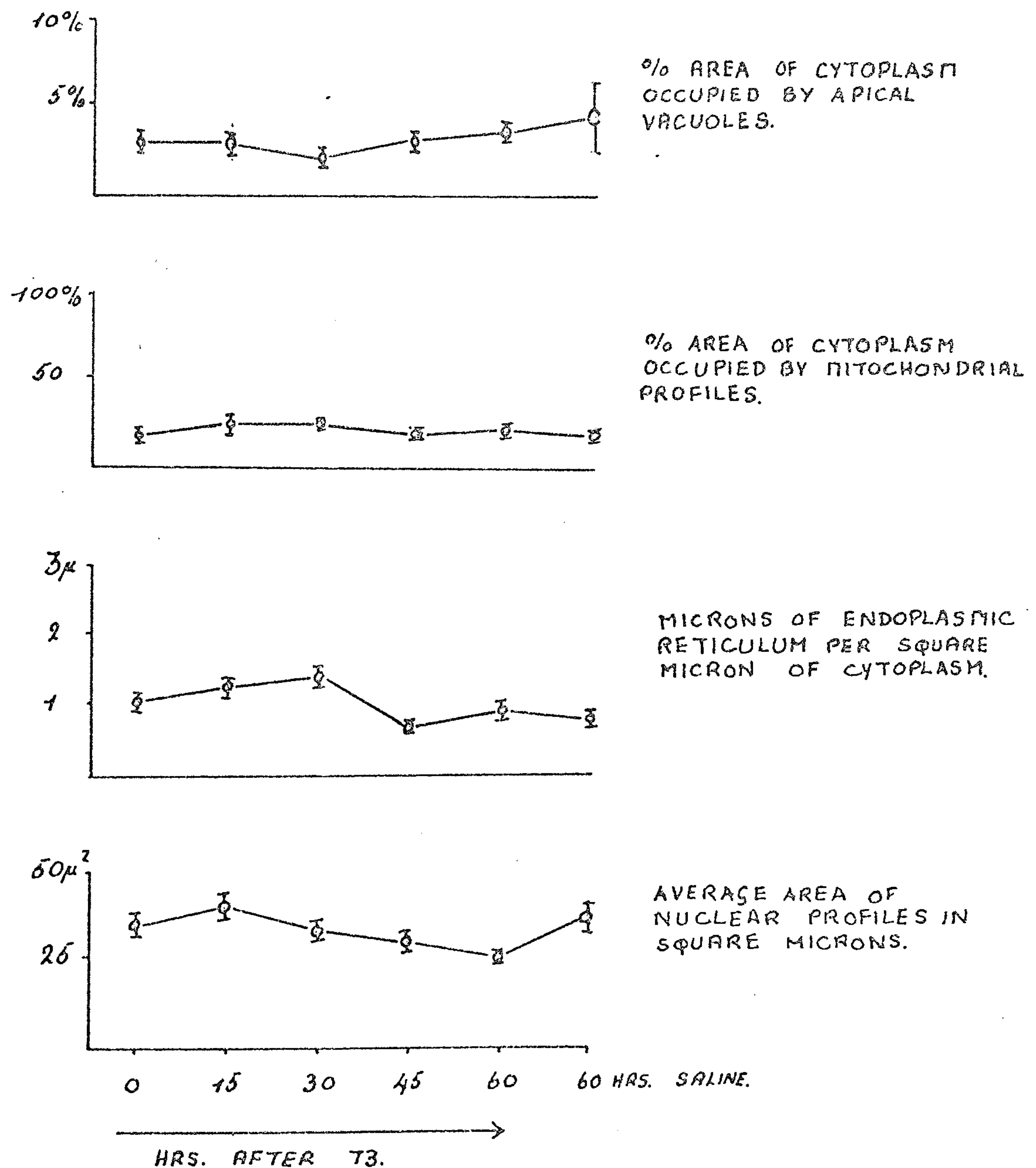
Table 14.

Statistical significance of the variations in cytoplasmic organelles in the kidney after a single dose of triiodothyronine.

	0 x 15	0 x 30	0 x 45	0 x 60	0 x C
Area of nuclear profiles	P > 0.1	P > 0.1	P < 0.05	P<0.01	P > 0.1
% area occupied by apical vacuoles	P > 0.1	P > 0.1	P > 0.1	P > 0.1	P > 0.1
% area occupied by mitochondria	P < 0.01	P > 0.1	P < 0.01	P < 0.05	P < 0.1
μ ER/μ^2 cytoplasm	P > 0.1	P < 0.02	P<0.001	P < 0.05	P < 0.1

Figure 8.

VARIATIONS IN THE PROPORTIONS OF CYTOPLASMIC ORGANELLES IN THE KIDNEY AFTER A SINGLE INJECTION OF TRI-IODOTHYRONINE.



in the size of the vacuoles rather than to alterations in the number present. There was no change in the size of the vacuoles during the first 15hrs, but after 30hrs there was a marked decrease in the size of the vacuoles, which then gradually returned to normal by 60hrs.

There was an increase in the percentage area of the cytoplasm occupied by mitochondrial profiles during the first 15hrs after hormone administration. The amount of mitochondrial profiles present remained slightly but significantly elevated up to 60hrs. Again there were slight but significant, variations in the concentrations of endoplasmic reticulum profiles present in the cytoplasm. There was a slight but significant increase in the concentration of profiles during the first 30hrs followed by a significant decrease, to below normal levels, between 30 and 45hrs. The concentration of endoplasmic reticulum profiles remained significantly depressed upto 60hrs after hormone administration.

Variations in the morphology of the nuclear envelope after a single injection of triiodothyronine.

Considerable variations were noted in the morphology of the nuclear envelope after prolonged treatment with either thyroid hormone or after thyroidectomy of experimental animals as compared to those examined from the liver and kidney of normal animals. These consisted of accumulations of obscuring material, associated with the nuclear envelope, and the development of small and large vesicles from the membranes of the nuclear envelope. These changes are examined in further detail in Chapter 4. The developments of these changes after a single injection of triiodothyronine was investigated, in order to associate their development with the hormone action.

Development of obscuring material associated with the nuclear envelope.

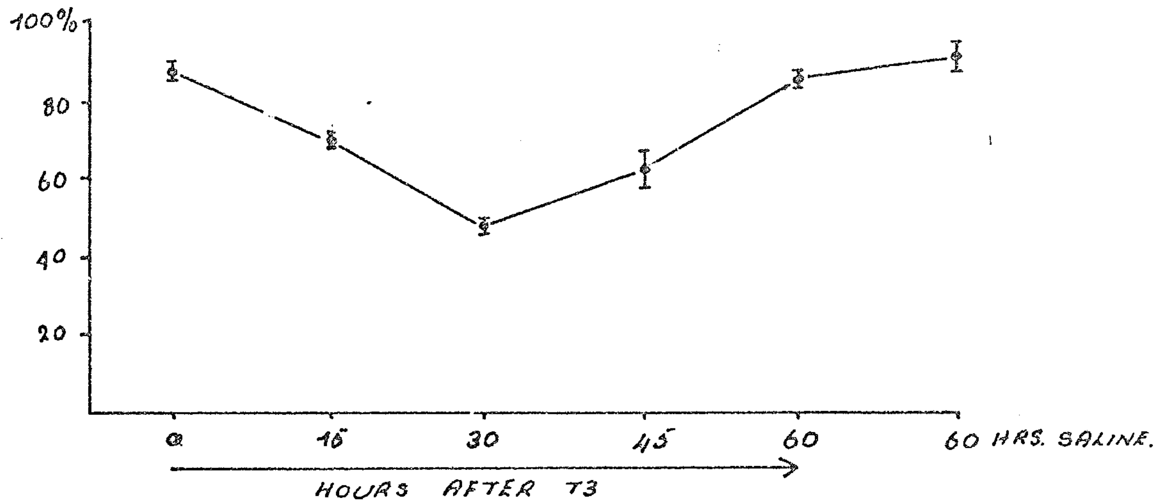
Nuclei from the liver showed considerable variations in the amount of obscuring material associated with the nuclear envelope during the first 60hrs after triiodothyronine administration. This was less obvious in the kidney. Initial visual examination suggested that there was an increased tendency towards the accumulation of material against the nuclear envelope during the first 45hrs after hormone administration. The number of nuclear membranes tended to increase after this time.

Counts were made of the numbers of nuclei at any one stage and the percentage not showing obscuring material was calculated. Nuclei were counted according to the criteria used in Chapter 4, All nuclei within three squares of the copper mounting grid were examined. Where the nuclear envelope could be clearly resolved at a magnification of x 10,000 the nuclei were counted as not obscured. Where less than half of the periphery of the nucleus was obscured the nucleus was counted as unchanged, where more than half was obscured the nucleus was counted as changed. The results are shown in Table 15 and Figure 9.

As is shown in Figure 9 there was a significant increase in the number of nuclei showing accumulations of obscuring material, during the first 15hrs after hormone administration. The numbers of obscured nuclei in the liver, increased to a peak at 30 hrs, where approximately 50% showed accumulations of obscuring material. The numbers of nuclei showing accumulations of obscuring material gradually returned to normal, but were still significantly more frequent than in the normal animals 60hrs after hormone administration. In the kidney the numbers of nuclei showing accumulations of obscuring material levelled off at approximately 30% between 15 and 30 hrs after hormone administration, and then gradually returned to normal by 60hrs, although nuclei with obscured nuclear envelopes were still significantly more frequent than in normal animals, at 60 hrs.

Figure 9.

VARIATIONS IN THE NUMBERS OF NUCLEI REMAINING UN-OBSCURED AFTER THE ADMINISTRATION OF A SINGLE DOSE OF TRI-IODOTHYRONINE.



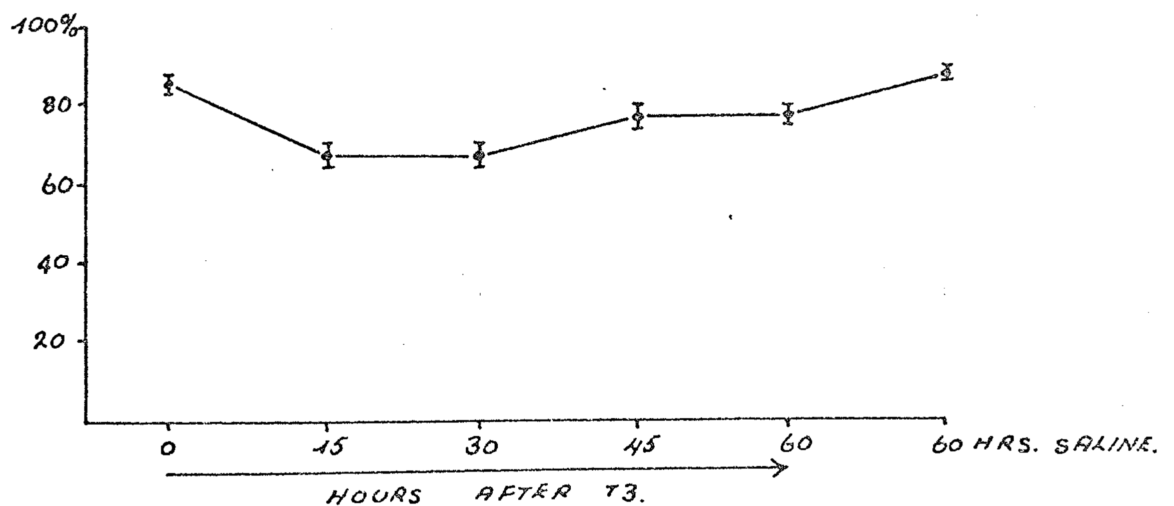


Table 16.

Percentage nuclei remaining unobscured at various times after the administration of a single dose of triiodothyronine.

	Hours after administration of T3					Control (60hrs saline)
	0	15	30	45	60	
Liver	89.90 ±3.59	71.86 ±1.86	48.40 ±1.85	63.86 ±5.25	85.06 ±2.29	93.46 ±4.28
Kidney	87.76 ±2.28	68.53 ±3.15	69.56 ±3.31	72.23 ±2.59	79.77 ±1.29	90.73 ±1.66

Table 17.

Statistical significance of the variations in numbers of nuclei showing accumulations of obscuring mterial,

	0 x 15	0.x 30	0 x 45	0 x 60	0 x C
Liver	$P < 0.05$	$P < 0.01$	$P < 0.1$	$P < 0.1$	$P < 0.1$
Kidney	$P < 0.05$	$P > 0.05$	$P < 0.1$	$P > 0.05$	$P > 0.1$

Developement of small vesicles from the outer membrane of the nuclear envelope, after a single injection of triiodothyronine.

Initial examination of the material suggested that there was a variation in the regularity of the nuclear membranes associated with the production of small vesicles during the 60hrs investigated after the administration of a single injection of triiodothyronine. Nuclear membranes tended to be less regular in profile 15 to 45 hrs after hormone administration than during the first 15 hrs.

In order to establish the patterns of development of

small vesicles from the nuclear envelope after thyroid hormone administration, counts were made of the total numbers of small vesicles occurring within four of their own diameters from the nuclear envelope. The results are shown below in Table 18 and in Figure 10.

Table 18.

Variations in the numbers of small vesicles associated with the nuclear envelope at various times after the administration of a single dose of triiodothyronine.

	Hours after administration of T3					Control (60hrs saline)
	0	15	30	45	60	
Liver	20.75 ± 4.64	16.81 ± 1.59	20.28 ± 2.39	16.78 ± 2.29	21.29 ± 5.86	15.50 ± 2.27
Kidney	13.83 ± 2.99	23.77 ± 2.61	20.50 ± 2.39	26.73 ± 3.09	28.8[illegible] ± 2.62	19.12 ± 3.63

Table 19.

Statistical significance of the variations in the numbers of small vesicles associated with the nuclear envelope after a single dose of triiodothyronine.

	0 x 15	0 x 30	0 x 45	0 x 60	0 x C
Liver	$P > 0.1$	$P > 0.1$	$P > 0.1$	$P > 0.1$	$P > 0.1$
Kidney	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P > 0.1$

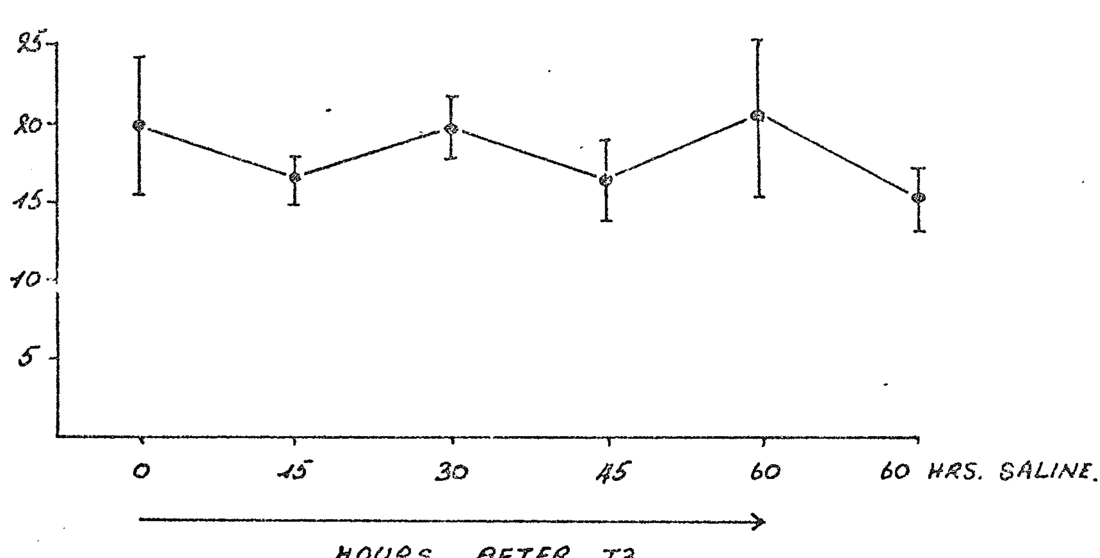
As is shown above there was no significant variation in the distribution of small vesicles around the nucleus at any time after a single injection of triiodothyronine in the liver. There was however, a significant increase in the numbers of small vesicles associated with the

Figure 10.

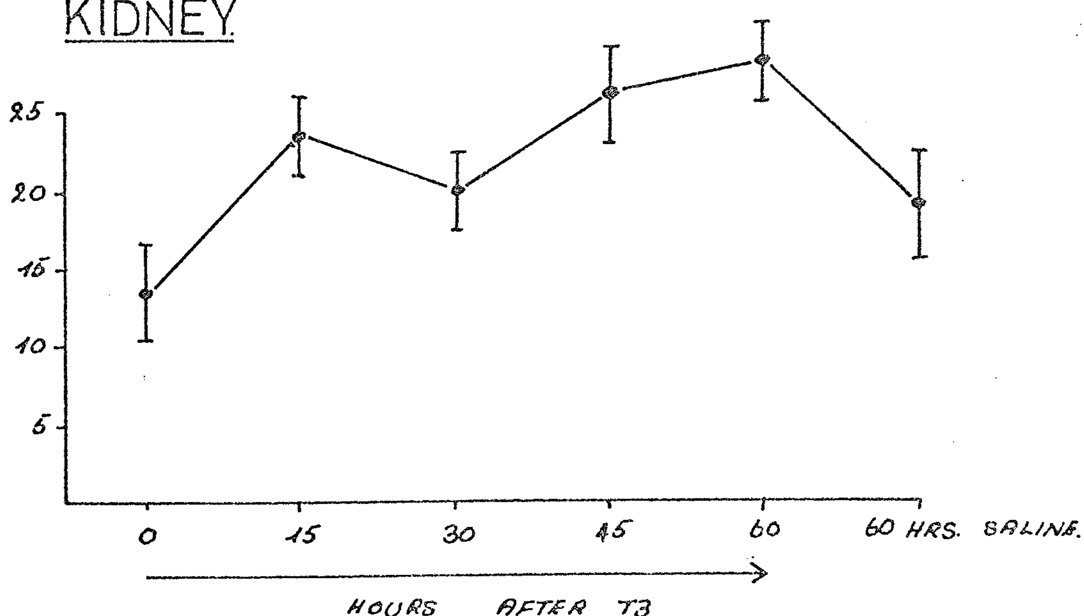
VARIATIONS IN THE PRODUCTION OF SMALL VESICLES FROM THE NUCLEAR ENVELOPE AFTER A SINGLE INJECTION OF TRI-IODO-THYRONINE.

(ESTIMATED AS TOTAL NUMBERS OF VESICLES OCCURRING WITHIN FOUR OF THEIR OWN DIAMETERS FROM THE NUCLEAR ENVELOPE.)

LIVER



KIDNEY



nuclear envelope in the kidney. The increase in the production of small vesicles from the nuclear envelope of the kidney was initiated during the first 15 hrs after hormone administration, and continued unto 60 hrs later.

Development of large vesicle structures from the nuclear envelope of the kidney, following a single injection of triiodothyronine.

Initial visual assessment did not suggest that any variation in the production of large vesicle structures from the nuclear envelope of kidney cells took place after the administration of a single dolse of triiodothyronine. However, in view of the observation that prolonged treatment with both triiodothyronine and thyroxine described in Chapter 4, caused an increase in the numbers of nuclei with associated large vesicle structures, it was decided to attemot a quantitative estimation of the numbers of nuclei with associated vesicle structures at various times after the administration of a single dose of thyroid hormone. The total number of nuclei occurring within three of the squares of the copper specimen grid were counted and the number with associated large vesicle structures was expressed as a percentage of the total number of nuclei counted. The results are shown in Table 20 and Figure 11.

As can be seen there was no overall accumulation of nuclei with associated vesicle structures after 60hrs of triiodothyronine, but there was a significant increase in the occurrence of large vesicle structures 15hrs after hormone administration, which had returned to normal at 30hrs.

Variation in the location of nucleoli after a single injection of triiodothyronine.

As is noted in Chapter 3, there was a significant variation in the numbers of nucleoli occurring in contact with the nuclear envelope after prolonged treatment with triiodothyronine.

Figure 11.

VARIATIONS IN THE NUMBERS OF LARGE VESICLES ASSOCIATED WITH KIDNEY NUCLEI AFTER THE ADMINISTRATION OF A SINGLE DOSE OF TRI-IODOTHYRONINE.

(EXPRESSED AS NUCLEI WITH ASSOCIATED LARGE VESICLES AS A PERCENTAGE OF THE TOTAL NUCLEI COUNTED)

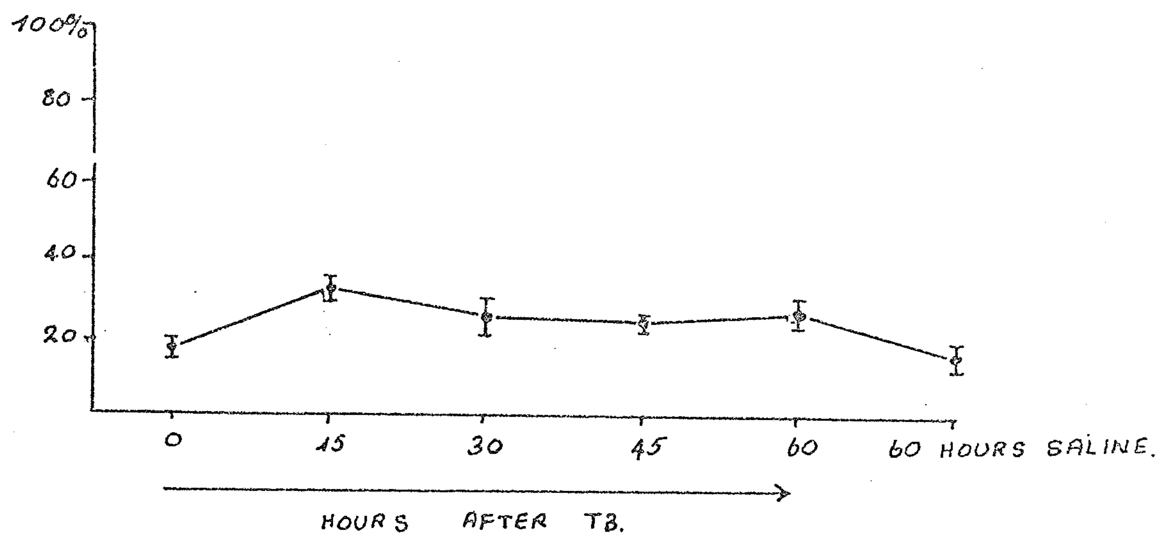


Table 20.

Variations in the development of large vesicle structures from the nuclear envelope of the kidney, after a single injection of triiodothyronine.

	Hrs. after administration of T3					Control (60hrs saline)
	0	15	30	45	60	
Kidney	16.24 ±2.10	32.44 ±2.70	25.53 ±4.68	24.06 ±2.04	27.91 ±3.63	15.32 ±2.72

Table 21

Statistical significance of variations in numbers of large vesicle structures after a single injection of triiodothyronine.

	0 x 15	0 x 30	0 x 45	0 x 60	0 x C
Kidney	$P < 0.05$	$P > 0.1$	$P > 0.1$	$P > 0.1$	$P > 0.1$

Table 22

Variations in the location of the nucleolus after a single injection of triiodothyronine.

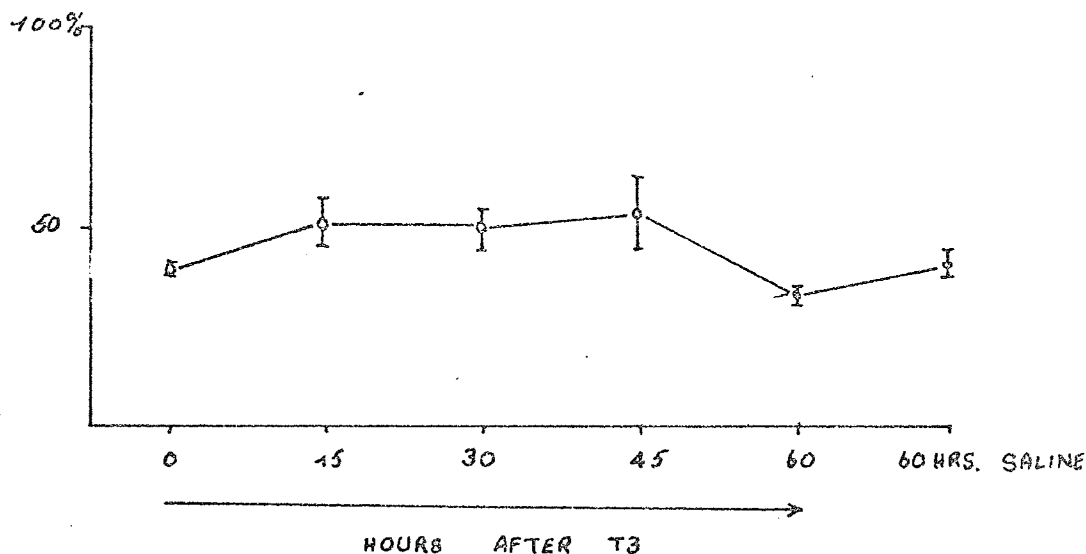
	Hrs after administration of T3					Control (60hrs saline)
	0	15	30	45	60	
Liver	40.25 ±2.48	52.22 ±6.35	51.43 ±5.05	55.20 ±8.09	34.89 ±1.04	42.57 ±2.83
Kidney	45.39 ±3.13	72.40 ±1.80	65.16 ±1.80	70.60 ±5.21	84.90 ±10.63	49.50 ±0.32

Figure 12.

VARIATIONS IN THE LOCATION OF NUCLEOLI AFTER A SINGLE DOSE OF TRI-IODOTHYRONINE.

(EXPRESSED AS PERCENTAGE OF NUCLEOLI WHICH OCCUR IN-CONTACT WITH THE NUCLEAR ENVELOPE.)

LIVER



KIDNEY

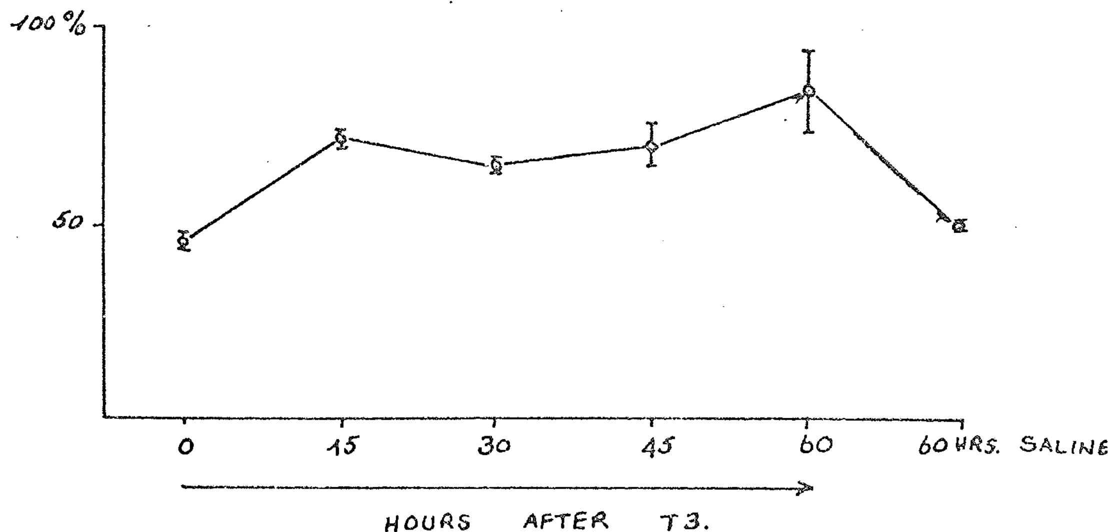


Table 23

Statistical significance of the variations in the location of nucleoli after a single dose of triiodothyronine.

	0 x 15	0 x 30	0 x 45	0 x 60	0 x C
Liver	$P > 0.1$	$P < 0.01$	$P < 0.01$	$P > 0.1$	$P > 0.1$
Kidney	$P < 0.1$	$P < 0.05$	$P < 0.05$	$P < 0.001$	$P > 0.1$

A quantitative estimation of the numbers of nucleoli occurring in close contact with the nuclear envelope, at various times after a single dose of hormone, in order to establish a relationship between thyroid hormone influence and the location of the nucleolus within the nucleus.

The numbers of nucleoli in contact with the nuclear envelope were counted within the nuclei of three of the squares of the specimen grid, and expressed as a percentage of the total numbers of nucleoli counted. The results are shown above in Table 22 and Figure 12.

There was a significant variation in the location of the nucleolus with respect to the nuclear envelope in both the liver and the kidney, although the effect was more pronounced in the kidney. The change in the location of the nucleolys appeared to be initiated within the first 15hrs of hormone action and continued throughout the following period. The numbers of nucleoli in contact with the nuclear envelope in the kidney was still significantly greater than in the normal tissues 60hrs after the injection with a single dose of triiodothyronine. although the numbers of nucleoli in contact with the nuclear envelope in the liver had returned to normal by 60hrs.

Discussion: The Sequence of Ultrastructural Changes Produced by the Influence of Thyroid Hormones.

The results of examination of mouse liver ultrastructure at various times after the administration of a single injection of triiodothyronine, parallel the biochemical results obtained by Tata and his co-workers, (Roodyn et al 1965; Widnell and Tata 1966; Tata et al 1963 and Tata 1970).although there are some interesting discrepancies. The results obtained from the kidney are similar to those obtained from the liver, although again there are some interesting differences.

One of the earliest detectable changes resulting from the administration of triiodothyronine was the accumulation of obscuring material associated with the nuclear envelope, in both the liver and the kidney. Accumulation of material associated with the nuclear envelope was initiated during the first 15hrs of hormone action, and continued in the liver until 50% of nuclei showed obscured nuclear envelopes 30hrs after the administration of triiodothyronine. The phenomenon was less extensive in the kidney, the number of nuclei with obscured envelopes levelling off at about 30% after 30hrs. The numbers of nuclei showing accumulations of material gradually declined after 30hrs, so that 90% of nuclei from both tissues resembled those of normal animals 60hrs after hormone administration.

The production of small vesicles from the nuclear envelope of the kidney also appeared to be initiated within the first 15hrs of hormone action. Production of small vesicles continued to increase up to 60hrs after hormone administration. Production of small vesicles from the nuclear envelopes of liver cells did not follow this pattern. There was no overall variation in the production of small vesicles, although there appeared to be regular fluctuations in the numbers of vesicles present at 15 hr intervals. It is probable that some other phenomenon, such as a diurnal variation in vesicle production is interfering with the effects

of thyroid hormone action. The production of large nuclear envelope vesicles from the kidney nuclei also appeared to be initiated during the first 15hrs of the hormone action. The numbers of nuclei with associated large vesicles then levelled off at approximately 10% above normal after 30hrs.

The changes in nuclear membrane morphology appear to be coordinated with the early effects of thyroid hormone action upon the nucleus, reported by Widnell and Tata (1966). They described an increase in the synthesis of rapidly labelled nuclear RNA detectable after 6hrs of thyroid hormone activity and an increase in the specific activity of nuclear RNA polymerase taking place 15hrs after the administration of a single dose of triiodothyronine. However the significance of the nuclear membrane changes in this respect is difficult to visualise unless they are connected with the accelerated transfer of material from the nucleus to the cytoplasm.

The significance of the variations in nuclear sizes, particularly in the kidney, are also difficult to understand. The variations in location of the nucleolus with respect to the nuclear envelope are coordinated with the variations in the morphology of the nuclear envelope. Nucleoli occurred in contact with the nuclear envelope more frequently after a single injection of triiodothyronine, the change in location also appeared to be initiated during the first 15hrs of hormone action, coordinated with the early effects upon the nucleus described by Widnell and Tata (1966). The change in location of the nucleolus under the influence of thyroid hormones, is probably also attributable to the increased turnover of material within the nucleus under the influence of thyroid hormones described by Tata (1964, 1965 and 1967) As is discussed in further detail in Chapter 3, the nucleolus is primarily concerned with the synthesis of ribosomal RNA, which is known to be influenced by thyroid hormone activity.(Scharrer and Darnell 1962; Penmann et al 1966; Perry 1962, 1963, and 1964; Widnell and Tata 1966; and Tata 1967). It appears

that a nucleolus actively concerned with RNA production has a tendency to migrate towards the nuclear envelope, possibly in order to discharge the products of its synthetic activity more readily to the cytoplasm.

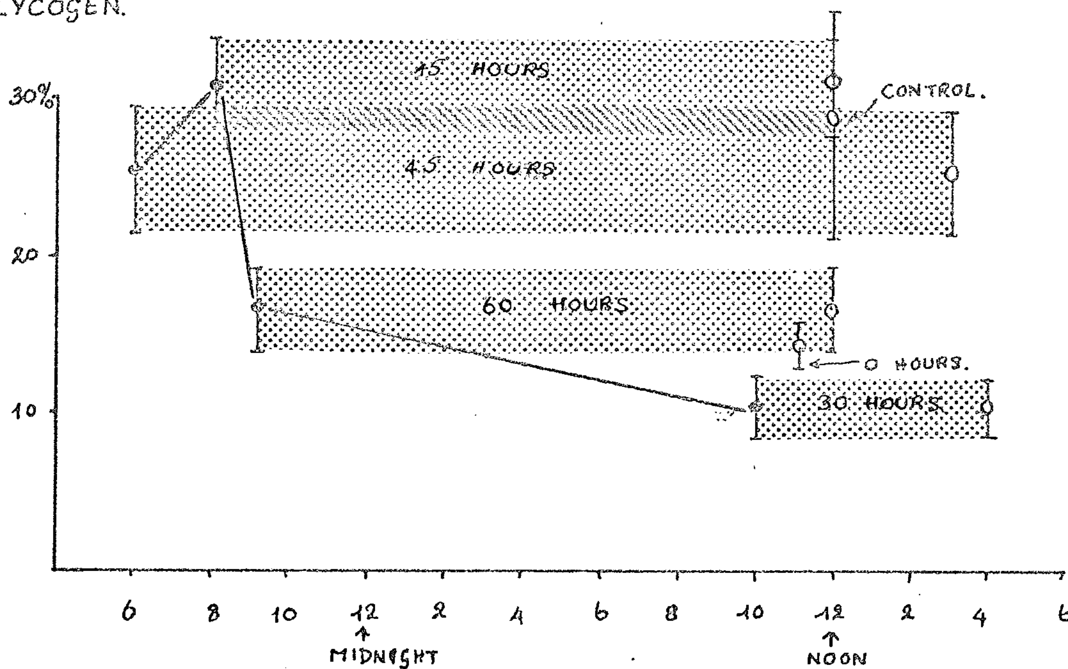
The reduction in glycogen content of the liver noted by Tata (1963) 24hrs after hormone administration was not obvious. There was a reduction in the glycogen content 30hrs after hormone administration but this was not statistically significant. The significant increase in glycogen content 15hrs after the administration of triiodothyronine and the apparent fluctuations at 15hr intervals after can probably be attributed to a diurnal fluctuation in the glycogen content of the liver, rather than to the action of the hormone. If the levels of glycogen are plotted against the time of injection rather than the time after hormone administration, as is shown in Figure 13, the levels appear to be higher before midnight and lower before midday. The time of death is unlikely to be affecting the content of glycogen since all animals were killed between 10 am and 4 pm. It would appear that thyroid hormone administration affects the diurnal variations in glycogen levels, and that its influence occurs between 15 and 45hrs after administration. There is a significant difference between the levels of glycogen present in the animals killed 15 and 60hrs after administration of hormone although there was only an hour between the times of injection. The levels appear to have decreased at 45hrs although this may be attributable to the diurnal variation, rather than to the hormone action.

The concentration of endoplasmic reticulum profiles in the liver appears to be similarly affected by the presence of a diurnal variation which is the inverse of the one noted for glycogen content, as is shown in Figure 13. The concentration of endoplasmic reticulum profiles was lowest in the animals which received an injection of triiodothyronine before midnight, and highest in the animals which received an injection before midday. Thus there appeared to be an increase in the amount of endoplasmic reticulum present after the administration of the thyroid

Figure 13

VARIATIONS IN THE CONCENTRATIONS OF GLYCOGEN AND ENDOPLASMIC RETICULUM IN THE LIVER WITH TIME OF INJECTION AND TIME OF DEATH.

TIME OF INJECTION = ●
TIME OF DEATH = ○



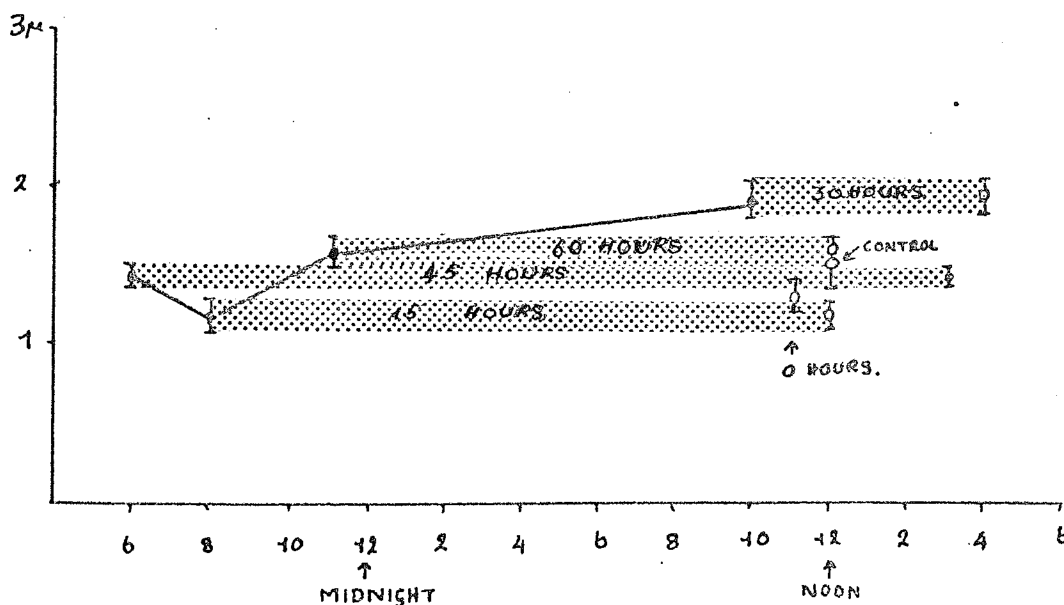
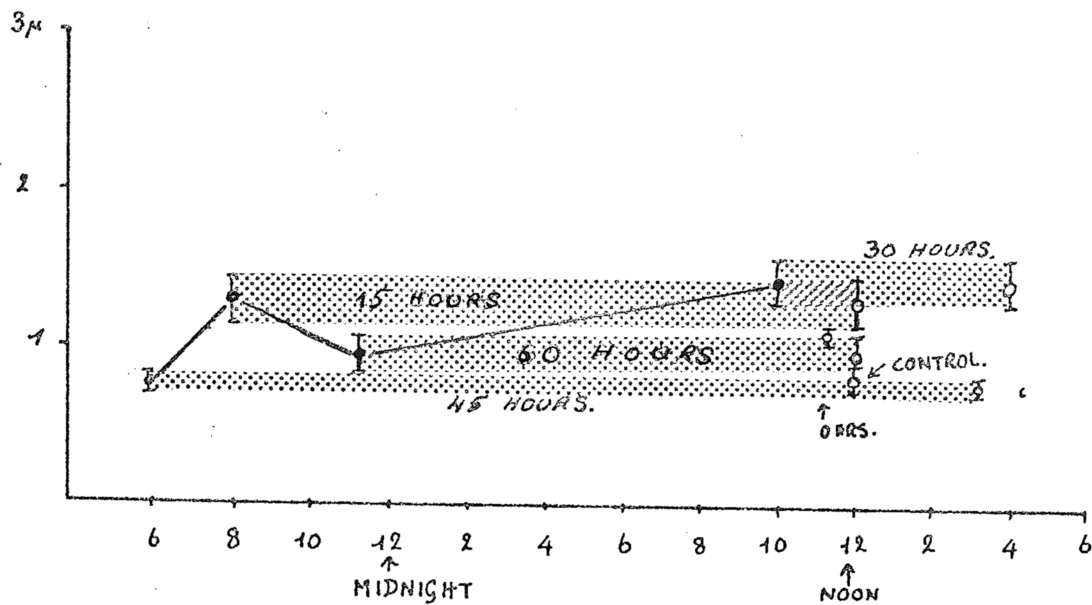


Figure 14.

VARIATIONS IN THE CONCENTRATION OF ENDOPLASMIC RETICULUM IN THE KIDNEY WITH TIME OF INJECTION AND TIME OF DEATH.

TIME OF INJECTION = ●

TIME OF DEATH = ○



hormone, and the increase appeared to be initiated between 15 and 45 hrs after hormone administration, which corresponds with the observation of Tata et al (1963) and Tata (1970) of the increase in synthesis of membrane phospholipids 20 hrs after hormone administration, and accumulation of ribosomes in the cytoplasm 24hrs after hormone administration. In the kidney, there also appears to be a diurnal variation in the concentration of endoplasmic reticulum profiles, which is the reverse of that seen in the liver, the concentration appears to be the greatest before midnight and least before midday, as is shown in Figure 11. The presence of a diurnal variation in the amount of endoplasmic reticulum present in the kidney appears to account for the apparent reduction in the amount of endoplasmic reticulum after prolonged thyroid hormone treatment, described in Chapter 1, and 45hrs after the administration of a single dose of triiodothyronine. It would appear that there is a significant increase in the concentration of endoplasmic reticulum between 15 and 30 hrs after thyroid hormone administration, which then returned towards normal.

The significance in the variation in the size of the apical vacuoles in the kidney is difficult to understand. The significant reduction noted at 30hrs after hormone administration could be attributed to hormone action and not to any diurnal fluctuation in the size of the vacuoles.

The increase in the size of mitochondrial profiles at 15hrs in the kidney and 30hrs in the liver, is difficult to understand. According to Roodyn et al (1965) changes in amino acid incorporating activity of the mitochondria were not detectable until 36hrs after hormone administration. The alteration in mitochondrial size is however, coordinated with the increased respiratory activity resulting in the increased basal metabolic rate detectable at 20hrs described by Tata et al (1963).

Although the changes in the ultrastructure of mouse liver and kidney produced by the influence of thyroid hormones could be placed

Table 24.

The sequence of ultrastructural changes following the administration of a single dose of triiodothyronine.

Time	Liver	Kidney
0hrs		
15hrs	Migration of nucleoli towards nuclear envelope	Migration of nucleoli towards nuclear envelope.
	Initiation of obscuration of nuclear envelope	Initiation of obscuration of nuclear envelope.
	Initiation of production of small vesicles from the nuclear envelope.	Initiation of production of of small vesicles from the nuclear envelope.
		Initiation of production of large vesicle structures from the nuclear envelope.
30hrs	Increase in concentration of endoplasmic reticulum profiles.	Increase in concentration of endoplasmic reticulum profiles.
	Decrease in glycogen content.	
		Decrease in size of apical vacuoles.
45hrs	Reduction in size of nuclei.	Reduction in size of nuclei.
	Increase in size of mitochondria.	Increase in size of mitochondria.

in some chronological order, as is shown in Table 24, a more detailed investigation would be necessary to substantiate this. Unfortunately the investigation of the effects of a single injection of triiodothyronine to normal animals upon the ultrastructure of the liver and kidney has meant that many of the effects of the thyroid hormone action were slight, and frequently not statistically significant. As was noted by Roodyn et al (1965) the effects of a replacement dose of triiodothyronine administered to a thyroidectomised animal was considerably greater than the efect of a dose of triiodothyronine administered to a normal animal.

Chapter 3.

The Effects of Thyroid Hormones upon Nuclear Morphology.

The relationships of structure and function within the nucleus are still not fully understood. A considerable amount of attention has been paid to the nucleolus and its involvement in the production of ribosomes is now generally accepted, but knowledge of the complex but confusing appearance of the structures present in the rest of the nucleoplasm, as they are revealed by electron microscopy, is yet in its infancy. A knowledge of the present understanding of nuclear structure and function is an essential prerequisite for an investigation of the influence of an external factor, such as the administration of a hormone, upon nuclear morphology.

Identification of nucleolar components.

The presence of nucleolus associated chromatin was demonstrated by Caspersson and Shultz (1940) by light microscopy, but the existance of intranucleolar chromatin was doubted until demonstrated by electron microscopy after enzyme digestion of thin sections (Hay and Revel 1963; Granboulan and Granboulan 1964). Using combined auto-radiography and enzyme digestion of thin sections, they were able to demonstrate replicating DNA which, it was suggested, might act as a template for nucleolar RNA synthesis.

It is now generally accepted that RNA is a major constituent of nucleoli. It has been suggested that nucleolar RNA is accumulated from multi-chromosal sites outside the nucleolus but the evidence from autoradiographic studies suggest that the nucleoli labell too rapidly for the accumulation of RNA to be derived from extra-nucleolar sites (Amano and Leblond 1960; Leblond and Amano 1962; Amano et al 1965; Perry 1962, 1964; Muramatsu et al 1961; Weisner et al 1965). It is

now accepted that nucleolar RNA synthesis occurs in situ during interphase, and is independent of the rate of RNA synthesis on extra-nucleolar chromatin (Perry et al 1961).

The nucleolus is also rich in proteins, of three types neutral, basic histones and non-histones, and residual proteins which together constitute up to 70 - 80% of the nucleolar dry weight (Vincent 1955; Maggio et al 1963; Busch et al 1963.) A protein synthetic capacity was attributed to the nucleolus by Caspersson (1950) but the controversy over whether or not the nucleolus could be involved in protein synthetic activity was not resolved until autoradiography demonstrated a rapid incorporation of H^3-amino acids by nucleoli. (Tixier-Vidal et al 1965). Birnstiel and Hyde (1963) demonstrated that the radioactivity was predominant in the residual fraction of the nucleolar proteins after incorporation of H^3-leucine by isolated nucleoli.

Much evidence has now accumulated supporting the idea that the nucleolar RNA resembles ribosomal RNA and represents some stage or stages in its production. The base composition of nucleolar RNA was found to be similar to that of ribosomal RNA from a variety of cells, and both were characterised by a high guanine-cytidine content (Edstrom 1960; Edstrom and Beerman 1962; Edstrom and Gall 1963; Hurlbert et al 1964; Liau et al 1965.). Brown and Gurdon (1969) discovered a mutant larvae of Xenopus laevis wich was unable to synthesise ribosomal RNA and was found to be lacking in nucleoli in all cells, and MacConkey and Hopkins (1964) have been able to demonstrate a concentration of DNA in the nucleolar chromatin of HeLa cells which was complementary to ribosomal RNA and could act as a template for ribosomal RNA synthsis.

45S ribosomal precurser RNA has been isolated from the nuclei of HeLa cells (Scharrer and Darnell 1962; Penmann et al 1966) and Perry has since demonstrated that it is synthesised in the nucleolus (Perry 1962, 1963, and 1964) Evidence from a number of sources showing

that synthesis occurs in the pars fibrosa and the products passed to the pars granulosa where they accumulate before being passed to the cytoplasm. Using a combination of enzyme digestion and electron microscopic microscopic autoradiography on cultivated monkey kidney Granboulan and Granboulan (1964) were able to demonstrate the uptake of H^3- uridine by intranucleolar and nucleolar chromatin, after 5mins of labelling the radioactivity appeared in the pars fibrosa and after 10mins. it began to accumulate in the pars granulosa. The radioactivity was completely removed after digestion with ribonuclease. Karasaki (1965) was able to demonstrate a similar sequence of events during the embryogenesis of Triturus pyrrhogaster. Gueskens and Bernhard (1966) were able to confirm the earlier findings on monkey kidney cells using autoradiography with the administration of Actinomycin D, which inhibited synthesis but did not affect the transport of material to the pars granulosa.

The conversion of 45S RNA into 35S and its subsequent cleavage into 18S and 28S is thought to take place within the nucleolus. Birnstiel et al (1963) has been able to extract 18S and 28S RNA from isolated pea nucleoli together with sub-ribosomal precurser particles and ribosomes. It seems that the linkage of the 18S and 28S RNA with protein to form 40S and 60S ribonucleoprotein particles may also take place within the nucleolus, although this is poorly substantiated. Birnstiel and Hyde (1963) demonstrated that a fraction of nucleolar proteins in respect of amino acid composition, and they were able to demonstrate that the uptake of H^3-leucine was greatest into the residual fractions of nucleoloprotein, ie. that fraction which most closely resembled ribosomal proteins.

Girard et al (1965) and Perry (1966) demonstrated what appeared to be the passage of 40S and 60S particles into the cytoplasm by biochemical techniques, and suggested that the 60S particles appeared to be retained in a nucleolar pool longer than did the 40S

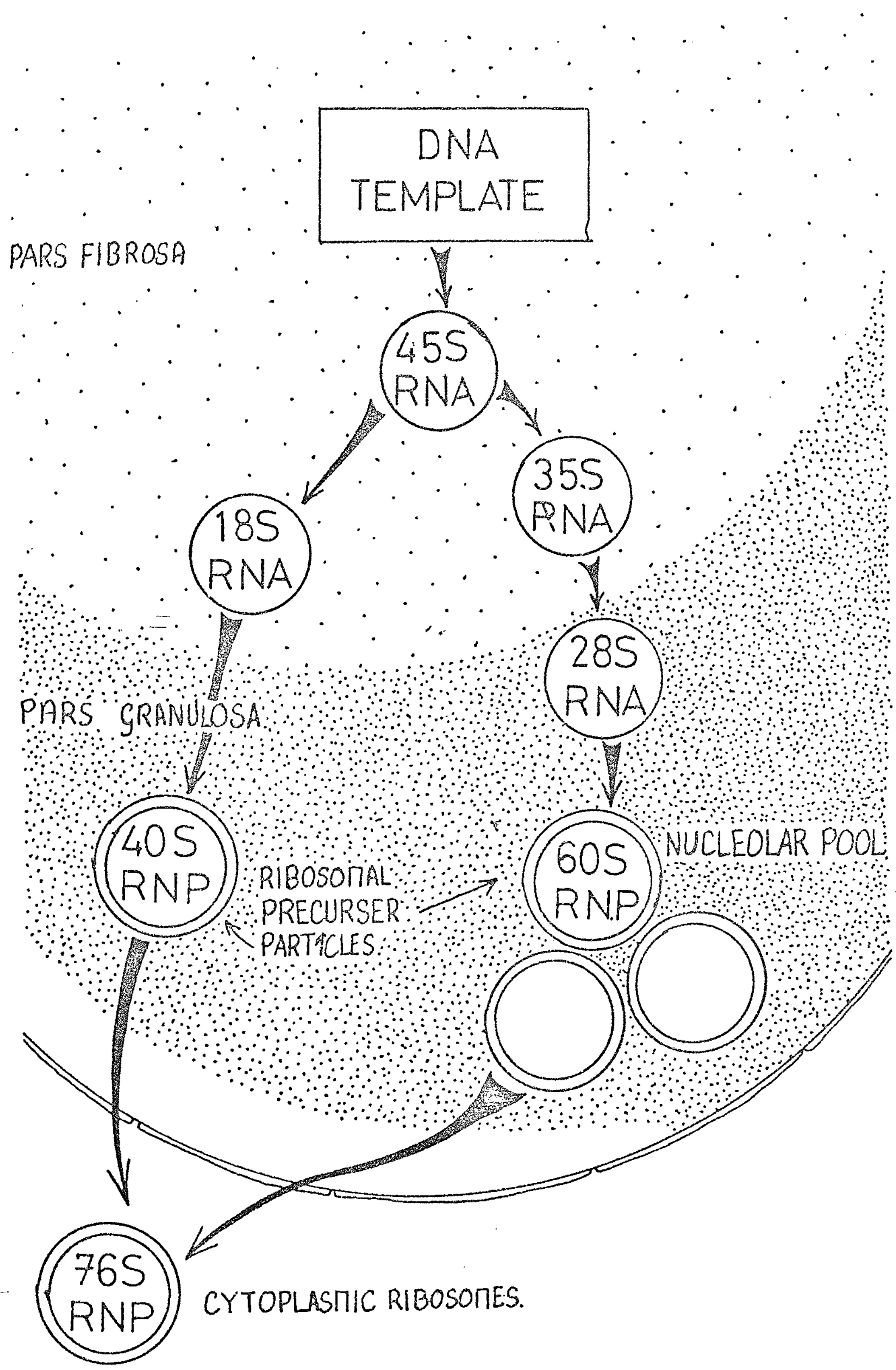
particles.

The present understanding of the relationships between function and structure of the nucleolus can be summarised as shown in Figure 15 on the following page. Various attempts have been made to relate the biochemical and autoradiographic evidence with the appearance of nucleoli in routine electen microscopic preparations. The pars pibrosa appears to be a dense, structureless mass at low magnifications, especially after aldehyde fixation, but higher magnifications reveal a filamentous network embedded in a proteinaceous matrix of similar electron density. The filamentous components have been estimated at approximately 50Å in diameter (Hay 1968) and resemble the filaments of deoxyribonucleoprotein distinguishable in the chromatin. It appears however, that the nucleolar filaments may be a form of ribonucleoprotein rather than deoxyribonucleoprotein (Swift 1963; Marinozzi 1964; Bernhard and Granboulan 1968.) It has been suggested that at least some of the fibrils represent 45S RNA in production before it becomes organised into a particle (Bernhard and Granboulan 1968). In which form they may already be linked to a protein.

The pars granulosa usually appears as a mass of granules of varying sizes associated with an underlying fine fibrillar network, which surrounds or is adjacent to the pars fibrosa. The underlying filaments appear to be continuous with those of the fibrosa and those of the nuclear chromatin, and probably represent a form of ribonucleoprotein (Bernhard and Granboulan 1968). The granular constituents of the pars granulosa are also ribonucleoprotein in composition, their contrast being markedly reduced by either pepsin or ribonuclease digestion, and almost completely lost by successive use of both enzymes (Bernhard and Granboulan 1968). The greater components are variable in size, and often difficult to measure according to Hay (1968) because of their frequent association with fibrils. However Hay estimated them as being 150Å in diameter as compared to 200Å diameter of cytoplasmic ribosomes,

Figure 15.

NUCLEOLAR INVOLVEMENT IN THE PRODUCTION OF RIBOSOMAL PRECURSER PARTICLES



showing their predominantly DNA constituent, although the contrast was not completely removed. Ris suggested that the remaining contrast was due to the histone fraction, known to be associated with the chromosomes.

Watson (1962) also described 'perichromatin' granules within the masses of condensed chromatin. These granules occurred as isolated spherical bodies approximately 300Å in diameter and surrounded by a clear halo approximately 250Å across. The granules were in general of slightly greater density than the surrounding chromatin. Their composition has not been established, but Watson suggested that they may contain both DNA and RNA or be of RNA alone, on the grounds of their reaction to different staining solutions.

The interchromatin space is much more complex and less well understood. The basic structure of this region of the nucleus is made up of a protein rich fibrillar network, closely resembling the underlying network of the nucleolus, with which it appears to be continuous (Bernhard and Granboulan 1968; Simard 1970). Of the heterogeneous collection of granules found within the interchromatin space only one component has been characterised, the 'interchromatin' granules of Swift (1959), Ris (1962) and Granboulan and Bernhard (1961). These granules were described as frequently angular, with high electron density and of approximately 200 - 250Å diameter. They occurred in groups, clusters and chains within sections. They have been shown to resist digestion by deoxyribonuclease, ribonuclease and pepsin when these enzymes were used on aldehyde fixed material, embedded in methacrylate.(Monneron 1966), although they tended to react to Bernhard's stain (Bernhard 1968) and to various fixation proceedures as though their main constituent was RNA (Simard 1970). The significance of these particles has not yet been established although Ris (1962) suggested that they may represent cross-sections through a course network of ribonucleoprotein fibrils, and it has been suggested that they may be ribosomes (Frenster et al 1960).

as estimated by Swift (1963). Lafontaine and Chounaird (1963), Marinozzi (1964) and Bernhard (1966) suggested that at least some of the granulosa particles represented the precurser particles of the 60S pool. This would account for the greater varia ility of size of nucleolar granules compared to cytoplasmic ribosomes, if it is borne in mind that these are probably still in a state of proteination.

Identification of nucleoplasmic constituents.

The identification of the constituents of the nucleolar regions of the nucleus and the biochemical significance is less well understood, mainly as a result of the difficulty in interpreting such a complex structure as the nucleus, in thin section.

After primary aldehyde fixation, followed by osmium post-fixation, the nucleus shows two main regions. The chromatin appears as a relatively electron dense mass of granules clumped against the nuclear envelope. Small masses are also present in the general nucleoplasm and surrounding the nucleolus. The distribution of the chromatin around the nuclear membrane seemed to suggest an artefactual arrangement but the presence of 'light channels' to the nuclear pores would indicate that this distribution does represent a condition present in the living cell and is not due to some factor in the preparation technique. The interchromatin space usually appears as an area of low general electron density containing a heterogeneous collection of particles and fibrillar components.

The identity of the various components of the nucleoplasm have been the result of considerable speculation. Ris (1962) has shown that the apparently granular nature of the chromatin represents cross-sections of closely coiled and twisted nucleo-histone fibrils of the chromosomes or heterochromatin. The contrast of these fibrillar cross-sections was considerably reduced by deoxyribonuclease digestion,

The other granular components seem to lack description in the literature, and there would appear to be no evidence of their identity or significance. It is possible that some may represent cross-sections of fibrillar material whilst others might be connected with the metabolic activity of the nucleus, although there is no evidence to support this idea.

The work of Amano and his group was able to establish some of the metabolic processes within the various regions of the nucleus, using light microscope autoradiography, but it has never been established, at the èlectron microscopic level, which of the particulate or fibrillar components were involved. Amano and Leblond (1964) demonstrated protein synthetic activity associated with the chromatin but not the nuclear sap or nucleoli of Purkinje cells of the cerebellum, liver cells and Sertoli cell nuclei of the rat. They were also able to demonstrate RNA synthesis associated with nucleoli and the chromatin but not the interchromatin space. They were also able to show that the turnover rate of RNA from nucleolar and non-nucleolar sites was comparable but that although nucleolar RNA acted as a precurser of the cytoplasmic ribosomes non-nucleolar RNA was turnover in situ, and that RNA synthesis in the two sites was independent.

Further work of Amano, Leblond and Nadler (1965) was able to demonstrate three separate pools of RNA analogous to messenger, soluble and ribosomal RNA with respect to specific activity, turnover times and amount present in the nucleus. The amounts of the three types of RNA present differed from the two sites, nucleolar and non-nucleolar, the nucleolar RNA being predominantly ribosomal in behaviour. Although they suggested that at least a third of the RNA from the non-nucleolar sites represented material of nucleolar origin migrating to the cytoplasm.

Variations in nuclear morphology with hormone treatments.

A number of studies have shown that thyroid hormones influence metabolic processes taking place within the nucleus. Tata (1968) found that the administration of Actinomycin D completely abolished the effects of thyroid hormones, and Paik and Cohen (1961) were able to show that the administration of thiouracil inhibited the production of certain enzymes under the influence of thyriod hormones, upon amphibia, as a result of its incorporation into 'useless' RNA.

It is now well established that the administration of thyroid hormones causes an increase in the ribonucleic acid synthesis early in their action.(Widnell and Tata 1963; Tata 1964; Tata 1967.) and it is now known that most of the newly synthesised RNA is ribosomal in character. (Widnell and Tata 1966; Tata 1967.)

Tata (1964), Tata (1965) and Tata (1967) showed that there was a rapid turnover of newly synthesised material under the influence of hormone action, which resulted in little accumulation of material within the nucleus. This effect was particularly prominent in the action of thyroid hormones in amphibian metamorphosis.

Thus it might be expected that there would be changes in nuclear morphology, particularly within the nucleolus, as a result of hormone action. Tata (1967) was unable to demonstrate an increase in the size or number of nucleoli in induced amphibian metamorphosis, when tadpole livers were examined in the electron microscope, although Siegel and Tobias (1966) demonstrated an increase in the number of cultured human kidney epithelial cells under the influence of thyroxine.

Various suggestions have been made that thyroid hoemones cause changes within DNA complexes, which might possibly reflected in conformational changes within the electron microscope. Tata (1966) suggested that there might be an interaction with the genetic template, connected with an increased histone synthesis, which it was suggested

might have a significant effect upon the activity of the nucleolus. Kim and Cohen (1966) demonstrated a modification of tadpole liver chromatin under the influence of thyroxine. There was a 20 - 50% increase in the template efficiency of thyroxine treated chromatin, which was abolished if the DNA was deproteinised.

Examination of nuclear morphology using various histo-chemical staining techniques.

Examination of nuclear morphology in glutaraldehyde and osmium fixed material revealed several features which might be influenced by the action of thyroid hormones. There appeared to be an increase in the amount of electron dense material present both in the nucleoplasm and associated with the nucleolus. In order to clarify the apparent hormone induced changes in nuclear morphology a classification system was devised, and nuclei scored according to their placing in this system. Silver staining following acrolein fixation was used in an attempt to distinguish nucleoproteins, and material stored in cold buffer was examined for its effects upon the extraction of material from the stored tissue blocks.

Small blocks of liver and kidney from thyroidectomised animals and euthyroid animals and animals rendered hyperthyroid with either thyroxine or triiodothyronine were prepared according to one of the following preparation proceedures;-

1. Routine glutaraldehyde and osmium fixation followed by section staining with uranium and lead salts.

2. Acrolein fixed material section stained according to the silver impregnation technique of Marinozzi (1963).

3. Glutaraldehyde fixed material stored in cold buffer for three months before subsequent post osmication and routine embedding. Sections were stained with uramium and lead salts.

4. Acrolein fixed material stored in cold buffer for three

months before routine embedding. Sections were stained according to the silver impregnation technique of Marinozzi (1963).

There also appeared to be an increase in the number of nucleoli occurring in close contact with the nuclear envelope after hormone treatment. for this reason an attempt was made to gain some quantitative evidence of the number of nucleoli adjacent to the nuclear envelope as compared to these occurring in the general body of the nucleus after each hormone treatment.

Location of nucleoli, within the nucleus.

There appeared to be a variation in the number of nucleoli in contact with the nuclear envelope in the nuclear profiles examined. The impression was gained that there were more nucleoli in contact with the nuclear envelope of nuclei from hormone treated animals than those from normal or thyroidectomised animals.

The numbers of nucleoli in contact with the nuclear envelope were counted and expressed as a percentage of the total numbers of nucleolar profiles counted from each experimental group, in an attempt to gain a quantitatitative estimate which would verify the initial visual impression. The results are shown in Table 25 and Figure 16 on the following pages.

As can be seen from Figure 16 there was a general trend towards a greater number of nucleoli in contact with the nuclear envelope with the raised levels of thyroid hormones, in both the liver and the kidney. However, the variation was statistically significant only in the liver, probably as a result of the rather small numbers of nuclei counted in each group.

There was a significant difference between the numbers of nucleoli in contact with the nuclear envelope in the thyroidectomised animals compared with the normal animals, and in both the hormone

treatments compared with the normal and thyroidectomised animals as is shown in Table 25, below.

Table 25

Numbers of nucleoli in contact with the nuclear envelope, as a percentage of the total numbers of nucleoli counted.

	TX	N	T3	T4
Liver	27.80 ±6.00	39.50 ±2.40	58.50 ±2.10	57.95 ±10.34
Kidney	49.06 ±10.45	58.93 ±8.73	64.96 ±5.04	71.96 ±8.77

Table 26

The statistical significance of the distribution of nucleoli shown above.

Liver

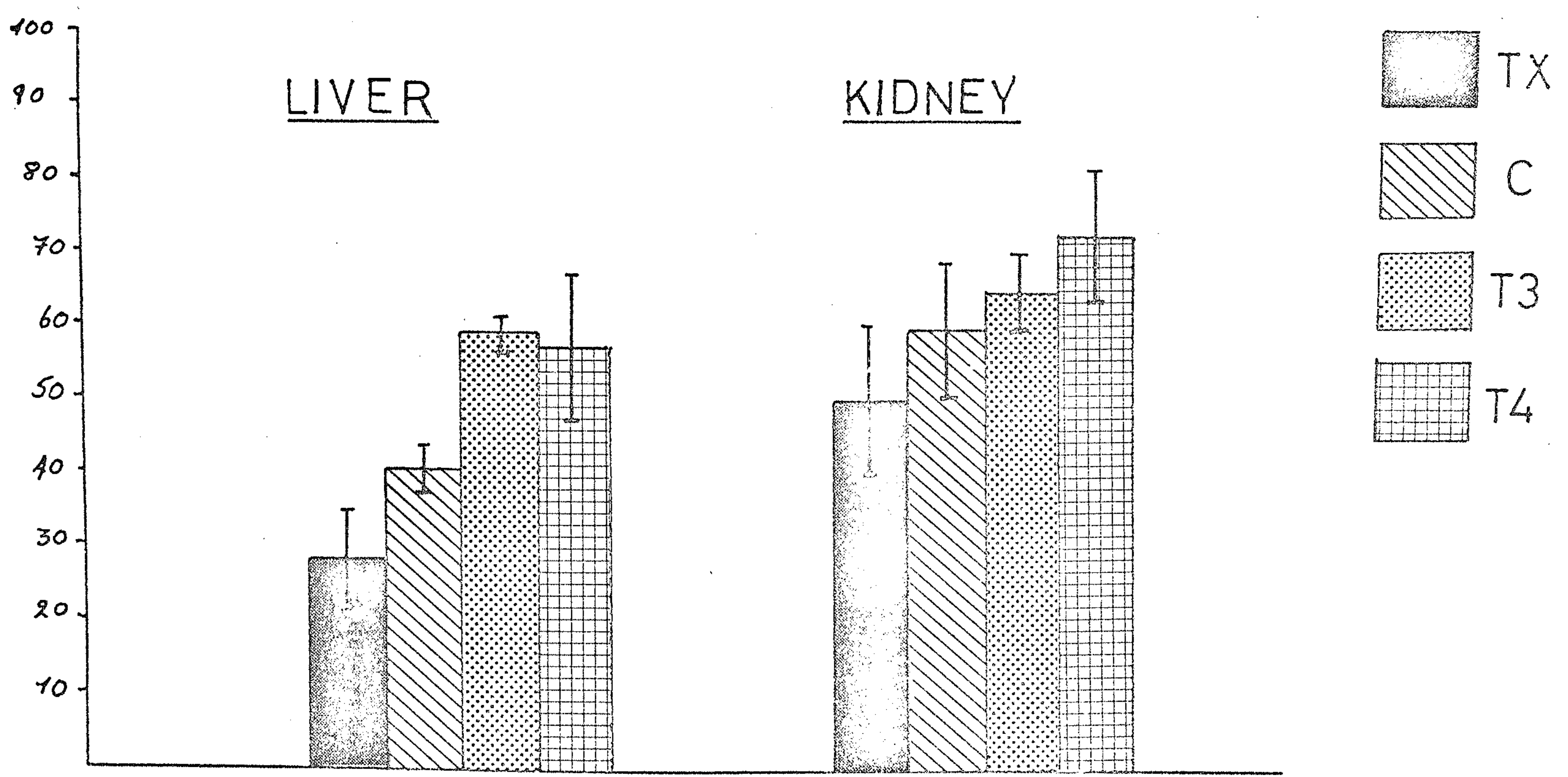
	TX	N	T3	T4
TX				
N	$P < 0.1$			
T3	$P < 0.02$	$P < 0.01$		
T4	$P < 0.02$	$P < 0.01$	$P < 0.02$	

Kidney

	TX	N	T3	T4
TX				
N	$P < 0.1$			
T3	$P < 0.1$	$P < 0.001$		
T4	$P < 0.02$	$P < 0.05$	$< P\ 0.01$	

Figure 16.

% NUCLEOLI IN CONTACT WITH THE NUCLEAR ENVELOPE.



Variations in nucleolar morphology with thyroid hormone treatment.

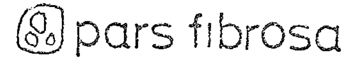
The initial observation of material from the different experimental groups suggested a possible variation in the morphology of the nucleolus, in particular the amount of pars granulosa material in association with the pars fibrosa. There appeared also to be a variation in the appearance of the pars fibrosa itself, from a relatively homogeneous aggregation of fine fibrils to a distinct nucleolonema.

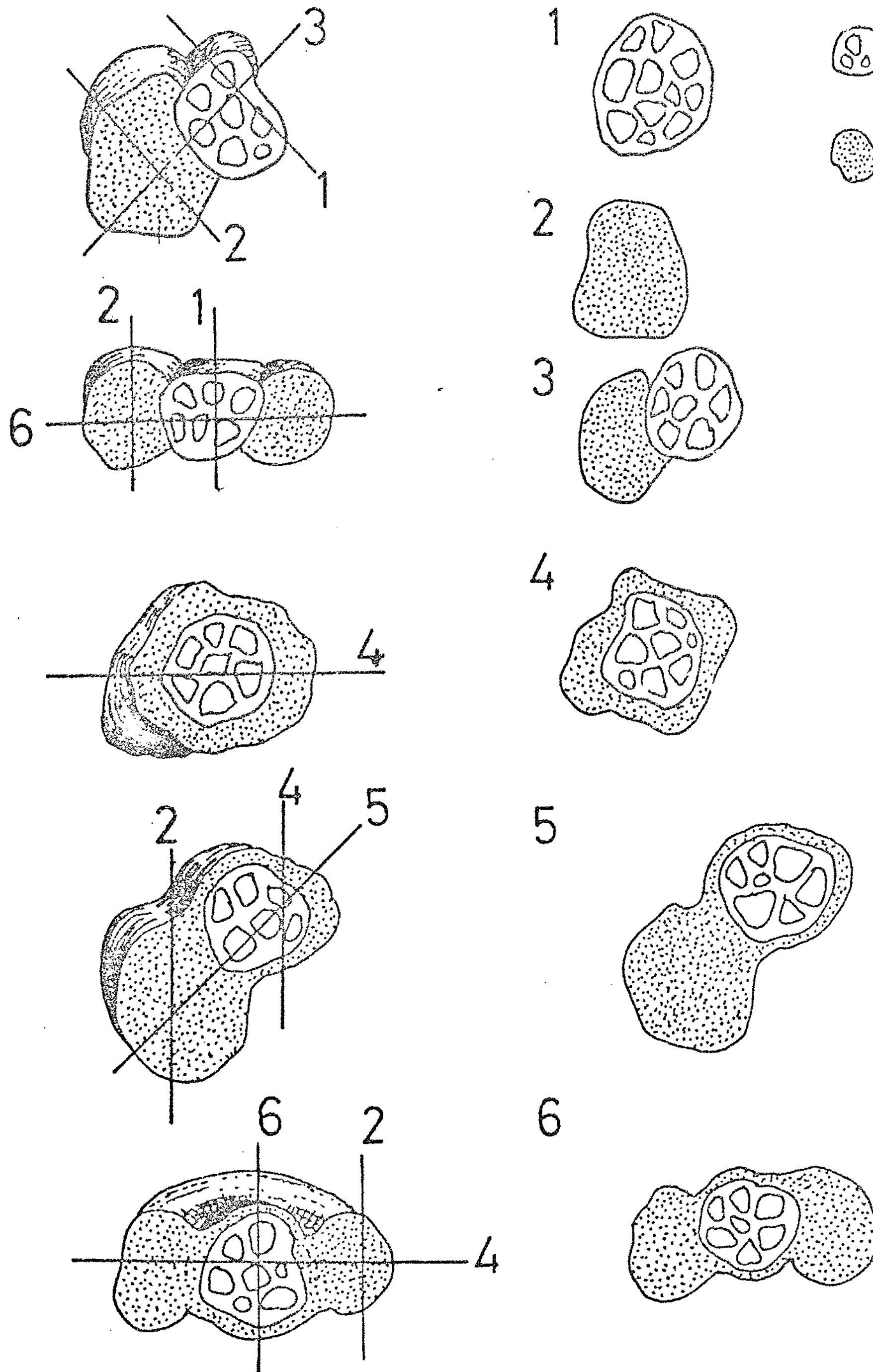
An endeavour was made to obtain quantitative estimation of the changes taking place. Nucleoli were classified into six basic types according to the appearance, and amount of pars granulosa material associated with the pars fibrosa, as is shown in Plates 28 - 33 Plate 28 shows a nucleolus typical of the appearance of the type called Category I. A well developed pars fibrosa is developed as a course reticulum or nucleolonema. There is little associated pars granulosa material present, with the exception of a few diffused peripheral granules, here termed 'halo particles'. Plate 29 shows the type of nucleolus included in Category II, where the nucleolus shows as a distinct mass of pars granulosa material not associated with a recogniseable pars fibrosa. It is questionable is some cases, whether such profiles are skimmed sections of nucleoli or are sections of chromatin aggregates. Plate 30 shows a nucleolus consisting of a descrete mass of pars granulosa associated with a descrete pars fibrosa, with little or no pars granulosa material extended around the pars fibrosa as 'halo particles'. This type of nucleolus was called Category III. Plate 31 shows a nucleolus typical of Category IV where the pars fibrosa is surrounded by pars granulosa, but where the pars granulosa does not show any aggregation into a desčrete mass. Plate 32 is typical of the type included in Category V. A distinct and separate body of pars granulosa is adjacent to and extended around a pars fibrosa. A nucleolus typical of Category VI is shown in Plate 33 . It is extremely difficult

Figure 17.

INFLUENCE OF SECTION PLANES UPON THE APPEARANCE OF NUCLEOLI IN THIN SECTION.

KEY





to distinguish between pars granulosa and pars fibrosa, although the two components are clearly present in the nucleolus.

To some extent the appearance of nucleoli will vary according to the plane of the section cut through it. As is shown on the preceeding pages in Figure 18 , there would appear to be four basic types of nucleolar form which may or may not be distinguishable from each other in the electron microscope. The four types being:-

1. A separate pars granulosa to one side of a pars fibrosa.

2. Two or more distinct bodies of pars granulosa associated with a pars fibrosa.

3. A pars granulosa with a pars fibrosa embedded in one side.

4. A pars granulosa extended around a pars fibrosa as a collar.

The category to which a nucleolar profile was allocated in this classification system based upon the profile appearance depended to some extent upon the plane of the section through it. This was borne in mind in the consideration of the data obtained from the use of this system.

Nucleolar profiles from four different preparation proceedures were examined. The numbers of nucleoli falling into each category, from each experimental group and preparation proceedure were counted, the results are shown in Tables 27 - 30 on the following pages.

The appearance of nucleoli from normal animals fixed in glutaraldehyde and osmium.

Almost all nucleoli consisted of two more or less distinct regions, a pars fibrosa, and a pars granulosa, as described by Hay (1968) and Marinozzi (1964) respectively.

The pars fibrosa usually occurrs as a dense more or less homogeneous non-descript mass, embedded in or adjacent to the pars granulosa. It is often possible to discern an underlying structure of

Classification of nucleoli between the type categories.

Table 27 .

Glutaraldehyde and osmium fixed material.

		1	2	3	4	5	6
Liver	TX	0	28	7	24	7	1
	N	1	15	1	24	14	2
	T3	0	18	1	14	6	0
	T4	1	8	1	13	7	0
Kidney	TX	0	22	2	16	5	3
	N	0	24	1	21	6	3
	T3	0	11	0	12	6	2
	T4	1	18	1	14	4	2

Table 28 .

Glutaraldehyde and osmium fixed material subjected to cold buffer storage.

		1	2	3	4	5	6
Liver	TX	9	19	4	4	0	0
	N	16	13	11	4	2	3
	T3	1	15	5	3	4	0
	T4	4	31	22	6	19	2
Kidney	TX	0	17	1	1	0	0
	N	2	13	6	1	1	0
	T3	0	4	7	0	2	1
	T4	0	3	7	0	3	2

Table 29 .

Acrolein fixed material stained with silver nitrate.

	1	2	3	4	5	6
Liver TX	1	16	15	14	11	0
N	5	15	7	19	8	1
T3	0	3	1	16	9	3
T4	0	21	2	20	20	0
Kidney TX	0	13	2	7	3	0
N	0	17	3	18	6	0
T3	0	12	5	21	9	0
T4	1	18	0	7	6	1

Table 30 .

Acrolein fixed material subjected to cold buffer storage and stained with silver nitrate.

	1	2	3	4	5	6
Liver TX	26	13	36	7	9	0
N	18	15	16	13	5	4
T3	11	10	8	14	14	4
T4	4	18	10	24	5	4
Kidney TX	0	2	0	0	0	0
N	6	6	1	1	4	0
T3	3	2	0	0	1	0
T4	2	3	1	3	1	0

closely woven, fine fibrils, with a matrix of some material of similar electron density. The fibrillar structure is usually organised into a nucleolonema, as is shown in Plate 28 , but this was not as well defined as some described elsewhere (Estable and Sotelo 1951, 1955.) . Some of the pars fibrosa observed were not so organised, and the appearance of these was similar to that shown in Plate 30 . Structures, which are best described as vacuoles usually occurred within the pars fibrosa, these are paler areas, usually filled with an electron transparent material within which can be discerned fine fibrils, as shown by the arrows (a) in Plate 34 . Occasionally these vacuoles are less well defined and may be almost completely filled with a material of almost the same electron density as the fibrosa itself, as is seen in Plate 33

The pars fibrosa occasionally appears to merge with the granular material itself as shown in Plate 33 , usually the two regions are separated by a narrow zone of low electron density as shown in Plates 30 and 31 .

The pars granulosa usually appears as a mass of more or less closely packed electron dense granules of similar size to cytoplasmic ribosomes. Where sections pass through the centre of a mass of granulosa the central zone is often so closely packed as to appear homogeneous, as is shown in Plate 30 . Peripheral areas are more loosely arranged and separate granules are readily discernible. Traces of an underlying fine fibrillar network can usually be traced within this region of more loosely arranged granules as is indicated by the arrows (b) in Plate 34 . In some nucleoli this fibrillar network appears to be continuous with the fibrils of the pars fibrosa on the one hand, and the chromatin on the other. Such fibrils resemble those of the fibrosa vacuoles and nucleoplasm as regards size and appearance. The loosely arranged peripheral granules usually extend around the pars fibrosa to form what have been described here as 'halo particles'.

Nucleoli from one nucleus, where more than one occurred,

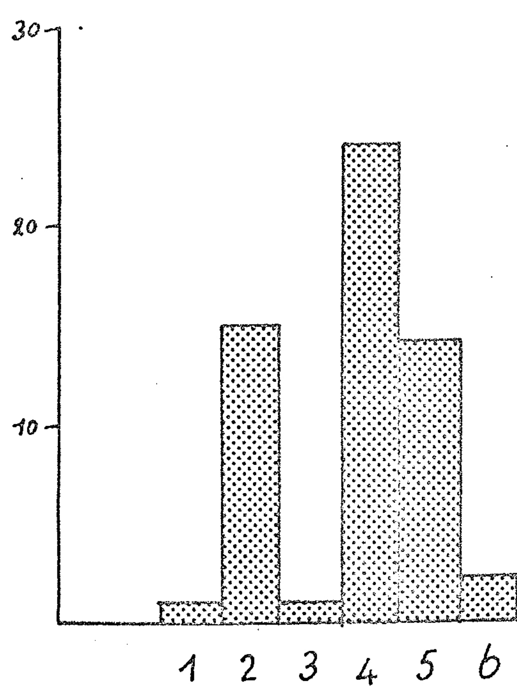
Figure 18.

NUCLEOLAR CLASSES

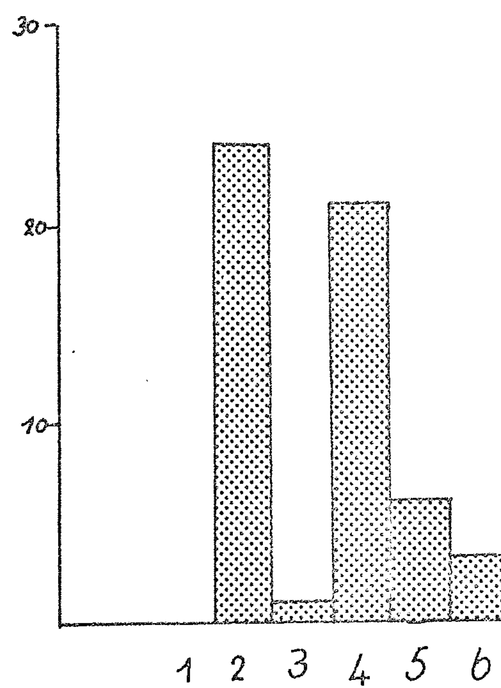
(Glutaraldehyde & Osmium fixation)

Control material

LIVER



KIDNEY



usually conformed to the same pattern, there being no apparent morphological differences, other than those attributable to different planes of section through the nucleolar body. Nucleoli from either the liver or the kidney also showed no apparent morphological differences.

The distribution of nucleoli between the categories in the classification system used is shown in Figure 18 on the preceeding page.

The effect of fixation proceedure upon the standard pattern of nucleolar morphology.

There were slight variations in the appearance of nucleoli after acrolein fixation followed by silver nitrate staining as compared to glutaraldehyde and osmium fixation followed by uranium and lead staining. Plates 35 to 40 shew the appearance of nucleoli corresponding to the six type categories after acrolein fixation and silver nitrate staining.

There was a greater distinction between the two components of the nucleolus after acrolein fixation compared to that seen after glutaraldehyde and osmium. The pars granulosa usually resembled the chromatin in electron density and the density of overlying silver deposits. Occasionally it was possible to distinguish between the nucleolar associated chromatin and the pars granulosa, as a result of differences in electron density, the pars granulosa material being less dense than the associated chromatin as is shown in Plate 37 .

The pars fibrosa was of considerably greater electron density than the pars granulosa in almost all of the material examined after the acrolein fixation proceedure, frequently having a higher electron density than any other tissue compenent. The pars fibrosa was almost always organised into a more or less distinct nucleolonema although there was considerable variation in the number of 'vacuoles' cut in a section, the extremes being shown by Plate 37 with only two

distinguishable vacuoles and Plate 35 where nearly twenty vacuoles are discernible. The vacuoles frequently varied considerably in size as is shown in Plate 40 where four very large vacuoles occur together with a number of small ones. Whether the nucleolonema always follows the same pattern, the varying appearance of the profiles being a result of sectioning through at different planes, or whether the development of a particular pattern varies from nucleolus to nucleolus or as a result of external influences, it is impossible to say, but nucleoli have been observed from different nuclei which show some degree of resemblance as regards shape and arrangement of vacuoles.

There were also variations in the appearance of the vacuoles. As was noted after glutaraldehyde and osmium fixation, some vacuoles were clear and distinct, such as those seen in Plates 38 , 39 and 40 , whereas others were filled with more electron dense material and appeared more as paler areas within the pars fibrosa than as distinct vacuoles, such as those shown in Plates 35 and 38 . It is possible that such vacuoles represent skimmed sections where the vacuole is visualised through a thin overlying layer of pars fibrosa material, as is shown in Figure 19

However, although this may not account for the appearance of some of the dark vacuoles it does not appear to account for all since in some cases, as is shown in Plates 41 and 42 , vacuoles were filled with material resembling pars granulosa material.

There was very little difference between the proportions of pars granulosa and pars fibrosa present in the nucleoli fixed in acrolein compared with those fixed in glutaraldehyde and osmium, and the results, when compared using a X^2 test were not significantly different as is shown below.

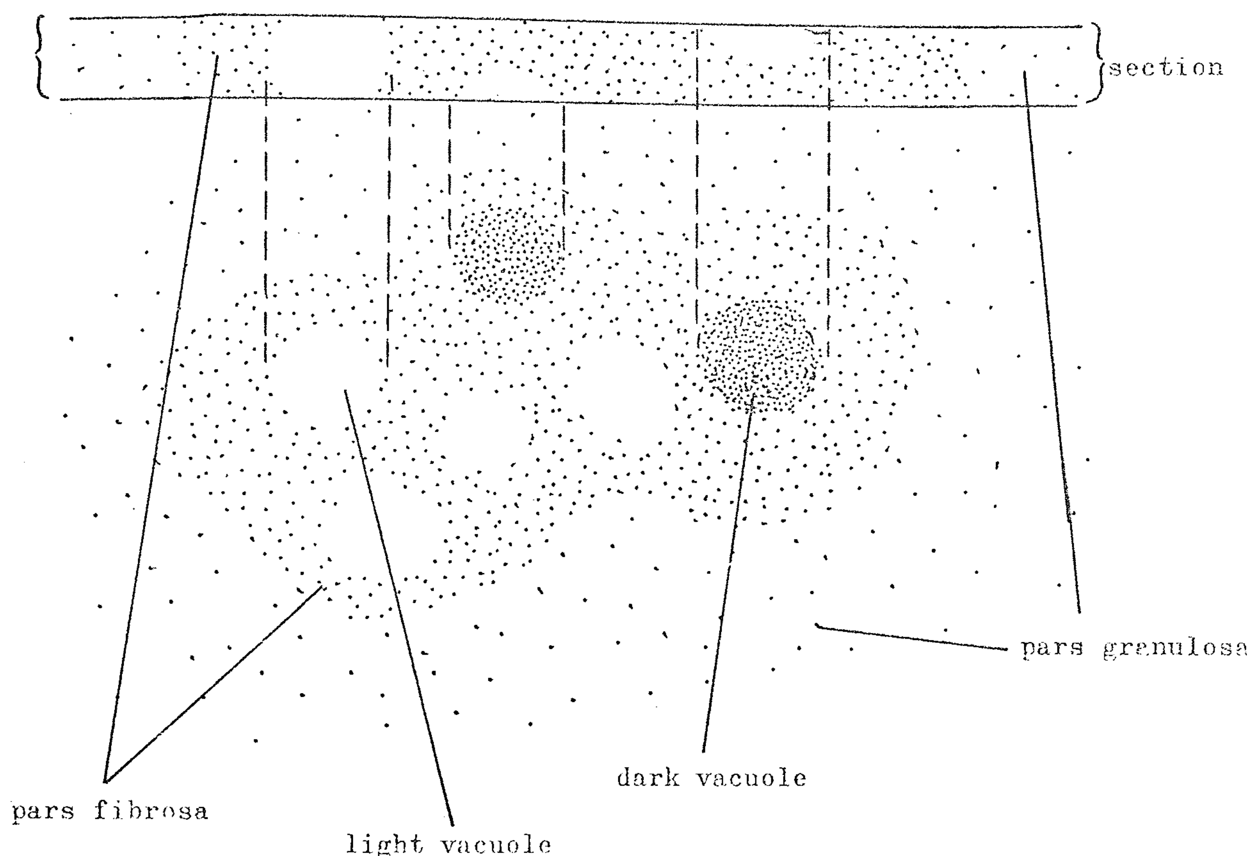
Glutaraldehyde and osmium x acrolein fixation

Normal x Normal

Liver $P < 0.1$ x Kidney $P < 0.8$

Figure 19 .

Possible explanation of the appearance of dark and light vacuoles in nucleolar profiles.



That this was so was not altogether suprising since both sets of material were prepared using primary aldehyde fixation.

Variations in results obtained after acrolein fixation and silver nitrate staining were due, in part to the variation in the appearance of material prepared in this way compared to the normally prepared material, as is shown by a comparison of Plates 35 to 40 with Plates 28 to 33 .

In general acrolein fixation resulted in a reduction in the amount of pars granulosa material preserved or demonstrated after silver nitrate staining of acrolein fixed material. In particular there was a reduction in the amount of 'halo particles' associated with the nucleoli. Instances where the pars fibrosa alone was cut in a section of a nucleolus (ie. Category I.) were also more frequent after acrolein

fixation than after glutaraldehyde and osmium.

The general if slight reduction in the amount of granulosa present after acrolein fixation may be due to one of several factors:-

1. Either acrolein fixation differs slightly from primary glutaraldehyde fixation, having less of a stabilising effect on the particles, so that some of the more diffuse particles have been removed from the section during subsequent dehydration and embedding.

2. Secondary osmium fixation helps stabilise the particles after aldehyde fixation so that better retention is obtained after osmium fixation.

3. Secondary osmium fixation results in better contrast of the particles, so that less electron dense ones are visualised.

4. The nature of the section staining used after acrolein fixation was such that the 'halo particles' were not sufficiently dense to produce an identifiable precipitation of silver over the section.

The phenomenon is probably to be explained by a combination of these factors, such as better retention of material by glutaraldehyde fixation and the granular nature of the section stain used after acrolein fixation. There may of course, be a histochemical reason for the slight difference in behaviour with the two twchniques. Glutaraldehyde and osmium fixation, as used here with uranium and lead section staining is a routine proceedure, which enhances the contrast of most cellular components but is not a histochemical technique, partly as a result of the number of contrasting agents used in the process. Acrolein fixation followed by silver nitrate section staining, however is a semi-histochemical proceedure, since it preferentially demonstrates nuclear proteins. The differences noted above may be a result of this. The 'halo particles' although resembling closely, granulosa deposits may have a low nucleoprotein content compared to the rest of the granulosa, or their nucleoprotein components may be masked by some other component,

so that they are obvious after routine preparation procedures but are not demonstrated by the acrolein and silver nitrate technique.

It is difficult to see the functional significance of the 'halo particles' if the above is true. and an investigation of their composition using enzyme digestion techniques would be necessary to confirm their composition.

The differences in the distribution of the nucleoli between the type categories after the different fixation procedures are shown in Figure 20 on the following page. There was little difference between the liver and the kidney in terms of the effect of fixative and subsequent staining on nucleolar morphology.

The effect of storage in cold buffer upon the standard pattern of nucleolar morphology.

Storage of material in cold buffer had a marked effect upon nucleolar morphology, after both glutaraldehyde and acrolein fixation as is shown by a comparison of Figure 21 . Statistical comparison of the results of glutaraldehyde fixed material with and without the period of storage in cold buffer and acrolein fixed material with and without the storage in cold buffer showed that there was a significant difference in the distribution of nucleoli between the different categories, as is shown below

Glutaraldehyde fixation x Glutaraldehyde fixation followed by storage in cold buffer.

Normal x Normal animals.

Liver $P < 0.001$ Kidney $P < 0.001$

Acrolein fixation x Acrolein fixed material stored in cold buffer

Normal x Normal animals

Liver $P < 0,05$ Kidney $P < 0.001$

There was a difference between the distribution of nucleoli between the

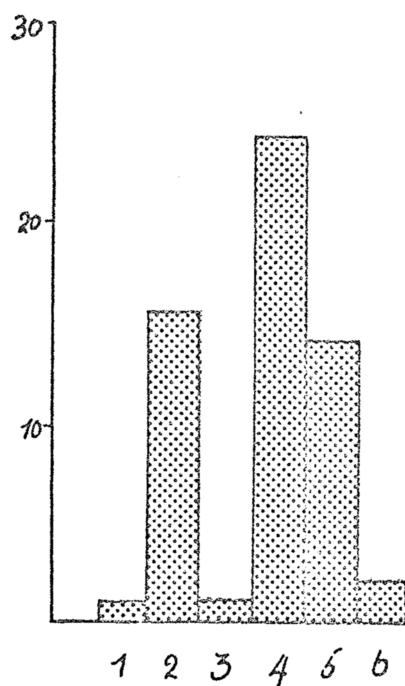
Figure 20.

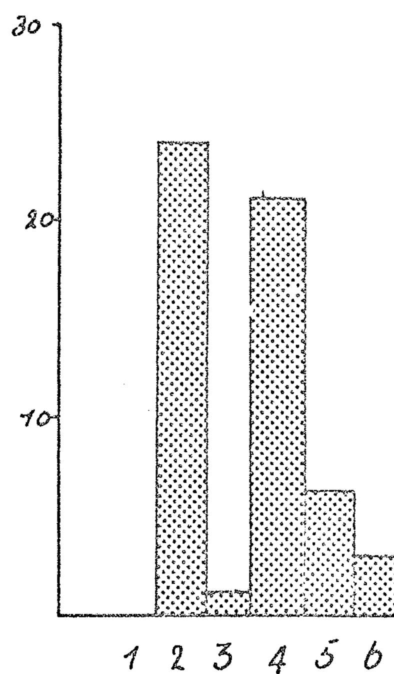
NUCLEOLAR CLASSES

Control material.

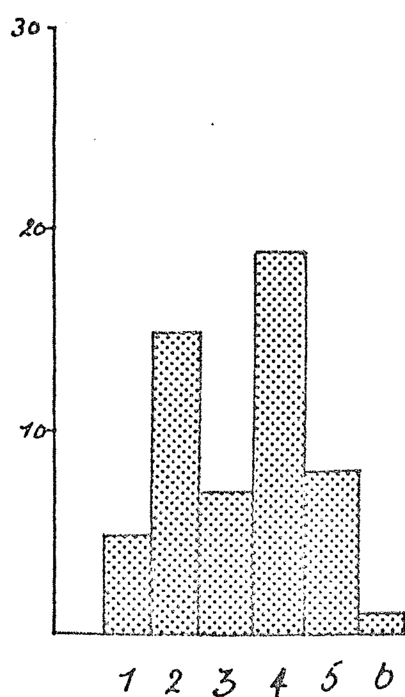
LIVER KIDNEY

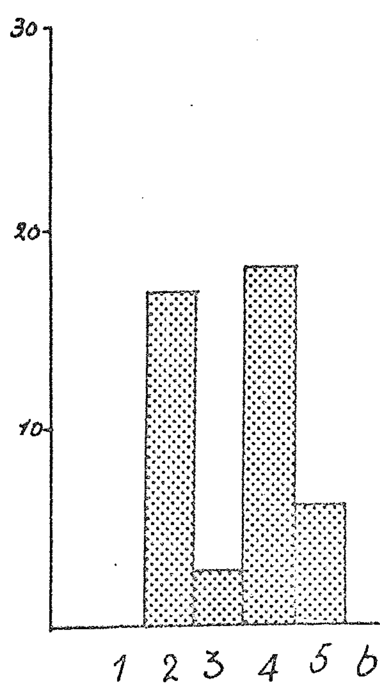
Glutaraldehyde & Osmium fixation.





Acrolein fixation.





categories after cold storage, with the different fixation proccedures.

Glutaraldehyde fixed material stored in cold buffer.	x	Acrolein fixed material stored in cold buffer.
Normal	x	Normal
Liver $P < 0.8$		Kidney $P < 0.2$.

This was surprising since although both acrolein and glutaraldehyde are aldehyde fixatives, glutaraldehyde is usually regarded as superior as far as tissue preservation and retention of constituents is concerned. There was little or no difference between the two tissues used.

There was a massive reduction in the amount of granulosa material particularly of the more diffused peripheral or 'halo' particles were usually absent. The general appearance ofnucleoli after cold buffer extraction, apart from this, resembled that of normally prepared material as is shown by a comparison of Plates 43, 44, 45, 46 and 47 . The number of nucleolar sections falling into Category I after storage in cold buffer as compared to normal fixation indicates that there must have been a vast leaching out of granulosa deposits during the cold storage period. There are two possible explanations for this, either the particles have been removed from the blocks during the storage period or their contrast and / or reactivity with subsequent contrasting agents was leached out or reduced. It would seem difficult for such large particles to be completely removed from the tissue, since cellular membranes appear to have remained intact. It would seem to be mote likely that some factor contributing either to the basic contrast of the particles or to their reaction with contrasting agents was removed. Examination of enzymatically digested blocks would be necessary before this could be elucidated.

Figure 21.

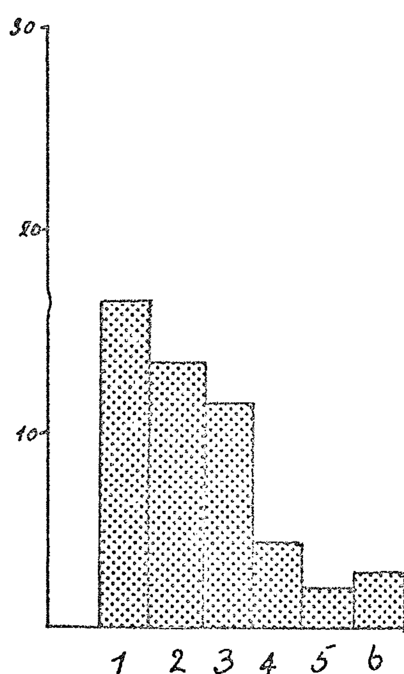
NUCLEOLAR CLASSES

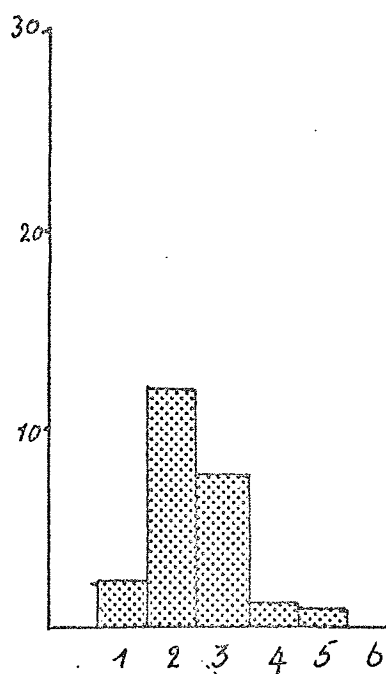
Control material.

Prolonged Cold Buffer Treatment.

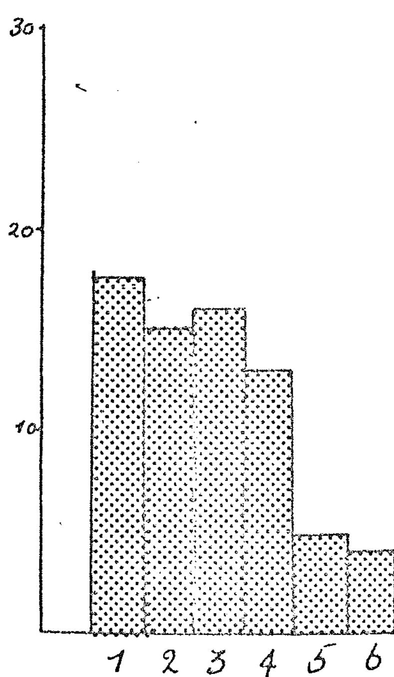
LIVER. KIDNEY

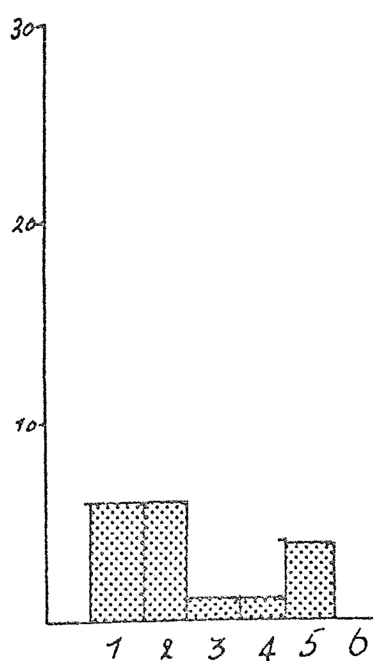
Glutaraldehyde & Osmium fixation.





Acrolein fixation.





The effects of thyroid hormones upon the distribution of nucleoli between the type categories.

1. Glutaraldehyde and osmium fixation.

Variation in the hormone status of the animals appeared to have little or no effect upon the nucleolar morphology after routine glutaraldehyde and osmium fixation, and there was no change in the distribution of nucleoli between the six type categories with the hormone treatment.

Table 31 .

Statistical significance of variations in distribution of nucleoli after hormone treatment, and glutaraldehyde and osmium fixation (X^2 test).

Liver

	TX	N	T3	T4
TX				
N	$P < 0.05$			
T3	$P < 0.8$	$P < 0.8$		
T4	$P < 0.2$	$P < 0.95$	$P < 0.9$	

Kidney

	TX	N	T3	T4
TX				
N	$P < 0.99$			
T3	$P < 0.90$	$P < 0.98$		
T4	$P < 0.95$	$P > 0.99$	$P < 0.90$	

Visual assessment revealed a slight increase in the amount of material present as 'halo particles' surrounding the nucleoli with the hormone treatment; there were no other apparent changes. It is possible, however, that changes occurred in the rate of turnover of nucleolar material in the hormone treated animals such that it keeps pace with the increased synthesis and no net accumulation of material in the pars granulosa took place. Thus hormone influences on nuclear or nucleolar activity nuclear or nucleolar activity would be reflected in increased activity of nucleo/cytoplasmic of other transport pathways, rather than in accumulation of material in the nucleolus.

It is possible that hormone induced changes occurred in

Figure 22.

NUCLEOLAR CLASSES

Glutaraldehyde & Osmium fixation

LIVER

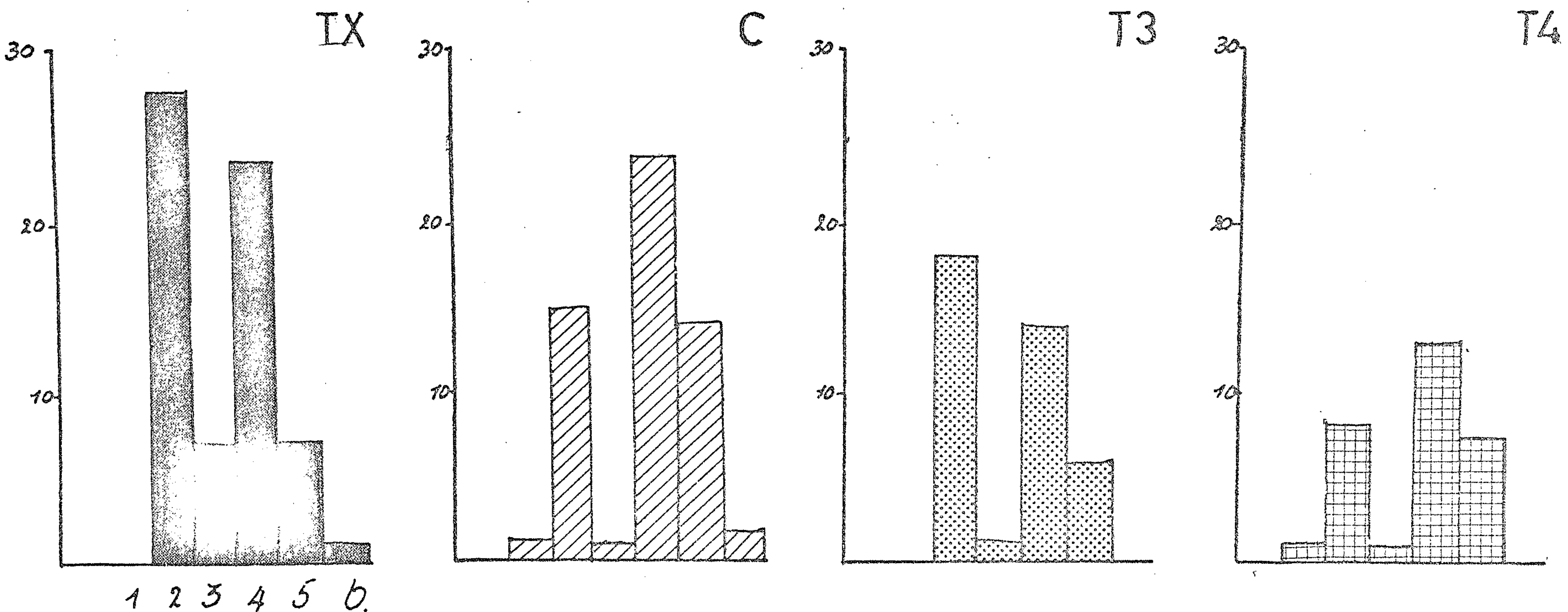
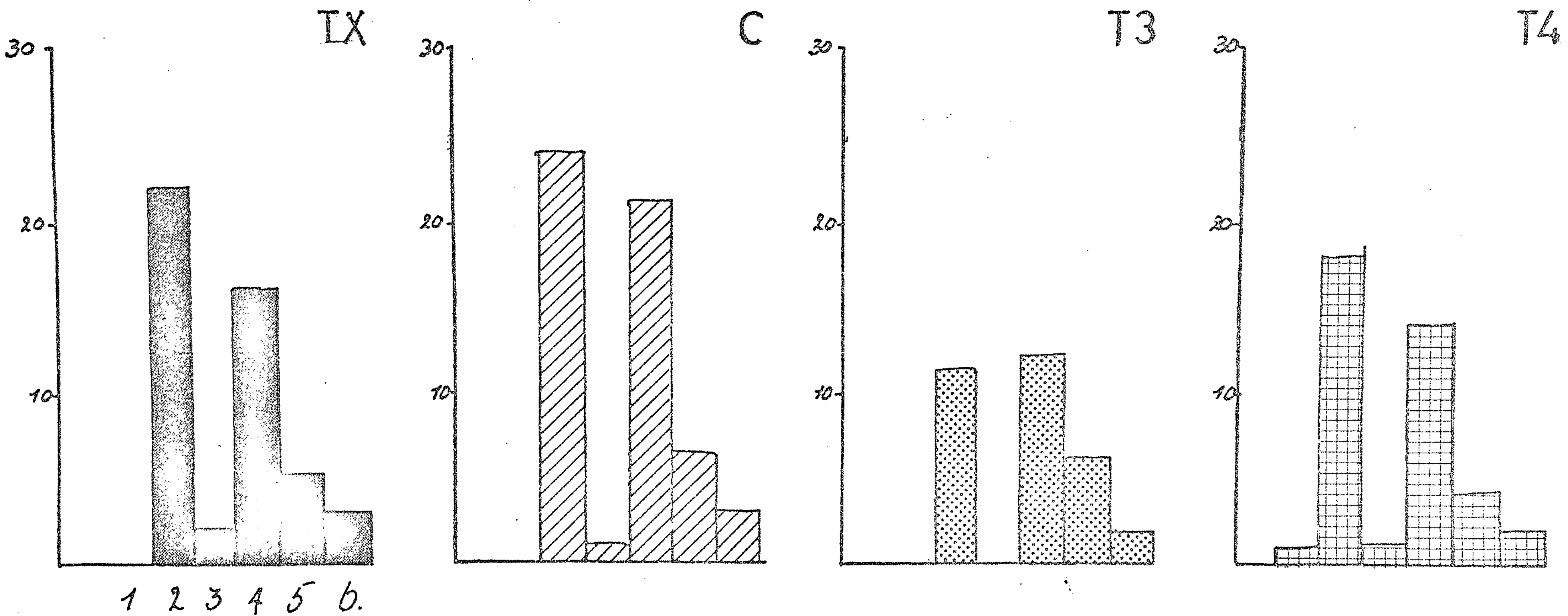


Figure 23.



the composition or staining capacities of constituents of the pars granulosa which were masked by the general reaction to osmium, lead and uranium used in the preparation procedure.

The distribution of the nucleoli between the type categories after hormone treatments are shown in Figures 22 and 23 .

2. Glutaraldehyde and osmium fixation , with storage in cold buffer.

Prolonged cold buffer extraction brought about a marked change in the distribution of nucleoli between the six type categories with the hormone treatments. There was an increase in the number of nucleoli falling into the Categories I and III. and a reduction in the proportion falling into Categories IV and V with the hormone treatments. However, this was only statistically significant when the data from the thyroidectomised animals was compared with that from the hormone treated animals in the liver. The lack of significance in the kidney was possibly due to the low numbers of nucleoli in some of the groups.

Table 32 .

Statistical significance of variations in distributions of nucleoli after hormone treatment, with glutaraldehyde and osmium fixation and storage in cold buffer.

Liver	TX	N	T3	T4
TX				
N	$P < 0.5$			
T3	$P < 0.2$	$P < 0.2$		
T4	$P < 0.001$	$P < 0.001$	$P < 0.8$	

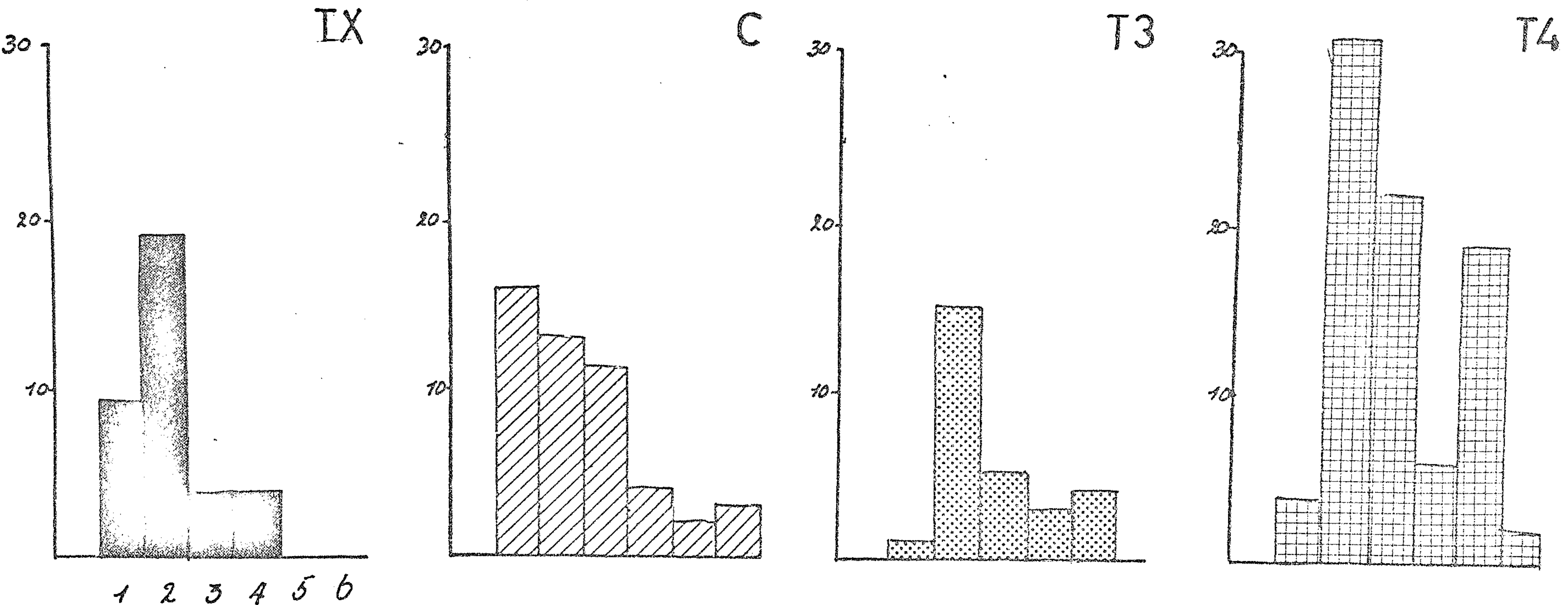
Kidney	TX	N	T3	T4
TX				
N	$P < 0.5$			
T3	$P < 0.5$	$P < 0.5$		
T4	$P < 0.5$	$P < 0.1$	$P > 0.99$	

It is interesting to note that the reduction in the amount of granulosa material after cold buffer storage, shown by observations and reflected

Figure 24.

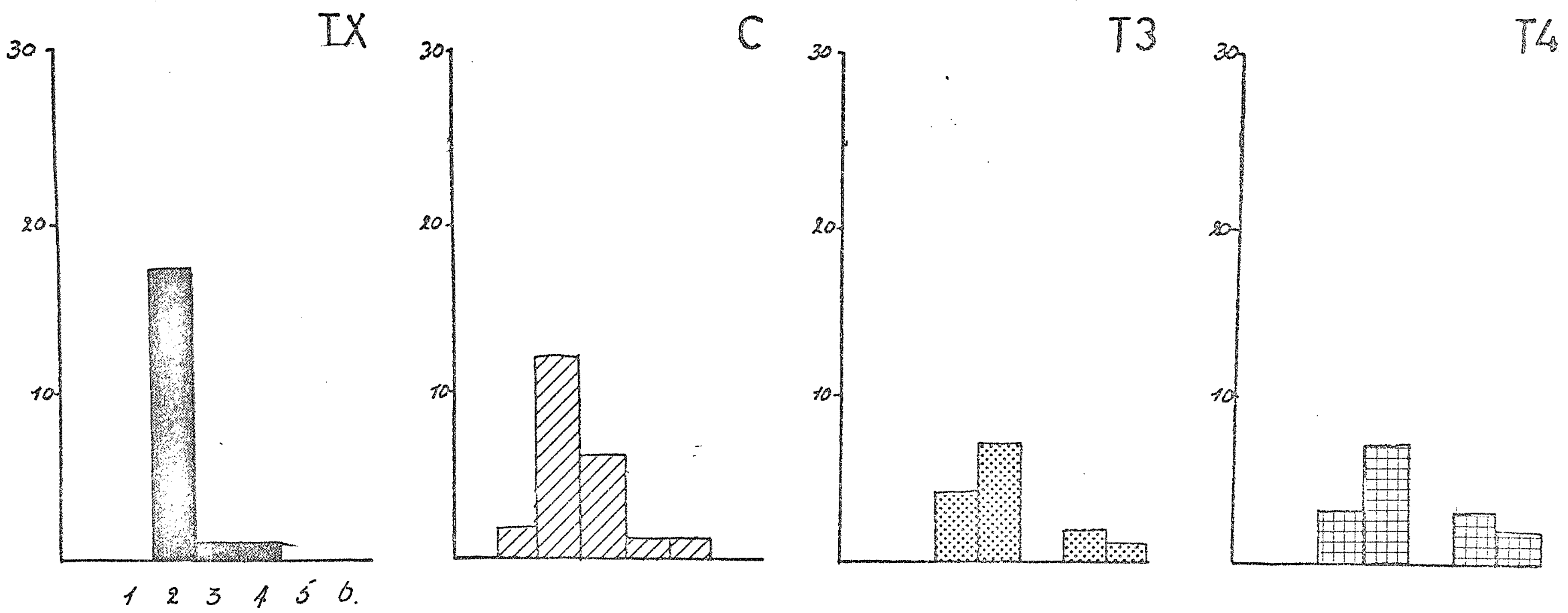
NUCLEOLAR CLASSES

Prolonged Cold-buffer Treatment.
Glutaraldehyde & Osmium fixation.
LIVER.



LIBRARY

Figure 25.



in the reduction of nucleoli falling into Categories IV and V was less marked in the hormone treated animals than in the normal or thyroidectomised animals, as is shown in Figures 24 and 25 on the preceeding pages. It is possible that particles produced under the influence of the hormone were more resistant to the leaching effects of the cold buffer than those present in the normal or the thyroidectomised animals.

3. Acrolein fixation and silver nitrate staining.

There was a difference in the effects of hormone treatment upon the argentophilic components of the nucleolus in the liver and the kidney. In the kidney there was little or no observable difference between nucleoli from the different experimental groups, and there was no dignificant difference in the distribution of nucleoli between the different categories with the hormone treatments. In the liver, however,

Table 33 .

Statistical significance of variations in distribution of nucleoli after hormone treatment, with acrolein fixation and silver nitrate staining.

Liver

	TX	N	T3	T4
TX				
N	$P < 0.2$			
T3	$P < 0.01$	$P < 0.001$		
T4	$P < 0.02$	$P < 0.02$	$P < 0.5$	

Kidney

	TX	N	T3	T4
TX				
N	$P > 0.99$			
T3	$P > 0.99$	$P < 0.01$		
T4	$P < 0.5$	$P < 0.1$	$P < 0.02$	

there was a marked increase in the amount of argentophilic material present in the pars granulosa in the hormone treated animals compared to the normal or thyroidectomised animals. The distribution of nucleoli between the different type categories was significantly different in the

Figure 26.

NUCLEOLAR CLASSES

Acrolein fixation

LIVER.

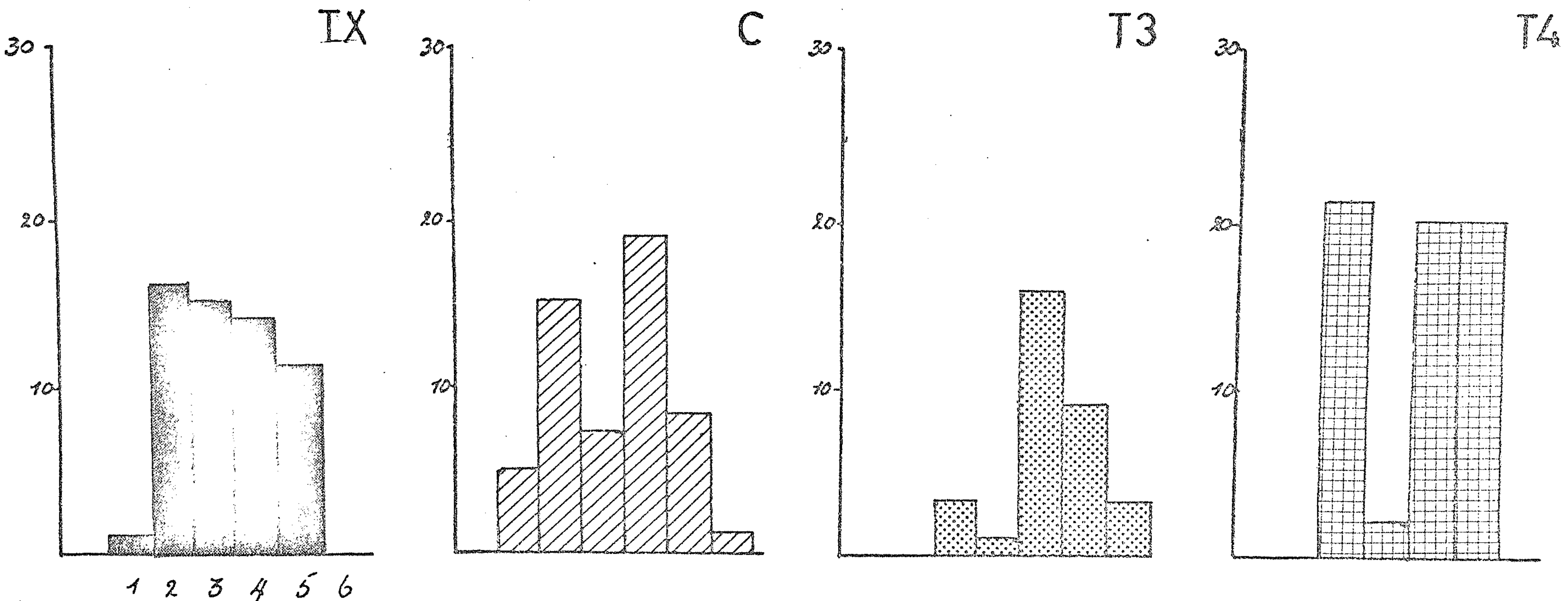
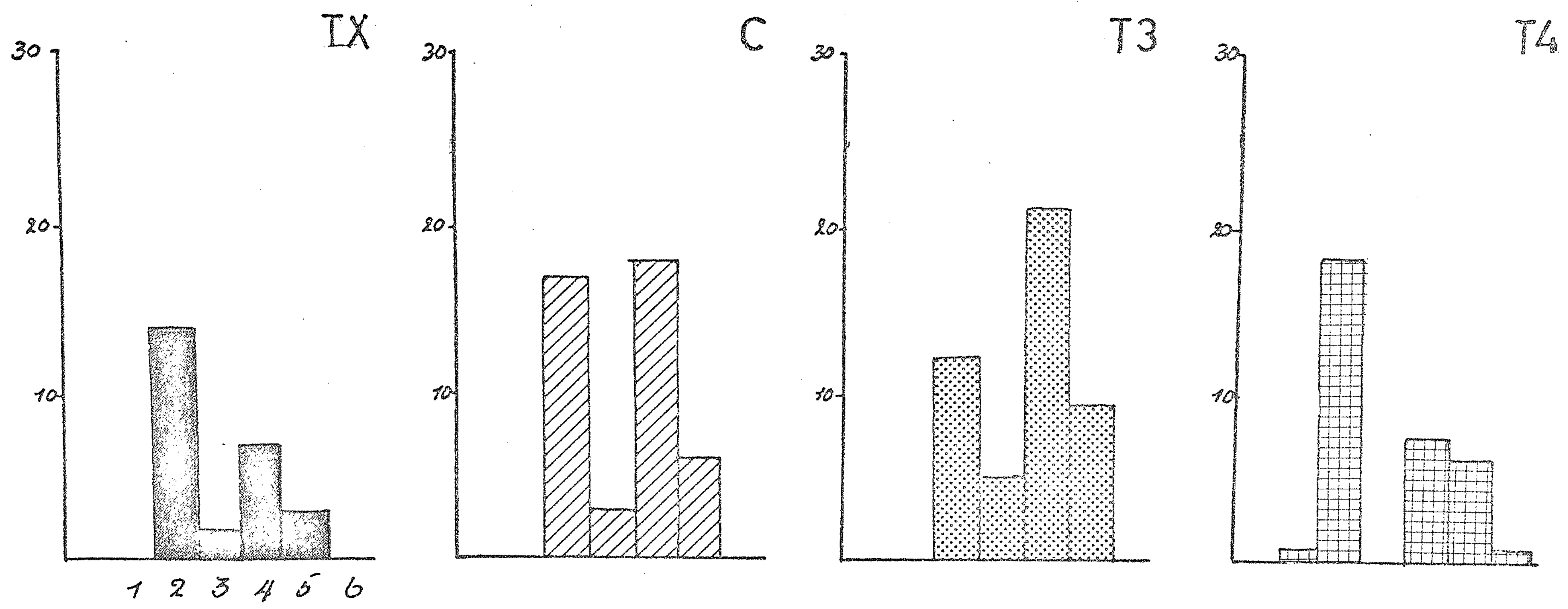


Figure 27.



hormone treated compared to the non-hormone treated groups as is shown in Table 33 above. Which suggests that particles produced under the influence of the hormone action are more argentophilic than those occurring in the normal or thyroidectomised animals, and that the hormone has a greater effect upon the nucleolus of the liver than that of the kidney. The distribution of nucleoli between the different type categories after hormone treatment and acrolein and silver nitrate staining are shown in Figures 26 and 27 on the preceeding pages.

4. Acrolein fixation with silver nitrate staining and cold buffer extraction.

The appearance of nucleoli after cold buffer extraction resembled those seen after normal acrolein fixation, but with an overall reduction in the amount of pars granulosa material present. Again there were differences in reaction of liver and kidney nucleoli to the effects of hormone treatment. Again there was no difference in the distribution of nucleoli between the type categories with the hormone treatments in the kidney, whereas there were marked differences in the liver, which were highly significant. There were marked increases in

Table 34.

Statistical significance of variations in distribution of nucleoli after hormone treatment, with acrolein fixation and cold buffer storage.

Liver	TX	N	T3	T4
TX				
N	$P < 0.01$			
T3	$P < 0.001$	$P < 0.2$		
T4	$P < 0.001$	$P < 0.02$	$P < 0.05$	

Kidney	TX	N	T3	T4
TX				
N	$P < 0.8$			
T3	$P < 0.8$	$P > 0.95$		
T4	$P < 0.8$	$P < 0.8$	$P > 0.01$	

Figure 28.

NUCLEOLAR CLASSES

Proloned Cold-buffer Treatment.

Acrolein fixation.

LIVER.

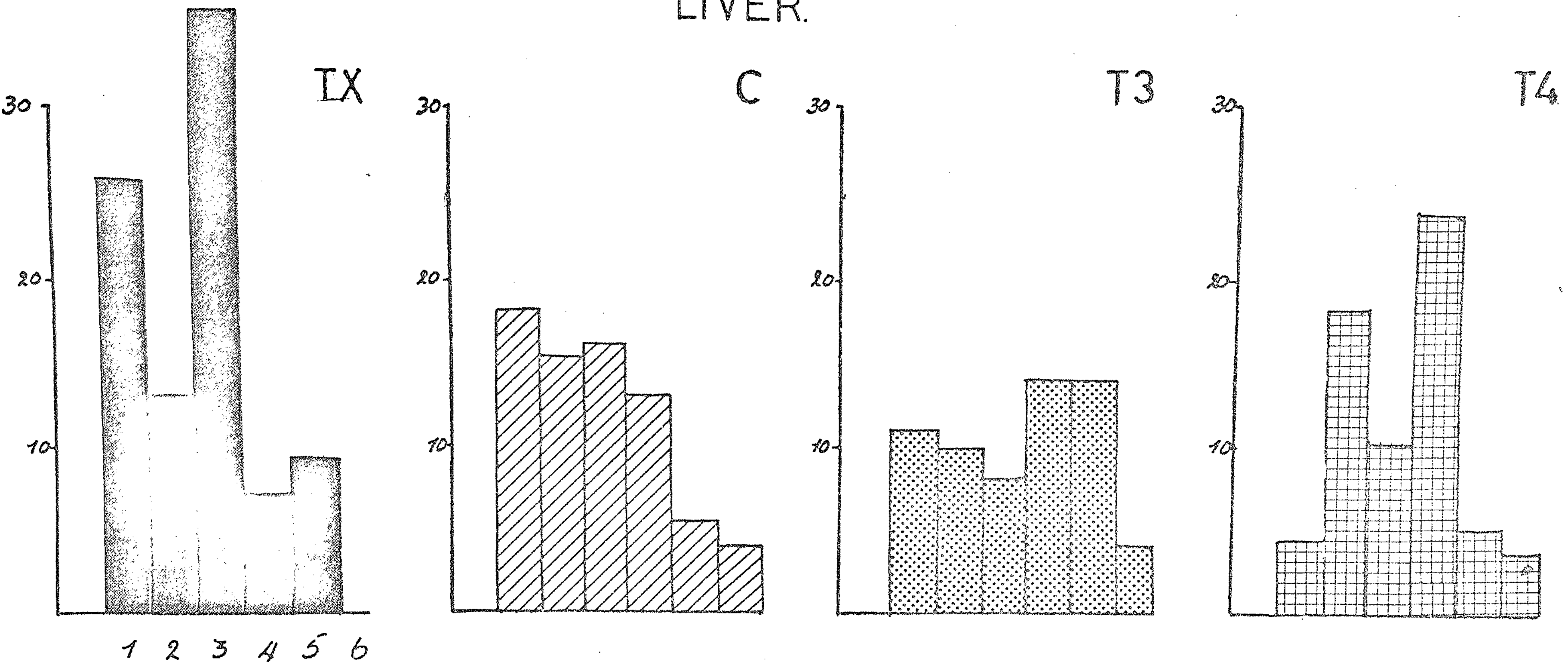


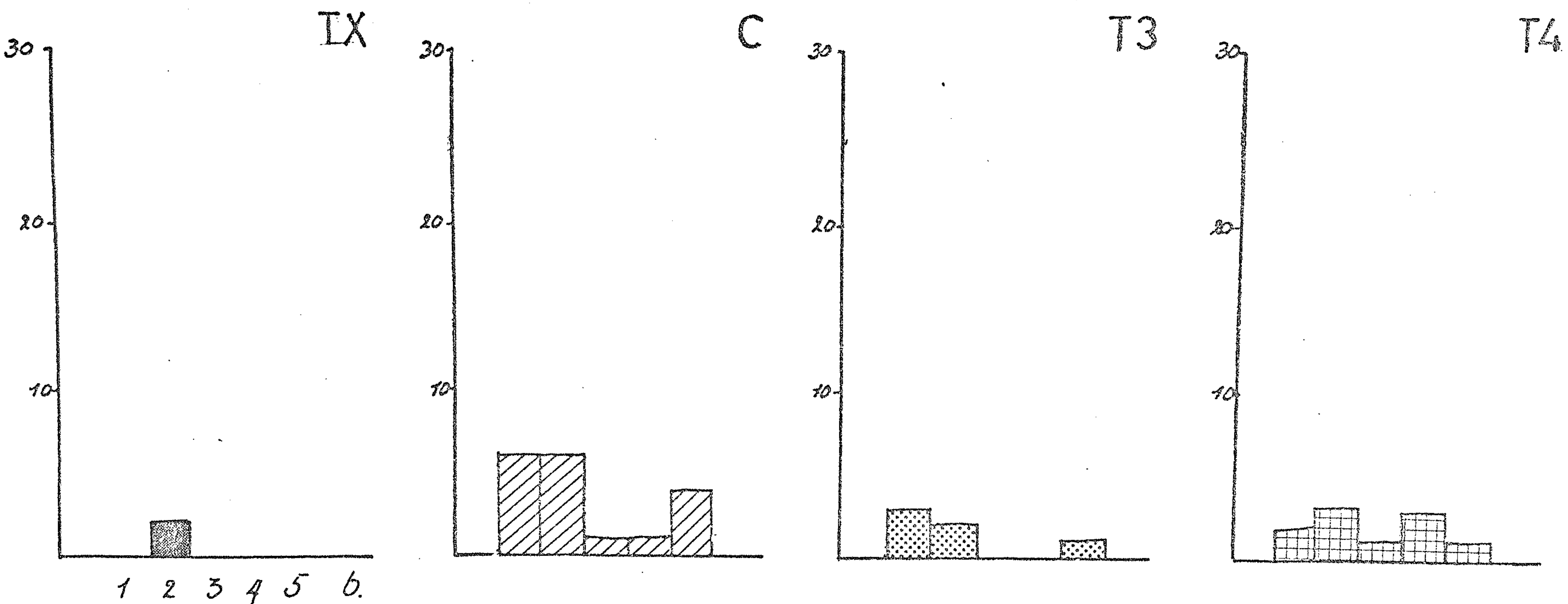
Figure 29.

NUCLEOLAR CLASSES.

Prolonged Cold-buffer Treatment.

Acrolein fixation.

KIDNEY.



the amount of argentophilic material which was resistant to cold buffer extraction in the nucleolus or hormone treated cells compared to those of normal or thyroidectomised animals, as is shown in Table 34 above. The distribution of nucleoli between the type categories after cold buffer extraction of acrolein fixed material is shown in Figures 28 and 29 on the preceeding pages.

The appearance of nucleoli after digestion with either ribonuclease or pepsin.

In order to clarify some of the hormone influenced changes which were visualised by variations in preparation proceedure material was examined after the inclusion of a stage of either ribonuclease or pepsin digestion in the preparation proceedure of routine glutaraldehyde and osmium fixation. It was hoped that some identification of the nucleolar components undergoing hormone influenced changes.

Ribonuclease digestion. There were significant variations in the appearance of nucleoli after ribonuclease digestion compared to those from normally prepared material. The division into two zones, pars fibrosa and pars granulosa, was more distinct due to a general reduction in the contrast of the pars fibrosa produced by the ribonuclease digestion.

The pars fibrosa was almost always present as a pale gray body associated with, but separated from pars granulosa material. Although some of the observed pars fibrosae were more or less homogeneous in appearance, most occurred as well developed nucleolonemae. The vacuoles within the nucleolonema were more distinct than after normal preparation, as is shown in Plate 48 , and dark vacuoles, although occasionally observed, were less frequent. Some of the vacuoles appeared to be filled with material resembling pars granulosa material, as is shown in Plate 49 (arrows). The greater clarity of the majority of vacuoles

after ribonuclease digestion would suggest that although some of the dark vacuoles do represent skimmed sections, the majority are 'dark' due to the presence of some ribonuclease digestible, electron dense matrix material.

The pars granulosa was less affected by ribonuclease digestion. In some cases there was a reduction in contrast so that the pars granulosa appeared paler than the chromatin but this effect was not marked and the pars granulosa was always distinct from the pars fibrosa by its greater electron density. There was a loss of underlying fibrillar network caused by the ribonuclease digestion, and the granular components appeared to be less densely aggregated, possibly as a result of the removal of electron dense material or as a result of the removal of part of the granular component.

There was little hormone affected variation in the ribonuclease digestible material within the pars fibrosa of the nucleolus, although there was a tendency towards more of the nucleoli from hormone treated liver and kidney to show vacuolated pars fibrosae after ribonuclease digestion than after normal preparation. Approximately 80% of nucleoli of liver nuclei, and 50% of nucleoli from kidney nuclei showed well developed nucleolonemal structures in material prepared routinely. After ribonuclease digestion approximately 95% of nucleoli from liver nuclei and 70% of nucleoli from kidney nuclei possessed a well developed nucleolonema within the pars fibrosa.

Classification of nucleoli from liver and kidney cells which had been subjected to ribonuclease digestion, into the six type categories did not reveal any differences in the distribution between the groups after hormone treatment, and there was no statistical difference between the distribution of nucleoli between the categories after hormone treatment, as is shown in Table 35 .

The distribution of ribonuclease digested material between the categories was compared with the distribution of nucleoli between

Table 35 .

Classification of nucleoli between the type categories.

Ribonuclease digested material.

		1	2	3	4	5	6
Liver	N	5	6	2	10	8	2
	T3	2	2	0	5	13	0
	T4	0	0	0	2	2	0
Kidney	N	0	2	0	10	6	2
	T3	1	5	1	4	3	1
	T4	1	6	0	13	4	0

Table 36 .

Statistical significance of variations in distribution of nucleoli after hormone treatment, and ribonuclease digestion. (X^2 test.)

Liver	TX	N	T3	T4
TX				
N				
T3		$P < 0.5$		
T4		$P < 0.8$	$P < 0.98$	

Kidney	TX	N	T3	T4
TX				
N				
T3		$P < 0.5$		
T4		$P < 0.5$	$P < 0.5$	

the categories after glutaraldehyde and osmium fixation, both with and without the period or cold buffer storage. and with acrolein fixation both with and without the cold buffer treatment, with interesting results.

There was no significant difference between the distribution

Figure 30.

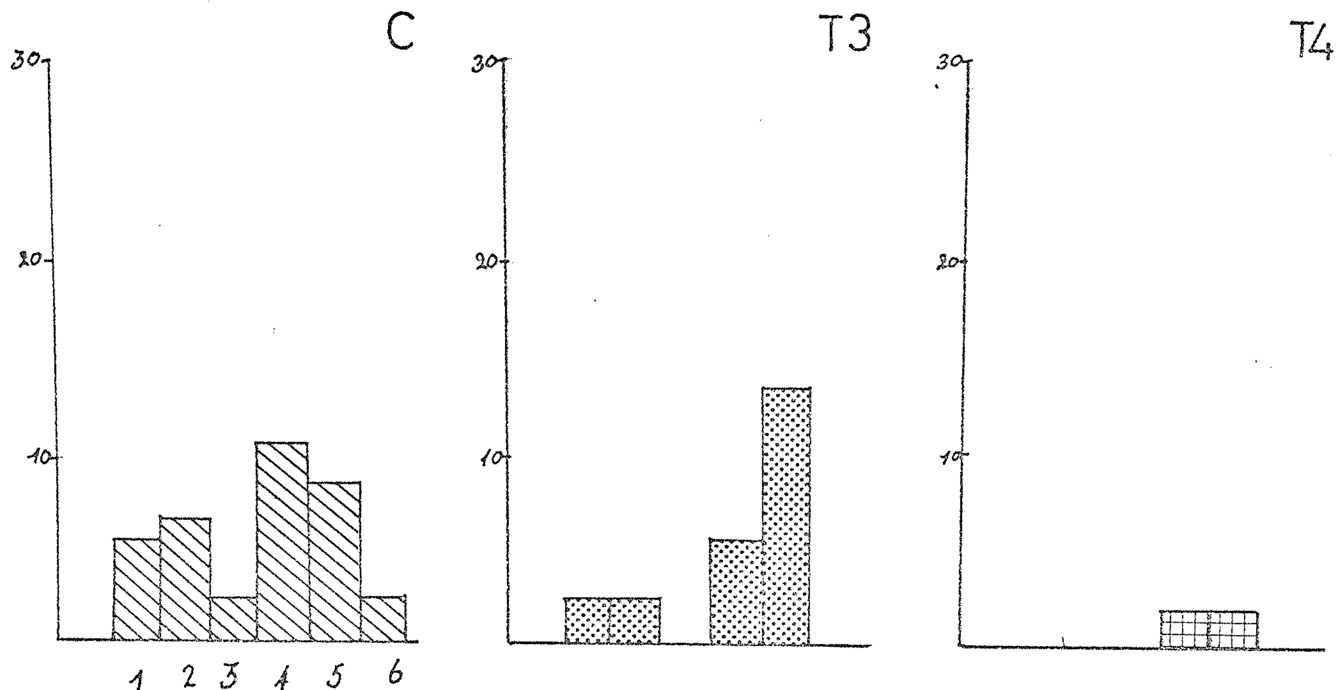
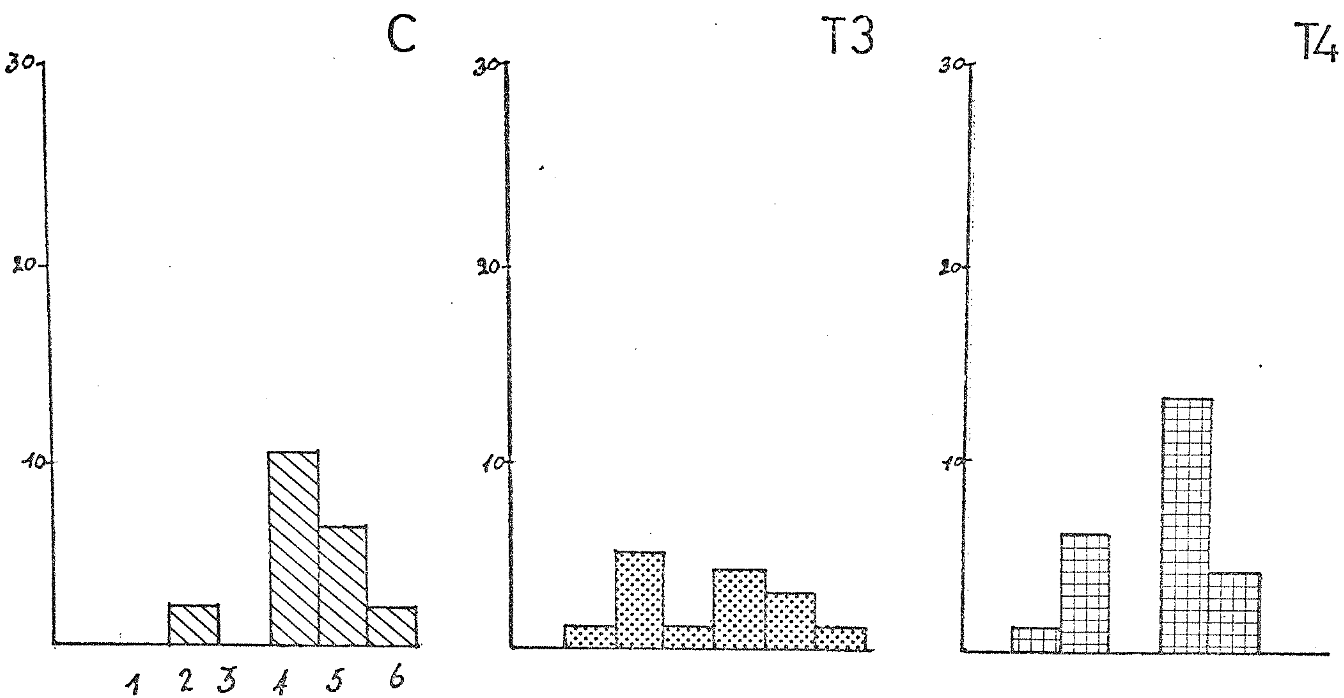


Figure 31.



of nucleoli between the categories after glutaraldehyde and osmium fixation, and ribonuclease digestion, although there was a considerable reduction in the amount of material present in the pars granulosa, especially of the 'halo particles' after ribonuclease digestion. However, there was a significant difference between the distribution of nucleoli after ribonuclease digestion and the distribution after cold buffer storage which would suggest that the material removed from the sections during the period of cold buffer storage is not ribonuclease digestible.

A similar pattern emerged after acrolein fixation, where there was no significant difference between acrolein fixed material and ribonuclease digested material, but there was a significant difference between the material fixed in acrolein and stored in cold buffer and ribonuclease digested material, although the difference between ribonuclease digested material and acrolein fixed material stored in cold buffer was not as pronounced as between ribonuclease digested material and glutaraldehyde fixed material stored in cold buffer.

Table 37.

Comparison of the distribution of nucleoli after ribonuclease digestion with the distribution after other preparation techniques.

	Normal		Normal
1.	Ribonuclease	x	Routine glutaraldehyde and osmium fixed material.
	Liver $>P$ 0.2		Kidney $P<$ 0.2
2.	Ribonuclease	x	Glutaraldehyde fixed material stored in cold buffer.
	Liver $P<$ 0.01		Kidney $P<$ 0.001
3.	Ribonuclease	x	Acrolein fixed material
	Liver $P<$ 0.8		Kidney $P<$ 0.1
4.	Ribonuclease	x	Acrolein fixed material stored in cold buffer.
	Liver $P<$ 0.05		Kidney $<P$ 0.01

Pepsin digestion. Pepsin digestion had a less marked effect upon the appearance of nucleoli than did ribonuclease. There was a slight reduction in the contrast of the pars fibrosa but this effect was less marked than the reduction in contrast produced by ribonuclease digestion. The main effect of pepsin digestion was in producing a loss of detailed structure within the nucleolus. It was difficult, if not impossible, to resolve the fibrillar nature of the pars fibrosa, or the basic granular structure of the pars granulosa.

Thus nucleoli, after pepsin digestion, usually resembled that shown in Plate 50 , and in Plate 51 , The pars fibrosa of kidney nucleoli usually showed poorly developed nucleolonemæ, whereas those of the liver were better developed. This was not affected by the pepsin digestion, there was no increase in the clarity with which the nucleolonema was visualised after pepsin digestion, in fact in many cases the development of the nucleolonema was less obvious as a result of the loss of detailed structure of the fibrillar components of the pars fibrosa. The pars granulosa was also less clear after pepsin digestion, usually appearing as a more or less homogeneous mass of electron dense material lacking the granular structure seen after normal preparation, however, there was usually no effect upon the underlying fibrillar network of the pars granulosa.

It would seen that pepsin digestion does remove some protein constituent of the nucleolus which results in a general loss of detail, It would seem probable that this protein component is a constituent of the fibrils of the pars fibrosa and granules of the pars granulosa rather than matrix material, since removal of matrix material would tend to result in an increase in the clarity rather than a reduction.

There was little detectable hormone influence upon the pepsin digestible material within the nucleolus. There was a slight increase in the number of nucleoli present in both the liver and the

Table 38 .

Classification of nucleoli between the type categories.

Pepsin digested material.

		1	2	3	4	5	6
Liver	N	1	6	0	5	4	0
	T3	0	6	2	16	12	3
	T4	1	5	0	11	8	0
Kidney	N	1	8	0	8	7	0
	T3	0	1	1	7	7	1
	T4	0	5	0	11	3	1

Table 39 .

Statistical significance of variations in distribution of nucleoli after hormone treatment and pepsin digestion (X^2 test).

Liver	TX	N	T3	T4
TX				
N				
T3		$P < 0.5$		
T4		$P < 0.95$	$P < 0.8$	

Kidney	TX	N	T3	T4
TX				
N				
T3		$P < 0.5$		
T4		$P < 0.8$	$P < 0.5$	

Figure 32.

NUCLEOLAR CLASSES.

Pepsin digestion
LIVER

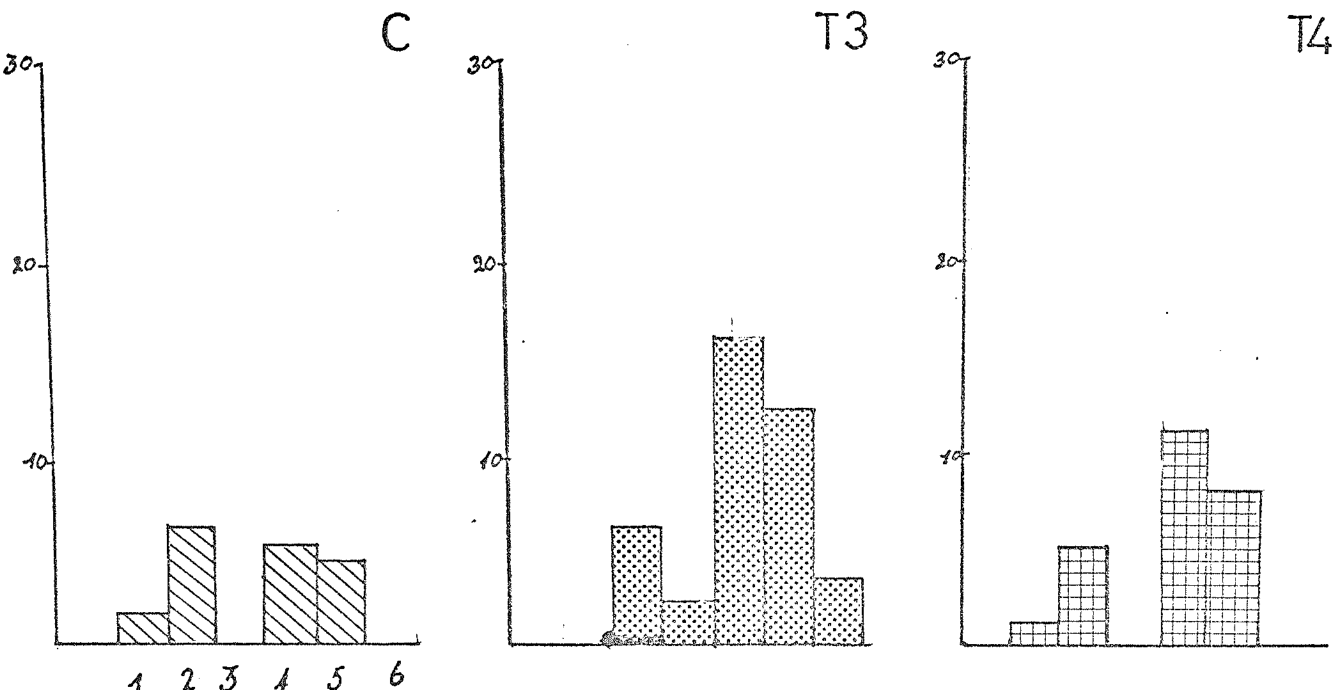
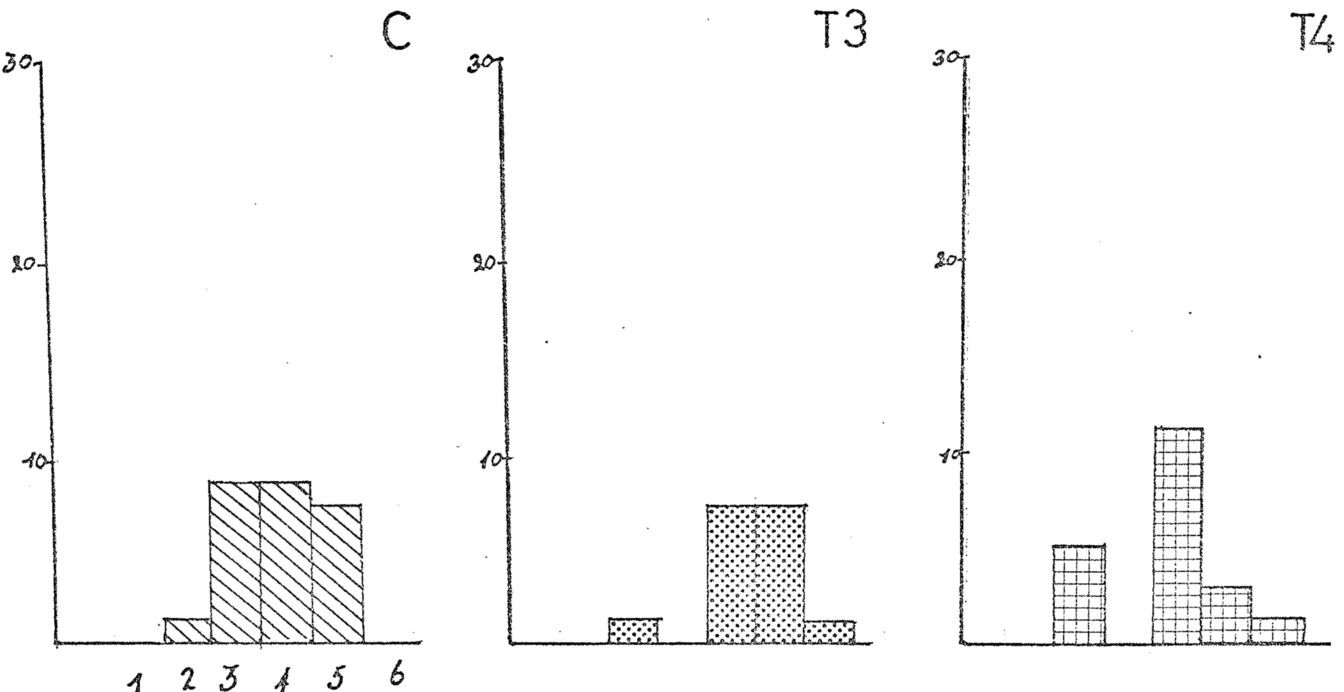


Figure 33.

NUCLEOLAR CLASSES.

Pepsin digestion

KIDNEY



kidney of hormone treated animals which showed some development of a nucleolonema within the pars fibrosa.

There did appear to be slight variation in the amount of pars granulosa material associated with the nucleoli after hormone treatment. There appeared to be an increased amount of granulosa material present especially of the loosely arranged 'halo particles' after hormone treatment. For this reason nucleoli from both liver and kidney were scored into the six type categories, but the classification of nucleoli from pepsin digested material did not reveal any hormone influenced change in the distribution of the nucleoli as is shown in Tables 38 and 39 .

Comparison of the distribution of nucleoli between the different categories after pepsin digestion with the distribution seen after the other preparation procedures used again revealed interesting results, as is shown in Table 40 . As was the case with ribonuclease digested material there was no significant difference between the distribution of nucleoli between the different categories of pepsin digested material and routinely prepared glutaraldehyde and osmium fixed material, whereas there was a significant difference between the pepsin digested material and the glutaraldehyde fixed material which had been subjected to a period of storage in cold buffer. Similarly there was no significant difference between the results of pepsin digestion and acrolein fixation, whereas there was between the material which had been pepsin digested and the acrolein fixed material that had been stored in the cold buffer.

Comparison of the results of ribonuclease digestion with those of pepsin digestion again showed that there was no significant difference in the distribution of nucleoli between the type categories after either treatment, which suggests that these two enzymes attack the same component of the nucleolus, which may be assumed to be a ribonucleoprotein component although the variation in the appearance of

Table 40.

Comparison of the distribution of nucleoli after pepsin digestion with the distribution after other preparation procedures.

	Normal	x	Normal
1.	Pepsin	x	Routine glutaraldehyde and osmium fixation.
	Liver $P < 0.9$		Kidney $P < 0,5$
2.	Pepsin	x	Glutaraldehyde fixed material stored in cold buffer.
	Liver $P < 0.01$		Kidney $P < 0.01$
3.	Pepsin	x	Acrolein fixed material.
	Liver $P < 0.8$		Kidney $P < 0.5$
4.	Pepsin	x	Acrolein fixed material stored in cold buffer
	Liver $P < 0.05$		Kidney $P < 0.02$
5.	Pepsin digested material	x	Ribonuclease digested material
	Liver $P < 0.8$		Kidney $P < 0,5$

the nucleolus after treatment with the different enzymes would suggest that they affect this component in different ways.

There would appear to be no hormone induced variation in either the ribonuclease digestible material present within the nucleolus after glutaraldehyde fixation or in the pepsin digestible material. This may however, be accounted for by the rather crude method of analysis in use. There were no discernible variations in the amount of pars granulosa material present after digestion with either enzyme and hormone treatment, this may have been due to changes taking place being too slight to be detectable. However, there was a variation in the appearance of the pars fibrosa, of the nucleolus associated with the

administration of thyroid hormones detectable after ribonuclease digestion which would suggest that although thyroid hormones exert an effect upon the nucleolus they do not cause an accumulation of either ribonuclease digestible or pepsin digestible material within the pars granulosa, which is in accord with the observations of Tata (1964, 1965 and 1967) which suggested that thyroid hormones increased the rate of turnover of ribonucleoprotein material within the nucleolus.

That there was a lack of statistical significance between the distribution of nucleoli between the type categories after digestion with either enzyme and after acrolein fixation followed by silver nitrate staining, would suggest that at least part of the nucleolar components stained by the acrolein / silver technique are ribonucleoproteins. Part of the components stained by the silver nitrate after acrolein fixation appear to be ribonucleoprotein but part do not, and it would appear to be that fraction that is affected by the action of thyroid hormones.

This however, leaves in question the identity of the hormone influenced fraction of components stained by acrolein and silver. Silver nitrate staining following acrolein fixation is known to stain deoxyribonucleoprotein components and possibly proteins, as well as ribonucleoproteins. Thus it is possible, although it seems to be unlikely that thyroid hormones induce changes in heterochromatin associated with the nucleolus rather than ribonucleoproteins. A more probable explanation would appear to be that they influence some protein component.

Again there is a significant difference between the distribution of nucleoli between the categories after digestion with either enzyme and after acrolein fixation followed by prolonged storage in cold buffer would suggest that the components removed during cold buffer storage after aldehyde fixation are not ribonucleoprotein, although it leaves in question their true identity.

Variations in the morphology of nucleoplasmic constituents with thyroid hormone treatment.

The non-nucleolar dark staining components, usually referred to as the chromatin consists mainly of granular material which occurs in close association with the nucleoplasmic reticulum. Most is seen to consist of masses of granules of about the same dimensions as the cytoplasmic ribosomes. A certain amount of this material consists of diffuse electron dense material of indeterminate structure. They are the equivalent of the perichromatin granules of Swift (1962) and Watson (1962) who estimated their size as 300 - 350 Å, or possibly of the interchromatinic granules of Swift (1959), Ris (1962), Granboulan and Bernhard (1963) and Bernhard and Granboulan (1963), which are approximately 200 - 250 Å in diameter. Detailed estimation of the sizes of these particles would be necessary to equate these particles with those described by other people.

It is interesting to note that the chemical nature of neither the interchromatinic or perichromatinic granules has been established, although it is possible that they consist of ribonucleo-protein with a protein coat. Smetana etal (1963) suggested that the interchromatinic granules might represent nucleolar ribonucleoprotein migrating to the cytoplasm, linked with a protein coat to prevent digestion by endogeneous ribonuclease.

In view of the effects of thyroid hormones on protein synthesis at the nuclear level it was decided to attempt an estimation of the amount of this granular material, after the various hormone treatments.

Nuclei were divided into five categories according to the amount and distribution of dark-staining material present as is shown in Plates 52 to 56 . Plate 52 shows a nucleus typical of those included in Category I. There was little obvious dark staining

Classification of nuclei between the type categories.

Table 41.

Glutaraldehyde and osmium fixed material.

		1	2	3	4	5
Liver	TX	1	8	18	14	0
	N	7	11	21	14	2
	T3	0	1	18	6	1
	T4	0	3	10	21	1
Kidney	TX	3	6	33	26	2
	N	0	10	24	17	0
	T3	0	0	14	17	1
	T4	0	4	21	33	2

Table 42.

Glutaraldehyde fixed material subjected to storage in cold buffer before osmication.

		1	2	3	4	5
Liver	TX	0	6	5	7	0
	N	3	6	13	9	0
	T3	0	3	7	3	0
	T4	0	3	18	11	0
Kidney	TX	3	8	5	6	0
	N	5	5	9	6	2
	T3	0	1	6	8	3
	T4	1	2	3	10	0

Table 43 .

Acrolein fixed material stained with silver nitrate.

		1	2	3	4	5
Liver	TX	0	7	6	7	0
	N	2	5	10	21	1
	T3	0	0	7	12	0
	T4	0	0	11	23	0
Kidney	TX	2	2	8	5	0
	N	0	0	18	19	0
	T3	0	0	17	27	0
	T4	0	1	8	13	1

Table 44 .

Acrolein fixed material subjected to cold buffer storage, stained with silver nitrate.

		1	2	3	4	5
Liver	TX	23	12	9	8	0
	N	13	11	11	10	3
	T3	2	3	25	23	0
	T4	0	4	20	16	1
Kidney	TX	4	0	0	2	0
	N	9	5	7	3	0
	T3	3	0	3	1	0
	T4	7	2	6	5	0

material present. Material that was present was fairly evenly distributed throughout the nucleoplasm. Plate 53 shows a nucleus of Category II, where there was an increased amount of dark staining material present. There was little accumulation within the nucleoplasm but there was a zone of sub-peripheral dark-staining material. Plate 54 is representative of nuclei of Category III. Instead of being evenly distributed throughout the nucleoplasm dark staining material is aggregated into small, loosely arranged groups of granules. A nucleus of Category IV is shown in Plate 55 where dark staining material was arranged in small, densely packed aggregations within the nucleoplasm. Plate 56 shows a nucleus of Category V where the aggregates of dark staining material were large and extensive.

The nuclei from each experimental group were scored according to which category they resembled most closely, The results are shown in Tables 41 to 44 .

The appearance of nuclei from normal animals fixed in glutaraldehyde and osmium.

Nuclei from material prepared with glutaraldehyde and osmium may be described as forming a standard pattern. Nuclei from normal animals usually resembled that shown in Plate 57 with some amount of granular dark staining material against the nuclear envelope and small loose aggregations of particles within the nucleoplasm. Peripheral deposits did not usually extend around the whole limmit of the nucleus but were interrupted by light channels adjacent to the nucleo-pores. Local aggregations often extended some distance into the nucleus. Small granular aggregations occurred throughout the nucleus and isolated granules or groups of a few granules were also fairly abundant.

Nuclei in which large aggregations of closely packed granules occurred, were seen in which case isolated granules were infrequent although small groups occurred throughout the nucleoplasm.

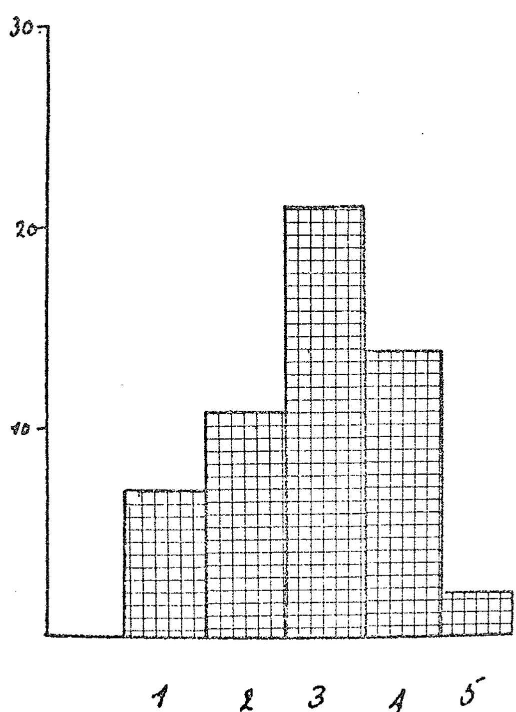
Figure 34.

CLASSIFICATION OF EXTRA - NUCLEOLAR MATERIAL.

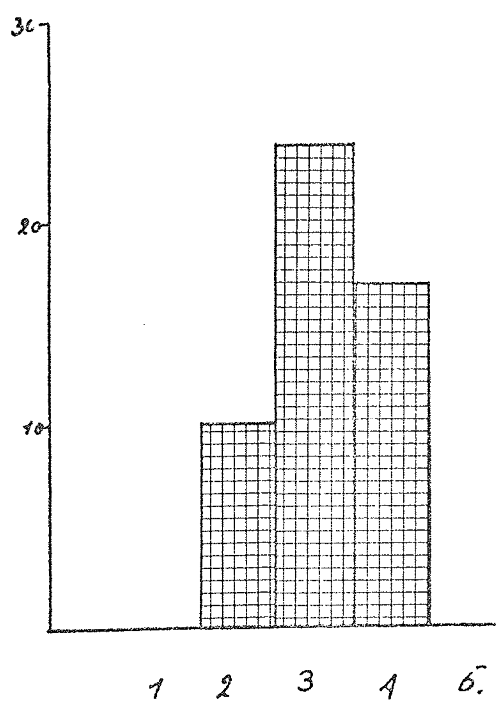
(Glutaraldehyde & Osmium fixation)

Control material.

LIVER



KIDNEY



Nuclei lacking in aggregations of dark staining material were infrequent.

Particle aggregates were associated with an underlying network of fine fibrils, as is shown by the arrows in Plate 57 . These fibrils were similar in size to those associated with the nucleolus This fibrillar network was aggregated into small, closely woven 'nodules' in places resembling the pars fibrosa of the nucleolus but of lower electron density. The interstices of the network appeared to be filled with a homogeneous material of low electron density. Such nodules can be seen in Plates 53 and 56 . It was occasionally possible to distinguish ill-defined strands of courser fibrils, usually associated with granular aggregations as is shown in Plates. 53.

The distribution of nuclei between the type categories in normal animals after routine preparation with glutaraldehyde and osmium is shown in Figure 34 .

The effect of fixation proceedure upon the standard pattern of nuclear morphology.

Variation of the fixation proceedure appears to have a marked effect upon the demonstration of nucleoplasmic dark staining material. Although the distribution of nuclei between the five categories after two fixation proceedures was not statistically significant as is shown below.

Glutaraldehyde and osmium	x	Acrolein fixation.
Normal	x	Normal
Liver P 0.95		Kidney P 0.1

The appearance of the five categories of nuclei after acrolein fixation and silver nitrate staining are shown in Plates 58 to 62 . The dark staining material tended to form extensive patches of loosely packed granules after acrolein fixation and silver nitrate staining as shown in Plate 60 and 61 , with extensive peripheral

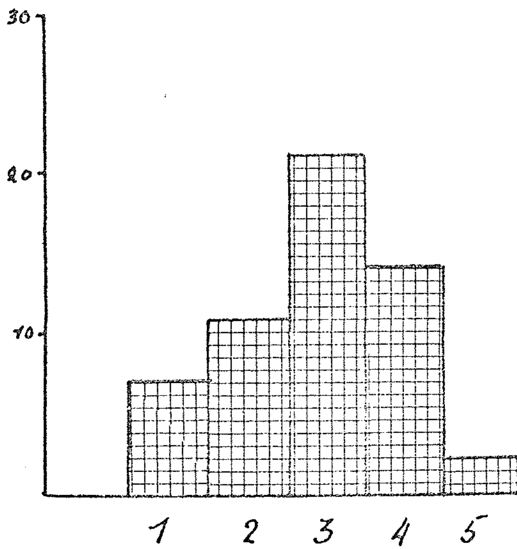
Figure 35.

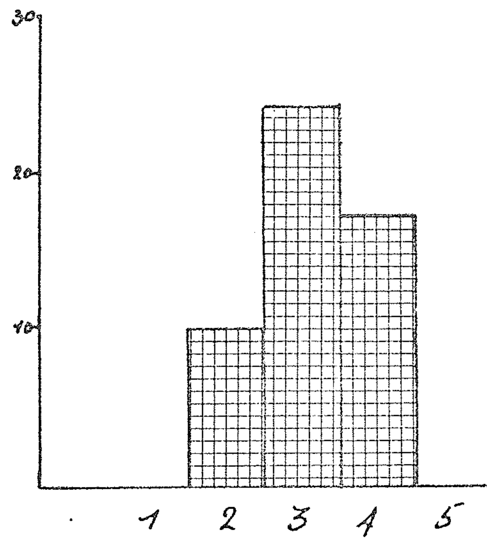
CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Control material.

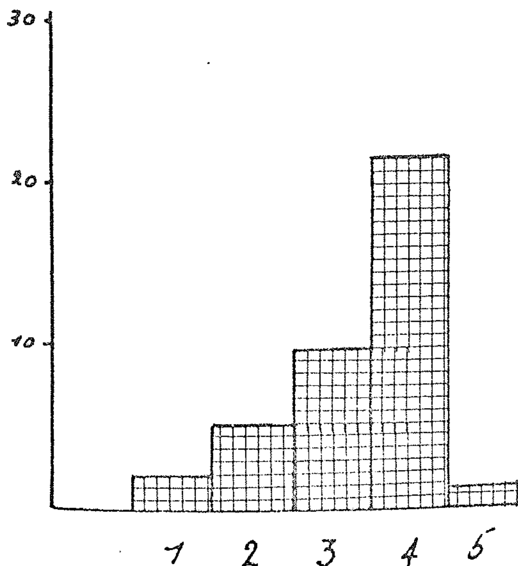
LIVER KIDNEY.

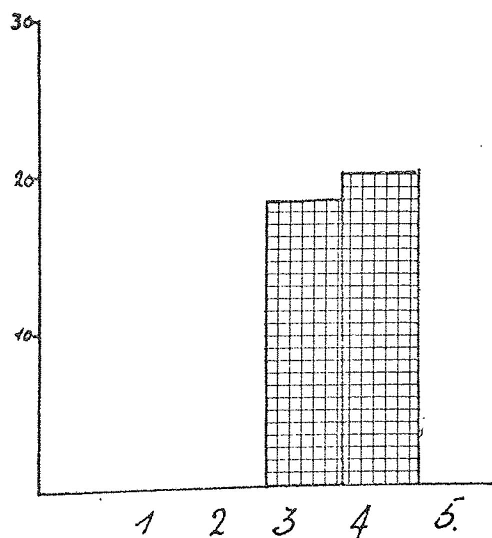
Glutaraldehyde & Osmium fixation.





Acrolein fixation.





deposits of densely packed granules. Some nuclei showed diffused granules across the whole nuclear section, with little or no peripheral accumulations as is shown in Plates 58 and 59 but such nuclei were not common. Other nuclei, assembling that seen in Plate 61 , where small densely packed aggregations of granules dominated the cytoplasm were more common.

There appeared to be a greater amount of stained material present after acrolein fixation and silver nitrate staining compared with that present after glutaraldehyde and osmium fixation. This was probably a result of the staining proceedure. The granules visualised after glutaraldehyde and osmium fixation represent structures present in the section, whereas granules visualised after acrolein fixation and silver nitrate staining are silver nitrate deposits on the surface of the section. Although silver nitrate deposits tend to occur over nucleoprotein components, which the granules seen after glutaraldehyde and osmium fixation probably are, they do not occur in the same proportion as the granules which gave rise to their deposition. Numerous granules of silver occur in association with only a few nucleoprotein granules.

There was no evidence of the detailed underlying fibrillar or granular structure of acrolein fixed material stained with silver nitrate due to the low background contrast of the material after aldehyde fixation without post staining, and due to the na ure of the silver stain.

The distribution of nuclei between the type categories after acrolein fixation from normal animals are shown in Figure 35 .

<u>The effect of storage in cold buffer upon the appearance of nuclei from normal animals.</u>

Again there appear to be differences due to the fixatives cold buffer extraction appear to have little effect upon glutaraldehyde fixed material, especially in the liver, as far as the distribution of

nucleoplasmic dark staining material is concerned. Although there does appear to be a slight reduction in the amount of granules occurring in concentrated masses in the kidney. A considerable amount of extraction appears to have taken place, however after acrolein fixation. The majority of nuclei fall either into Category I where all the granular dark staining material was removed or not stained, or Category II where only diffused granular deposits and a narrow zone of sub-peripheral granules remained. Statistical comparison of the results of acrolein fixation with and without storage in cold buffer shows that there was a significant redistribution of nuclei between the five categories in both tissues. There was no statistical significance in the results obtained from material fixed in glutaraldehyde and osmium in the liver and the kidney when they were compared to those from glutaraldehyde fixed material and stored in cold buffer, as is shown below.

Glutaraldehyde fixation x Glutaraldehyde fixation followed by storage in cold buffer.

Normal x Normal

Liver P 0.9 Kidney P 0.01

Acrolein fixation x Acrolein fixation followed by storage in cold buffer

Normal x Normal

Liver P 0.01 Kidney P 0.001

Thus it seems probable that acrolein is a poorer fixative that glutaraldehyde resulting in extraction of poorly stabilise granules, or dark staining granular component during subsequent processing, although there was no significant difference between the distribution of nuclei between the five categories after cold buffer treatment, as is shown above. It seems likely that this may occur during normal embedding proceedures and is merely exaggerated during cold buffer storage, and may account for the apparent differences in specificity between the

Figure 36.

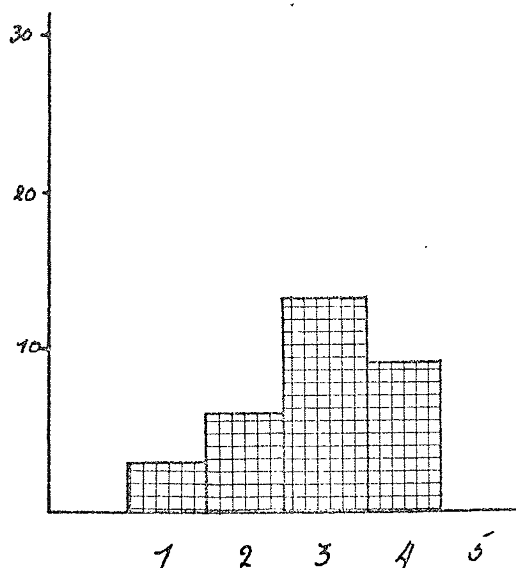
CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

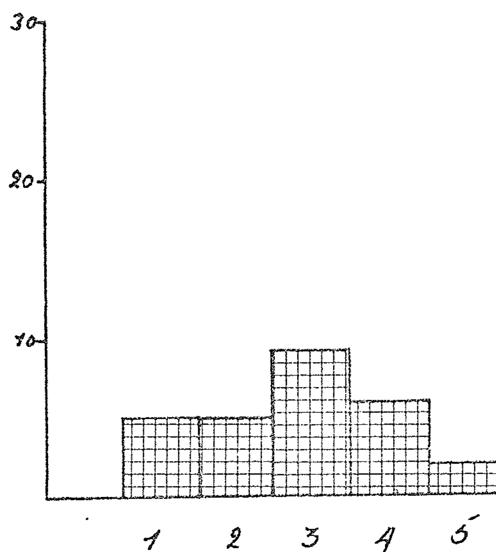
Control material.

Prolonged Cold-buffer Treatment.

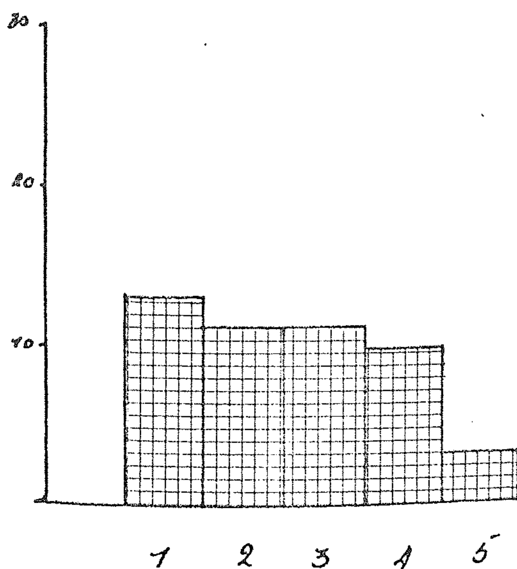
LIVER KIDNEY

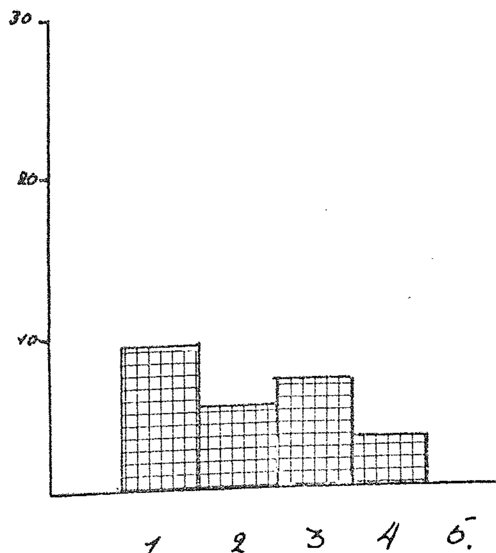
Glutaraldehyde & Osmium fixation.





Acrolein fixation.





two proceedures, glutaraldehyde and acrolein, during normal processing.

Glutaraldehyde fixation followed by storage in cold buffer,	x	Acrolein fixed material followed by storage in cold buffer.
Normal	x	Normal
Liver P> 0.99		Kidney P> 0.99.

The distribution of nuclei between the type categories after storage in cold buffer following either acrolein or glutaraldehyde fixation are shown in Figure 36 .

The effects of hormones upon the distribution of nuclei between the type categories.

1. Glutaraldehyde and osmium fixation.

The distribution (it is questionable how accurate this method of quantitisation was for indicating amount) of nucleoplasmic dark staining material varied quite markedly with the hormone status of the animals.

As was noted previously, nuclei tended to be normally distributed between the five type categories in euthyroid liver and kidney. There was a change in the distribution of nuclei between the five categories, with the hormone treatment so that there tended to be a greater proportion of nuclei in Categories III and IV in the hormone treated animals than in the normal or thyroidectomised animals. This change in the distribution was more significant in the liver than in the kidney as is shown in Table 45 . In hypothyroid or normal animals the nucleoplasmic dark staining material usually occurred as small, loosely arranged groups of particles or as isolated granules, a number of nuclei were found where no apparent aggregations of particles occurred and a number where the only aggregations were against the nuclear envelope. Increased hormone levels produced an apparent increase in the amount of nucleoplasmic dark staining material which was reflected as a change in the degree of aggregation of particles. Large groups of more or less

Figure 37.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Glutaraldehyde & Osmium fixation.

LIVER.

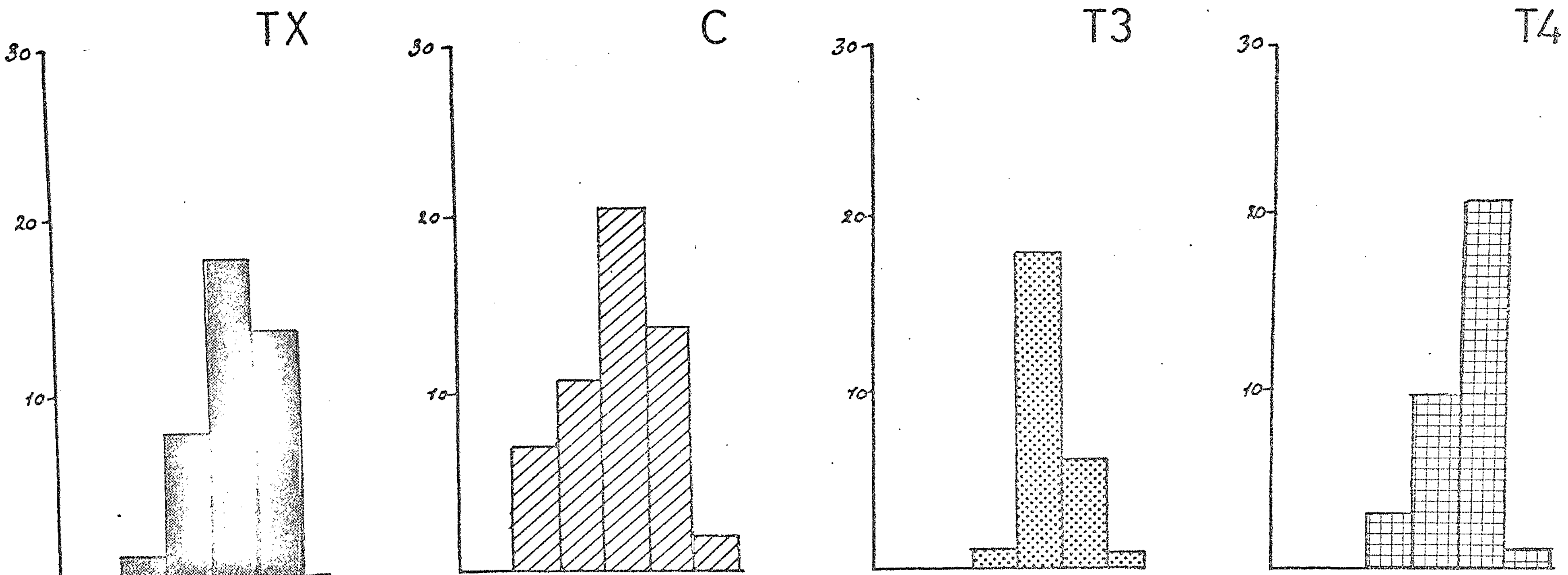


Figure 38.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Glutaraldehyde & Osmium fixation

KIDNEY.

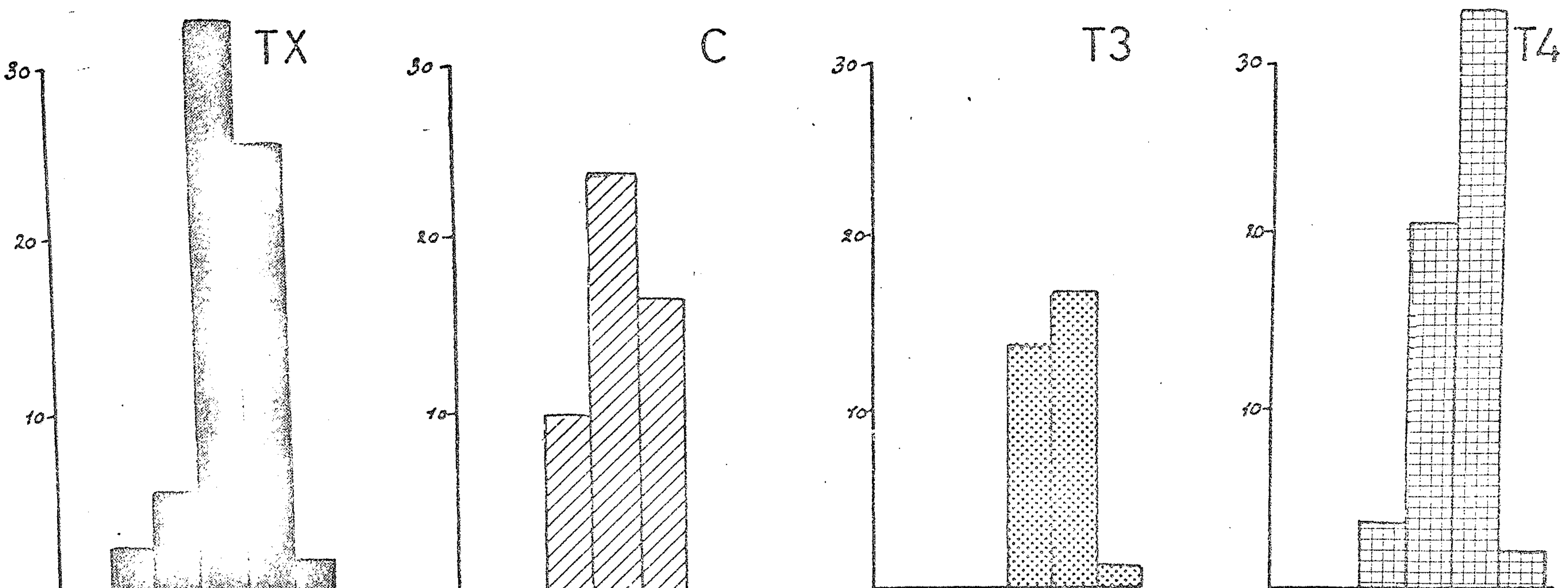


Table 45 .

Statistical significance of variations in distribution of nuclei after hormone treatment and glutaraldehyde and osmium fixation (X^2 test).

Liver				
TX				
N	P < 0.5			
T3	P < 0.2	P < 0.05		
T4	P < 0.2	P < 0.01	P < 0.05	
	TX	N	T3	T4

Kidney				
TX				
N	P < 0.2			
T3	P < 0.2	P < 0.05		
T4	P < 0.1	P < 0.05	P < 0.8	
	TX	N	T3	T4

closely packed granules were common and occurred throughout the nucleus. Sub-peripheral deposits often extended some way into the nucleus.

The distribution of nuclei between the type categories after hormone treatment are shown in Figures 37 and 38 .

2. Glutaraldehyde and osmium fixation, with storage in cold buffer.

There was no significant difference between the distributions of nuclei between the five categories in the different experimental groups in either liver or kidney, as is shown in Table 46 .

However, there appeared to be a marked reduction in the general contrast of the granules constituting the nucleoplasmic dark staining material after the period of cold buffer extraction. It is therefore possible that the staining capacities of the particles are altered after hormone treatment, so that after hormone treatment a greater proportion of the nucleoplasmic particles are susceptible to 'contrast reduction' with cold buffer storage. It is otherwise difficult to visualise why there should be a significant difference in the

Figure 39.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Prolonged Cold-buffer Treatment.

Glutaraldehyde & Osmium fixation

LIVER.

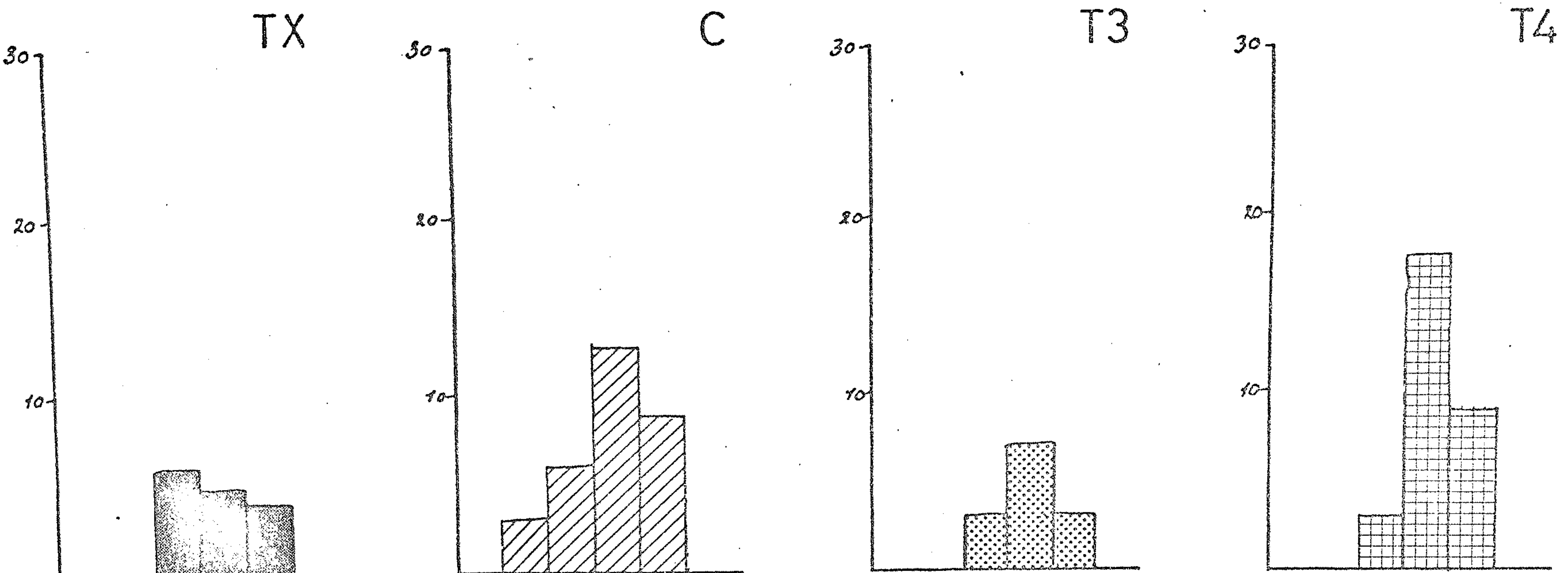


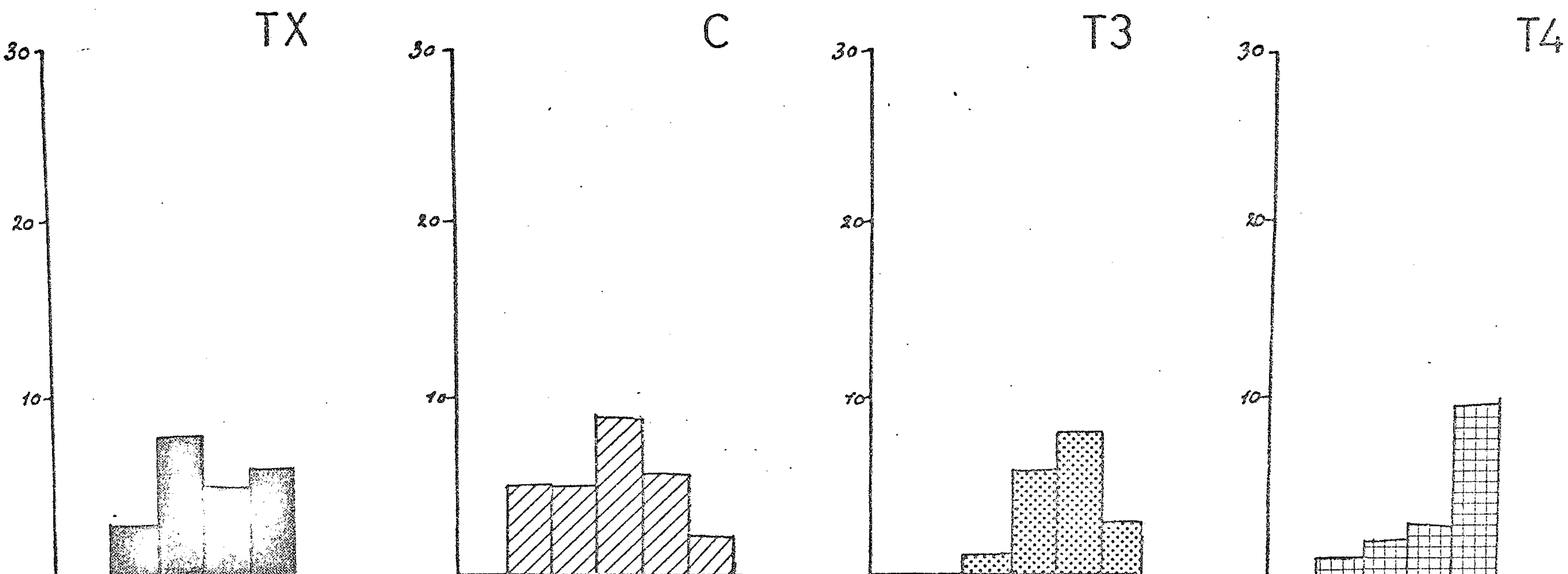
Figure 40.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Prolonged Cold-buffer Treatment

Glutaraldehyde & Osmium fixation

KIDNEY.



distribution of nuclei between the different categories with the hormone treatments with routine glutaraldehyde and osmium fixation which is lost when a prolonged buffer storage period is interposed between the two fixation stages.

Table 46.

Statistical significance of variations in the distribution of nuclei after hormone treatment with glutaraldehyde and osmium fixation and storage in cold buffer (X^2 test).

Liver	TX	N	T3	T4
TX				
N	P< 0.8			
T3	P<0.98	P<0.8		
T4	P<0.99	P<0.5	P<0.8	

Kidney	TX	N	T3	T4
TX				
N	P<0.5			
T3	P<0.05	P<0.2		
T4	P<0.2	P<0.2	P< 0.5	

3. Acrolein fixation and silver nitrate staining.

Visual assessment of material fixed with acrolein and stained with silver nitrate suggested that hormone treatment caused an increase in the amount of argentophilic material in the nucleoplasm in the liver, and to a lesser extent in the kidney. There appeared to be an alteration in the distribution of argentophilic material, particularly in the liver with the changed hormone levels. Argentophilic material tended to be dispersed throughout the nucleoplasm in thyroidectomised and normal animals, but tended to occur as small aggregations after hormone treatment. The distribution of nuclei between the five categories after acrolein fixation in shown in Figures 41 and 42 .

This overall pattern, however, does not show up in the

Figure 41.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Acrolein fixation

LIVER.

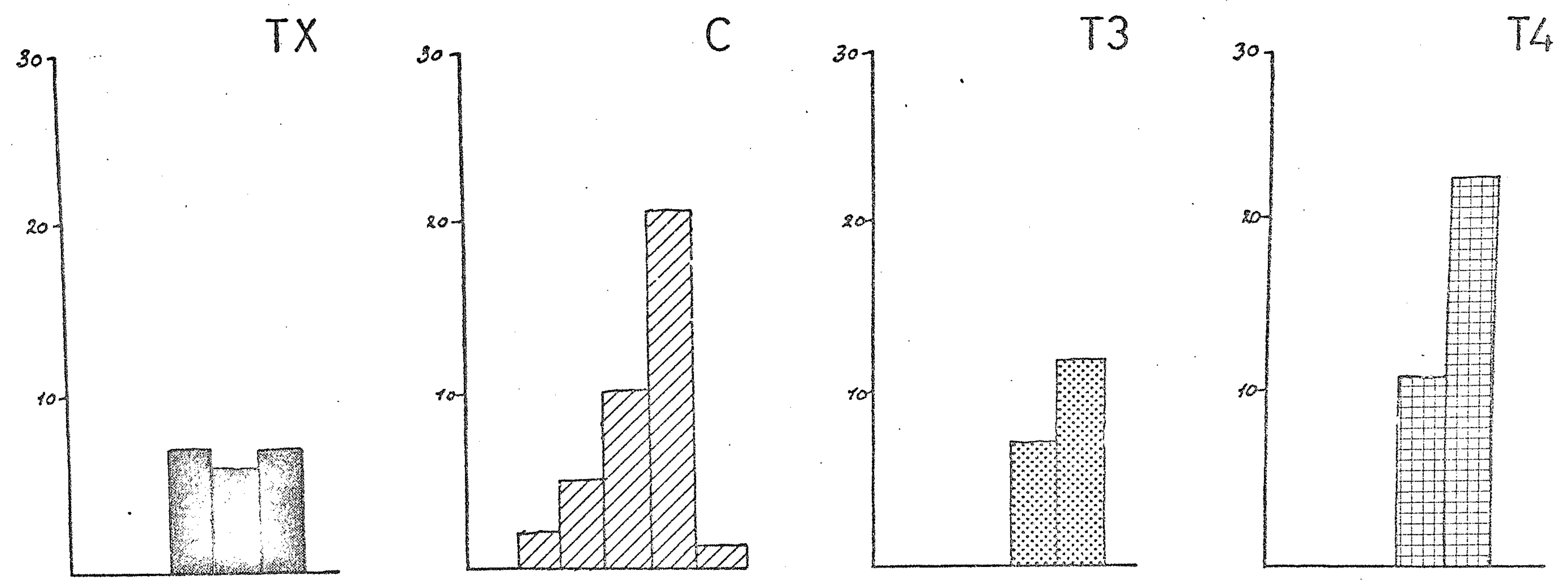


Figure 42.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Acrolein fixation

KIDNEY.

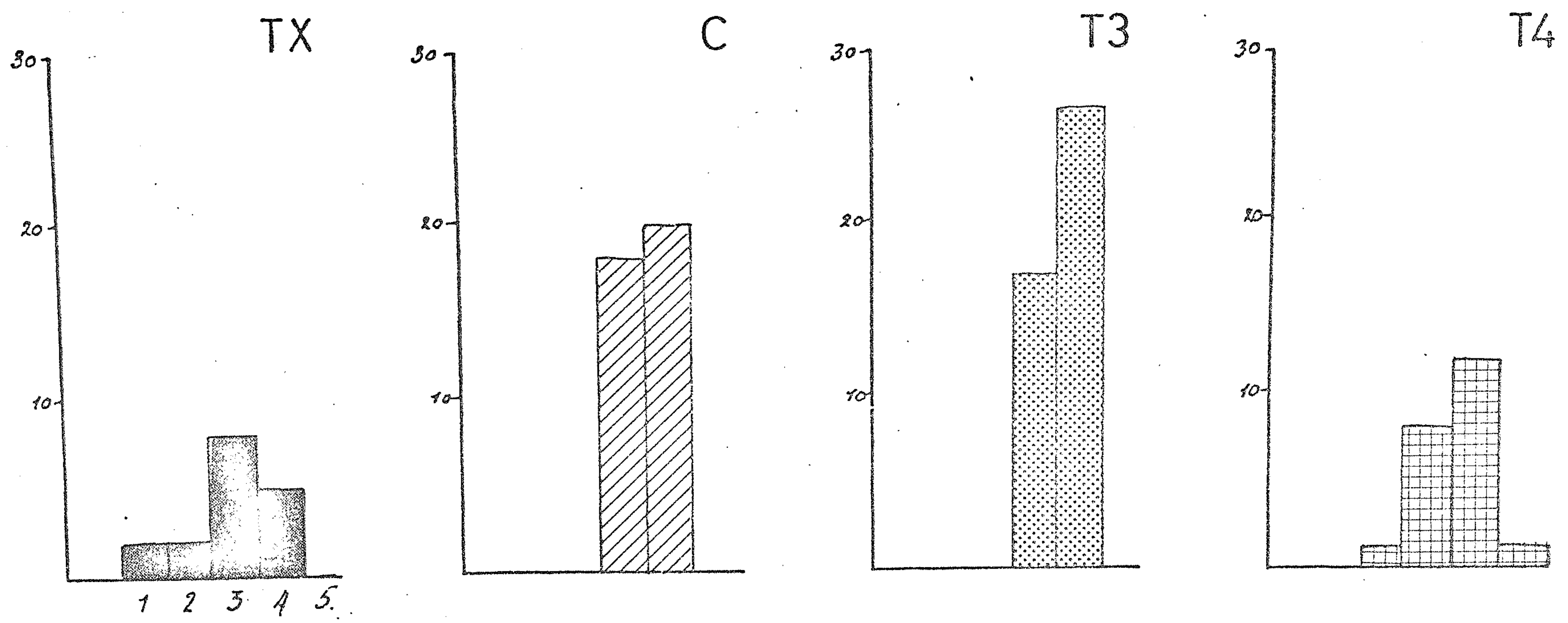


Table 47.

Statistical significance of variations in the distribution of nuclei after hormone treatment with acrolein fixation and silver nitrate staining (X^2 test.)

Liver	TX	N	T3	T4
TX				
N	P< 0.5			
T3	P <0.1	P< 0.5		
T4	P< 0.01	P <0.1	P< 0.99	

Kidney	TX	N	T3	T4
TX				
N	P< 0.05			
T3	P <0.02	P <0.95		
T4	P <0.5	P< 0.5	P< 0.5	

results of the attempt at quantitative assessment, probably due to difficulties in assessing material stained with silver nitrate as compared to routinely prepared material, and as due to the fact that although there was a tendency towards increased aggregation of particles the aggregates were still relatively small. There was little statistical significance in the distribution of nuclei between the categories with the hormone treatments in either the liver or the kidney, as is shown above in Table 47 .

4. Acrolein fixation with silver nitrate staining and cold buffer extraction.

The variation in appearance and distribution of argentophilic material in the nucleus after hormone treatment was more marked in the liver than the kidney. There was no apparent difference in the distribution or degree of aggregation of argentophilic material after hormone treatment, in the kidney and no significant difference in the distribution of nuclei between the five type categories. Whereas

Figure 43.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Prolonged Cold-buffer Treatment.

Acrolein fixation.

LIVER.

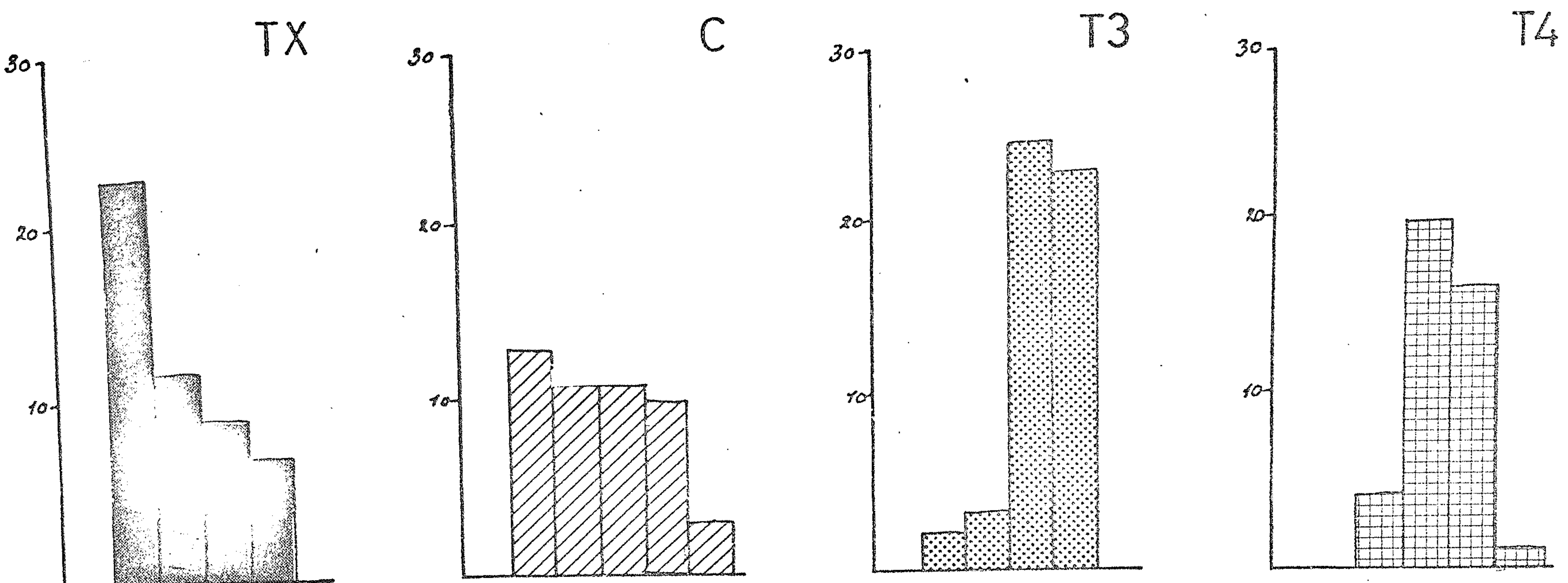


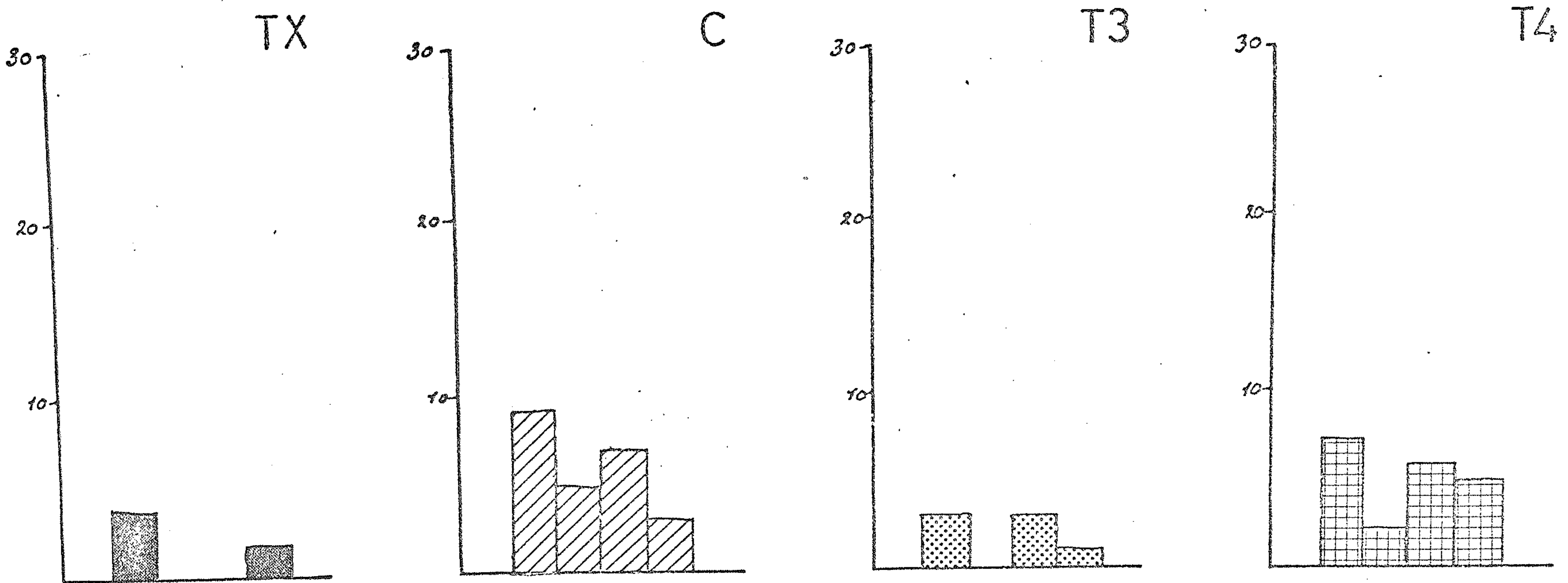
Figure 44.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Prolonged Cold-buffer Treatment.

Acrolein fixation

KIDNEY.



in the liver there was a marked change in the distribution of nuclei after hormone treatment and in their appearance as is shown below, in Table 48 and in Figures 43 and 44 .

Table 48 .

Statistical significance of variations in distribution of nucleoli after hormone treatment, with acrolein fixation and storage in cold buffer. (X^2 test).

Liver	TX	N	T3	T4
TX				
N	P < 0.2			
T3	P < 0.001	P < 0.001		
T4	P < 0.001	P < 0.001	P < 0.5	

Kidney	TX	N	T3	T4
TX				
N	P < 0.5			
T3	P < 0.8	P < 0.8		
T4	P < 0.5	P < 0.8	P < 0.95	

There was an overall increase in argentophilic material in the liver with the hormone treatments, but the most marked effect was upon the distribution of the particles. A large proportion of the nuclei from thyroidectomised animals possessed little argentophilic material and much of that which was present was restricted to a narrow sub-peripheral zone. After hormone treatment a considerable proportion of the nuclei showed material aggregated into clumps throughout the nucleoplasm.

The appearance of nuclei after digestion with either ribonuclease or pepsin.

Material which had been prepared with glutaraldehyde and osmium fixation but included a stage of either ribonuclease or pepsin digestion between the two fixation stages, was examined in order to

gain some identification of the nuclear components affected by thyroid hormone action.

Ribonuclease digestion. As well as significant variations in the appearance of nucleoli, ribonuclease digestion had a significant effect upon the appearance of non-nucleolar zones of nuclear profiles. The general appearance of nuclei after ribonuclease digestion resembled those of normally prepared material. Chromatin was usually unaffected by the ribonuclease digestion, and occurred as obvious, electron dense deposits of granular material against the nuclear envelope, adjacent to the nucleolus and as isolated clumps of granules within the nucleus. The major effect of the enzyme was upon the interchromatinic space as is shown by Plates 63 and 64 , which show nuclei after ribonuclease digestion. The underlying fine fibrillar network was usually removed by the ribonuclease digestion, especially in the liver. Thus the interchromatin space appeared as a relatively homogeneous area occupied by material of medium electron density, within which occasional fine fibrils could be discerned, and small groups of chromatin granules. Interchromatin granules were occasionally visible as is shown in Plate 63 (arrows). Where they did occur they were more obvious than in normally prepared material.

Little apparent difference could be discerned between nuclei from either liver or kidney, from either experimental group, after ribonuclease digestion, upon preliminary observation. In order to gain further clarification of this impression nuclei were scored according to the five type categories. The results are shown in Table 49 and in Figures 45 and 46 . However, there was no significant difference between the distribution of nuclei between the categories, which could be attributed to hormone influence.

The distribution of nuclei between the type categories after ribonuclease digestion were compared with the distributions after

Figure 45.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Ribonuclease digestion

LIVER

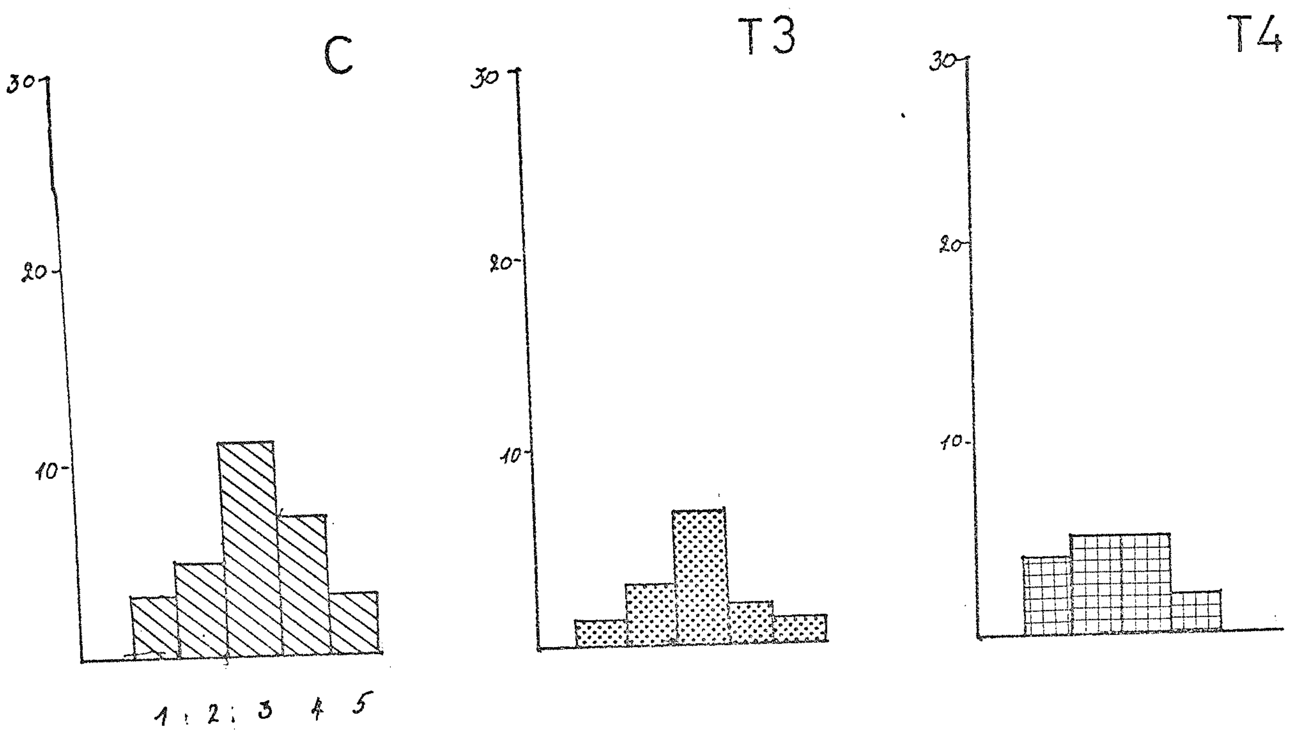


Figure 46.

CLASSIFICATION OF EXTRA - NUCLEOLAR MATERIAL.

Ribonuclease digestion

KIDNEY

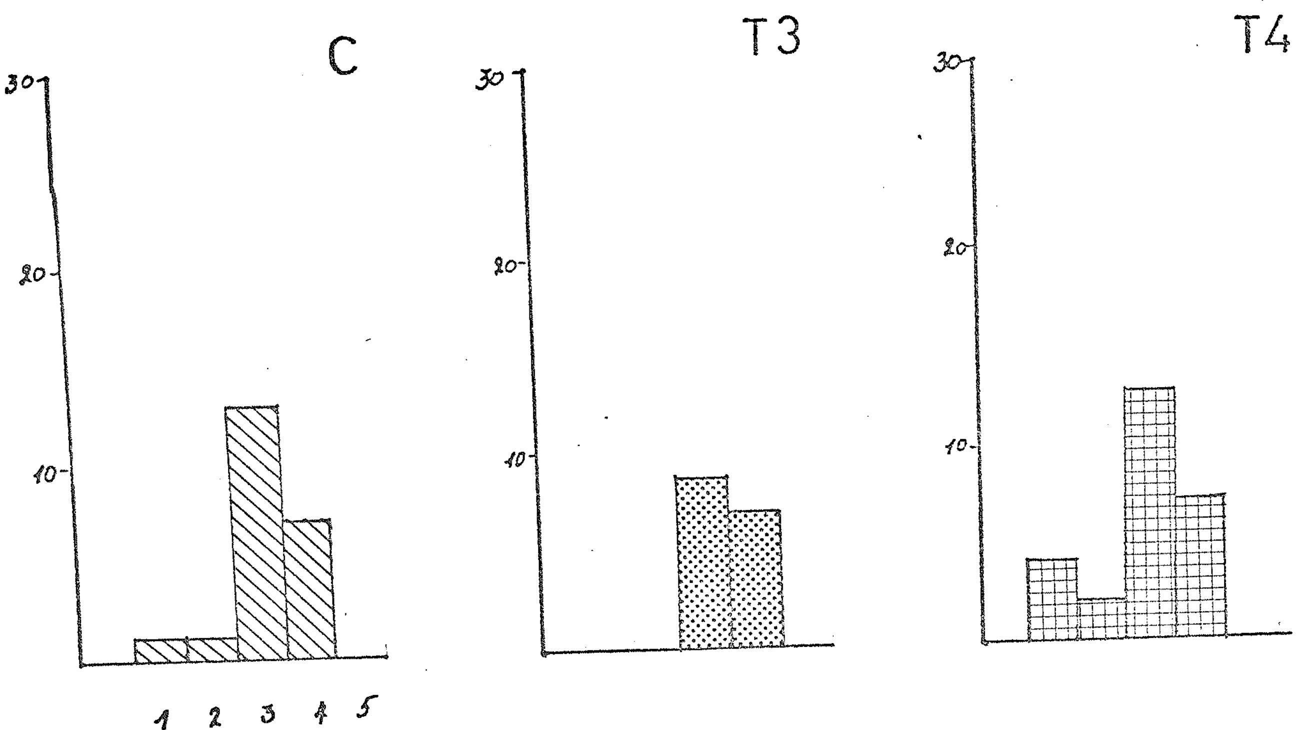


Table 49.

Classification of nuclei between the type categories.

Ribonuclease digested material.

		1	2	3	4	5
Liver	N	3	5	9	7	3
	T3	1	3	7	2	1
	T4	4	5	5	2	0
Kidney	N	1	1	13	7	0
	T3	0	0	9	7	0
	T4	4	2	13	7	0

Table 50.

Statistical significance of variations in distribution of nuclei after hormone treatment and ribonuclease digestion (X^2 test)

Liver

	TX	N	T3	T4
TX				
N				
T3		$P < 0.95$		
T4		$P < 0.8$	$P < 0.8$	

Kidney

	TX	N	T3	T4
TX				
N				
T3		$P < 0.9$		
T4		$P < 0.9$	$P < 0.5$	

other preparation proceedures as is shown in Table 49.

There was no detectable difference between the distribution of nuclei after ribonuclease digestion and the distribution of nuclei between the categories after normal glutaraldehyde and osmium

fixation, or between ribonuclease digestion and glutaraldehyde fixation followed by cold buffer storage. However, the differences in distribution of the nuclei between the categories after ribonuclease digestion compared to the distribution seen after glutaraldehyde fixation and cold buffer storage appears to be greater in the kidney, than in the liver. This observation is interesting in view of the obervation that the effect of the cold buffer was greater upon th. kidney than the liver, and would suggest thatthat fraction of nuclear dark staining material which was subject to cold buffer extraction was not ribonuclease digestible.

Table 51 .

Comparison of the distribution of nuclei after ribonuclease digestion with the distribution after other preparation techniques.

	Normal		x	Normal	
1.	Ribonuclease	Liver P 0.9	x	Routine glutaraldehyde and osmium fixed material.	Kidney P 0.5
2.	Ribonuclease	Liver P 0.8	x	Glutaraldehyde and osmium fixed material stored in cold buffer.	Kidney P 0.2
3.	Ribonuclease	Liver P 0.2	x	Acrolein fixed material.	Kidney P 0.5
4.	Ribonuclease	Liver P 0.8	x	Acrolein fixed material stored in cold buffer.	Kidney P 0.05

That there was no significant difference between the distribution of nuclei between the categories after ribonuclease digestion, compared to acrolein fixation is not surprising, in view of the fact that there was also no significant difference between acrolein as a fixative and glutaraldehyde as regards nuclear appearance. This

would suggest that the nuclear dark staining material visualised after glutaraldehyde and osmium fixation followed by uranium and lead staining and by acrolein followed by silver nitrate, is not ribonucleoprotein and thus not subject to ribonuclease digestion.

That the difference between the results obtained for acrolein fixation followed by prolonged treatment in cold buffer, differed so slightly from those obtained after ribonuclease digestion is interesting, and may be partly explained by the small amount of data available for both groups. However, it would appear that, at least a small fraction of the material subject to extraction by cold buffer is also subject to ribonuclease digestion. This does raise the question why if part of nuclear dark staining material subject to the staining by silver nitrate after acrolein fixation is ribonuclease digestible there was not a greater difference between the results obtained from acrolein fixation and those obtained from ribonuclease digestion. This may however be explained by the fact that the fraction of nuclear dark staining material affected by ribonuclease digestion is so small that variations in it are almost indetectable.

Pepsin digestion. Pepsin digestion, did not appear to have such a marked effect upon nuclear morphology, as did ribonuclease digestion. Nuclei of both liver and kidney resembled those of material prepared routinely with glutaraldehyde and osmium fixation as is shown by Plates 65 and 66 which show nuclei of liver and kidney, respectively.

There was a slight reduction in the clarity with which the granular constituents of the chromatin could be visualised, in the majority of nuclei. There was commonly a reduction in the electron density of the matrix material, so that nuclear profiles appeared to be paler than after normal preparation and stood out in sharp contrast with the surrounding cytoplasm. In some cases the underlying fibrillar network was more difficult to visualise than after normal

preparation, but this effect was not marked, and was not as extensive as after ribonuclease digestion.

Initial observation did not reveal any apparent variation in the amount of dark staining material in the nucleus after pepsin digestion, with the hormone influence. For this reason nuclei were classified according to the five type categories. The results of the classification into the categories are shown in Table 52 and Figures 47 and 48 . As was seen after ribonuclease digestion, no significant differences were found between the distributions of the nuclei between the categories after any hormone treatment, as is shown in Table 53 .

Table 52 .

Classification of nuclei between the type categories.

Pepsin digested material.

		1	2	3	4	5
Liver	N	1	1	5	2	1
	T3	4	4	10	6	1
	T4	2	2	13	4	2
Kidney	N	3	3	11	7	1
	T3	0	1	10	5	1
	T4	2	0	15	13	1

Again the results of classifying nuclei according to the type categories after pepsin digestion were compared with those obtained from nuclei from the other experimental proceedures, in order to obtain some identification of the composition of the dark staining material. The results are shown in Table 54 .

There was no significant difference between the distribution of nuclei between the categories when either normally prepared glutaraldehyde fixed material or glutaraldehyde fixed material which

Figure 47.

CLASSIFICATION OF EXTRA - NUCLEOLAR MATERIAL.

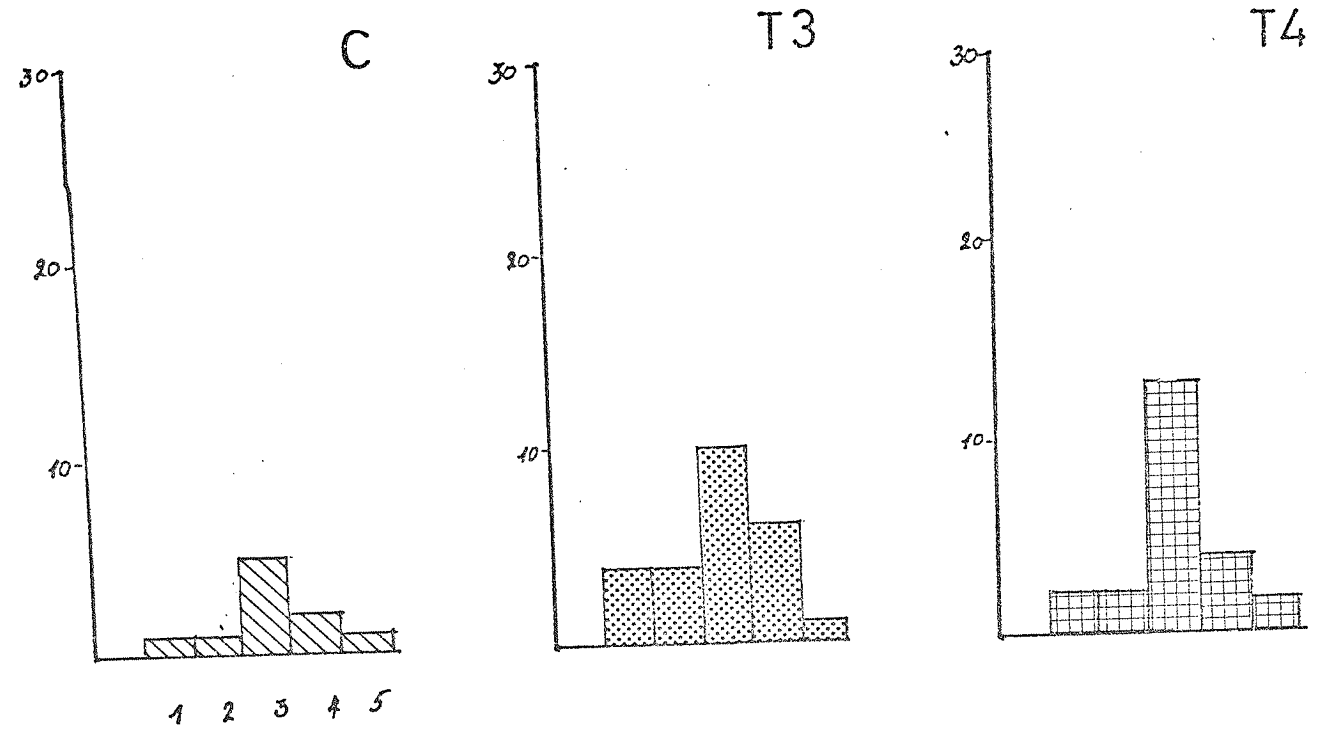


Figure 48.

CLASSIFICATION OF EXTRA-NUCLEOLAR MATERIAL.

Pepsin digestion

KIDNEY

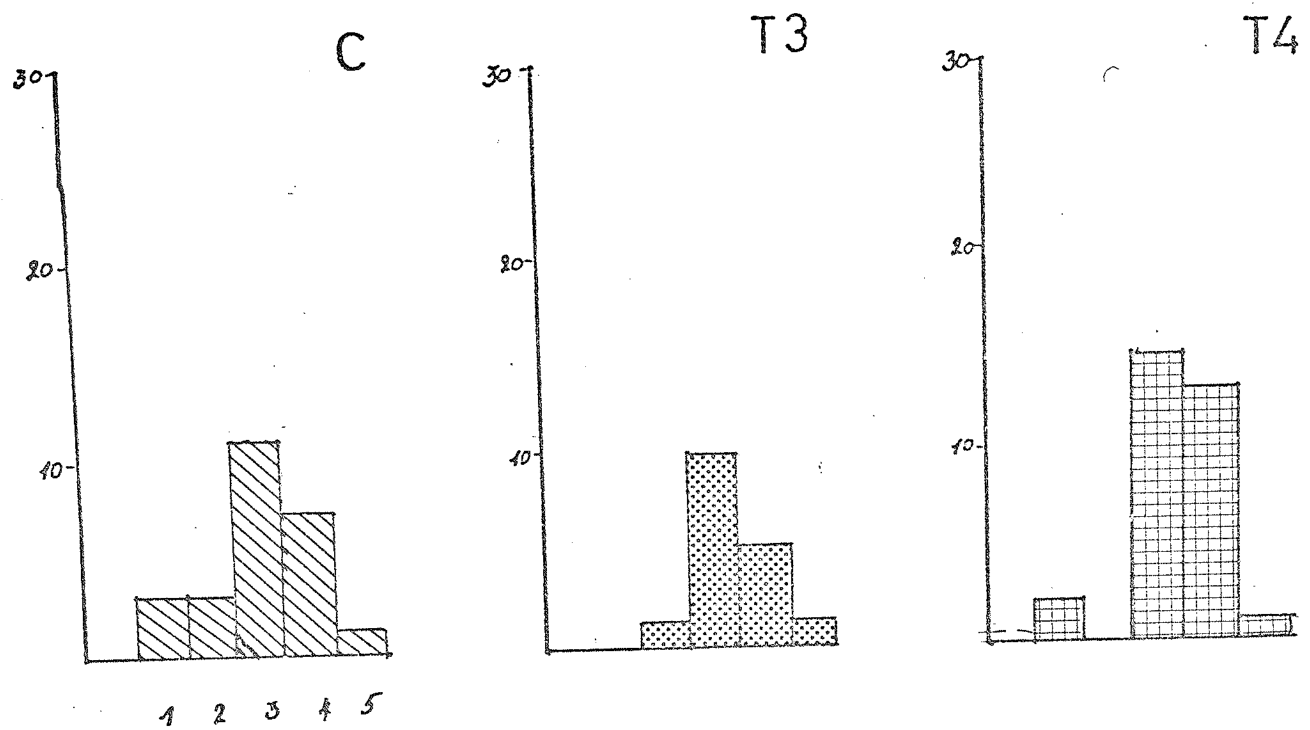


Table 53 .

Statistical significance of variations in distribution of nuclei after hormone treatment and pepsin digestion. (X^2 test)

Liver

	TX	N	T3	T4
TX				
N				
T3		P < 0.98		
T4		P < 0.99	P < 0.90	

Kidney

	TX	N	T3	T4
TX				
N				
T3		P < 0.80		
T4		P < 0.50	P < 0.80	

had been subjected to prolonged cold buffer treatment were compared with results obtained from pepsin digested material, which would suggest that pepsin digestion had no effect upon the amount of dark staining material present in the nucleus, although it tended to alter the appearance somewhat.

Again there was no significant difference between the pepsin digested material and that which had been fixed in acrolein which would again suggest that pepsin has no effect upon the dark staining material present which reacted with the acrolein / silver technique. However, the significant difference between the results obtained from pepsin digestion and acrolein fixation followed by cold buffer treatment would suggest that the nuclear components affected by cold buffer extraction are not protein in composition, since they are not subject to pepsin digestion.

There was no significant difference between the effects of ribonuclease or pepsin, as is shown in Table 54 .

Thus neither ribonuclease digestion nor pepsin

Table 54.

Comparison of the distribution of nuclei after pepsin digestion with the distribution after other preparation proceedures.

	Normal	Liver		Normal	Kidney
1.	Pepsin	$P < 0.95$	x	Routine glutaraldehyde and osmium fixation.	$P < 0.2$
2.	Pepsin	$P < 0.8$	x	Glutaraldehyde fixed material stored in cold buffer.	$P < 0.95$
3.	Pepsin	$P < 0.5$	x	Acrolein fixed material	$P < 0.5$
4.	Pepsin	$P < 0.1$	x	Acrolein fixation followed by storage in cold buffer.	$P < 0.05$
5.	Pepsin	$P < 0.98$	x	Ribonuclease digestion	$P < 0.8$

digestion have a marked effect upon the distribution of dark staining material within the nucleoplasmic areas of nuclear profiles, and it may be assumed that the dark staining material constituting the chromatin does not contain any detectable ribonucleoprotein constituent. The underlying fibrillar network of the interchromatin space did appear to be basically ribonucleoprotein in composition, and the reduction in electron density of the interchromatinic space after pepsin digestion would appear to suggest the presence of some proteinaceous matrix material.

There were no detectable variations in the appearance of nuclear profiles after either ribonuclease or pepsin digestion. However, since the major variations produced by thyroid hormone activity would be expected to take place in the ribonucleoprotein constituents this was not altogether surprising.

Discussion: The effects of thyroid hormones upon nuclear morphology.

Although the validity of the method of analysis used to obtain quantitative estimations of the hormone induced changes in electron dense nuclear material is open to question, it has certain advantages over subjective visual assessment. The division of photographic records of ultrastructural observations into categories according to morphological types obscures detailed differences and opposing trends. It imposes precise, artificial barriers across a gradual change, and obscures trends in morphological patterns which diverge from that trend originally selected by the observer. But the method has the advantage of simplifying the interpretation of masses of photographic data, and of eliminating to some extent, the almost inevitable confusion. If the initial categories are carefully selected, some semi-quantitative evidence can be to substantiate or dismiss initial visual impressions. General patterns of change can then be subjected to more detailed examination.

The allocation of different patterns of nuclear and nucleolar morphology into categories according to the extent of electron-dense material, or deposits of silver nitrate present has revealed several varying morphological patterns influenced by thyroid hormones. They can be summarised in diagrammatic form, as is shown in Figures 49, 50 , 51 and 52 .

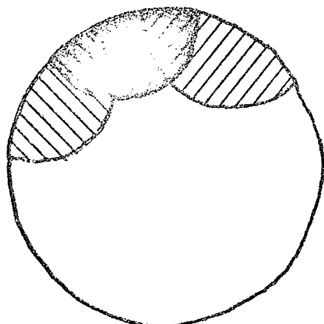
Thyroid hormones did not appear to affect the appearance of nucleoli or the amount of pars granulosa present in either liver or kidney prepared by routine fixation with glutaraldehyde and osmium and section staining with lead and uranium. There was an increase in the amount of material present which caused precipitation of silver from a silver nitrate staining solution after acrolein fixation of the liver. The was no hormone associated change detectable in this material in kidney cell nucleoli.

Figure 49.

REACTION OF PARS GRANULOSA MATERIAL TO DIFFERENT PREPARATION TECHNIQUES.

LIVER.

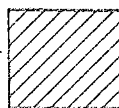
TX



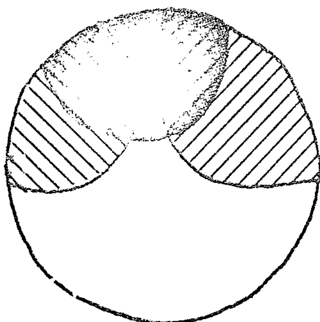
KEY.

TOTAL = THOSE PARTICLES VISUALISED AFTER ROUTINE PREPARATION.

ARGENTOPHILIC PARTICLES (ACROLEIN FIXATION.)

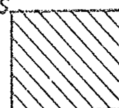


C

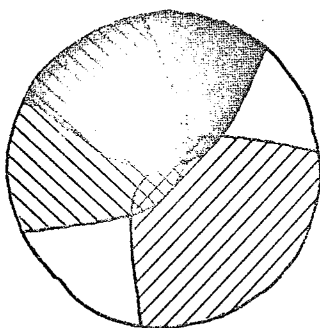


GLUTARALDEHYDE FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION.

ACROLEIN FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION.



T3



T4

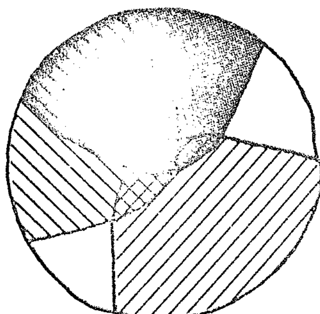
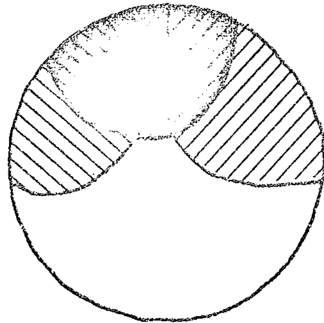


Figure 50.

REACTION OF PARS GRANULOSA MATERIAL TO DIFFERENT PREPARATION TECHNIQUES.

KIDNEY.

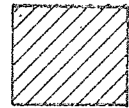
TX



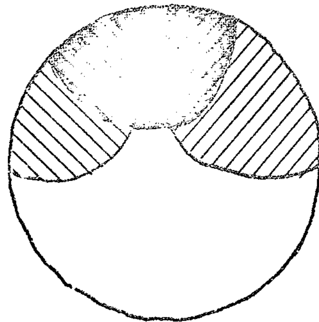
KEY.

TOTAL = THOSE PARTICLES VISUALISED AFTER ROUTINE PREPARATION.

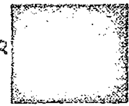
ARGENTOPHILIC PARTICLES (ACROLEIN FIXATION)



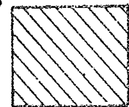
C



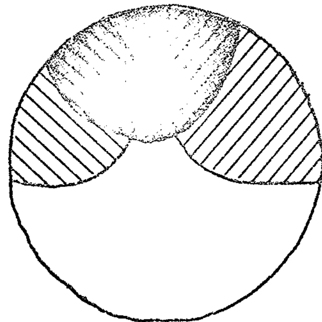
GLUTARALDEHYDE FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION.



ACROLEIN FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION.



T3



T4

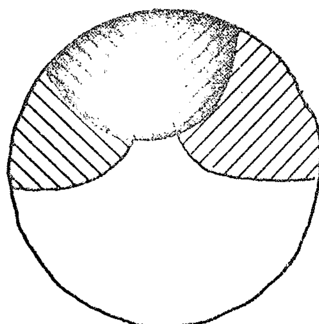
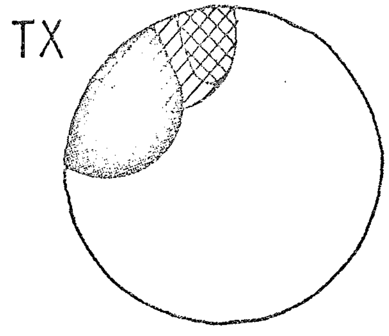


Figure 51.

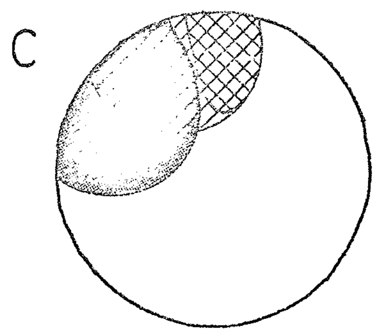
REACTION OF NUCLEAR PARTICLES TO DIFFERENT PREPARATION TECHNIQUES

LIVER

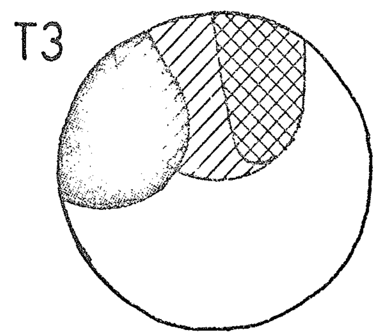
TX



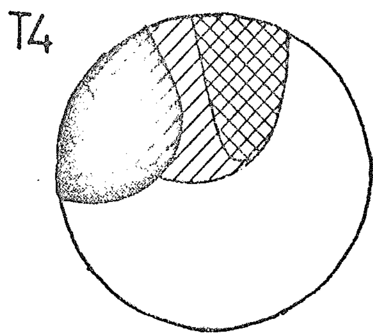
C



T3



T4

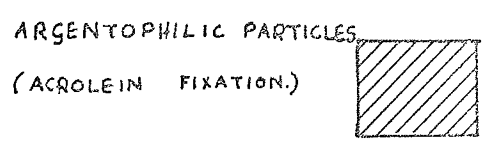


TENDENCY TOWARD INCREASED AGGREGATION OF PARTICLES.

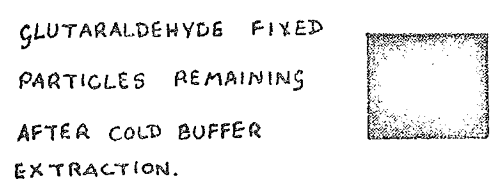
KEY.

TOTAL = THOSE PARTICLES VISUALISED AFTER ROUTINE PREPARATION.

ARGENTOPHILIC PARTICLES (ACROLEIN FIXATION.)



GLUTARALDEHYDE FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION.



ACROLEIN FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION.

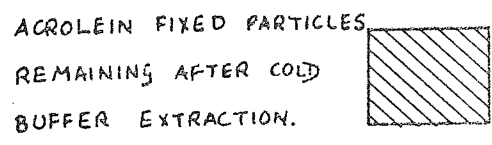
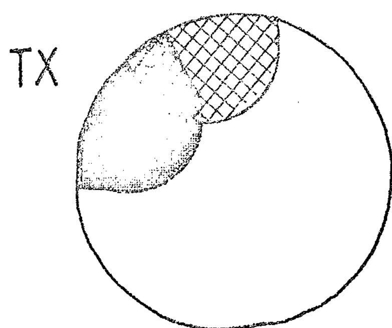


Figure 52.

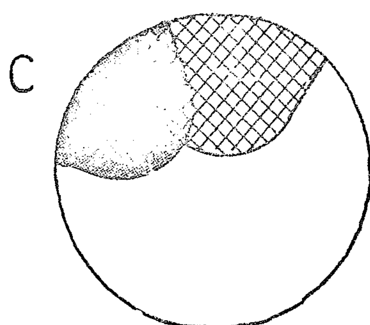
REACTION OF NUCLEAR PARTICLES TO DIFFERENT PREPARATION TECHNIQUES.

KIDNEY.

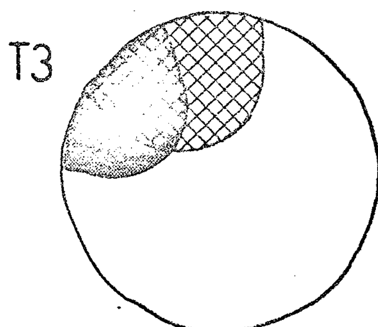
TX



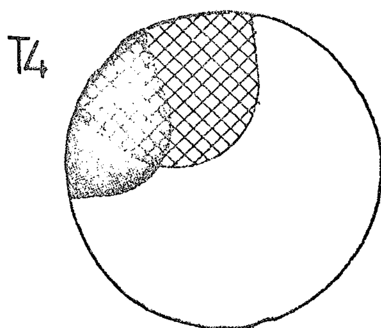
C



T3



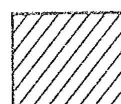
T4



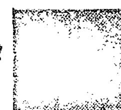
KEY.

TOTAL = THOSE PARTICLES VISUALISED AFTER ROUTINE PREPARATION.

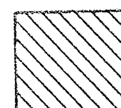
ARGENTOPHILIC PARTICLES (ACROLEIN FIXATION.)



GLUTARALDEHYDE FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION



ACROLEIN FIXED PARTICLES REMAINING AFTER COLD BUFFER EXTRACTION.



TENDENCY TOWARD INCREASED AGGREGATION OF PARTICLES.

There appeared to be an increasing tendency toward aggregation of apparently particulate components of the nucleoplasm of the nucleus after routine preparation of both liver and kidney cells. This effect was also noted after acrolein fixation and silver nitrate staining where an impression was obtained of a general increase in the amount of argentophilic material present, but this material did not appear to be ribonucleoprotein in composition.

The administration of thyroid hormones to the animals also appeared to affect the amount of material removed from the tissue blocks which had been stored for three months in cold buffer solutions. Glutaraldehyde appeared to be a better fixative than acrolein in that less material was removed after glutaraldehyde fixation than after acrolein fixation, but the same general pattern emerged. More dark staining material, as visualised by uranium and lead staining after glutaraldehyde and osmium fixation, remained associated with the nucleoli after hormone treatment in tissues from normal or thyroidectomised animals. More argentophilic material remained associated with the nucleoli of cells of hyperthyroid than of cells of euthyroid or hypothyroid animals after cold buffer extraction of acrolein fixed material.

Similarly, in the nucleoplasmic zones of the nucleus, although there was no setectable change in the amount of dark staining material visualised by uranium and lead staining of glutaraldehyde and osmium fixed tissue there was a greater amount of argentophilic material retained in tissues from hormone treated animals after cold buffer extraction of acrolein fixed tissues. In all cases there was no apparent change in the amount of material remaining in the nuclei after cold buffer storage of kidney tissue.

Thus there would appear to be present in nuclei of both liver and kidney cells, an apparently particulate component which is visualised by uranium and lead staining following glutaraldehyde and osmium fixation, which will precipitate silver from a silver nitrate

staining solution following acrolein fixation, and which is affected by the levels of thyroid hormones present in the animals. Not all of the particles present are susceptible to cold buffer extraction, or react to the staining solutions to the same extent.

The question of the identity of the particulate component now arises. There are two main possibilities. It may either represent ribosomal precurser particles produced in the nucleolus, before transfer across the nucleoplasm to the cytoplasm or it may represent profiles of chromatin fibrils cut transversley in thin section. It would be impossible to state definately which, if either, identity was the correct one due to the present state of understanding of nuclear fine structure, and to the fact that both structures would stain with lead and uranium salts and would precipitate silver from a silver nitrate staining solution after acrolein fixation; both deoxy- and ribonucleoproteins are argentophilic in this preparation proceedure. Removal of some constituent responsible for the staining reactions by cold buffer extraction could be expected to affect both components, although perhaps to differing extents.

However, it is possible to predict which might be a feasible explanation as is summarised in Figure 53 . It is unlikely that all the particles visualised in the electron microscope are either ribosomal precurser particles or profiles of chromatin strands. It is likely that most of the particles observed in the pars granulosa of the nucleolus are ribosomal precursers although some may represent profiles of chromatin strands of the nucleolus associated chromatin. It seems likely that at least some of the particles observed in the nucleoplasm represent profiles of chromatin strands whilst part represent ribosomal material of nucleolar origin migrating toward the cytoplasm. Examination of material digested with either ribonuclease or pepsin suggested that at least part of the pars granulosa was composed of ribonucleoprotein, however, the effect of the enzymic digestion was slight and the results

Figure 53.

Particle populations revealed by analysis of photographs of material prepared in different ways, by categories according to appearance.

Particle site	Probable Identity of particle	Susceptibility to cold buffer extraction	Reaction to silver nitrate.	Probable major constituent of particle.
NUCLEOLUS	Incomplete ribosomal precurser particles.	Particles which are more resistant to extraction in cold buffer.	Argentophilic particles	Ribonucleoprotein particles
	Complete ribosomal precurser particles	Particles which are less resistant to extraction in cold buffer.		
NUCLEOPLASMIC SITES OF THE NUCLEUS,	Ribosomal precurser particles of nucleolar origin, migrating across to the cytoplasm.	Particles less resistant to extraction by cold buffer.	Argentophilic particles	Ribonucleoprotein particles
	Chromatin strands with altered staining capacities as regards heavy metal stains.	Apparent 'particles' which are more resistant to extraction by cold buffer.	Non-Argentophilic particles.	Profiles of deoxyribonucleoprotein strands.

inconclusive. There was no evidence of a ribonucleoprotein component within the chromatin of the nucleoplasm.

Hormone action at the nuclear level could be expected to affect several aspects of nuclear morphology. Hormone induced depletion of a nucleolar pool of ribosomal precursers would be expected to be reflected in alterations in the amount of pars granulosa material associated with the nucleolus. Hormone induced synthesis of material in the nucleolus might give rise to alterations in the appearance of the nucleolus as a result of its changed functional state, with or without accumulation of material in a nucleolar pool, which would be seen as an alteration in the amount of material within the pars granulosa. Neither hormone induced alteration in the amount of material present in the pars granulosa, or nucleolar changes which might be expected to reflect a changed functional state were seen to take place after normal preparation proccedures, but were visualised after ribonuclease digestion, where there were significant changes in the appearance of the pars fibrosa. That little hormone influenced variation in the amount of pars granulosa material present, does not exclude the possibility that hormones do increase the rate of nucleolar synthesis. If thyroid hormones increase the rate of transfer of material to the cytoplasm as seems probable (Tata 1964, 1965 and 1967) then no accumulation of nucleolar material would be expected. However, an alteration in the susceptibility of nucleolar material to extraction by cold buffer and increase in the amount of argentophilic material present suggests an alteration in the composition of nucleolar constituents associated with the hormone activity. There is an increase in the amount of material resistant to extraction and incomplete might be expected to be more tightly bound to a tissue than complete particles. The increase in the amount of argentophilic material suggests an increase in the amount of nucleoprotein present, at least part of which appeared to be ribonucleoprotein.

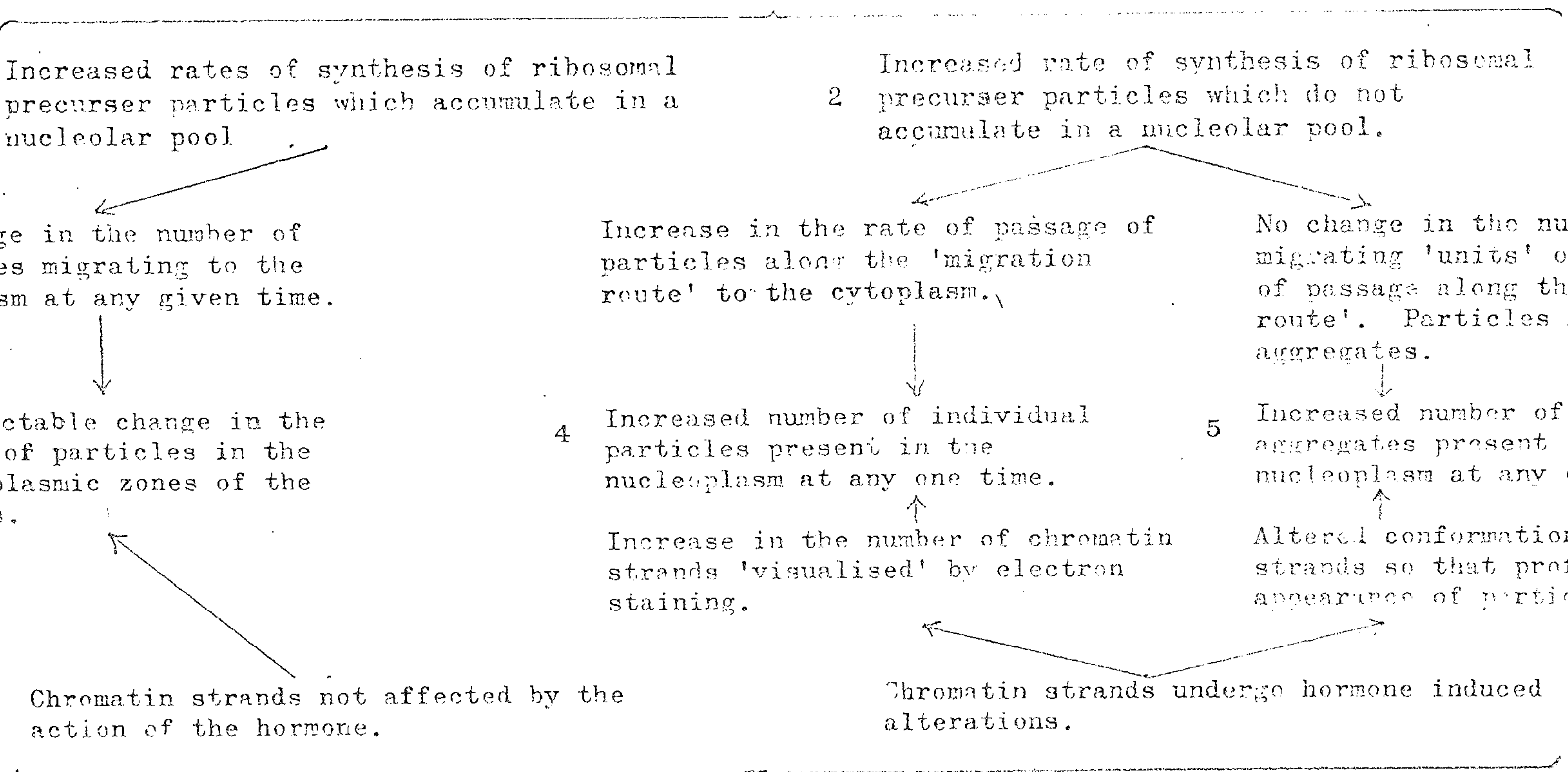
An increased rate of turnover of ribosomal material within the nucleolus would be reflected in an increase in the amount of ribosomal material migrating to the cytoplasm, whether as a result of the increased load of material pushing preformed material down a 'migration route', or as a direct result of the hormone action on a transfer mechanism. Increased rate of migration of particles could take place, either as an increase in the number of particles travelling at a constant rate, or as an increase in the speed at which particles move across the nucleoplasm, both of which would be reflected in an increased number of particles of nucleolar origin, present in the nucleoplasm at any one time. Particles may tend to migrate as aggregates rather than as individual particles which would be reflected in a tendency for particles present in the nucleoplasm to aggregate under the influence of thyroid hormones. However, some of the apparent aggregates of particles observed in the nucleoplasm are relatively large and it is difficult to imagine how any such aggregates could move through the network of fine fibrils which constitutes the 'skeleton' of the nucleoplasm.

Although it is possible that some of the aggregates represent migrating precurser particles it seems more likely that the tendency toward the formation of apparent particle aggregates under the influence of thyroid hormones reflects a hormone induced conformational change in the chromatin strands. This could either represent a change in the detailed structure of the fibrils which affects their reaction to different staining techniques and their visualisation in the electron microscope, or as a change in the arrangement of individual strands. A group of associated strands, closely apposed for some functional process or a complexly but loosely coiled strand cut in thin section would give the appearance of an aggregate of small particles. It is possible to predict what functional process, induced by hormone action this might represent.

Since no ribonucleoprotein material could be detected

Figure 54.

Possible effects hormone action at the nucleus might have upon nuclear morphology.



within the dark staining material using ribonuclease digestion, it would appear that the latter interpretation is the more likely. However this does not exclude the possibility that hormone action causes an increase in the amount of ribonucleoprotein material migrating across the nucleoplasm, if it is accepted that the total amount of ribonucleoprotein present is too small to be detectable, or that for some reason it is not accessible to digestion by ribonuclease. The possible effects of hormone action at the nuclear level are summarised in Figure 54 .

The differing influences thyroid hormone action appear to have upon nuclear morphology of liver and kidney cells can be attributed to the differing biochemical effects the hormones have been shown to have on the two tissues. Liver has been shown to be a major target organ of thyroid hormone action. As such thyroid hormones may be expected to have a significant effect upon at least some of the cellular processes reflected in changes in detailed morphology. Although thyroid hormones exert some influence, either directly or indirectly, upon renal function the kidney is not a major target organ for thyroid hormone action, and as such hormone influenced changes in nuclear morphology would not be expected to be as marked as those taking place in the liver.

Chapter 4.

The Effects of Thyroid Hormones upon the Appearance of the Nuclear Envelope.

There was considerable variation in the appearance of the nuclear envelope of both liver and kidney nuclei between the different experimental groups. The membrane elements of the nuclear envelopes of liver and kidney cells from normal animals were fairly easily resolved, and regular with a regular perinuclear space. Nucleopores were clearly distinguishable and fairly frequent. The nuclear envelopes from cells of both the liver and kidney of thyroidectomised animals appeared much 'crisper', were very regular, and the perinuclear space was reduced in width, The appearance of the nuclear envelope from both liver and kidney cells of hormone treated animals showed considerable variations. The membranes of the envelopes of both liver and kidney cells tended to be obscured by some material, and thus were difficult to resolve although the effect was more marked in the liver. The envelopes from kidney nuclei were more easily resolved but tended to be puckered and indented. The two effects were not restricted in the two tissues, kidney nuclei showed some degree of obsuration and liver nuclei, where their membranes could be discerned also tended to be more irregular than those from normal animals.

Various aspects of nuclear membrane morphology were thus examined in greater detail in order to establish fully the effects of thyroid hormone administration. The aspects studied in further detail are as follows:-

1. The frequency of nuclear pores in profiles of the nuclear envelope.

2. The obscuration of the nuclear envelope.

3. The development of irregularities in the nuclear envelope.

The frequency of nucleopores in profiles of the nuclear envelope.

A visual impression was gained of a variation in the number of nuclear pores per nuclear profile, there being, apparently, more pores per profile in the hormone treated tissues than in those from normal or hypothyroid animals. As a result an attempt was made to gain some quantitative estimation of the numbers of pores per profile in each tissue and treatment. The results are shown below in Table 55.

Table 55 .

The frequency of nuclear pores in nuclear profiles per hormone treatment.

	TX	N	T3	T4
Liver	16.67 $\pm$2.21	12.50 $\pm$1.80	24.67 $\pm$5.40	14.00 $\pm$3.50
Kidney	10.00 $\pm$1.20	10.10 $\pm$0.94	13.28 $\pm$1.41	8.86 $\pm$0.77

Table 56 .

Statistical significance in the frequency of nuclear pores per profile with thyroid hormone treatment.

Liver

	TX	N	T3	T4
TX				
N	$P > 0.1$			
T3	$P > 0.1$	$P < 0.02$		
T4	$P > 0.1$	$P > 0.1$	$P > 0.1$	

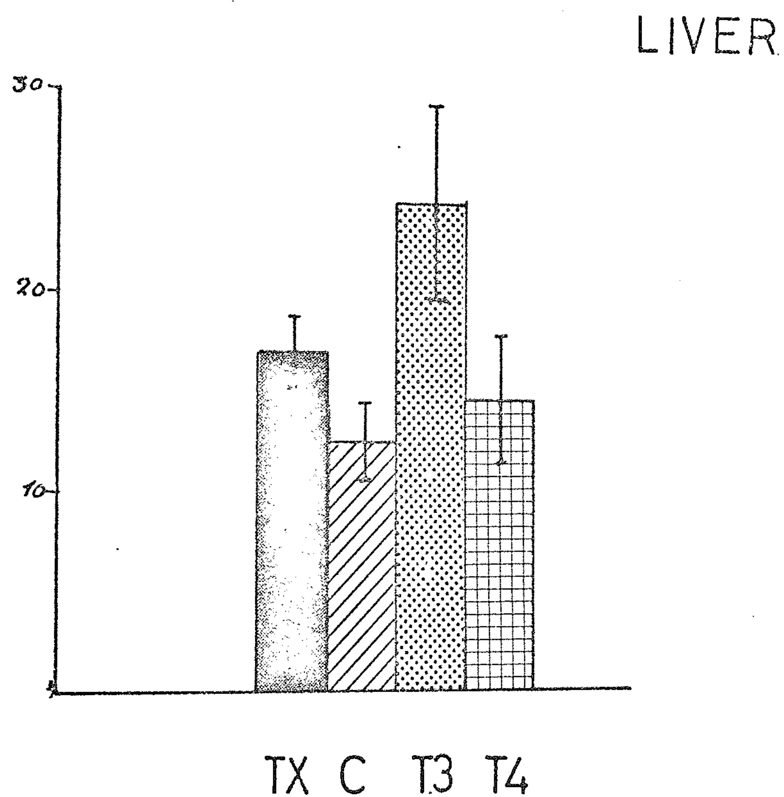
Kidney

	TX	N	T3	T4
TX				
N	$P > 0.1$			
T3	$P > 0.1$	$P < 0.01$		
T4	$P > 0.1$	$P < 0.01$	$P < 0.01$	

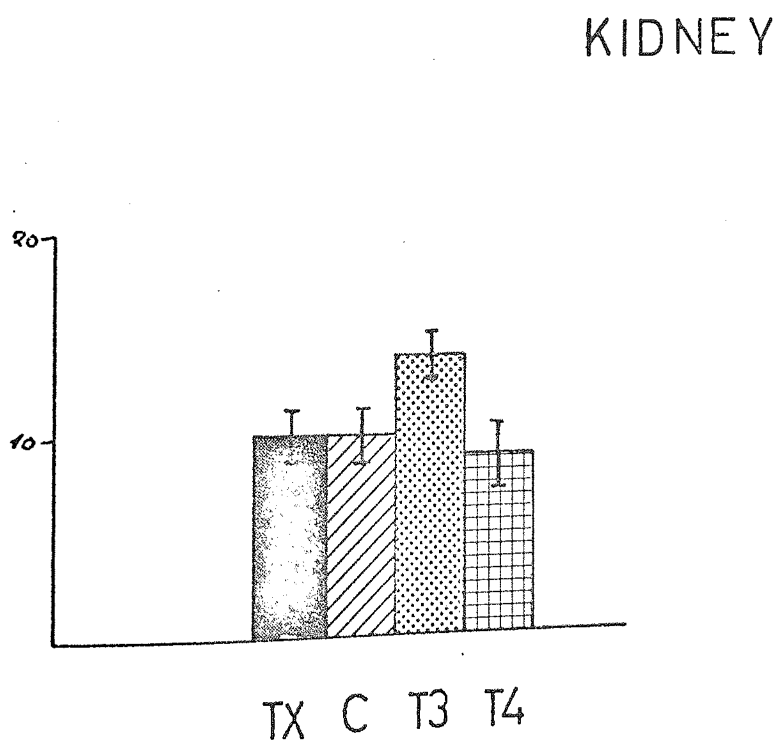
Figure 55

NUCLEO-PORES PER NUCLEAR SECTION

LIVER



KIDNEY



Inspite of difficulties experienced in estimating the number of pores present in the nuclear profiles in upto 20% of the nuclei from both liver and kidney after hormone treatment due to the presence of obscuring material. It is possible that the nuclei which could be counted, were those relatively unaffected by the hormone treatment, a pattern of change associated with hormone influence which reflected the pattern of change noted in Chapter 1, upon glycogen and mitochondria in the liver. There were no differences in the numbers of nuclear pores per nuclear section from either thyroidectomised animals or thyroxine treated animals from both the liver and the kidney. However there was a significant increase in the number of pores per nuclear profiles in both liver and kidney after triiodothyronine treatment. This effect was particularly marked in the liver, as is shown in Figure 55 .

The obscuration of the nuclear envelope.

The most obvious effect of thyroid hormone administration was the obscuration of the nuclear envelope of the liver, and to a lesser extent the kidney. The majority of nuclei from the livers of thyroid-ectomised or normal animals were surrounded by a clear double membrane structure, as is shown in Plates 67 and 68 . The double membrane structure was particularly clear if lead salts were used after uranium in the section staining proceedure, as the comparison of Plates 67 and 68 shows.

A considerable proportion of the nuclei from hormone treated animals were bounded by indistinct nuclear envelopes of which those shown in Plates 69 and 70 are characteristic. Instead of the double membrane structure being clear and obvious it is obscured in some way. In some cases, as is shown, for instance by the nuclear envelope in Plate 70 , the perinuclear space was of relatively high electron density and the nuclear membranes appeared diffused. In other

cases, especially after the use of lead as a post-stain, the double membrane structure could be discerned through a halo of material of increased electron density, as is shown in Plate 69 .

This obscuring of the nuclear envelope was also noted in the kidney, where however the effect was less obvious, the nuclear membranes showing less obvious deposits of obscuring material. The nuclear envelopes bounding kidney nuclei from thyroidectomised or normal animals resembled those from liver nuclei of similarly treated animals in being distinct and obviously composed of a double membrane as is shown in Plate 71 .

A considerable proportion of nuclear envelopes from hormone treated cells again showed obscuring deposits, as is shown in Plates 72 and 73 . The obscuration tended to be less intense in the kidney, although the double membrane structure was frequently obliterated, and was often less extensive. Many of the kidney nuclei showed obscuration around only part of their periphery, whereas liver nuclei tended to be obscured around their whole circumference.

In order to establish a pattern of development of the deposits of obscuring material with the normonal influence, counts were made of nuclei showing changed and unchanged nuclear envelopes. Counts were made of each nucleus of three grid squares of the copper mounting grid. Grid squares were selected at low magnifications on grounds of gross technical quality. Nuclear envelopes distinguishable as a distinct double membrane structure at magnifications of x10,000 were counted as unchanged. Where the nuclear membranes could not be resolved at x10,000 were counted as changed even if the individual elements could be distinguished at higher magnifications. Where only part of the nuclear envelope showed deposits of obscuring material it was counted as unchanged if less than half its circumference was obscured and changed if more than half of its circumference showed deposits of obscuring material. Initially counts were made on tissue which had been routinely

prepared using glutaraldehyde and osmium fixation followed by uranium section staining. The results of these counts are shown in Table 57 and Figure 56 , and are expressed as unchanged nuclei as a percentage of the total nuclei counted.

Table 57 .

Percentage nuclei remaining unobscured after thyroid hormone treatment. (Material section stained with uranium only)

	TX	N	T3	T4
Liver	80.15%	89.69%	56.00%	52.46%
	±3.90	±3.59	±7.01	±3.59
Kidney	93.57%	91.24%	63.68%	66.52%
	±1.84	±1.34	±1.83	±2.25

Table 58 .

Statistical significance of variations in the appearance of the nuclear envelope with thyroid hormone treatment, after uranium staining.

Liver

	TX	N	T3	T4
TX				
N	$P < 0.01$			
T3	$P < 0.02$	$P < 0.01$		
T4	$P < 0.001$	$P < 0.001$	$P > 0.1$	

Kidney

	TX	N	T3	T4
TX				
N	$P > 0.1$			
T3	$P < 0.001$	$P < 0.001$		
T4	$P < 0.001$	$P < 0.001$	$P > 0.1$	

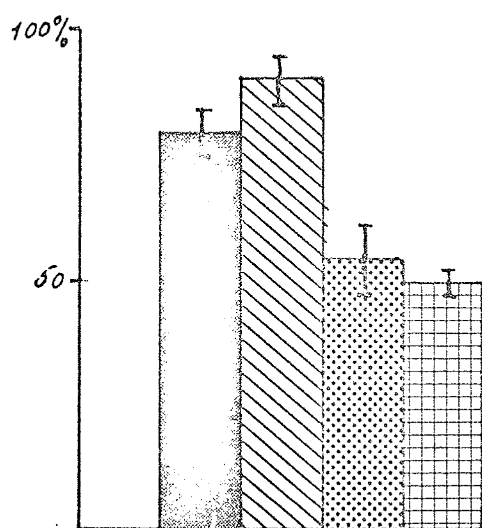
As is shown above there was a significant difference between the number of unchanged nuclei from normal or thyroidectomised animals compared to nuclei from hormone treated animals in both the

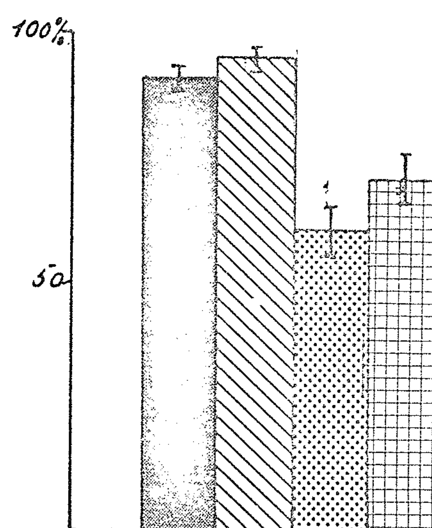
Figure 56.

% NUCLEAR ENVELOPES NOT SHOWING OBSCURING MATERIAL.

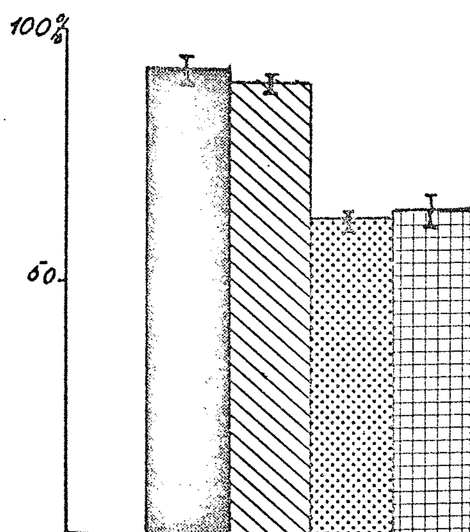
LIVER KIDNEY

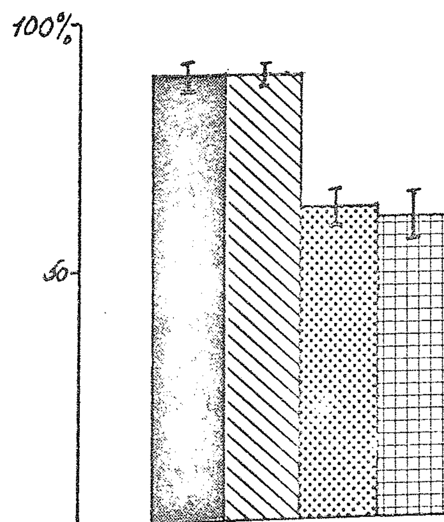
Section stain = uranyl acetate.





Section stain = uranyl acetate & lead tartrate.





TX C T3 T4

TX C T3 T4

liver and kidney.

Later, examination was made of material post-stained with lead in an attempt to gain better resolution of the membrane elements of the obscured nuclear envelopes. It was found that it was possible in a number of cases, to distinguish elements of the nuclear envelope through the obscuring deposits, thus the change in the nuclear envelope appearance connected with the hormone treatment was due to deposits of some obscuring material rather than to a breakdown in the membrane structure.

Nuclei were again scored according to the same criteria and the results are shown in Table 59 and Figure 56. Inspite of being better able to resolve the elements of the nuclear envelope, the results show the same statistical significance.

Table 59 .

Percentage nuclei remaining unobscured after thyroid hormone treatment. (Material stained with uranium and lead).

	TX	N	T3	T4
Liver	89.63% ±2.15	94.79% ±1.45	57.08% ±4.38	67.09% ±4.62
Kidney	91.35% ±2.27	92.32% ±1.11	63.90% ±3.38	59.38% ±4.48

That there is no significant difference in the numbers of nuclei with obscured nuclear envelopes from thyroxine and triiodothyronine treated tissues after either staining technique is not surprising since both hormones act in a similar way, differing only in their time course, and since both had been administered in such a way as to minimise differences arising from this. However, the significant

Table 60 .

Statistical significance of variations in the appearance of the nuclear envelope with thyriod hormone treatment, after staining with uranium and lead.

Liver

	TX	N	T3	T4
TX				
N	$P < 0.02$			
T3	$P < 0.001$	$P < 0.001$		
T4	$P < 0.001$	$P < 0.001$	$P > 0.1$	

Kidney

	TX	N	T3	T4
TX				
N	$P < 0.1$			
T3	$P < 0.001$	$P < 0.001$		
T4	$P < 0.001$	$P < 0.001$	$P > 0.1$	

difference between the numbers of obscured nuclear envelopes from the livers of thyroidectomised and normal animals requires further explanation. It must be pointed out that although the nuclei of tissues from thyroidectomised animals were counted as obscured, their nuclear membranes often being not readily distinguishable according to the selected criteria, they did not show accumulations of obscuring material when examined at magnifications above x10,000. The reason they were not readily resolved at magnifications of x10,000 being that the nuclear membranes tended to be very regular, with a narrow perinuclear space narrower than those of nuclear envelopes from normal animals.

Material was also examined after fixation in either glutaraldehyde or osmium in order to establish that the obscuring of the nuclear envelope was not due to arefacts arising out of fixation. Counts were again made of changes in the appearance of the nuclear envelope and the results are shown in Table 61 .

The appearance of nuclear membranes after glutaraldehyde fixation are shown in Plates 76 and 77 . Again the same pattern of

Table 61 .

Percentage nuclei with unobscured nuclear envelopes after variations in the fixative proceedure.

	Liver		Kidney	
	N	T3	N	T3
Glutaraldehyde	92.00% ±1.52	64.20% ±1.89	91.36% ±2.47	68.93% ±2.39
Osmium	-	-	-	-

change emerges with glutaraldehyde used as the only fixative.

Unfortunately, it was found impossible to make meaningful counts of changes in the appearance of the nuclear envelope after osmium fixation because of the general quality of osmium fixed material, as is shown in Plates 74 and 75 . The even contrast resulting from the fixation proceedure made it difficult to distinguish the nuclear envelope, and the nuclear membranes appeared so regular and closely apposed that the individual elements were often difficult to distinguish at x20,000 and it was found impossible to separate them according to the criterion of resolvability at x10,000.

Thus although the phenomenon of nuclear membrane obscuration may be a result of dammage during processing of material for examination, it seems it does reflect a process taking place in the cell under the influence of thyroid hormones. It is possible that the hormones may affect the way that the tissue reacts to fixation proceedures but it is more likely that it is a reflection of a build up of material around the nuclear envelope or an alteration in the membrane composition produced by the hormone influence.

Identification of obscuring material: An attempt was made to identify

the material causing the obscuring of the nuclear envelope. Tissues have been examined after digestion with either ribonuclease or pepsin.

Nuclear membranes of 90% of nuclei from all treatments of both liver and kidney after both pepsin and ribonuclease digestion resembled those of unobscured nuclei from routinely prepared material. Plate 78 shows a portion of a nuclear envelope from a cell of a proximal convoluted tubule which was characteristic of those of both liver and kidney which had been digested with ribonuclease. Both elements of the nuclear envelope were clearly visible and the perinuclear space not obscured by electron dense material.

Plate 79 shows the nuclear envelope of a liver cell which had been treated with pepsin and which shows the characteristic appearance of material after pepsin digestion, where again the nuclear membranes were clearly visible and the perinuclear space not obscured. Although a small proportion of nuclei of material so treated showed less clear nuclear membranes many of the nuclear membranes showed a reduction in electron density compared to those of material which had been routinely prepared.

There appeared to be no difference in the appearance of nuclear membranes from any of the treatments after either ribonuclease or pepsin digestion. Comparison of counts made of obscured nuclear envelopes from each hormone treatment after digestion with both enzymes showed that there was no statistical difference between the groups, as is shown in Table 63 .

Statistical comparison of the data in Table 62 with that obtained from material prepared routinely with glutaraldehyde fixation and uranium and lead staining showed that there was no significant difference between the counts of normal animals from either treatment, but there was a significant difference between the counts obtained from the hormone treated animals after both enzyme digestion techniques and the routinely prepared material, as is shown in Table 64 . Comparison

Table 62 .

Percentage nuclei with unobscured nuclear envelopes after variations in thyroid hormone levels and enzyme digestion.

	N	T3	T4
Ribonuclease digestion.			
Liver	90.66%	89.98%	91.41%
	±1.09	±1.56	±1.08
Kidney	95.96%	94.84%	94.40%
	±2.72	±2.25	±2.46
Pepsin digestion			
Liver	94.31%	92.32%	88.80%
	±2.63	±1.02	±0.62
Kidney	91.86%	91.98%	96.38%
	±1.45	±1.76	±4.70

Table 63 .

Statistical significance of variations in the appearance of the nuclear envelope with thyroid hormone treatment, after enzyme digestion.

Liver

	TX	N	T3	T4
TX				
N				
T3		P > 0.1		
T4		P > 0.1	P>0.1	

Kidney

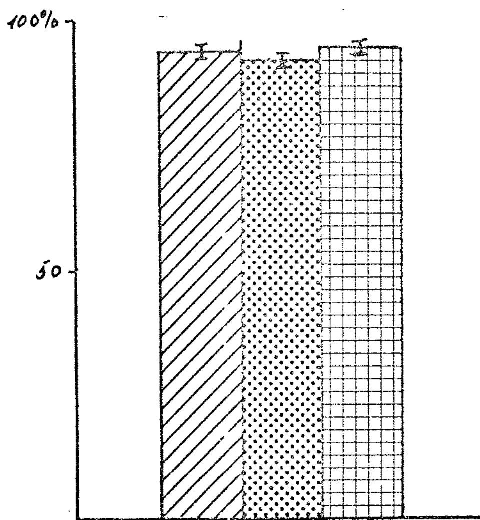
	TX	N	T3	T4
TX				
N				
T3		P > 0.1		
T4		P > 0.1	P < 0.01	

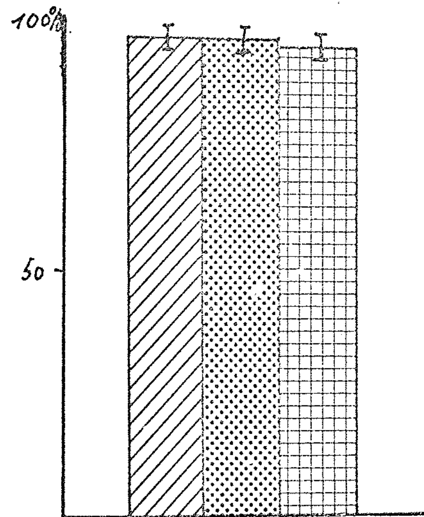
Figure 57.

% NUCLEAR ENVELOPES NOT SHOWING OBSCURING MATERIAL.

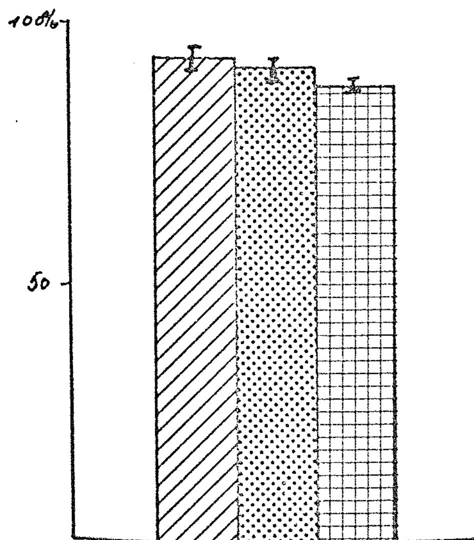
LIVER KIDNEY

Ribonuclease digested material.





Pepsin digested material.



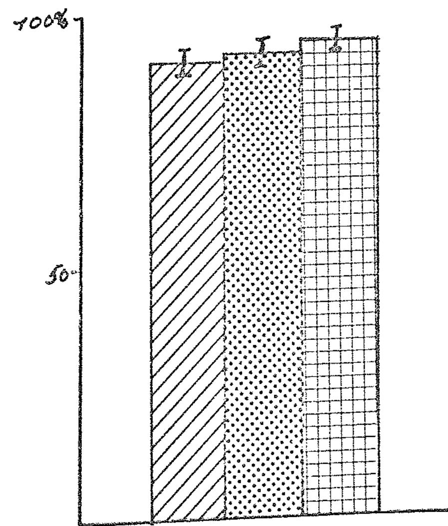


Table 64 .

Statistical comparison of the results obtained from ribonuclease and pepsin digested material with those obtained from normally prepared material.

	N x N	T3 x T3	T4 x T4
Ribonuclease digestion x routine preparation.			
Liver	P > 0.1	P < 0.001	P < 0.001
Kidney	P < 0.1	P < 0.001	P < 0.001
Pepsin digestion x routine preparation			
Liver	P > 0.1	P < 0.001	P < 0.01
Kidney	P > 0.1	P < 0.001	P < 0.001
Routine preparation x Control preparation for enzyme digestion .			
Liver	P < 0.05		
Kidney	P > 0.1		

of routinely prepared material with the material prepared as a control for the enzyme digestions showed that there was no significant difference so that it can be concluded that the differences oberved in the enzyme digested material were due to the action of the enzyme and not due to the incubation in the buffer solutions. Thus it would appear that the material obscuring nuclear envelopes after thyroid hormone treatment is digestible by both ribonuclease and pepsin and would thus seem to be identified as of ribonucleoprotein composition.

The development of irregularities in the nuclear envelope.

Although the nuclear envelopes of kidney nuclei showed obscuring deposits associated ith the thyroid hormone treatment, a more obvious effect of the thyroid hormones was upon the regularity of the membrane constituents of the nuclear envelope.

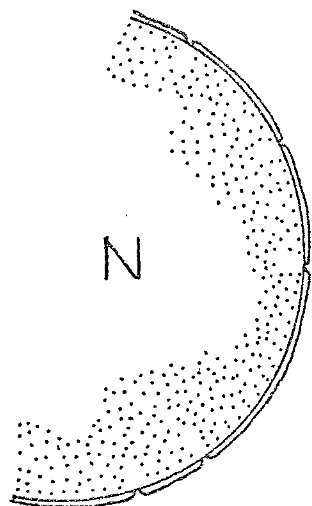
Membranes of nuclei from the kidneys of thyroidectomised animals were very regular in profile and the perinuclear space was even in width around its whole periphery, as is shown in Plate 80 . The nuclear envelopes from normal kidney cells, of which Plate 81 is an example, showed some degree of irregularity in the appearance of the membrane constituents, width variations in the width across the perinuclear space. However, after hormone treatment the nuclear membranes were often grossly irregular and often widely separated, with considerable variations in the width across the perinuclear space. This was particlularly marked in the cells of the proximal convoluted tubule, as is shown in Plates 82 and 83 .

This effect could also be seen in the liver, although it was less pronounded, and to some extent masked by the occurrence of obscuring material associated with the nuclear envelope. However variations in width of the perinuclear space and irregularities in the profiles of the outer membrane element of the nuclear envelope could often be distinguished in material stained with lead salts. Plate 34 shows the nuclear envelope of a liver cell of a normal animal where the constituent membranes are very regular in profile. Plates 85 and 86 show the nuclear envelopes of liver cells from hormone treated animals where it is possible to see marked irregularities in the profiles of the nuclear envelope, when compared to that seen in Plate 84 .

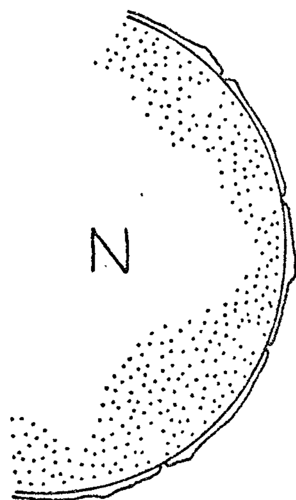
In order to gain some quantitative indication of the variation in regularity and separation of the membrane elements of the nuclear envelope, measurements were made at 10 randomly selected points

Figure 58.

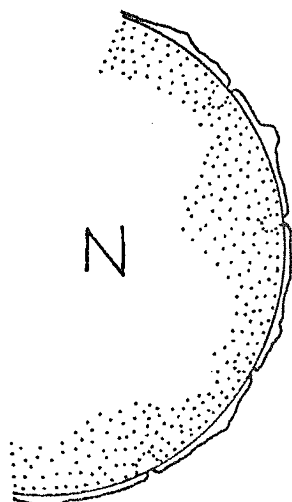
NUCLEAR MEMBRANE CHANGES.



THYROIDECTORISED ANIMALS.



CONTROL ANIMALS.



HORMONE TREATED ANIMALS.

around the nuclear envelope of 10 randomly selected nuclei from each experimental group. Measurements were corrected for magnification using the following formula:-

$$\frac{x \times 10^7}{M} = y\text{Å}$$

where x = the measured length on a print.

M = the total magnification of the print.

y = the un-magnified distance in Å.

All measurements were made on material fixed in glutaraldehyde and osmium and double stained with uranium and lead. The results are shown in Table 65 .

Table 65 .

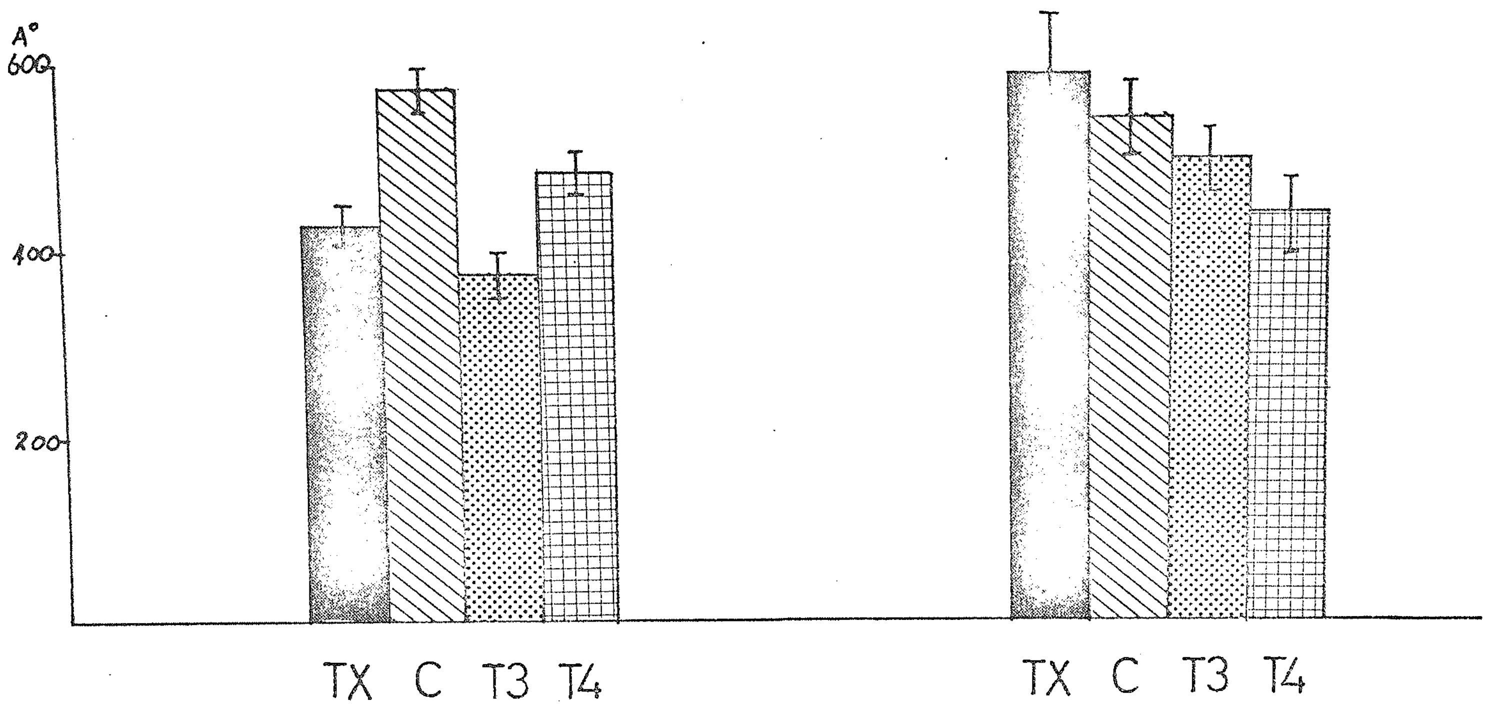
The variation in the width across the nuclear envelope after thyroid hormone treatment. (Measurements in Å).

	TX	N	T3	T4
Liver	424.34 ±2.16	573.47 ±2.31	384.42 ±2.10	497.8 ±2.94
Kidney	592.51 ±5.61	547.98 ±2.91	500.69 ±2.94	446.06 ±3.66

Although the results of each experimental group of the liver are significantly different there is no overall pattern which appears meaningful in terms of the influence of thyroid hormone action. This may be to some extent explained by the extreme difficulty experienced in making accurate measurements across the nuclear membranes from cells of hormone treated animals. It is possible , that in the case of the liver, the sampling of nuclei and of points of measurement around the nuclear envelopes, was not sufficiently random for the full pattern of change to emerge, due to the complication of obscuring

Figure 59.

WIDTH ACROSS THE NUCLEAR ENVELOPE.



material around a significant proportion of the nuclei.

Table 66 .

Statistical significance of variations in the width of nuclear envelopes after hormone treatment.

Liver	TX	N	T3	T4
TX				
N	$P < 0.001$			
T3	$P < 0.001$	$P < 0.001$		
T4	$P < 0.001$	$P < 0.01$	$P < 0.001$	

Kidney	TX	N	T3	T4
TX				
N	$P < 0.001$			
T3	$P < 0.01$	$P > 0.1$		
T4	$P < 0.001$	$P < 0.05$	$P > 0.1$	

In the case of the kidney there is a regular pattern of variation with the different experimental groups, which is however, the reverse of the result expected from the initial observation. The mean distance across the perinuclear space being reduced with the hormone treatment rather than increased. This result would tend to through doubt on the method of measurement rather than upon the existence of the variation in width across the nuclear envelope with thyroid hormone treatment.

Vesiculation of the nuclear envelope.

Closer examination of the nuclear envelopes of hormone treated liver and kidney cells, revealed that the puckering of the outer element was associated with the production of vesicles. These vesicles were of two types, small single membrane bound electron transparent vesicles were found in both the liver and the kidney. Larger,

vesicles, bounded by a double membrane and frequently containing granular material tended to involve both the elements of the nuclear envelope. These were restricted to the nuclei of the kidney.

The development of small nuclear membrane vesicles; The outer nuclear membrane was frequently extended into finger like projections and pockets which pinched off to form single membrane bound vesicles, with the same relative contrast as the nuclear envelope, the lumen resembling the perinuclear space in general contrast and electron density. No evidence was found that these vesicle profiles represented sections of microtubules or lamellae derived from the nuclear envelope. The vesicles were always small, being between 500 and 1200Å in diameter and rarely, up to 1500Å in diameter.

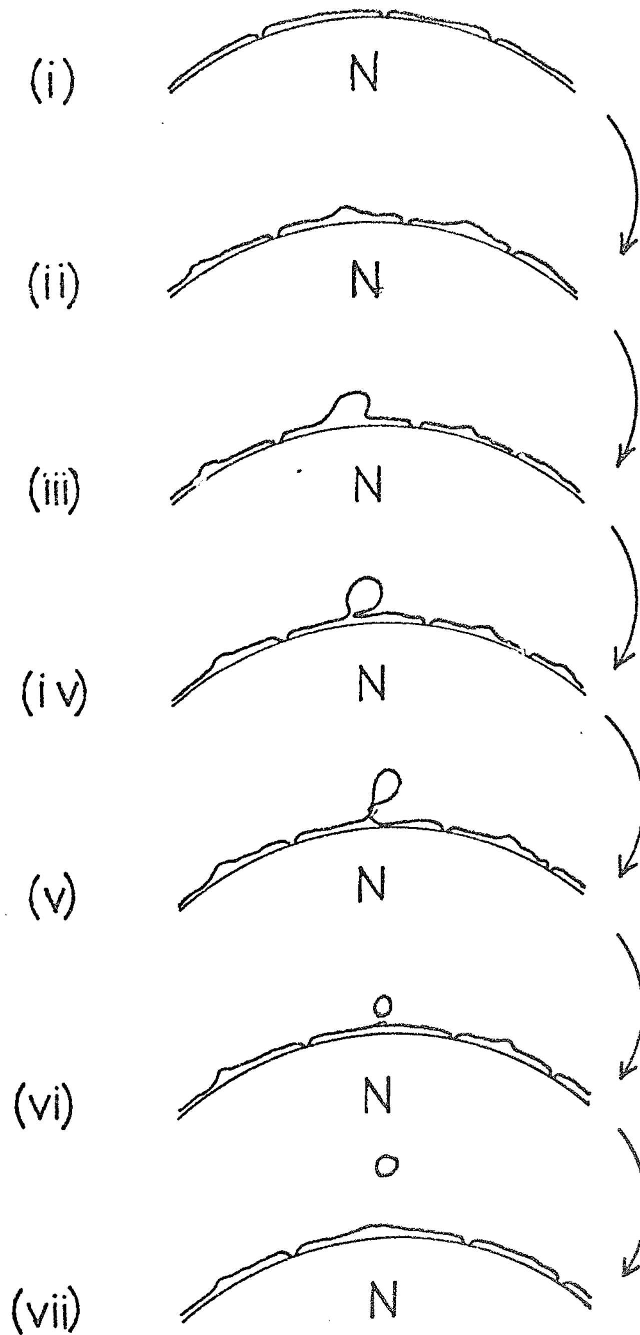
Figure 60 represents a hypothetical series of developmental stages of a small vesicle from the outer element of the nuclear envelope. Profiles of vesicles representing the stages shown were observed in the material examined, suggesting that the series shown does in fact occurr naturally.

Stage (i) in Figure 60 represents the regular appearance of the nuclear envelope of normal or thyroidectomised animals, shown in Plates 81 , 80 and 84 . Stages (ii) and (iii) represent the undulations and outpushings of the outer element of the nuclear envelope of many of the nuclei of hormone treated tissues, Such nuclear membranes being shown in Plates 82 and 83 . Stages (iv) and (v) represent the pinching off of a protrusion of the outer membrane to form a vesicle, and can be seen in Plates 87 and 88 . Stages(vi) and (vii) representing finallly separated vesicles can be seen in Plates 89 and 82 .

Small vesicles were obvious in the kidney where the nuclear membranes were clearly distinguishable, but were more difficult to see in the liver. However, they were obvious around the nuclear

Figure 60.

ORIGIN OF MEMBRANE VESICLES.



envelopes of those liver cells which were not obscured by the occurence of electron dense material, and they were frequently distinguishable through the deposits of obscuring material of other nuclei especially in material post stained with lead salts. Plates 90 and 91 show vesicles associated with the nuclear envelopes of liver cells, which are distinguishable through the obscuring material.

The relationships of the small vesicles to cytoplasmic structures was not obvious. There was no apparent association between the small vesicles and mitochondria or the Golgi apparatus but there was an occasional juxtaposition of points of vesicle production with lamellae of the endoplasmic reticulum. Small chains of vesicles were found produced from the nuclear envelope of kidney cells which were associated with the endoplasmic reticulum. In the liver lamellae of the endoplasmic reticulum often occur in parallel layers concentric with the nuclear envelope, and rows of vesicles were often found between the innermost lamella of the endoplasmic reticulum and the nuclear envelope. Vesicles associated with the endoplasmic reticulum were often flattened suggesting conversion into lamellae of the endoplasmic reticulum, but no evidence was found of fusion of vesicles with constituents of the endoplasmic reticulum.

There are, however, difficulties in assessing the relationships of the vesicles with other cytoplasmic structures, partly as a result of their size and partly because of their simple structure. One vesicle is of such dimensions as to be included within the thickness of one thin section so that examination of serial sections is impossible. Their simple structure makes it impossible to identify one vesicle profiles from one section to another, so that interpretation of structure must rely on profile shapes rather than upon tracing profiles through serial sections. However, no evidence has been found which would suggest that profiles represent anything other than small vesicles.

Again their simple structure makes it impossible to

equate vesicle profiles occurring any distance away from a nucleus with those of obvious nuclear membrane origin. So that it is impossible to ascertain the fate of such vesicles if they move out into the cytoplasm. However, small vesicles were found fairly frequently in the general cytoplasm,

The frequency with which small vesicles occurred in association with the nuclear membrane appeared to vary between the experimental groups, and inorder to establish a pattern of change, counts were made of the frequencies of the vesicles. Counts were made only of those vesicles occurring in association with the nuclear envelope. This was assessed as those vesicles occurring within four of their own diameters of the outer membrane element of the nuclear envelope. Counts were made of vesicles in the following groups:.

(i) The total numbers of vesicles occurring within 4 of their own diamters of the nuclear envelope.

(ii) Those vesicles occurring within 2 diameters of the nuclear envelope.

(iii) Those vesicles occurring between 2 and 3 diameters from the nuclear envelope.

(iv) Those vesicles occurring between 3 and 4 diameters from the nuclear envelope.

The results are shown in Table 67 , and in Figures 61 and 62 , and the results of a statistical comparison of the data is shown in Table 68.

There is a trend towards greater vesicle production from the outer membrane of the nuclear envelope of cells of hormone treated animals, both from the liver and the kidney. However, there was a statistical significance only when the counts of hormone treated animals were compared with those of the thyroidectomised animals, which is probably accounted for by the small number of nuclei counted.

Table 67 .

The distribution of single membrane vesicles in relation to the nuclear envelope after the influence of thyroid hormones.

(i) The total numbers of vesicles occurring within 4 of their own diameters of the nuclear envelope.

(ii) Those vesicles occurring within 2 diameters of the nuclear envelope.

(iii) Those vesicles occurring between 2 and 3 diameters of the nuclear envelope.

(iv) Those vesicles occurring between 3 and 4 diameters of the nuclear envelope.

		TX	N	T3	T4
Liver	(i)	11.05 ±1.41	12.08 ±1.65	22.71 ±6.28	19.67 ±1.96
	(ii)	6.32 ±0.98	7.83 ±0.90	8.07 ±1.51	16.46 ±7.95
	(iii)	3.44 ±0.59	3.75 ±1.19	8.08 ±4.31	5.14 ±0.72
	(iv)	2.62 ±0.47	2.20 ±0.79	4.53 ±0.72	4.50 ±1.96
Kidney	(i)	10.83 ±4.88	21.54 ±3.15	30.50 ±5.50	27.00 ±4.08
	(ii)	9.00 ±4.32	13.00 ±2.77	20.00 ±3.00	15.83 ±2.65
	(iii)	2.50 ±0.65	4.00 ±0.60	6.00 ±1.00	22,50 ±8.87
	(iv)	3.33 ±0.34	2.75 ±0.49	4.50 ±2.50	7.00 ±1.59

Figure 61.

DISTRIBUTION OF SINGLE MEMBRANE VESICLES IN RELATION TO THE NUCLEAR ENVELOPE IN THE LIVER.

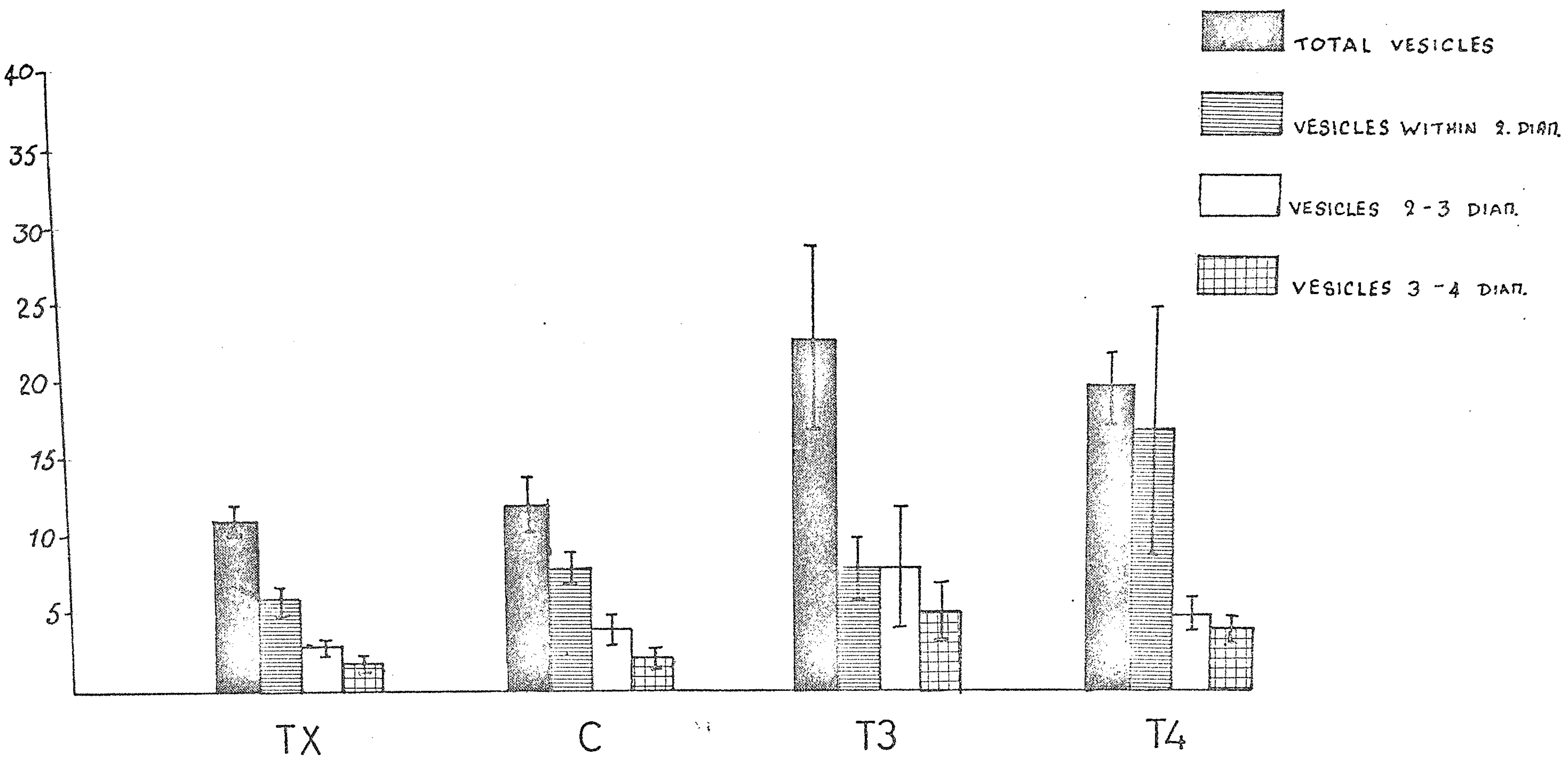


Figure 62.

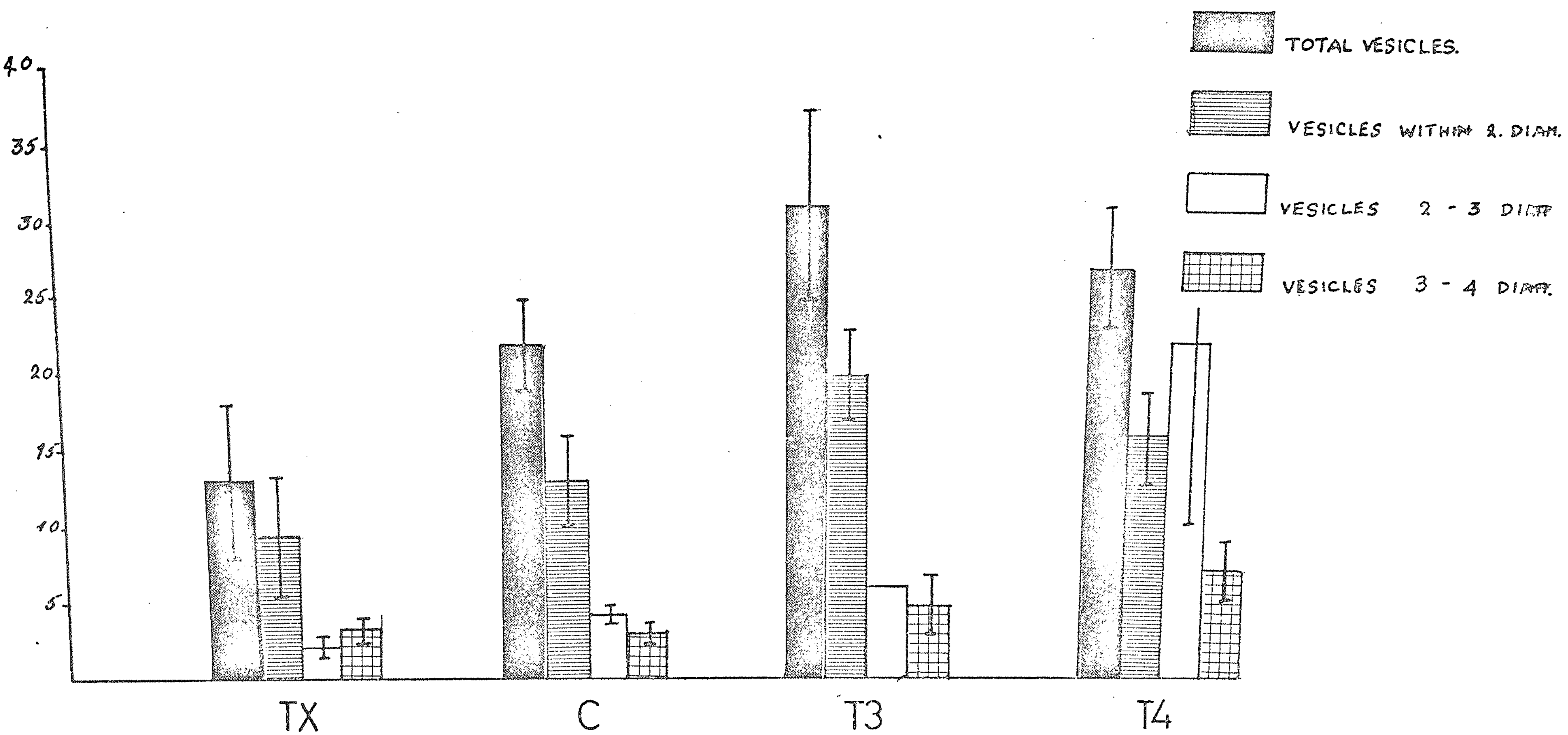


Table 68 .

Statistical significance of the variations in numbers of vesicles occurring within 4 diameters of the nuclear envelope with thyroid hormone treatment.

Liver

	TX	N	T3	T4
TX				
N	$P > 0.1$			
T3	$P < 0.05$	$P > 0.1$		
T4	$P < 0.05$	$P > 0.1$	$P > 0.1$	

Kidney

	TX	N	T3	T4
TX				
N	$P > 0.1$			
T3	$P > 0.1$	$P > 0.1$		
T4	$P < 0.01$	$P < 0.02$	$P > 0.1$	

The development of large nuclear membrane vesicles: Production of large vesicles from the nuclear envelope was almost completely restricted to the kidney and to the nuclei of cells of the proximal convoluted tubule. Both elements of the nuclear envelope were involved in the production of the large vesicles, and granular material resembling karyoplasm was frequently included within the inner constituent membrane. The large membrane vesicles were almost always irregular in shape, with the membrane elements widely separated, and the included material poorly defined.

A complete developmental series, such as that postulated in Figure 63 , could be traced from an outpushing of the outer element of the nuclear envelope to a well developed blister containing a membrane bound mass of karyoplasm, frequently within different regions of the one section. The first stage in the development of a large vesicle, as shown in stages (ii) to (iv) in Figure 63 appears to be either the production of a blister from the outer element of the nuclear envelope, or as an outpushing of both nuclear membranes, which separate later,

Figure 63.

ORIGIN OF GRANULE-CONTAINING VESICLES.

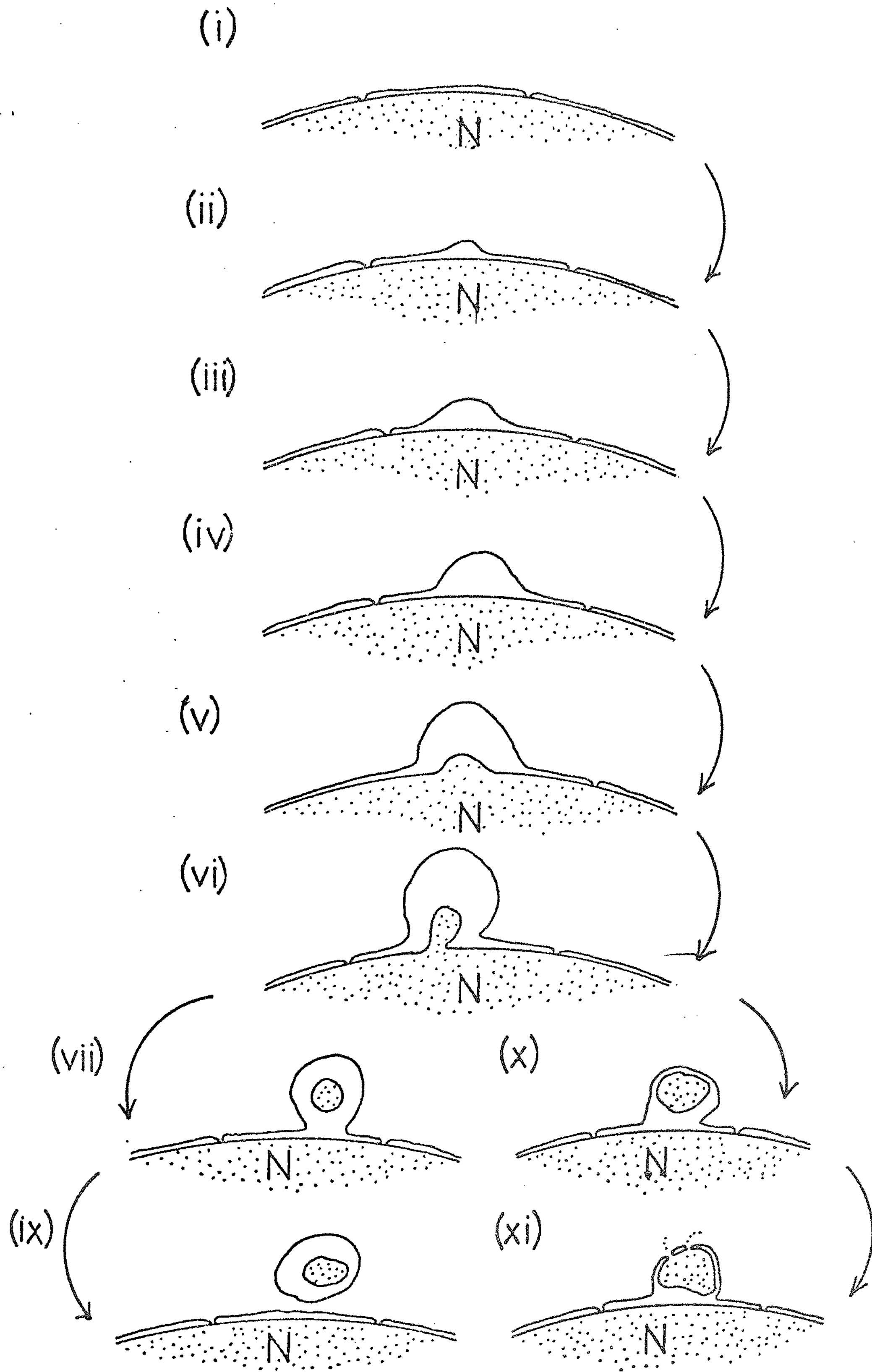
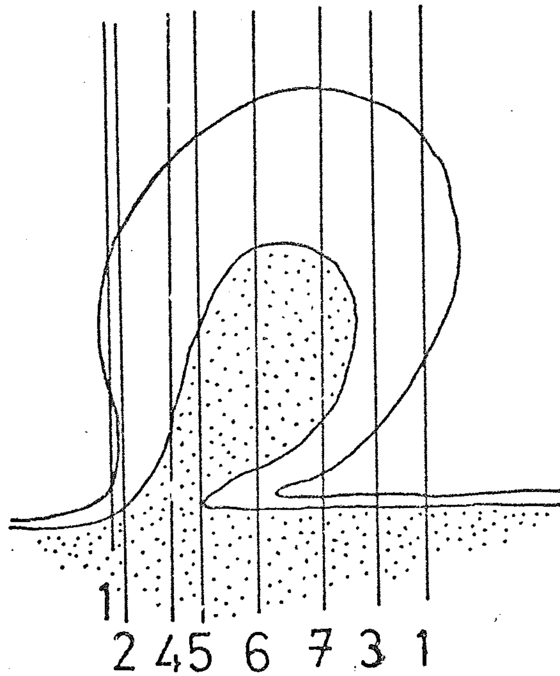
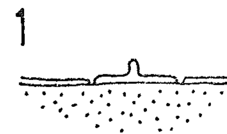


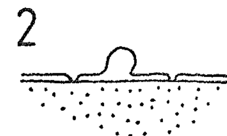
Figure 64.

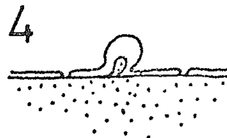
INFLUENCE OF SECTION PLANES UPON THE APPEARANCE OF NUCLEAR MEMBRANE VESICLES.

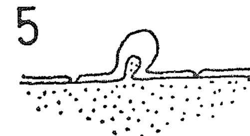


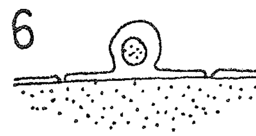
GRANULE - CONTAINING VESICLES.

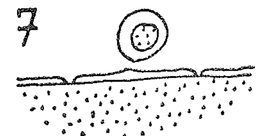




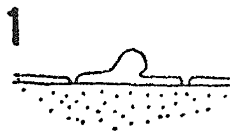


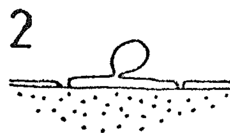


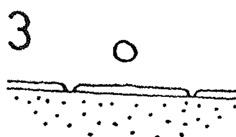




SINGLE MEMBRANE VESICLES.







as is shown in Plate 92 . The outpushing of the outer membrane is followed by an outpushing of the inner membrane which is represented by stages (iv) and (v) in Figure and in Plates 94 and 95 . The puckering of the inner membrane carries with it, material from the karyoplasm, which is pinched-off to form a membrane bound packet of karyoplasm free in the expanded perinuclear space, as is shown in Stages (vii) and (x) and Plate 95 .

It is only after the inner element of the nuclear envelope appears to have completely re-united across the surface of the nucleus that the outer nuclear membrane begins to pinch-off with its contents to produce a large vesicle such as that represented by stage (ix) and Plate 96 . The final separation of the double membrane bound vesicle from the nuclear envelope does not appear to be necessary for the complete function of the structure. In many cases the outer margins of the vesicle appear to breakdown, as is shown in Plates 97 and stage (xi), as soon as the inner element of the nuclear envelope is restored across the surface of the nucleus, and the granular contents of the vesicle appear to be released at this point. In fact it is questionable if the vesicles are ever finally separated from the nucleus, as few were found free in the cytoplasm, and none were identifiable at any distance from the nucleus.

The appearance of well developed double membrane vesicles adjacent to nuclei can probably be accounted for in terms of section planes, as is shown in Figure 64 ,thus it seems probable that the developmental stages of large vesicles shon by stages (vii) and (ix) in Figure 64 , do not normally take place, and there presence in thin sections is a result of the plane of section.

Large vesicles or blisters were observed associated with the nuclear membranes of kidney cells in material from all experimental treatments, but they appeared to be far more frequent after hormone treatment, than in thyroidectomised or normal animals. Counts were

made of the numbers of nuclei in each experimental group. All the nuclei within three of the squares of a copper mounting grid were counted, and those with vesicles associated with the nuclear envelope were also counted and expressed as a percentage of the total number of nuclei counted. The results are shown in Table 69 .

Table 69 .

Percentage nuclei with large vesicle structures associated with the nuclear envelope after thyroid hormone treatment.

	TX	N	T3	T4
Kidney.	8.39 ± 1.47	14.31 ± 2.78	24.10 ± 4.92	16.12 ± 3.28

Table 70 .

The statistical significance of the distribution of large vesicle structures with the hormone treatments.

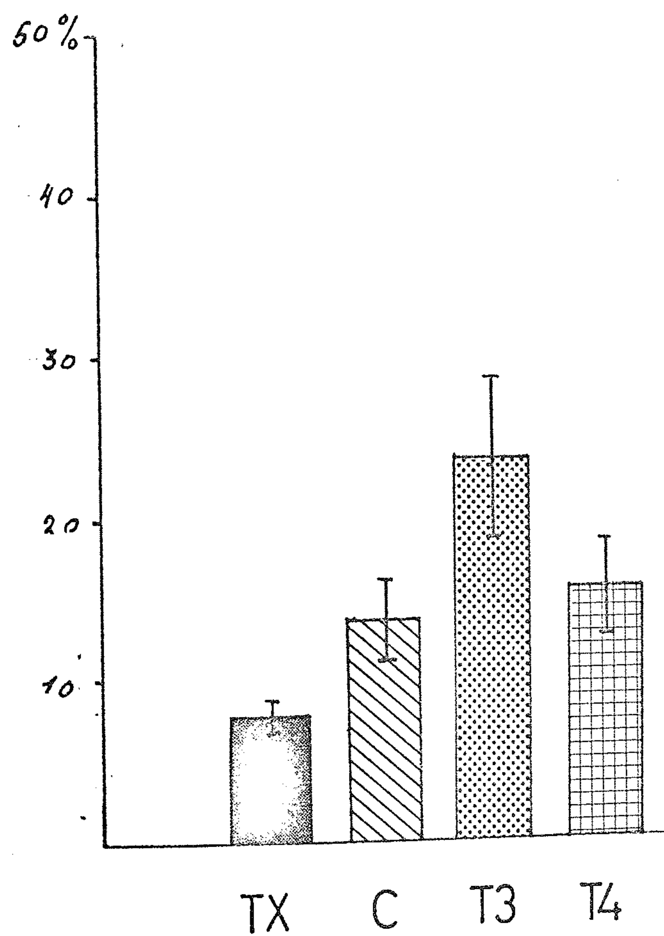
Kidney	TX	N	T3	T4
TX				
N	$P<0.001$			
T3	$P<0.001$	$P<0.001$		
T4	$P<0.001$	$P<0.001$	$P>0.1$	

Thus there was a significant increase in the number of nuclei which developed large vesicle structures, after the hormone treatment, and there was a significant reduction in the number of developed large vesicles in the kidney cells of thyroidectomised animals.

The identification of the contents of large vesicles: After routine preparation of material using glutaraldehyde and osmium followed by uranium and lead staining, the contents of the large vesicles appeared to resemble the karyoplasm. The included material was of lower electron density than the chromatin, but frequently resembled it in appearance, as is shown in Plates 96 and 95 . The contents frequently consisted

Figure 65.

% NUCLEAR PROFILES WITH LARGE VESICLES IN THE KIDNEY



of groups of granules, or fibril profiles of the same dimensions as those composing the chromatin and the underlying fine fibrillar network of the nucleus. Frequently small fibrillar components similar in dimensions to those of the karyoplasm could be discerned. Occasionally the included material appeared to be wholly fibrillar in composition.

Material was examined after acrolein fixation and silver nitrate staining and the reaction of the vesicle contents to the silver stain was compared with that of the karyoplasm. It was hoped that some indication of the chemical composition of the included material might be obtained. However, certain difficulties were encountered in interpreting the appearance of material prepared with acrolein and silver nitrate, since the silver was seposited over the section as a fine granular deposit, and not bound to the tissue constituents as are more conventional stains. Since the distribution of silver granules was restricted, to nucleoprotein components, it was necessary to rely upon the inherent contrast of the tissue for the identification of other components. Although acrolein imparts sufficient electron density to the tissue to allow identification of gross cytoplasmic structures, the contrast was no sufficient to allow detailed analysis, since membranes were rarely visible. Thus large vesicles were frequently difficult to identify since their membranes were not visible it was difficult to ascertain the relationships to the nuclear envelope. However, the nuclear envelope was frequently identifiable as a pale, narrow zone surrounding the nucleus, against which nuclear silver deposits abruptly ceased. The outer element of the nuclear envelope could usually be identified by the presence of silver granules over attached ribosomes. Structures which could be assumed to represent large vesicles could be identified as local expansions of the pale perinuclear zone, where there was a discontinuity in the silver deposits over the ribosomes attached to the outer nuclear membrane. Such structures usually appeared to be deveid of contents, but occasionally material could be discerned within them.

This material frequently appeared continuous with the nucleoplasm, in the process of diffusing from nucleus to vesicle. It was usually of similar electron density to the fibrillar components of the nucleoplasm, and relatively devoid of deposits of silver, as is shown in Plate 98 . Although occasional granules were detected, no vesicles were found in which the contents resembled chromatin, in having dense, overlying aggregations of particles. The surface of chromatin masses frequently continued across apparent vesicles, as is shown in Plate 99 , chromatin masses were never seen protruding into vesicle structures, even where material was present within the vesicle space.

Thus the contents of vesicles, where identifiable, resembled the fibrillar components of the nucleoplasm in electron density, and non-deposition of silver granules rather than chromatin. From which it might be assumed that vesicle included material is not deoxyribonucleoprotein but could be either protein or ribonucleoprotein of nuclear origin.

Material was also examined after digestion with either ribonuclease or pepsin. Ribonuclease digestion did not have a very marked effect upon the relative contrast of the large vesicle inclusions. The contents of vesicles resembled those of routinely prepared material, in this respect. However, as is shown in Plates 100 and 110 , there was a general loss of structure of the contents. The vesicle inclusions usually appeared as homogeneous gray masses of material with no apparent fibrillar or granular structure. This would suggest that part at least of the included material is ribonucleoprotein in composition. The lack of apparent effect upon the contrast of the contents would suggest the presence of some other components.

Pepsin digestion had little apparent effect upon the included material of large vesicle structures, as is shown by examination of Plate 102 . There was an effect upon neither the relatice contrast or the structure of the contents. Which would suggest that either protein

is not a major component of the vesicle inclusion or that protein components are not accessible to pepsin digestion.

Discussion: The effects of thyroid hormones upon the appearance of the nuclear envelope

The occurence of the observed nuclear membrane changes could be accounted for by arefactual preparation or to a natural phenomenon. The variation of the membrane changes with the thyroid hormone influence suggests that their occurence is not artefactual. The lack of variation with the variation in fixation proceedure also tends towards this conclusion.

A number of references have been made to various alterations in the nuclear envelope which are affected by physiological conditions, which again suggests that the changes observed under the influence of thyroid hormone action are not artefactual. Meek and Moses (1961) have recorded a microtubulation of the nuclear envelope. Large blebs surrounded by a complex wall composed of both nuclear membranes have been recorded from a number of different sources; Vielkind and Vielkind (1970) in fish melanomas; Huhn (1967) in normal monocytes; Smith and O'Hara (1969) in nomal leukocytes; Orwin (1969) in wool follicles; Ahearn, Campbell and Luce (1967) in human bone marrow cells; Toro and Olah (1966) in guinea pig thymus; McDuffie (1967) in human leukemic cells and Mollo, Canese and Stamignoni (1969) in epithelial cells and connective tissue. Szollosi (1965), Baker and Franchi (1967) and Adams and Hertig (1964) have noted cytoplasmic vesicles of nuclear envelope origin produced during certain stages of the meiotic process, in various mammalian oocytes, in which substances resembling the chromatin occurred. Scharrer and Wurzelmann(1969) noted changes similar to those in the kidney under the influence of thyroid hormones in *Protopterus* oocytes which were apparently

connected with the transport of material across the nuclear envelope. Kilarski and Jasinski (1970) noted a variation in the production of vesicles from the nuclear membranes of gas gland cells of Perca fluviatilis which was connected with variation in the protein synthetic activity of the gas gland cells.

Thus it would seem probable that the changes observed under the influence of thyroid hormones also represent a natural phenomenon connected with a physiolgical process which is influenced by thyroid hormone action. The natural candidate for such a physiological process is protein synthesis since thyroid hormones are known to act through protein synthesis. It is interesting to note that the changes observed by Kilarski and Jasinski (1970) and Scharrer and Wurzelmann (1969) were also connected with protein synthesis. The connection between vesicle production from the nuclear envelope and protein synthesis is obscure but several possible suggestions arise, as follows;-

1. An increase in surface area of the nucleus connected with protein production which could be connected with dissipation of energy, or exchange of small molecules.

2. Production of vesicles or outpushings of the nuclear envelope increasing the surface of the membrane itself rather then of the nucleus, to facilitate more rapid exchange of small molecules across the perinuclear space.

3. Production of small vesicles from the nuclear membranes, which contain small particles from the nucleoplasm or other nuclear componenent, the vesicle then being pinched off from the nuclear envelope and carried off into the cytoplasm.

4. Production of membrane components from the nuclear envelope for the endoplasmic reticulum or Golgi apparatus, which might be synchronised with the production of new ribosomal material in the nucleus.

It is probable that a combination of several of these suggestions would provide the explanation for the observed phenomena.

Vesicles of two types were observed, small single membrane bound vesicles derived from the outer element of the nuclear envelope, and large double membrane bound vesicles derived from both elements of the nuclear envelope and containing material of nuclear origin. The most likely explanation for the occurrence of small membrane vesicles is that they are connected with the production of new endoplasmic reticulum synchronised with the increased production of ribosomes within the nucleus. This seems probable for two reasons. The endoplasmic reticulum and the nuclear envelope appear to be of similar structure and are regarded as representing an interconnecting channel within the cell. The nuclear membrane is derived from the innermost lamellae of the endoplasmic reticulum after cell division, thus it is possible the two membrane systems contribute one to another during the normal functioning of the cell. Relationships between small vesicles and lamellae of the endoplasmic reticulum have been observed fairly frequently, suggesting that the vesicles might be contributing to the endoplasmic reticulum. Small vesicles might also be carrying electron transparent material or small molecules over to the cytoplasm from the perinuclear space or the nucleus itself, or over to the endoplasmic reticulum. It is also possible that the production of small vesicles from the nuclear envelope might act as a signal to the endoplasmic reticulum to produce more membrane lamellae, although this seems less likely.

There have been several suggestions that the production of the large membrane vesicles from the nuclear envelope represents a secondary route across the nuclear envelope when conditions within the cell are such that the nuclear pores cannot cope with an increased load of material for transport from the nucleus to the cytoplasm. (Scharrer and Wurzelmann 1969) Such an increased load of nuclear material might be represented by ribosomal precurser particles, which are possibly of too great a size to be easily coped with by the nuclear pores.

It has never been fully established that the nuclear pores

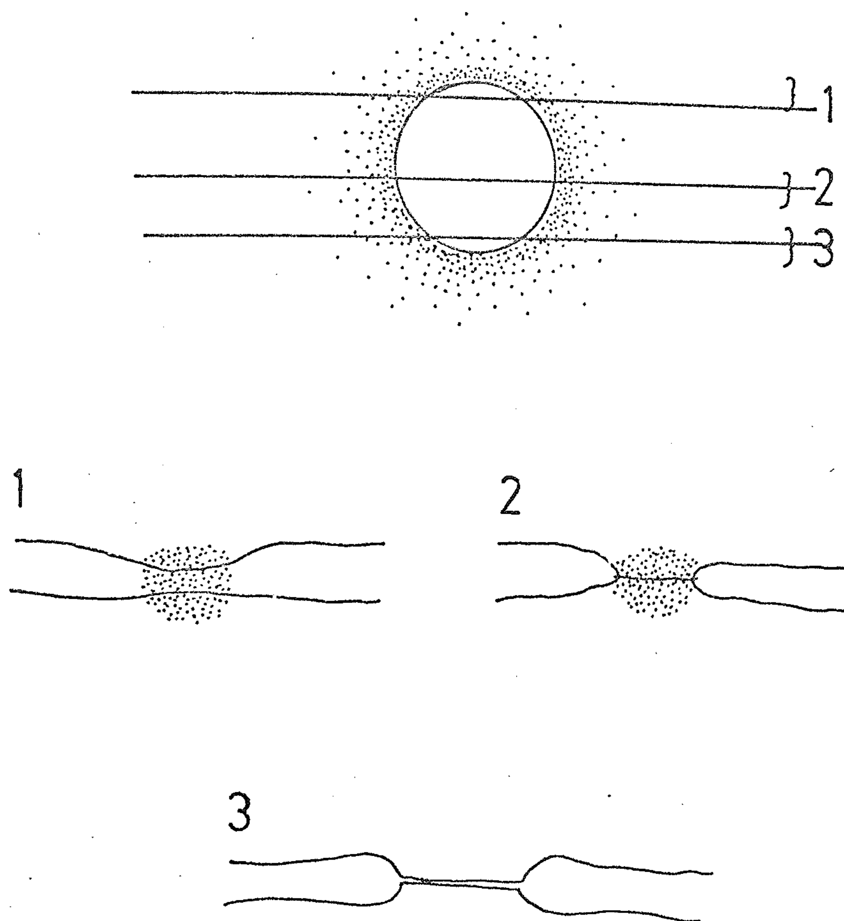
represent the usual route for the transport of large particles such as ribosomal precursers across the nuclear envelope. In which case it seems probable that such particles as ribosomal precurser particles would have to pass out via a route such as a vesicle. In which case increased production of ribosomal precursers or other small particles by the nucleus would be expected to cause an increase either in the size of particle containing vesicles, or an increase in the number produced. However, it would seem probable that the nuclear pores cope with the normal load of nuclear material transported to the cytoplasm, including ribosomal precurser particles, although they may not provide a sufficient means during periods of rapid production, and a secondary route of 'bulk' transport would then be necessary. Such a route could be provided by the double membrane bound large vesicles.

Lowenstein (1964) has shown that the electrical resistance of the nuclear envelope presents a formidable barrier to the direct passage of material across it, and that this resistance is destroyed if the continuity of the membrane is broken. Thus vesicle production would present a means of transporting material across the nuclear membrane without at anytime causing a break in both elements of the envelope, and a consequent loss of the diffusion barrier. Thus, if the vesicles represent bulk transport of material across the nuclear membrane, the question arises of how the granular contents leave the vesicles and enter the cytoplasm. However, it appears that these vesicles are rarely pinched off from the nuclear envelope, before breakdown of the vesicle membrane has begun. Breakdown of the outer vesicle membranes and release of contents appears to take place as soon as the repair of the inner membrane of the nuclear envelope across the point of vesicle production is complete. Thus transport of material via this route may be rapid.

This leads to the probability that if greater nucleo-cytoplasmic transfer takes place under the influence of the thyroid

Figure 56.

INFLUENCE OF SECTION PLANES UPON THE APPEARANCE OF NUCLEAR PORES IN THIN-SECTIONS



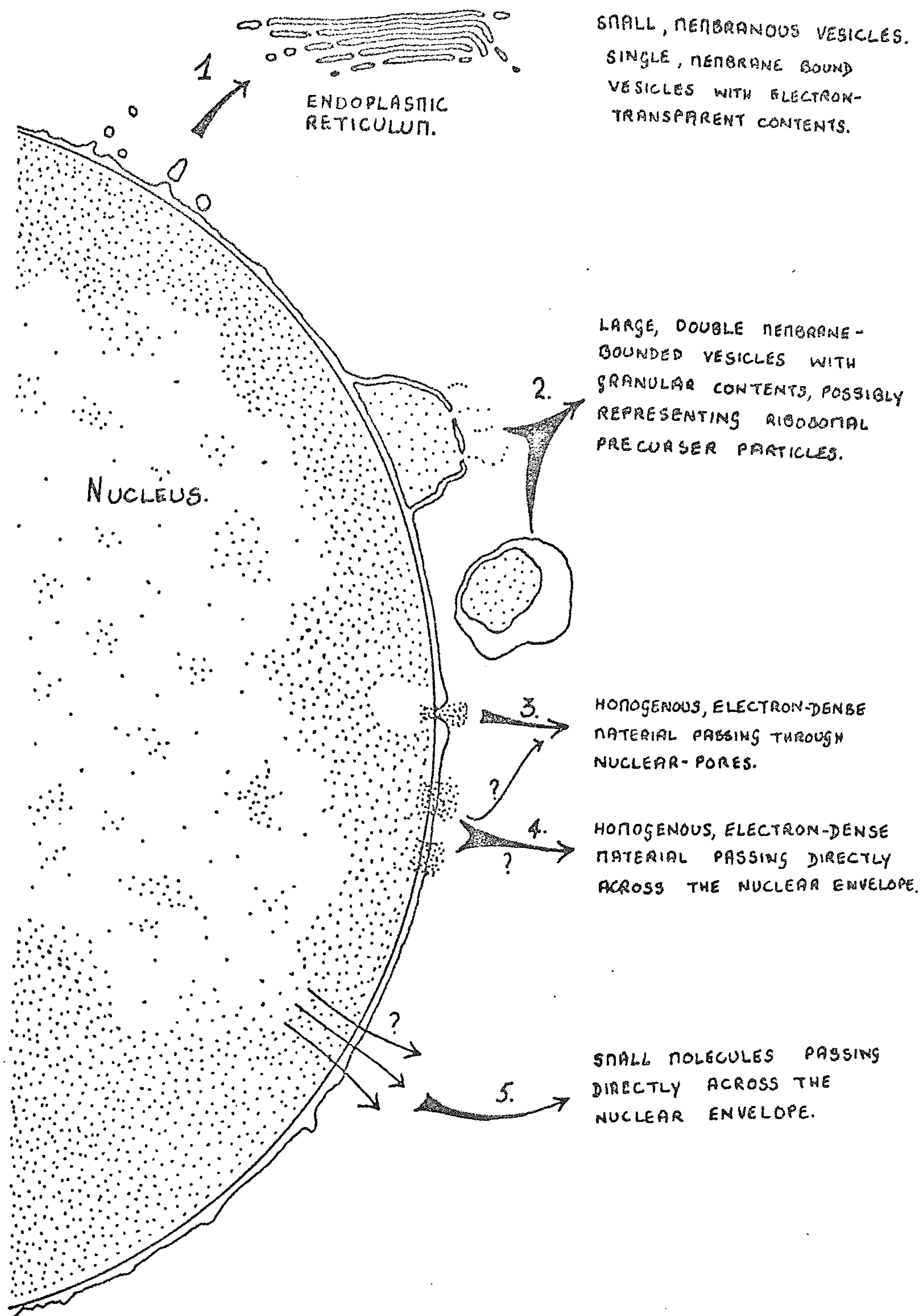
hormones, it must either be by the increased activity of nuclear pores or by some other route directly across the nuclear envelope, such as would be provided by vesicle production. Close examination of pores from the different treatments reveals that there may be a difference in their activity.

The appearance of nuclear pores in thin sections varies according to the plane of section, as is shown in Figure 66 , and according to the amount of material associated with it. Pores of type 3 were more frequently found in the thyroidectomised animals than in material of either of the other experimental groups, and most of the pores seen in nuclear sections from thyroidectomised animals appeared to conform to this pattern. Pores of types 2 and 3 occurred in material from normal animals, in which case the associated material or 'pore deposit' was quite diffuse. Material from the hormone treated animals showed pores almost exclusively of type 2, where the 'pore deposit' was frequently electron dense. Pores resembling those of type 1 were rarely if ever seen in material from the thyroid hormone treated animals. The possibility arises of an association between the electron dense material associated with the nuclear pores of material from hormone treated animals and the electron dense obscuring material associated particularly with the nuclear envelope of liver after hormone treatment, although the connection is difficult to visualise.

There would thus appear to be several possible routes for the transport of material across the nuclear envelope, which might possibly be influenced by thyroid hormone administration. Routes 3 and 5 probably represent those in use by a normally functioning cell. That small molecules pass freely across the nuclear envelope itself, either by diffusion or by an active transport mechanism, and that larger particles pass out to the cytoplasm through the nuclear pores seems probable, although some transport may take place by the means of small membrane vesicles represented by Route 1 even in the normal cell. There

Figure 67.

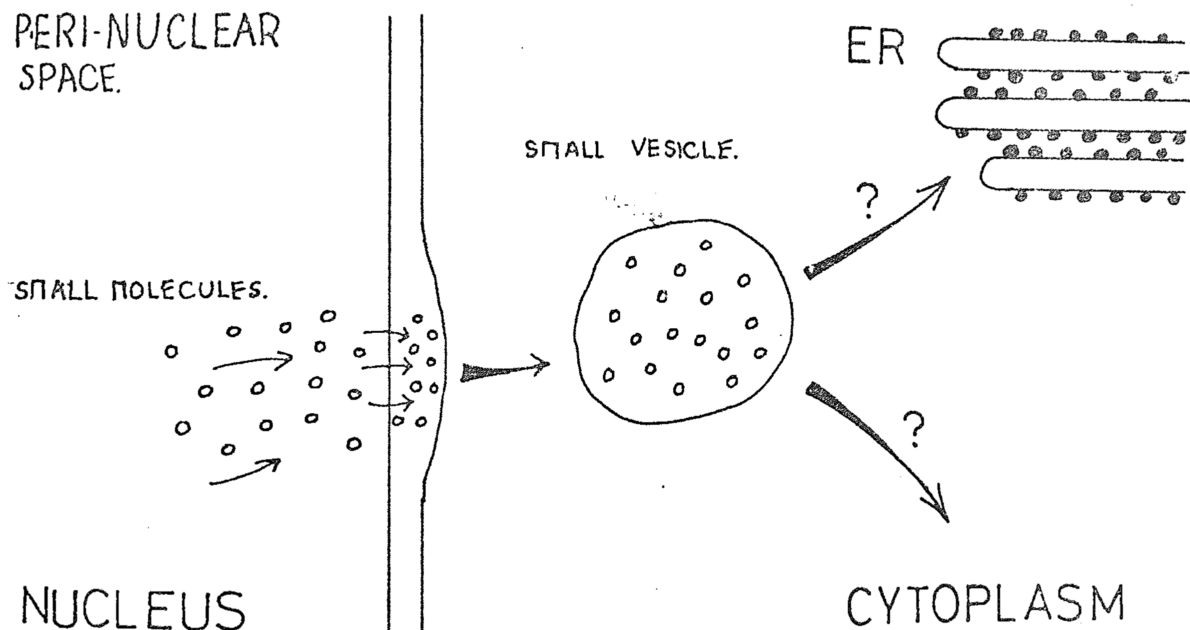
POSSIBLE ROUTES OF NUCLEO-CYTOPLASMIC TRANSFER.



has been some question as to whether or not small molecules can pass directly across the electrical barrier presented by the nuclear envelope (Loewenstein 1964). This may account for the occurrence of the small single membrane vesicles. Small molecules may diffuse freely into the perinuclear space, and are then pinched off in small vesicles from the outer elements of the nuclear envelope, which may then contribute to some other cytoplasmic organelle such as the endoplasmic reticulum, as is shown in Figure 68 . This explanation would also account for the

Figure 68 .

Possible significance of small, single membrane vesicles in the transport of small molecules across the nuclear envelope.



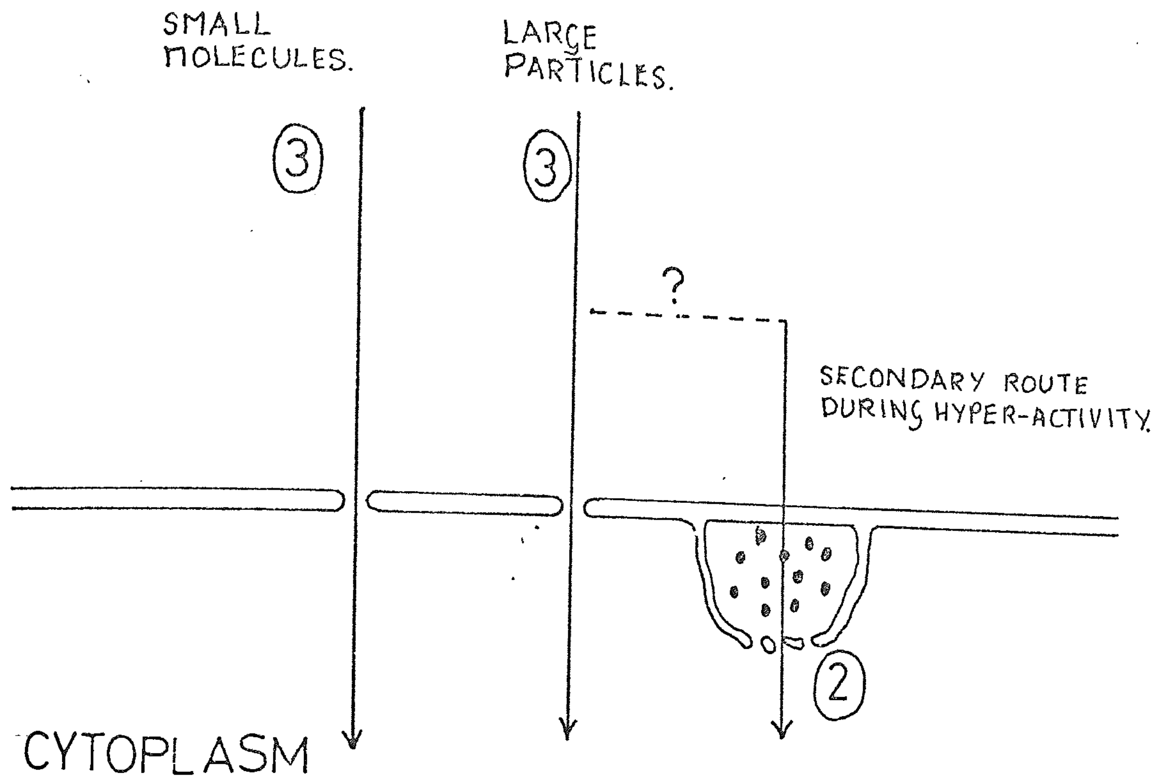
occurrence of small numbers of electron transparent vesicles in normal and thyroidectomised animals, and their increased frequency in the material from hormone treated animals.

Although there was a slight increase in the numbers of nuclear pores occurring after hormone treatment, it would seem that increased nucleo-cytoplasmic transfer necessitates the use of a secondary route of bulk trasport across the nuclear envelope, such as that shown in Figure 69 .

The pathway shown as Route 4 in Figure 67 remains something of an anomaly. The functional significance of the electron

Figure 69 .

The possible significance of large vesicle structures in the transport of particulate material across the nuclear envelope.



dense obscuring material associated with the nuclear envelope, particularly in the liver, of thyroid hormone treated animals, is difficult to visualise. It is possible that it represents accumulations of material against the nuclear envelope prior to transfer to the cytoplasm. The possibility that it represents some breakdown of the nuclear membrane structure to facilitate transfer directly across the envelope, seems unlikely in view of the observations on the significance of the nuclear envelope as a diffusion barrier. (Loewenstein 1964).

Chapter 5.

Discussion: The Effects of Thyroid Hormones upon the Ultrastructure of Liver and Kidney.

Biochemical analyses of the cellular changes resulting from thyroid hormone action have yeilded a considerable amount of information regarding the mode of action of the two hormones. This investigation has shown that many of the alterations in metabolism are accompanied by detectable ultrastructural modifications, although the changes in the ultrastructure of many of the cellular organelles were not as marked as would be expected on the basis of the biochemical data. This is probably attributable to some extent to the use of normal animals rather than thyroidectomised ones for the hormone treatments. As was pointed out by Roodyn et al (1965) thyroidectomised animals made a considerably greater response to the administration of thyroid hormones than did normal animals. However, due to the difficulties experienced in obtaining thyroidectomised mice, use of normal animals was unavoidable.

Thyroid hormone administration is known to result in increased protein synthetic activity in both the liver and the kidney (Tata et al 1963; Michel et al 1963). This was obvious from the cellular changes in both tissues used in this investigation, as well as from the biochemical data obtained by other workers. The change in metabolic activity in the liver, towards more protein synthetic activity was obvious in the depletion of glycogen and increase in the concentration of endoplasmic reticulum, which could be demonstrated within 30hrs of hormone administration, inspite of the complication of apparent diurnal fluctuations in glycogen content and concentration of endoplasmic reticulum. The effect was less marked in the kidney although increases in the concentration of endoplasmic reticulum profiles could be

demonstrated in the first 30hrs of hormone action, when apparent diurnal variations in concentration of endoplasmic reticulum was considered. Alterations in mitochondrial activity demonstrated by Tata et al (196_) and Roodyn et al (1965) were not reflected in alterations in the mitochondrial structure, although there was a detectable increase in the cytoplasmic area occupied by mitochondrial profiles 45hrs after hormone administration which followed the peak in amino acid incorporation by mitochondria demonstrated by Roodyn et al (1965). The changes in cytoplasmic organelles detectable after a single dose of triiodothyronine were sustained and often increased after a prolonged treatment with either triiodothyronine or thyroxine.

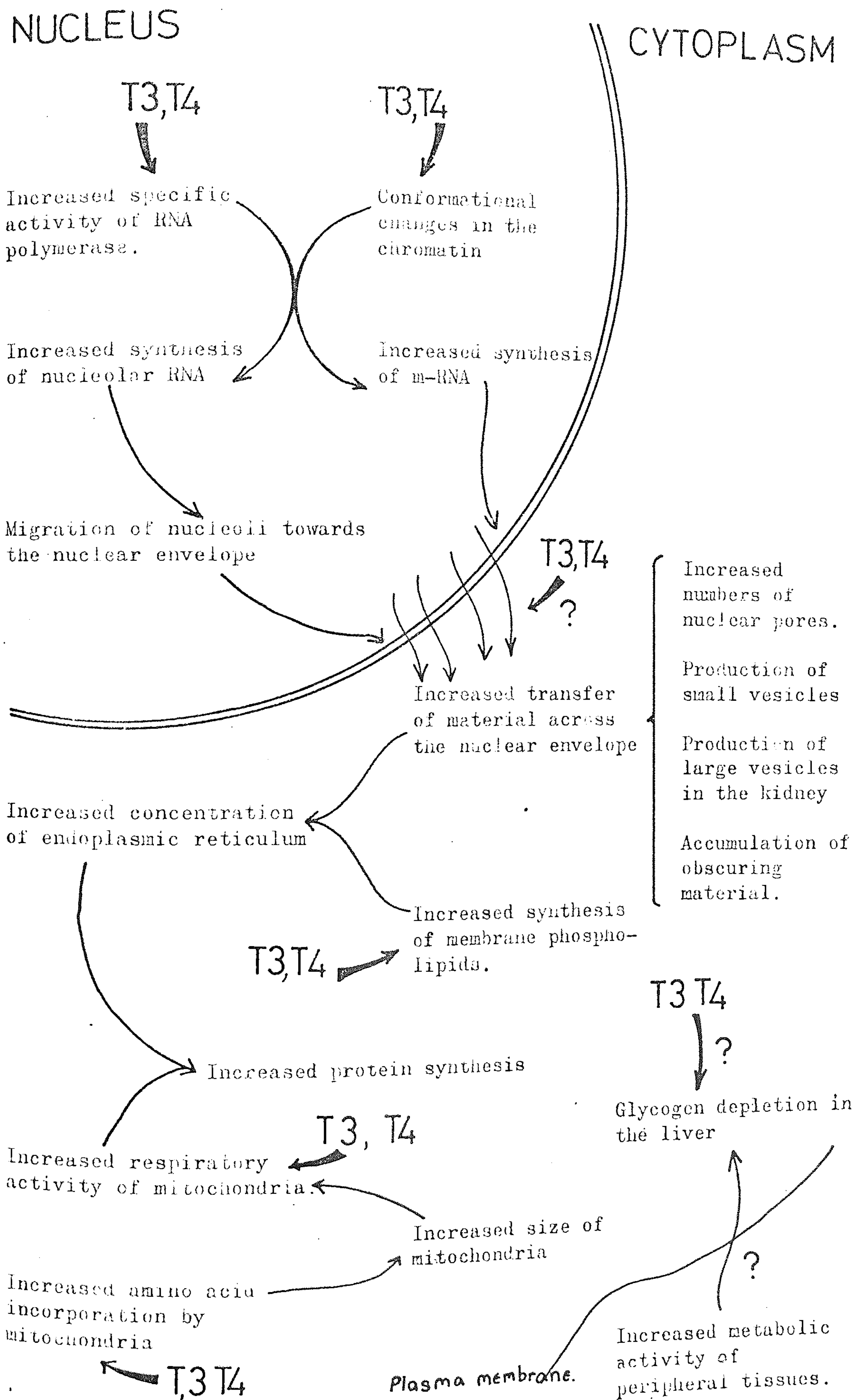
The early effects of thyroid hormones upon the nucleus were not accompanied by obvious changes in nuclear ultrastructure, although there were significant reductions in nuclear size 60hrs after hormone administration which was not sustained after prolonged hormone treatment. The significance of the alterations in nuclear size is difficult to visualise. Apart from slight alterations in the amount of chromatin visualised in nuclei of both the liver and the kidney associated with hormone treatment there were no alterations in nuclear constituents which were immediately apparent. There was no apparent alteration in the appearance of nucleoli which could be attributed to a changed functional state, in either the liver or the kidney associated with the hormone treatment. There were however, hormone associated alterations in both the material associated with nucleoli and in the nucleoplasmic constituents detectable after variations in the preparation proceedure. For instance, there was an increase in the argentophilic material associated with the nucleoli of the liver after hormone treatment, and hormone treatment resulted in an increase in the amount of material resistant to cold-buffer extraction present both in the nucleoplasm and associated with the nucleolus. These alterations were slight however, and their significance difficult to visualise,

due to the difficulty of further identifying the nature of the components. That alterations, however slight, can be detected in the nucleus suggests that thyroid hormones do exert an influence upon nuclear components, which are potentially detectable in the electron microscope, but which cannot be fully appreciated with the techniques available for histochemical analysis of ultrastructural components at present.

That the detectable variations in nuclear components are so slight suggests that hormone influence might act upon the rate of transfer of nuclear material towards the cytoplasm as well as upon the nuclear synthetic activity, as was suggested by Tata (1964, 1965, 1967) The tendency of nucleoli to start migration towards the nuclear envelope during the first 15hrs after triiodotyronine administration tends to support this view. The products of nucleolar synthesis are known to be mainly ribosomal (Scharrer and Darnell 1962, Penman et al 1966, Perry 1962, 1963, 1964) and migration of nucleoli towards the nuclear envelope would conceivably facilitate the rapid transfer of ribosomal material to the cytoplasm. Migration of nucleoli occurs at about the time when increased nuclear synthetic activity is first detectable (Widnell and Tata 1966).

Alterations in the morphology of the nuclear envelope, which can be related to increased activity of nuclear / cytoplasmic transfer mechanisms also occurr within the first 15hrs after triiodothyronine administration, and are sustained after prolonged treatment with either triiodothyronine or thyroxine. There was a significant increase in the number of nuclear pores after triiodothyronine treatment, and there was more electron dense material associated with the nuclear pores after either triiodothyronine or thyroxine treatment than before. Hormone treatment produced a significant increase in the production of small vesicles from the outer element of the nuclear envelope, and of large vesicle structures from

The Effects of Thyroid Hormones upon Ultrastructure and Metabolism at the Cell Level.



the nuclear envelope of kidney nuclei detectable with 15hrs of hormone administration, which could be associated with transfer of material across the nuclear envelope. The significance of the accumulation of electron dense material across the nuclear envelope, which also began within the first 15 hrs of hormone action, is difficult to visualise, inspite of its apparent ribonucleoprotein composition, in view of the un-likelihood of any material passing in bulk directly across the nuclear envelope (Loewenstein 1961). That this material represents an alteration in the structure of the nuclear membranes themselves is unlikely in view of the apparent identity of the material and of the fact that the membranes themselves remain detectable despite the presence of the obscuring material.

Thus the influence of thyroid hormones upon cellular metabolism summarised in Figure 1, is paralleled by effects upon the ultrastructure of cells. The observations summarised in Figure 1 can be extended to include the data obtained from this investigation of the ultrastructural effects of thyroid hormone influence, as is shown in Figure 70. The results of increased synthesis of ribosomal material and membrane phospholipids were obvious in the accumulation of endoplasmic reticulum, particularly in the liver. The increased mitochondrial activity was accompanied by increases in mitochondrial size, but was not reflected in any alteration in the structure of mitochondria. The depletion of liver glycogen after hormone treatment was obvious. The early influence of thyroid hormones upon nuclear synthetic activity was not reflected in any significant alteration in the appearance of any of the nuclear components. The influence of thyroid hormones upon the turnover of the products of nuclear synthesis had significant effects upon processes connected with the transfer of material from the nucleus to the cytoplasm, visualised in the electron microscope. Increased nucleo / cytoplasmic transfer was reflected in gross changes in the appearance of the nuclear envelope.

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APPENDICES.

Appendix I.

Morphological Changes in the Nuclear Envelope of the Liver, Kidney and Uterus of the Rat during the Oestrous Cycle.

In view of the possibly artefactual origin of the observed changes in the morphology of the nuclear envelope under the influence of thyroid hormones, an investigation of the morphology of the nuclear envelope was undertaken in another experimental system. The liver, kidney and uterus of rats at different stages in the oestrous cycle were selected for study. Samples of the three tissues were taken from rats in pro- and di-oestrus for comparison as these were readily available, and it has been shown that there is a 104% increase in the aldosterone and a 119% increase in the corticosterone secretion rate at pro-oestrus at a time when oestrogen secretion was also maximal (Hinsall and Crocker 1970; Yoshinaga 1969). It appears that the steriods aldosterone, corticosterone and oestrogen act via protein synthesis at a similar point in the process as that at which thyroid hormones exert their influence (Sharp and Leaf 1966; Williams-Ashman and Reddi 1071; Castles and Williamson 1965). Widnell and Tata (1965) showed that certain steroids and thyroid hormones act via a similar route. Thus it was thought that the oestrous cycle would provide a simple but comparable experimental system to the influence of thyroid hormones in which to investigate some of the effects of hormones upon the morphology of the nuclear envelope.

Methods.

The stages of the oestous cycle were determined by taking vaginal smears daily, between 8.30 and 9.30am. for fourteen days before death. Small pieces of liver, kidney and uterus from each of four

pro-oestrous and four di-oestrous animals whilst they were uder phenobarbital anaesthesia. The tissues were fixed routinely in glutaraldehyde and osmium tetroxide. Sections were stained with lead and uranium before examination in the electron microscope.

The appearance of liver nuclei.

The nuclei from cells of the di-oestrous liver were rounded with occasional indentations, there was little dark staining material present and nucleoli tended to be prominent but small. In contrast the nuclei from the livers of animals at pro-oestrus were large prominent and rounded. There was much dark staining material present, and the nucleoli were large and well developed.

The nuclear envelopes surrounding nuclei from the livers of di-oestrous animals were well defined, evenly separated and regular as is shown in Plate 103. The nuclear envelpes surrounding nuclei from the liver of animals in pro-oestrus tended to be obscured by an electron dense material resembling that previosly described after thyroid hormone treatment of mice, as is shown in Plate 104.

The nuclei showing obscured membranes were counted according to the criteria used in estimating the extent of the obscuring material in the mouse. The results are shown below. There was a

Percentage nuclei with unobscured nuclear envelopes at pro- and di-oestrus.

Di - oestrus	Pro - oestrus
76.60 $\pm$ 2.39	39.35 $\pm$ 12.93

significant difference between the results of the two experimental groups with $P < 0.001$. Thus there was a marked tendency towards an accumulation of some obscuring material against the nuclear envelope of liver cells

during pro-oestrus, in the rat.

It is interesting to note that after lead post staining of material prepared from thyroid hormone treated mice it was often possible to distinguish the nuclear membrane elements through the obscuring material, this was rarely possible in the case of the rat in pro-oestrus. Where the membrane elements were distinguishable in the mouse they were frequently puckered and irregular, where they were distinguishable in the rat they were usually regular and even in profile.

The appearance of kidney nuclei.

The nuclei of both the proximal and distal convoluted tubules of di-oestrous kidneys were rounded, dark staining material was restricted to a narrow sub-peripheral zone. Nucleoli were prominent but rounded and small with only minimal amounts of associated pars granulosa material present. The nuclei of the pro-oestrus kidneys showed varying responses to the circulating hormones. The nuclei of the proximal convoluted tubules remained comparatively unchanged, whereas those of the distal convoluted tubules showed marked morphological variations. There were marked increases in the amount of dark staining material present and nucleoli tended to be larger and better developed.

The nuclear envelopes surrounding nuclei of the proximal convoluted tubule cells of the di-oestrous animals tended to be crisp regular and with evenly separated membrane elements. They were relatively unchanged during pro-oestrus, their appearance is shown in Plate 105. The nuclear envelopes surrounding the nuclei of the distal convoluted tubules during pro-oestrous were irregular with the production of small vesicles from the outer membrane element, as is shown in Plate 106. During di-oestrus they were regular with no production of small vesicles, as is shown in Plate 107. Very few nuclei of either proximal or distal convoluted tubule showed any accumulation of obscuring material during pro-oestrus, and the extent of the vesicle

production and the irregularity of the nuclear envelope were not as marked as in the mouse kidney after thyroid hormone treatment.

The appearance of the nuclei of the uterus.

Attention was restricted to the nuclei of the endometrium, which was identified in thick sections stained with toluidine blue before the thin sections were cut for electron microscopic examination.

The nuclei of the di-oestrus endometrial cells were elongated and irregular in outline, those of the epithelial layer were more rounded but still idented. The nucleoplasm was homogeneous, with little dark staining material present, nucleoli were small, with a prominent nucleolonema but little associated pars granulosa. Pro-oestrus nuclei were more regular in outline, possessed a greater amount of dark staining material and there were greater amounts of granulosa material associated with the nucleolus.

The nuclear envelopes of the endometrial cells during di-oestrus were crisp and regular, a few of the epithelial cell nuclei showed extensive local expansions of the perinuclear space devoid of contents. There was a marked accumulation of obscuring material across the nuclear envelope of the pro-oestrous material, which was absent from the epithelial cell layer nuclei. The epithelial cell nuclei tende to show increases in the numbers of outer membrane puckers.

Discussion: Variations in the appearance of the nuclear envelope during the oestrous cycle.

The observations on the variations in the appearance of the nuclear envelope during the oestrous cycle appear to confirm that steroid hormones, in particular those circulating during the oestrous cycle, oestrogen, corticosterone and aldosterone, act at a point in protein synthesis similar to that at which thyroid hormones act, in

order to produce similar effects. Th t the g eatest ultrastructural alterations in the didney during pro-oestrus occurred in the distal convoluted tubules is of interest in view of the observation of Vander et al (1958) that aldosterone is maximally active upon the distal convoluted tubule.

That there are similar alterations in the appearance of the nuclear envelope during the oestrous cycle of the rat to those occurring in the mouse as a result of thyroid hormone influence suggests that the changes are the result of a physiological process and are not the result of some fault in the processing of material for electron microscopy.

Appendix II.

Solutions used in the Preparation of Material for Electron Microscopy.

FIXATIVES.

1. Glutaraldehyde.

(a) 5% glutaraldehyde in 0.05M sodium cacodylate, pH 7.4

(b) 2.5% glutaraldehyde in phosphate buffer, pH 7.4

2. Acrolein.

10% in 0.05M sodium cacodylate, pH 7.4

3. Caulfield's osmium tetroxide.

2% osmium tetroxide	-	10ml.
veronal acetate buffer	-	4ml.
0.1N HCl.	-	4ml.
distilled water	-	2ml.

adjusted of pH 7.4 with 0.1N Cl.

plus 0.045gm. sucrose/ml. fixative solution.

BUFFER SOLUTIONS.

1. Phosphate buffer.

M/15 $Na_2HPO_4.2H_2O$ (Sorenson's salt)	-	72ml.
M/15 $KH_2PO_4.H_2O$	-	28ml.

adjusted of pH 7.4

2. Sucrose / phasphate washing medium.

0.44M sucrose solution	-	100ml.
0.2M phosphate buffer, pH 7.4	-	100ml.

3. Veronal acetate.

Sodium veronal (sodium barbitone)	-	2.94gm.
Sodium acetate	-	1.94gm.

made up to 100ml. with distilled water.

EMBEDDING MEDIA.

1. Epon 812.

A. Epikote resin - 62ml.

Dodecenyl succinic anhydride - 100ml.

B. Epikote resin - 100ml.

Methyl nadic anhydride - 89ml.

For use:- 70% A } 30% B

+ 1.5% DY062 araldite accelerator.

Appendix III.

Preparation Schedules used in the Processing of Material for Electron Microscopy.

OSMIUM FIXATION.

1.	Caulfield's 2% osmium tetroxide.		60min.
2.	Veronal acetate		5min.
3.	Ethanol dehydration		75min.
	(50% ethanol	5min.	
	70% ethanol	5min.	
	90% ethanol	5min.	
	absolute alchohol	30min.	
		30min.)	
4.	Infiltration with embedding medium		180min.
	(Epoxy propane	30min.	
	50% epoxy resin in epoxy propane	30min.	
	Un-polymerised epoxy resin.	30min.	
		30min.)	

GLUTARALDEHYDE FIXATION.

1.	5% glutaraldehyde in sodium cacodylate	180min.
2.	Sodium cacodylate wash.	60min.
3.	Dehydration	75min.
4.	Infiltration in embedding medium.	180min.

GLUTARALDEHYDE AND OSMIUM, DOUBLE FIXATION

1.	5% glutaraldehyde in sodium cacodylate	180min.
2.	Sucrose /phosphate buffer wash	4 changes in 24hrs.
3.	Caulfield's 2% osmium tetroxide	60min.
4.	Veronal acetate wash	5min.
5.	Dehydration	75min.
6.	Infiltration with embedding medium	180min.

ACROLEIN FIXATION

1.	10% acrolein in sodium cacodylate	60min.
2.	Sodium cacodylate wash	60min.
3.	Dehydration	75min.
4.	Infiltration with embedding medium	180min.

ENZYME DIGESTION

(a) Ribonuclease digestion

1.	2.5% glutaraldehyde in phosphate buffer	15min.
2.	Sucrose/phosphate buffer wash	4 changes in 24hrs.
3.	Ribonuclease (1mg/100ml.) in phosphate buffer, pH 6.4	180min.
4.	Phosphate buffer wash	5min.
5.	Caulfield's 2% osmium tetroxide	60min.
6.	Veronal acetate	5min.
7.	Dehydration	75min.
8.	Infiltration with embedding medium	180min.

(b) Pepsin digestion

1.	2.5% glutaraldehyde in phosphate buffer	15min.
2.	Sucrose/phosphate buffer wash	4 changes in 24hrs.
3.	Pepsin (0.5gm./100ml.) in 0.1N HCl	20min.
4.	Phosphate buffer wash	5min.
5.	Caulfield's 2% osmium tetroxide	60min.
6.	Veronal acetate	5min.
7.	Dehydration	75min.
8.	Infiltration with embedding medium	180min.

SECTION STAINS.

URANIUM STAINING.

(a) Saturated methanolic uranyl acetate

25% uranyl acetate in methanol.

Stain for 30min.

Wash in methanol

(b) Aqueous uranyl acetate

2% uranyl acetate in water, adjusted to pH 4.5 with NaOH

Stain for 60min.

Wash in distilled water.

LEAD STAINING.

A. lead tartrate 63gm/l. distilled water

B. sodium hydroxide 14.5gms.

sodium potassium tartrate 2.5gm/500ml. water.

To use:- equal parts of A and B, filter and stand.

Stain for 15min.

Wash briefly in N/100 NaOH and distilled water.

SILVER NITRATE.

10ml 0.3% $AgNO_3$ adjusted of pH 7.8 - 9 with

5% borax; made up to 30ml. with distilled water.

Stain before mounting en copper grids, for 45mins. at 70°C

Wash briefly in 0.5% thiosulphate and water.

TOLUIDINE BLUE (for light microscopical examination of thick sections)

2% in aqueous 2% borax

Fix sections to glass slides using heat

Heat a drop of the staining solution on the slide

Wash in running water.

Blot dry.

PHOTOGRAPHIC PROCESSING.

Photographic plates:- 'Ilford' N 50 contrasty plates

or 'Ilford' EM 50 plates

DEVELOPMENT.

1. Hydroquinone developer, 'Maxicon' (2+1) 2min.
2. Fixer, 'Kodafix' (1+4) 4min.
3. Continuous wash in running tap water 30min.
4. Dried at 60°C.

All procedures carried out at 60°C.

PHOTOGRAPHIC PRINTING.

'Ilfobrom', single weight papers, Grades 1 - 5 as necessary.

DEVELOPMENT.

1. 'Bromophen' developer (1+7) approx. 2min. (twice the clearing time)
2. 'Kodafix' (1+3) 5min.
3. Continuous wash in running tap water 30min.
4. Dried on rotary glazer.