The Effect of Calcium on Thyroid Function

Halina Elizabeth Krowinski

B.Sc. (Hons)

University Department of Medicine, Royal Infirmary,

Glasgow

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FACULTY OF MEDICINE

Errata

Page 626	The fees for overseas students should be amended from £1010 to £1230.
Page 644	The degree of Master of Science is now also offered by the Faculty of Medicine as M Sc (Medical Science).

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Summary

This thesis investigates the role of calcium in the production of goitre in the rat.

In the initial dietary experiments excess calcium carbonate was added to a standard Remington low iodine diet which contains 1% calcium carbonate. No goitrogenic effect of excess calcium was found.

However, when calcium carbonate was added in differing concentrations to a calcium deficient low iodine diet, the thyroid weight increased with increasing dietary calcium. The total calcium content of these thyroids increased and the total iodine content decreased. The serum thyroid-stimulating hormone increased with added calcium although the serum thyroxine did not show any significant change.

In other experiments rats were made increasingly iodine deficient before starting the calcium diets. Iodine deficiency appeared to decrease the goitrogenic effect of calcium.

In rats made goitrous by administration of propyl-thiouracil or potassium perchlorate, the addition of 1% calcium carbonate diminished the goitre size.

In vivo and in vitro experiments show that the thyroid is capable of accumulating radioactive calcium. In vivo the thyroid uptake of ^{45}Ca was increased by feeding a low iodine diet.

The mechanism of the action of calcium in goitrogenesis is a complex interaction mediated via TSH. Calcium does not affect the trapping of iodide by the thyroid but to a small extent it inhibits protein binding of iodide. No binding of calcium to thyroglobulin could be detected in vitro. Calcium did not increase the urinary secretion of iodine.

The role of calcium in the iodine metabolism of the thyroid is discussed but the precise mechanism of the goitrogenic action of calcium is still not clear.

CHAPTER 1

Section 1

General Introduction

The thyroid is an endocrine gland found in all vertebrates. It was first named "the thyroid" or "oblong shield" (thyreos in Greek) by Wharton in 1656 who suggested that its function was to beautify the neck by filling the spaces about the larynx. Experimental thyroidectomy was used in the 19th century to study thyroid function. It was then recognised that this produced symptoms similar to those found in myxoedema which was first noted by Curling (1850). It was found that these symptoms could be relieved by feeding sheep thyroid (Murray 1920). This led to the idea of an internal secretory function for the gland. Baumann in 1896 discovered that the thyroid contained a large concentration of iodine. Subsequent work led to the crystallisation of L-thyroxine (T_4) from hydrolysates of thyroid tissues by Kendall in 1915. A more potent hormone containing 3 iodine atoms, triiodothyronine (T_3) was discovered later (Gross & Pitt-Rivers (1952)). It is now known that thyroid hormones regulate the rate of cellular oxidation in virtually all tissues.

The normal thyroid consists of two lobes lying on either side of the trachea attached to it by loose connective tissue. The lobes are connected by an isthmus which lies anterior to the trachea just below the cricoid cartilage. Two pairs of parathyroid glands are normally situated on or beneath the posterior surface of the thyroid lobes. The thyroid is encased by a thin fibrous capsule and it is very well vascularised.

Using a light microscope, the gland is seen to contain closely packed spheroidal sacs called follicles. Each follicle is filled with a clear, viscous fluid which constitutes the main thyroid mass. This colloid consists of a large glycoprotein, thyroglobulin. The follicle wall is lined by a single layer of epithelial cells which surround the lumen.

The thyroid epithelium which rests upon a well-defined basement membrane consists of two types of cells: follicular cells and light cells. In the electron microscope, the apical surface of the follicular cell is characterised by numerous microvilli extending into the colloid. The cytoplasm contains many vesicles composed of a single membrane to which ribosomes are attached. There is a large nucleus usually situated

in a basal position. The Golgi apparatus is generally apical in location and is often associated with colloid droplets which are also dispersed throughout the cell. Lysosomes are present toward the base of the cell and mitochondria are scattered diffusely throughout the cytoplasm.

Light cells or C-cells lie in thyroid follicles within the basement membrane. They do not contain colloid droplets nor do they come into contact with the colloid. The cytoplasm is packed with vesicles and the Golgi zone is conspicuous. The nuclei, mitochondria and lysosomes are similar to those found in follicular cells.

The main function of the thyroid gland is to secrete adequate supplies of hormone to the peripheral tissues. In order to do this, there must be available to the gland, enough iodine to synthesise a normal amount of hormone. Normally iodine balance is maintained by iodine ingested from food and water and this varies greatly throughout the world depending on the iodine content of the soil and water. Iodide is ingested in both the organic and inorganic form but it is mainly the inorganic form that is made available to the thyroid.

The iodide in the body is mainly confined to the extracellular fluid from which it is removed by two sites. The major clearance of iodide occurs via the thyroid and the kidney although small quantities are lost through the skin and in expired air. Iodide filtered through the kidney is largely reabsorbed. Factors affecting the uptake of iodide by the thyroid such as thyroid stimulating hormone (TSH) and drugs such as thiocyanate do not affect iodide clearance by the kidney.

The thyroid contains the largest pool of body iodide, mostly in the form of iodinated amino acids. Plasma iodide is actively transported through the basal membrane surface into the follicular cell. Intrathyroidal iodide may then be oxidised and organified or it may diffuse back into the extracellular fluid but normally the rate of inward clearance exceeds the combined rates of organic-binding and back diffusion and thus intrathyroidal concentration gradients for iodide greater than unity are maintained within the gland. TSH stimulates iodide transport which is dependent on oxidative phosphorylation. It is competitively inhibited by some monovalent anions, for example, perchlorate, thiocyanate.

After being transported into the thyroid, the iodide is first oxidised probably by hydrogen peroxide at a site near to the surface of the apical microvilli. It is thought that this reaction is mediated by a thyroidal peroxidase. Iodide oxidation is followed by the iodination of tyrosine which is contained in the thyroglobulin molecule to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). Organic iodinations are also controlled by TSH.

Hormonally active iodothyronines are then synthesised from iodotyrosines by a coupling reaction. Two DIT molecules are coupled to yield a molecule with two iodinated rings linked by an ether bridge. An alanine side chain is lost from the ring (β) which ultimately contains the phenolic hydroxyl group. The coupling reaction seems to occur within the thyroglobulin molecules and it requires an oxidation process. It may be mediated by the same peroxidase that catalyses the oxidation of iodide since virtually all agents that inhibit organic binding also inhibit coupling although the coupling reaction is much more sensitive to inhibitors. Iodine deficiency and the lack of TSH impair the synthesis of iodothyronines more than the synthesis of iodotyrosines.

A large amount of hormone is stored in the thyroid in the form of a glycoprotein, thyroglobulin. This protein with a molecular weight of 660,000 is synthesised in the follicular cells and is thought to be iodinated either in the cells or at the cell-colloid interface after which it is extruded into the follicular lumen. Its sedimentation coefficient ($S^{o}20$, w) is approximately 19.4S and the polypeptide is composed of about 5650 amino acid residues and about 9% by weight carbohydrate. The iodine content of thyroglobulin is variable and it depends on the iodine intake. Other iodoproteins are found in the thyroid including a dimer of thyroglobulin which has a sedimentation coefficient of 27S, and also proteins with sedimentation coefficients less than 19S under some conditions. From results of in vitro experiments thyroglobulin biosynthesis is thought to proceed by the following sequence:

n (3-8S) molecules --> $\frac{n}{2}$ (12S) molecules --> $\frac{n}{4}$ noniodinated thyroglobulin --> matured iodinated thyroglobulin where n represents the number of polypeptide chains.

In order to release the hormones into the blood, thyroglobulin must first be hydrolysed. The hydrolysis is carried out by proteases and peptidases and it is greatly influenced by the action of TSH. After the formation of pseudopodia at the apical surface of the follicle, endocytosis of colloid occurs to yield colloid droplets. Fusion of these droplets with lysosomes allows the digestion of the protein to release iodothyronines into the blood. A variety of agents, including iodine can inhibit the processes of proteolysis and release of hormones.

Iodotyrosines liberated by the hydrolysis of thyroglobulin are rapidly deiodinated by an enzyme, "iodotyrosine deiodinase". The iodine arising from this is recycled into the iodinating mechanism and incorporated into newly synthesised thyroglobulin.

Control of thyroid function

Nearly every stage in the process that leads to synthesis and secretion of thyroid hormones is under the control of TSH. The regulation of TSH release by the pituitary is influenced by thyroid hormone feedback and by neural influences. The hypothalamus secretes thyrotropin releasing hormone (TRH) which acts on the pituitary to promote a rapid discharge of TSH. This

reaction requires calcium ion for activation. The TSH released then stimulates the thyroid follicular cell to synthesise and release more hormone. T_4 exerts a negative feedback control of the system by inhibiting the release of TSH by the pituitary.

Thyrocalcitonin

Another hormone which can be released by the thyroid is thyrocalcitonin. It has been shown that this is produced in the C-cells of the thyroid of many species including man. Calcitonin acts on the skeleton to inhibit bone resorption and thus plays a part in calcium homeostasis by lowering the serum calcium.

Section 2

Occurrence and causes of Goitre

The condition known as goitre which simply means enlargement of the thyroid gland occurs in every country of the world in which it has been sought. Areas of endemic goitre, defined as those in which more than 10% of the population suffer from goitre, include the northwestern and Great Lakes areas of the U.S.A., the mountainous areas of Central and South America, several regions of Central Europe, especially the Alps, the Pyrenees and also parts of Finland, Yugoslavia and Greece;

several areas of Africa, particularly in the Congo; the Himalayas, China and in New Guinea.

Endemic goitre has existed in these areas for many thousands of years. There are references to it in ancient Hindu scriptures dating back to 2000 BC, and the Chinese Emperor Shen-Vung is reputed to have treated it with iodine-rich seaweed in 3000 BC. Pre-Columbian ceramic figurines showing the disease may be seen in museums in Central and South America.

...search into the cause of this enlargement has been carried out for many years. One of the first workers to suggest that there was a connection between iodine deficiency and the occurrence of goitre was a French chemist, Chatin, who in 1850 carried out iodine analysis of food, soil and water. It is now widely accepted that endemic goitre occurs in areas where the iodine content of the water, soil and locally grown food crops is low. Surveys carried out in many parts of the world have confirmed this to be the main cause of goitre in these areas.

Studies carried out in Venezuela by Roche (1959) show that in the goitrous areas a high thyroid uptake of radio-active iodine and a low urinary excretion of ^{131}I was found in most individuals. A low concentration of iodine in drinking water was also found. The

greater thyroid avidity for iodine is in accordance with the theory that lack of iodine is an important factor in the production of goitre but in every endemic area there are individuals who do not develop goitre although they do have a high thyroid uptake of ^{131}I. Therefore other factors are also involved in goitre production.

Investigations in South East Asia have shown similar results (reviewed by Ramalingaswami (1973)). Radioiodine uptakes were elevated, TSH levels were elevated, serum protein-bound iodide levels were reduced and the organic iodine content of glands examined at autopsy were low. The thyroids of goats from a goitrous area of India were also studied and similar results were found. Adding potassium iodide or potassium iodate to domestic salt substantially reduced the incidence of goitre in the Himalayan endemic goitre zone. This treatment also reduced the incidence of goitre in South America (reviewed by Stanbury (1969)).

The presence of a goitre in an individual causes little discomfort when small but large goitres may cause respiratory distress that requires surgery. However, in areas of endemic goitre other defects including deaf-mutism, cretinism and mental retardation are also found. Endemic cretinism is the most important aspect

of endemic goitre. It only occurs in association with severe endemic goitre and it probably arises due to foetal deprivation of thyroid hormone during critical phases of development. There does not seem to be a strong genetic contribution to endemic goitre although a higher prevalence of goitre was found in children whose parents had goitre than among those whose parents did not.

Goitre is caused by a failure of the thyroid to produce enough hormone. This causes a rise in secretion of TSH which increases the thyroidal clearance to enable the thyroid to accumulate a sufficient quantity of iodine. Then the thyroid begins to enlarge, giving increased cell mass with decreased follicular size. The iodination level and the thyroxine content of thyroglobulin also decrease, thus causing another increase in TSH. The initial cause of goitre may be the lack of dietary iodine, ingestion of an anti-thyroid substance which prevents the synthesis or release of sufficient thyroid hormone or a genetically determined enzyme defect.

An excess of dietary iodine will usually prevent the formation of a goitre in a normal individual but when the available iodine is at a critically low level, then the presence of a goitrogen may favour thyroid

enlargement. Sporadic goitre can occur due to the ingestion of some goitrogenic substance even when there is sufficient iodine.

Iodine may be displaced by the other halogens if they are present in excess and fluoride has been reported as being one of the factors responsible for goitre in South Africa and in the Punjab (Wilson (1941)). A number of drugs have been found to exert a goitrogenic action. Perchlorate which can be concentrated by the thyroid inhibits the active transport of iodide into the gland and when the iodine intake is low this leads to goitre. Thiocyanate also inhibits iodide transport but by a different mechanism. It does not appear to be concentrated by thyroid tissue but it is actively metabolised by the gland. It can also inhibit thyroid iodinations at slightly greater concentrations than are required for inhibition of iodide transport.

Delange, Thilly and Ermans (1968), as a result of studies of iodine metabolism in Idjwi Island, Zaire, concluded that iodine deficiency is not the direct cause of the higher prevalence of goitre found in North Idjwi compared to that in the South. Delange and Ermans (1971) linked this to the consumption of cassava grown in the goitrous area. The antithyroid action is probably the

result of the production of thiocyanate from cyanide arising from a cyanogenic glucoside contained in the cassava.

The organification of iodide is inhibited by a group of compounds containing the group $S = \overset{R}{\underset{|}{C}} - N =$. This includes thiourea, thiouracil, propylthiouracil and carbimazole. The coupling of iodotyrosines to form iodothyronines is most susceptible to the action of these compounds followed by the iodination of MIT to form DIT, then iodination of tyrosines to form MIT. Since iodide transport is not inhibited there is an accumulation of perchlorate dischargeable iodine. The mechanism of action of thionamides is not known but probably involves inhibition of peroxidases. Unlike perchlorate, increasing the dose of iodine does not prevent their action.

A number of drugs, mainly aminoheterocyclic compounds and substituted phenols used for purposes other than treatment of the thyroid have been shown to be goitrogenic. Some foods e.g. cassava as mentioned above are also known to contain goitrogenic substances. Chesney in 1928, found that cabbage caused goitre in rabbits. Other members of the Brassica family which contain thionamides are goitrogenic. Excessive iodine in the diet has also been shown to cause goitre. This

was found in Japan (Suzuki et al., 1965) in a coastal region where the inhabitants consumed large quantities of iodine-rich seaweed.

Calcium as a goitrogen

For more than a century there have been reports that there is an association between the production of goitre and the ingestion of excess calcium. In 1831 Boussingault (1831) after carrying out surveys in Colombia advanced the theory that excess calcium may cause goitre. Surveys by Orr (1931) in Great Britain and by Stott in India (1932) have shown that goitre often occurs in limestone regions. In 1948, Murray et al., (1948) published the results of a survey of thyroid enlargement and the iodine and mineral content of water in the British Isles. They suggested that the higher incidence of goitre in certain areas of England as compared with that in certain Scottish areas having water of similar iodine content, may be due to the hardness of English water compared to the softness of Scottish water. A more recent survey (1969-1971) in Zambia (Nwokolo (1974), Nwokolo and Ekejiuba (1974), Nwokolo and Khodorovsky (1974)) also suggests that a high calcium content of food, water and soil may be important in the aetiology of endemic goitre when the iodine content of

the environment is low. Soils of limestone and dolomite derivation which are rich in calcium are very extensive in Zambia and water from most wells has a very high calcium content. Liming of soil decreases entrance of iodine into plants and high calcium content diminishes the mobility of iodine in the soil. In the body calcium is thought to decrease the metabolic effect of thyroxine (Roche and Lissitzky, 1960). However, there is no general agreement on the goitrogenic role of calcium in the reported experimental studies.

Section 3

Development of a low iodine test diet

In order to investigate the production of goitre in experimental animals, it was necessary to formulate a suitable diet. The basic test diet used in the feeding experiments in this work is that developed by Levine, Remington and Von Kolnitz (1933). They were led to choose this diet which is based on the rachitogenic diet of Steenbock and Black (1925) because Krauss and Monroe (1930) found that it also produced enlarged thyroid glands. In order to provide a source of vitamin D to prevent rickets, Remington added irradiated yeast to this diet, the formulation of which is given in table 1. Analysis showed that this diet

contained 15 µg iodine/kg. When fed to rats, 35 days on the diet produced an average thyroid weight of 53.2 mg/100 g body weight. The addition of 400 µgI/kg to the diet produced a thyroid weight of 12.6 mg/100 g body weight.

The enlarged thyroids were dark red in colour with little or no iodine-containing colloid. Comparison of the dry thyroid weights showed that the enlargement obtained is primarily an actual gain in tissue weight and not merely accumulation of fluid although the hyperplastic glands do have a higher water content. The iodine concentration of the goitrous glands was about 0.88 µg iodine per gland compared to 10 µg iodine per gland for the rats fed iodine supplements.

The Steenbock-Black diet contains a large amount of calcium carbonate ($CaCO_3$) in order to give the abnormally high calcium : phosphorus ratio which causes rickets in the rat. Remington did not find any indication that either the high calcium content nor the abnormal Ca : P ratio were responsible for the goitrogenic activity of the diet. In subsequent investigations Remington (1937), therefore, used 1% Ca CO_3 instead of 3% Ca CO_3.

Levine, Remington diet (1933)		Sharpless' diet (1943)	
Yellow corn	- 76%	Soybean flour	- 75%
wheat gluten	- 20%	butterfat	- 5%
calcium carbonate	- 3%	calcium carbonate	- 0.5%
sodium chloride	- 1%	sodium chloride	- 1%
yeast	- 0.2%	brewers yeast	- 3%
		sucrose	- 15.5%
Iodine content 15 µg/kg		Iodine content 40 µg/kg	

Hellwig's diet (1935)		Taylor's diet (1954)	
Corn meal	- 50%	Yellow-maize meal	- 80.5%
rolled oats	- 50%	Bemax	- 10%
		low vitamin casein	- 6%
Iodine content not stated		sodium chloride	- 2%
		dried liver	- 0.5%
		Iodine content 87.6 µg/kg	
		Calcium content 3.58 g/kg	

Gandra and Coniglio's diet (1961)	
Corn meal	- 79%
wheat gluten	- 18%
dried liver	- 2%
sodium chloride	- 1%
Iodine content not stated	

Table 1

Composition of diets used for the production of goitre in the rat.

The effect of Remington's diet was further investigated by Money, Rall and Rawson (1952) who used the change in thyroid weight and the uptake of radioactive iodine by the thyroid as an indication of change in thyroid status. Since their diet contained three times as much iodine (45.0 μg/kg) as Remington's diet it was only slightly goitrogenic and even after 106 days (15 weeks) on the diet their rats had a thyroid weight of only 25.3 mg. The ^{131}I uptake of these glands was 36.4% and the average total iodine content was 5.3 μg per gland. They carried out experiments in which each component of the diet was omitted in turn (except for the corn meal). This decreased the thyroidal uptake of ^{131}I in every case but it did not change the thyroid weight significantly. The largest decrease in ^{131}I uptake occurred when $CaCO_3$ was omitted from the diet. From this they concluded that their diet did not contain a positive goitrogen but that the increase in thyroid weight was caused by a decrease in iodine intake.

From their results they suggest that, when dietary iodine is low, an increase in thyroid radioiodine uptake occurs first, followed by a decrease in the level of inorganic serum iodine. As the diet is continued, the thyroid iodine level decreases as the gland secretes stored hormone. When the level of circulating hormone decreases, the pituitary secretes more TSH, the results of which are then observed as an increase in thyroid weight.

Section 4

The effect of calcium on the rat thyroid

Several workers have investigated the goitrogenic role of calcium using various diets containing differing amounts of iodine and calcium.

Sharpless et al., (1943) using a diet that produced a slight thyroid enlargement investigated the goitrogenic effect of several calcium salts, namely calcium chloride, calcium phosphate, calcium carbonate and calcium lactate. The formulation of their basic diet is given in table 1. 2% $CaCl_2$ or the equivalent amount of calcium in the other salts was added to this diet. In some experiments they added 1% $CaCl_2$ to the drinking water instead of supplementing the food. Thyroids twice the normal size but half the size of those formed when the diet was not supplemented with iodine were produced by the basic diet. None of the calcium salts produced a real enlargement of the thyroids which all contained the same amount of iodine (1.2-2.1 µgI/gland).

Sharpless et al., also investigated the effect of adding 2% $CaCl_2$ to the drinking water of rats fed Hellwig's diet (see table 1). To prevent tetany it was necessary to supplement this diet with vitamin D for the animals not receiving calcium and with yeast for those receiving

2% $CaCl_2$. No significant differences in thyroid weight were observed.

The effect of adding vitamin D which is important in the absorption and utilisation of calcium was also investigated by the same workers. This did not change the thyroid weights when $CaCO_3$ was administered but it caused an increase when the calcium salt used was $CaCl_2$. They concluded that calcium does not influence the size of the thyroid gland but if fed with vitamin D, $CaCl_2$ can act as a goitrogenic agent. The mechanism of action proposed was that the chloride causes some loss of iodine from the gland followed by an increase in thyroid weight when an excess of calcium is absorbed.

Since some low iodine diets used for the production of goitre contain 1 or 2% $CaCO_3$, Taylor (1954) used a diet low in both calcium and iodine content. Its composition is given in table 1. He compared the thyroid weights and percentage radioiodine uptake of rats on this diet with those receiving 2% $CaCO_3$ or 2 µgI/day supplements. Significantly larger thyroids were found in the rats receiving calcium supplements than in the other groups, and they also had a greater uptake of ^{131}I. He showed that in the presence of $CaCO_3$ the radioiodine could still be absorbed from the gut. The increased avidity of the

gland for iodine showed that the trapping mechanism was not inhibited. Some protein binding still existed as shown by autoradiography but an injection of sodium thiocyanate did discharge some of the ^{131}I trapped in the gland. Excess iodine prevented the goitrogenic action of calcium. His conclusion is that the addition of 2% $CaCO_3$ to a low iodine diet produces a larger, more hyperplastic thyroid but the mechanism for this is not clear. It does not act like perchlorate since the trapping mechanism is not inhibited. It only acts when there is a low iodine intake and protein binding is only partially inhibited. Therefore, he suggested that calcium exerts its goitrogenic effect by inhibiting the synthesis of T_4 in the thyroid or by increasing iodide clearance by the kidney.

Similar experiments were carried out by Gandra and Coniglio (1961). Their experimental animals were fed a low iodine, low calcium diet, which is essentially that of Remington as can be seen in table 1. This they supplemented with (a) 260 µgI/kg, 15 g $CaCO_3$/kg and (b) 30 g $CaCO_3$/kg for 30 days then 60 g $CaCO_3$/kg for a further 10 days. They found that rats receiving excess dietary calcium had larger thyroids than the controls after 42 days (6 weeks) on the diets. An average thyroid weight of 34.8 mg was obtained for those receiving calcium as opposed to 23.7 mg for those on the basic low iodine, low calcium diet. This enlargement

was accompanied by increased content of inorganic iodine and decreased content of bound iodine in the thyroid although the total radioiodine uptake was less for those receiving calcium.

Incubations of rat thyroid slices in a buffer medium containing ^{131}I were also carried out by these workers. $CaCl_2$ was added to half the flasks and this decreased the total ^{131}I uptake of the slices by interfering with the conversion of free to bound iodine. The trapping of ^{131}I by the slices was not inhibited. Thus the goitrogenic action of calcium is due at least, in part, to a direct effect on the thyroid tissue. Blocking of the preliminary iodide oxidation step in the thyroxine-oxidase system seems to be a probable site of action of calcium. Less probable is the hypothesis that calcium activates a protease which degrades thyroglobulin or a deiodinase which removes iodine from tyrosines.

The calcium content of the thyroid and other tissues has been measured by Kaellis and Goldsmith (1965). They ashed the tissues in an incinerator after drying to constant weight, dissolved the ash in 1 M hydrochloric acid and then titrated the solution using the method of Bachra, Dauer and Sobel (1958) in order to measure the calcium content. The thyroid contained more calcium in mg/100 g wet weight of tissue than either the liver,

submaxillary gland, kidney or serum (mg/100 ml). They showed that the calcium concentration of the thyroid fell if the rats were fed thiourea but it rose with a high calcium low iodine diet (Remington diet +2% $CaCO_3$ for 8 weeks). The concentration remained the same as the controls on a normal diet when the rats were fed the Remington low iodine diet without calcium supplements. They do not state whether calcium exerted a goitrogenic effect or not in their dietary experiments. They concluded that the thyroid captures calcium since the normal thyroid level is 3½ times that of the serum. Some of this calcium may be complexed as calcium-thyroglobulin. This complex is possibly on tyrosine which resists iodination or on an iodinated tyrosine and interferes with thyronine formation.

Section 5

Localisation of calcium in the thyroid

Since it has been shown that the thyroid contains a high concentration of calcium various efforts have been made to determine the form and location of calcium in the gland.

40% of normal human thyroids have been shown to contain discrete crystals of calcium oxalate and in some abnormal glands calcium-containing structures termed psammoma bodies have been found. Using the technique of electron-probe microanalysis Robison, Van Middlesworth and Davis (1971)

determined the distribution of calcium, iodine and phosphorus, in various human thyroid glands. Iodine is uniformly distributed throughout the colloid which is surrounded by phosphorus areas, indicative of epithelial cells. Calcium is distributed throughout the colloid region and is also highly concentrated in the follicular cells. The distribution of phosphorus indicates that most of the calcium is not present as calcium phosphate. Since the calcium concentration and distribution from frozen sections and formalin-fixed embedded sections were identical some of the calcium is likely to be bound to thyroglobulin. This would give support to Taylor's hypothesis that the synthesis of T_4 is inhibited by calcium-binding to thyroglobulin. High calcium areas such as is observed in nodular goitre could thus be formed by the precipitation of calcium as the lumen becomes smaller.

Similar results have also been obtained by Dupuy, Rouais and Blanquet (1976) who used the (glyoxal bis 2 hydroxyanilcalcium) complex to localise the calcium in the rat thyroid. Again the colloid calcium content seems more important than that of epithelium.

The distribution of calcium and the effect of various substances on it have been investigated using dog thyroid slices incubated in vitro with ^{45}Ca by Rodesch, Bogaert,

Dumont (1976). Their work suggests that thyroid tissue calcium is distributed in at least two compartments. One compartment (A) may be the extracellular space since the release of $^{45}Ca^{2+}$ taken up by it was not influenced by the calcium ionophore A-23187 which increases cell permeability to calcium or by metabolic inhibitors or by TSH. Incubation at $0^{o}C$ increased the uptake of ^{45}Ca by this compartment but decreased the release. The second compartment is a slowly exchangeable store which is probably a mitochondrial location since the release of ^{45}Ca was accelerated by ionophore A-23187, antimycin A and also by TSH.

Section 6

Influence of calcium on thyroid metabolism

It seems reasonable to consider that the large concentration of calcium in the thyroid is playing an active role in the metabolism of the gland.

Most stages in the synthesis and release of the thyroid hormones are controlled by TSH. Hachiya et al (1976) have shown that TSH will stimulate the thyroidal uptake of radioactive ^{45}Ca both in vitro and in vivo. Feeding a low iodine diet for one or two weeks to increase the endogenous TSH, increased the uptake of ^{45}Ca into mouse thyroid two or three times respectively in comparison to controls. The addition of TSH to

glands incubated with ^{45}Ca increased the uptake about twice. Similar effects were also produced by long-acting thyroid stimulator (LATS) which is another thyroid stimulator with some of the properties of TSH, Therefore the enhancement of calcium uptake by the gland may be important for the action of TSH on the stimulation of hormone production.

It has been shown that calcium is involved in the stimulus-secretion coupling in several secretory systems. The secretory mechanism is activated when the normal physiological stimulator combines with a membrane receptor promoting the influx of calcium ions. Removing calcium ions from the incubation medium will, therefore, block secretion from a number of endocrine cell types.

However, Williams (1972) showed that removal of calcium or magnesium ions from the incubation media did not affect the release of thyroidal ^{131}I but that elevated levels of either cation inhibited both TSH and cyclic adenosine monophosphate (c AMP) stimulated release of ^{131}I. Willems, Rocmans and Dumont (1971) have also shown that calcium is not required for thyroid secretion but that it is required for the TSH-stimulated oxidation of C-1-glucose and also for the organification of iodide.

Several of the functions of the thyroid, including iodide binding to proteins and secretion are regulated through the activation of the adenylate cyclase system by TSH. It has been suggested by Zor et al., (1968), that calcium is essential for the action of c AMP rather than its formation since the effects of dibutyryl cyclic AMP (DB - cAMP) on glucose oxidation was also reduced in the absence of Ca^{2+} ions. The calcium ionophore A-23187 has been used to study some of these functions. Yamashita et al., (1976), showed that calcium plus a high concentration of ionophore enhanced ^{14}C-1-glucose oxidation but it lowered the tissue/medium (T/M) radioiodine ratio in slices under conditions where organic binding was inhibited by methimazole. They also obtained a similar depression in the T/M ratio with TSH and DB cAMP. The ionophore increased the cyclic guanosine monophosphate (cGMP) level in the slices and slightly decreased the basal but not the TSH-stimulated cAMP level.

On the other hand, Van Sande et al., (1976), showed that calcium at high concentration plus ionophore inhibited the accumulation of cAMP, hormone secretion and pseudopod formation in TSH-stimulated slices but enhanced iodide organification and C-1-glucose oxidation. They found that calcium plus ionophore also increased the

accumulation of cGMP. Agents, for example, acetylcholine, carbomylcholine which enhance cGMP levels had no effect in calcium depleted thyroid slices.

Therefore intracellular calcium modulates the cGMP level. Thus increased Ca^{2+} ions and/or cGMP act like cAMP in their activation of iodination and act in opposition to cAMP in that they depress secretion. It is not known whether the growth effect of TSH is mediated by cAMP.

Section 7

Purpose of this work

The main aim of this study was to define the goitrogenic role of calcium in the rat and to try to explain the mechanism of its action.

Chapter 2

Materials and Methods

Section 1

Materials

The materials and the suppliers used for the animal feeding experiments are as follows:-

Materials	Suppliers
Male Sprague-Dawley rats, 120-150g body weight and also weanlings	A. Tuck and Son, Rayleigh, Essex
Remington low iodine diets	ICN Nutritional Biochemicals Corporation and Teklad Test Diet
Calcium deficient, low iodine diet	Teklad Test Diets
Dietary additives	
Calcium carbonate ($CaCO_3$)	British Drug Houses Ltd.
Potassium perchlorate ($KClO_4$)	British Drug Houses Ltd.
Propylthiouracil (PTU)	Koch-Light Laboratories Ltd.
Potassium iodide (KI)	British Drug Houses Ltd.

Dietary regimes

Control experimental diet

Diet 41B (Modified) (pelleted) from Oxoid Ltd. This diet is stated to contain 0.84% calcium and at least 2300 µg iodine/kg diet.

Preliminary experimental diets

Low iodine diet (LID) - Remington low iodine diet as supplied by Nutritional Biochemicals. This diet is stated to contain 0.4% calcium. The precise iodine content of this diet is unknown. Its composition is as follows:-

Yellow corn meal (grown in iodine deficient area)	78%
Wheat gluten	18%
Brewers yeast	2%
Calcium carbonate	1%
Sodium chloride	1%

This diet was supplemented:-

Diet X = LID + 2.5g $CaCO_3$/kg diet

Diet Y = LID + 5g $CaCO_3$/kg diet

Diet Z = LID + 20g $CaCO_3$/kg diet

Distilled water was supplied ad libitum.

Definitive experimental diets

The basic diets in these experiments were supplied by Teklad Test Diets.

Diet A - calcium deficient, low iodine diet supplied by Teklad Test Diets.

This diet is stated to contain 0.035% calcium and less than 50µg iodine/kg diet and its formulation is:-

Ground whole yellow corn	79%
Vital wheat gluten	18%
Brewer's yeast powder	2%
Sodium chloride (iodine free)	1%

It is thus identical in composition to the Remington low iodine diet but with the omission of 1% $CaCO_3$. This diet was supplemented:-

Diet B = Diet A + 5g $CaCO_3$/kg

Diet C = Remington low iodine diet i.e., Diet A + 10g $CaCO_3$/kg

Diet D = Diet A + 20g $CaCO_3$/kg

PTU treated rats

Diet A + 0.2g PTU/kg diet

Diet C + 0.2g PTU/kg diet

$KClO_4$ treated rats

Diet A + 20g $KClO_4$/kg diet

Diet C + 20g $KClO_4$/kg diet

Unless stated otherwise, all animals received distilled water. In some experiments 2% calcium chloride ($CaCl_2$ $6(H_2O)$) or KI (1.31 mg KI/l) were given in the drinking water.

Buffer solutions

Krebs No. 2 buffer

83 ml 0.15M sodium chloride

4 ml 0.15M potassium chloride

1 ml 0.16M potassium dihydrogen phosphate

1 ml 0.16M magnesium sulphate

3 ml 0.15M sodium bicarbonate

18 ml of a sodium phosphate buffer solution containing 100 ml 0.1M Na_2HPO_4 and 25 ml 0.1M NaH_2PO_4.

The pH was adjusted to 7.40 by the addition of 0.12M Na_2HPO_4

Phosphate buffered saline (PBS).

0.15M sodium chloride in 0.01M potassium phosphate buffer pH 6.8.

Section 2

Methods

Determination of tissue calcium

The calcium content of the thyroids of rats from the dietary experiments was measured after chloric acid digestion by atomic absorption spectrophotometry (A.A.S). The calcium concentrations of the thyroid, liver, kidney, heart and muscle of rats on diet 41B were also determined in the same manner.

Chloric acid was prepared as suggested by Leffler (1954) for the measurement of protein-bound iodine. Four to six rat thyroids were digested together to provide a sufficient concentration of calcium for measurement. The glands were sliced while still frozen and heated with 1 ml distilled, deionised water and 3-4 ml chloric acid in a 50 ml beaker on a hot plate at a low temperature (90°C). If an iodine estimation was also to be carried out, 0.5 ml of 0.5% sodium chromate solution was also added to keep the iodine in the oxidised form. After complete digestion of the glands which took about 1¼ hours, the digestion beakers were left overnight in the fume cupboard to allow volatilisation of the excess oxidising agent. The digests were then made up to 10 ml with distilled, deionised water in volumetric flasks. A 1:5 dilution with 0.1% lanthanum chloride (BDH reagent) was made before reading the calcium concentrations of the digests on the A.A.S. This type of spectrophotometry measures the absorption of a beam of monochromatic light by atoms in a flame. The energy absorbed is proportional to the numbers of atoms present in the optical path and therefore the concentration of the element in the sample can be determined. The instrument used was a Perkin-Elmer Model 403 AA spectrophotometer.

The heart and muscle were sliced while frozen and digested as above with 5 ml chloric acid. The liver and kidneys were homogenised to facilitate digestion before the addition of 5 ml chloric acid.

All glassware used in the determination of calcium was soaked in chromic acid for 48 hours before use to remove traces of calcium since initially a very high concentration of calcium was found in distilled, deionised water blanks. For each set of analyses, two distilled, deionised water blanks and a range of calcium standards prepared from BDH standard calcium chloride solution for atomic absorption (containing 1 mg Ca/ml) were included with the digestion of the test samples and their calcium concentration was measured. Before the method was used for tissue analysis, a large number of calcium standards were processed in order to determine the accuracy and reproducibility of the assay. The standard deviation for standards containing 0.62 mmol Ca/l, 1.25 mmol Ca/l and 2.50 mmol Ca/l was $\pm$ 0.02 mmol Ca/l. The dilution of the samples with lanthanum chloride solution before reading on the spectrophotometer was necessary to prevent interference from phosphate ions which can depress the calcium signal.

The measurement of serum calcium was also carried out by atomic absorption after a 1:50 dilution of the serum by 0.1% lanthanum chloride solution.

Determination of thyroid iodine

The iodine concentration of the chloric acid digests of the thyroids from some of the dietary experiments was measured by the Department of Medicine, Glasgow Western Infirmary. The method used was an automated procedure given in the Technicon-Auto Analyzer Methodology Manual No PB/D-1. The iodine concentration of the blanks and potassium periodate (KIO_3) standards included in the digestion process were also assayed.

Care had to be taken during the acid digestion to prevent loss of iodine which would give low results. The coefficient of variation for the repeated assay of the standards used (1.6μmol I/l, 3.2 μmol I/l, 40 μmol I/l, 80 μmol I/l) was between 5-10% of the standard.

Determination of serum thyroxine (T_4) and serum thyroid-stimulating hormone (TSH)

The T_4 and TSH in the rat serum were measured by a radioimmunoassay (RIA) method. RIA is a protein-binding method of analysis. Specific antibodies against the compound to be estimated are prepared. When a known excess of radio-actively labelled antigen and an unknown amount of stable antigen in the test serum are mixed, they compete for the binding sites available from a

defined amount of the antibody. The greater the amount of stable antigen present, the fewer of the labelled molecules of the antigen will bind to the antibodies and therefore more of the labelled molecules will be present in the free state. At equilibrium a certain ratio of free to bound molecules will exist and by measuring the free or the antibody-bound labelled molecules it is possible to establish standard curves to measure the unknown quantity of unlabelled compound present. In the two RIA methods used here, the bound antigen is separated from the free molecules by binding the antigen-antibody complex to a second antibody and the radioactivity in the bound fraction is measured to plot the standard curve.

Radioimmunoassay of rat thyroxine

The reagents used in this assay were supplied by the Radioimmunoassay Unit in the Biochemistry Department, Glasgow Royal Infirmary.

T_4 stock standard solution was diluted to give a range of working standards containing 0 to 400 nmol T_4/l serum. 100 µl of 0.1% bovine serum albumin (BSA) in 0.05M barbitone buffer pH 8.6 were pipetted into each sample tube. 25 µl test rat serum were then added in duplicate. 25 µl T_4-free rat serum were added to each of the tubes containing 100 µl standard. To displace

T_4 from its binding proteins 400 µg 1-anilinonaphthalene-8-sulphonic acid (magnesium salt) (ANS)/100 µl barbitone buffer was added to each tube. 500 pg ^{125}I-labelled T_4 and then T_4 antisera prepared in rabbits to give a final dilution of 1:2000 were added. Finally the second antibody, donkey-antirabbit (DAR), in a final dilution of 1:96 and normal rabbit serum in a final dilution of 1:2000 were added to each tube. The assay tubes were incubated overnight at 4°C. They were centrifuged at 2000 rpm for 30 minutes on the refrigerated MSE to give a pellet containing the antibody bound T_4. The supernatants were aspirated off at the water pump and the pellets were counted on a Wallac gamma counter for long enough to obtain 10,000 counts in the assay tubes containing the total amount of label added.

The results were calculated using a computer programme and a standard curve of percentage bound versus concentration of T_4 in the standards was plotted.

In each assay, tubes not containing any T_4 antibody were also processed in the same manner as the test samples to determine the non-specific binding of the labelled T_4. Tnree serum samples, containing high, normal and low concentrations of T_4 which had been assayed several times by the RIA unit, were included in each assay as quality controls.

A typical standard curve for the assay of rat serum T_4 is shown in fig. 1. The standard curves obtained were good smooth curves with maximum binding greater than 46%. Non-specific binding was usually less than 1.6%. The sensitivity of the assay was very good since a T_4 concentration down to about 7 nmol T_4/l could be detected and measured. The mid-point of the assay was between 55-58 nmol T_4/l which is in the normal range. The standard deviation of the assay duplicates which is calculated by the programme used for the results was 1.7% which is acceptable for a manual assay. The coefficient of variation for the three known quality controls is about 6%.

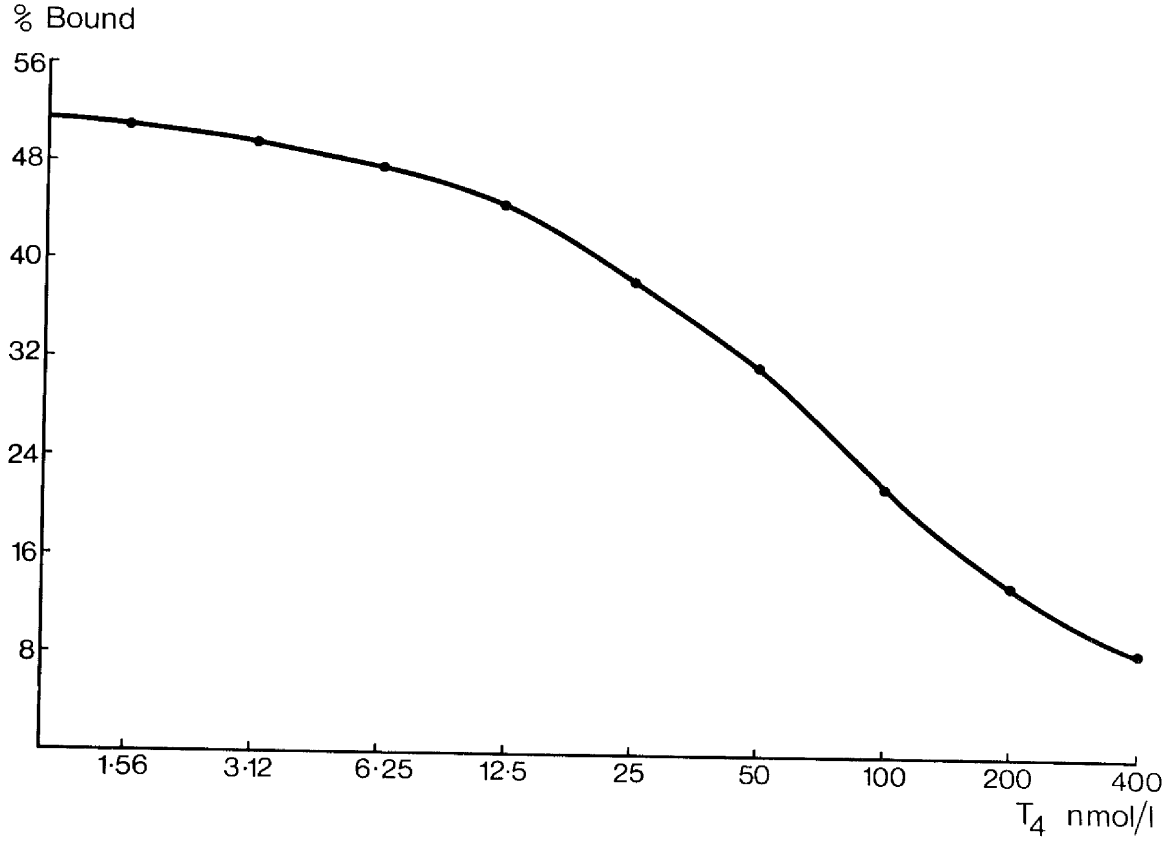
The normal range of serum T_4 for control rats on diet 41B for various lengths of time was found to be 33-75 nmol T_4/l with an average of 49.5 nmol T_4/l (39 sera analysed). 70% of the normal values were between 40-60 nmol T_4/l.

Radioimmunoassay of Rat Thyroid Stimulating Hormone (TSH)

Purified rat TSH for radioiodination, antiserum to rat TSH prepared in rabbits and rat TSH reference preparation (TSH-RP-1) were provided by the National Institute of Arthritis and Metabolic Diseases. The

FIGURE 1

A typical standard curve for RIA of rat T_4


% Bound
56
48
40
32
24
16
8
1·56
3·12
6·25
12·5
25
50
100
200
400
T4 nmol/l

iodination of rat TSH was carried out every two weeks using a standard Chloramine T reaction. Two aliquots each containing 2.5 µg TSH in 25 µl buffer (0.01M phosphate, 0.15M NaCl + 0.01% merthiolate pH 7.6) were allowed to thaw and combined in a Henley centrifuge tube. 25 µl of 0.5M phosphate buffer were added. Using a syringe 15 µl (1.5 mCi) of Na ^{125}I were added at the same time as 25 µl of a Chloramine T solution containing 15 mg Chloramine T in 10 ml of 0.05M phosphate buffer pH 7.6. The reaction tube was gently mixed on the Vortex whirlimix for 40 seconds. Then 50 µl of a solution of 24 mg sodium metabisulphite in 10 ml 0.05M phosphate buffer were added and the solution was again mixed for a further 40 seconds. The oxidation reaction was stopped by adding 100 µl of normal rabbit serum to the mixture which was kept on ice.

To assess the percentage incorporation of ^{125}I an aliquot of the reaction mixture was diluted 1:10 and 10 µl was applied to two paper electrophoresis strips. The strips were run at 5mA for 50 minutes in a barbitone buffer. The remaining reaction mixture was applied to a Sephadex G-75 column with a 2 cm plug of Amberlite IRA-400 ion exchange resin on the top of the Sephadex. This column had been previously coated with 2 ml of 2% bovine serum albumin (BSA) in the phosphosaline buffer

and then washed with 20 ml buffer. The 0.01M phosphosaline buffer was used to elute the fractions. Twenty 0.5 ml fractions were collected in tubes containing 200 µl of 1% BSA in this buffer. The numbered fraction tubes were stoppered and counted at fixed geometry in a hand gamma counter. The four fractions containing the maximum radioactivity, (that is, the peak, tube preceding and two following) were kept stored in ice.

The purity of the labelled TSH was assessed by applying 10 µl of the peak column fraction to each of two electrophoresis strips which were run as stated above. All four strips were dried, cut into 1 cm fractions and counted for 10 seconds on the gamma counter.

The protein and iodine peaks were located in each strip and the percentage radioactivity in the protein peak relative to the total radioactivity on the strip was calculated. The percentage incorporation of iodine was between 42%-53% and the percentage purity of the labelled TSH with regard to unbound iodine was greater than 90%. The specific radioactivity was also calculated from the first set of strips since in each assay a known mass (50 pg) of label is added rather than a known amount of radioactivity.

For each assay a set of standards was prepared from the standard TSH supplied. The TSH was diluted in 1% BSA in 0.01M phosphosaline buffer and 11 dilutions containing 8 μU/ml to 500 μU/ml were used.

Assay procedure

To each of the standards, 100 μl thyrotoxic human serum were added. 100 μl test samples were pipetted in duplicate into tubes containing 100 μl 1% BSA in phosphosaline. 50 μl of the antibody solution were diluted with 40 ml 0.1% BSA in a buffer containing 0.01M phosphate, 0.15 M NaCl, 0.01% merthiolate + 0.05M EDTA (pH 7.6). 200 μl of this antibody solution were then added to each tube in the assay. They were then mixed on the Whirlimix and left to incubate for 24 hours at room temperature.

50 pg of ^{125}I-TSH in 100 μl buffer were added to each tube. After mixing they were again incubated for 24 hours at room temperature.

To precipitate the antibody complex and thus to separate the free TSH from antibody-bound TSH, solutions of normal rabbit serum in a final dilution of 1:1000 and then donkey anti-rabbit serum were added in a final dilution of 1:80. Starch at 1 mg/tube was also added with the DAR solution to stabilise the pellets obtained.

The assay tubes were incubated overnight at 4°C after the addition of the second antibody.

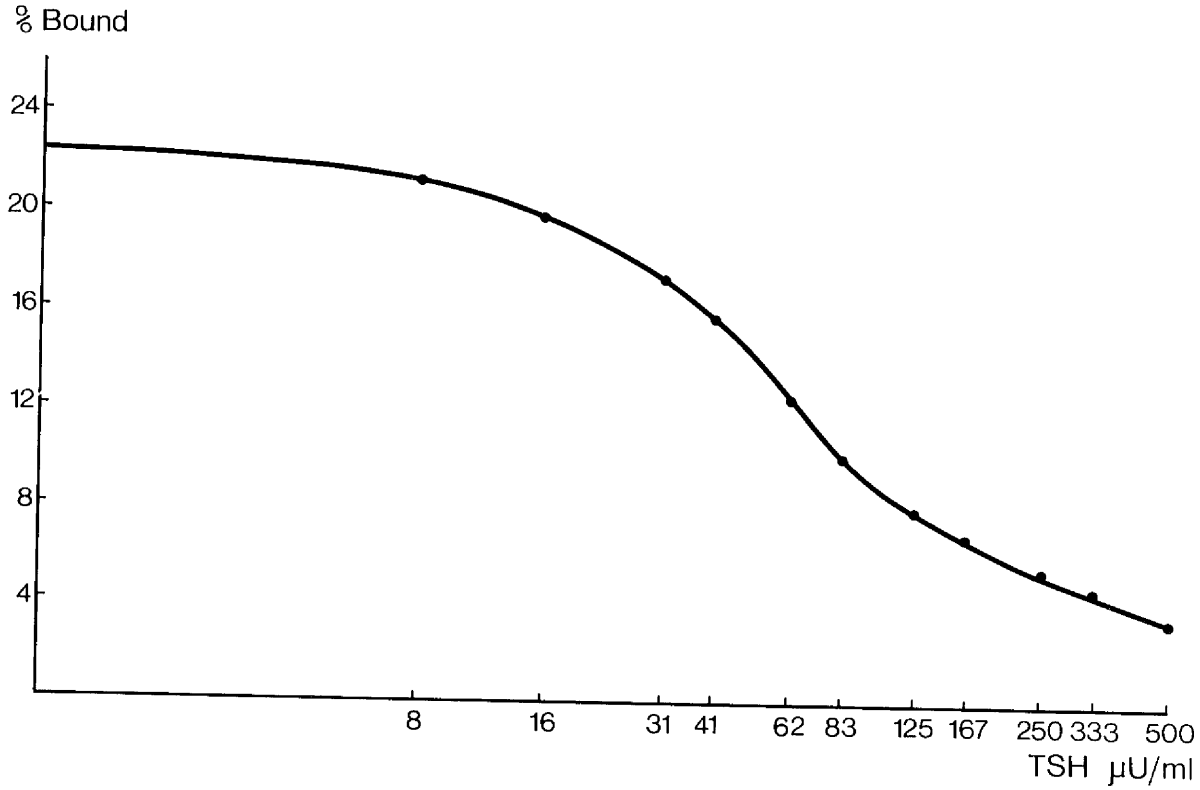
The tubes were centrifuged at 2,000 rpm for 30 minutes in the refrigerated MSE. The supernatants were aspirated off and the pellets were counted on a Wallac gamma counter for sufficient time to accumulate at least 10,000 counts for tubes containing 50 pg of ^{125}I-TSH only. Tubes containing buffer, label but no TSH-antibody were also included in each assay to assess the non-specific binding of the label.

The results were calculated using a computer programme and the standard curve was plotted on semilog paper.

A typical standard curve for the assay of rat serum TSH is shown in fig. 2. The sensitivity of this assay was also good. The lowest limit of the assay was usually between 14-19 μU/ml TSH. Since all of the test serum samples were from rats on a low iodine diet which should raise the TSH concentration, their TSH level was within the range detectable by the conditions described. Maximum binding was usually greater than 20%. The mid-point of the assay was 55-67 μU/ml TSH which is in the normal range. The S.D. for the assay duplicates was about 1.5% which is acceptable. Non-specific binding which measures the binding of the label

FIGURE 2

A typical standard curve for RIA of rat TSH


% Bound
24
20
16
12
8
4
8
16
31
41
62
83
125
167
250
333
500
TSH µU/ml

in the absence of its antibody was about 6-7%. When the same sample was assayed several times in the same assay the coefficient of variation was about 7%. Inter-assay variation was also within acceptable limits (coefficient of variation - about 11%) although the greater the amount of TSH in the serum, the less reproducible the results since the standard curves flatten out above 250 μU TSH/ml. Rats on the normal diet 41B exhibit a wide range of TSH values - from 42 μU/ml to 150 μU/ml with an average of 94.1 μU/ml (33 analyses). Therefore, many of the rats on low iodine diets had serum TSH that was in this range. Animals that received the goitrogens PTU or $KClO_4$ had very high levels of TSH and thus their serum had to be diluted usually 1:1 with buffer before measurement.

Lowry Protein Estimations

Lowry et al., (1951)

The solutions used in this method were :-

(1) 2% Na_2CO_3 in 0.1 MNaOH

(2) 1% Cu SO_4 $5H_2O$

(3) 2% Na K tartrate

Solution A = 100 ml (1) + 1 ml (2) + 1 ml (3)

Solution B = 5 ml Folin-Ciocalteu's reagent + 9 ml distilled H_2O. To 1 ml of protein solution of a suitable dilution 5 ml of solution A were added. This was

mixed and left for at least 10 minutes. 0.5 ml of solution B was then added and mixed immediately. The tubes were allowed to stand for 30 minutes and then the optical density at 750 nm was read on a Gilford spectrophotometer SP 500.

A distilled water blank was included in each assay and a standard curve was prepared from solutions of BSA in distilled water containing from 25 to 400 µg protein/ml. Each assay sample and each standard was assayed in duplicate.

A standard graph was drawn and the unknown concentrations read from this.

In vitro incubation studies

Fresh sheep thyroid glands were obtained from the abattoir and kept in ice until required. The glands were freed from fat and thin slices were prepared using a razor blade. Samples of tissue weighing 50-100 mg were placed in 25 ml-conical flasks containing 2 ml Krebs No. 2 buffer which had been gassed previously with oxygen. The flasks were put into a shaking water bath at 37°C in an oxygen atmosphere to equilibrate for 15 minutes. 10 µCi of radioactive label were then added to each flask. Incubations were carried out using ^{45}Ca or ^{125}I in the medium.

At the end of the incubation period, the medium was immediately poured off, the slices washed briefly in 2 ml cold PBS, blotted on filter paper and reweighed. They were then homogenised in 2 ml PBS and centrifuged on the refrigerated MSE for 10 minutes at 20,000g. The medium was centrifuged likewise.

0.05 ml medium and 0.5 ml supernatant from the homogenised slices were counted in a Packard Tricarb liquid scintillation counter after the addition of 5 ml Instagel (Packard) when ^{45}Ca (a beta emitter) was added to the medium. ^{125}I which is a gamma emitter was counted on a Packard gamma counter.

The insoluble pellets were digested in 0.5 ml Soluene-100 (Packard) or 0.2 ml Soluene-350 (Packard) at 50°C for one hour and counted after the addition of 5 ml Dimilume (Packard).

In four incubations the slices were heated at 85°C before the addition of ^{45}Ca or ^{125}I.

Chapter 3

Results of Dietary Experiments

Section 1. Effect of dietary calcium on thyroid weight

1. Preliminary feeding experiments.

In the initial set of dietary experiments, the Remington low iodine diet (LID) from Nutritional Biochemicals (NBC) was used as the basic experimental diet to which calcium carbonate ($CaCO_3$) was added to give diets X, Y, and Z as stated above. In each experiment male Sprague-Dawley rats of about 120-150 g body weight were arbitrarily divided into three or four groups each containing five or six rats. Initially all the animals were given LID for four or eight weeks to cause a slight degree of iodine deficiency in order to make the thyroid more sensitive to the effect of a mild goitrogen. After this time, one group remained on LID, while the others were given either diet X, diet Y or diet Z. These diets were administered for varying lengths of time ranging from 4 to 16 weeks.

At the end of the experimental period, the animals were killed by ether anaesthetic, weighed and the thyroids were carefully removed. The glands were also weighed then stored in the freezer at -20°C till their calcium content could be measured.

The rat body weights, the thyroid weights and the thyroid calcium concentrations (mg Ca/100 g fresh thyroid weight) obtained for animals on diets LID, X, Y, Z for various times are shown in table 2.

Diet	No. of rats	Body weight (g)	Thyroid weight (mg)	Thyroid calcium (mg/100g)
Experiment 1.	Rats on LID for 4 weeks + 8 weeks on diets			
LID	5	331	28.84	-
X	5	332	23.28	-
Z	5	308	30.72	-
Experiment 2.	Rats on LID for 4 weeks + 12 weeks on diets			
LID	5	352	30.49	-
X	5	352	31.86	-
Z	6	326	35.45	-
Experiment 3.	Rats on LID for 8 weeks + 4 weeks on diets			
LID	11	366	26.78	18.1
X	5	421	28.20	-
Y	6	337	28.62	17.2
Z	11	320	27.11	20.4
Experiment 4.	Rats on LID for 8 weeks + 8 weeks on diets			
LID	11	374	31.82	20.9
X	5	436	38.95	18.5
Y	7	341	30.30	23.6
Z	11	379	34 .42	18.7

Table 2

Results of preliminary feeding experiments

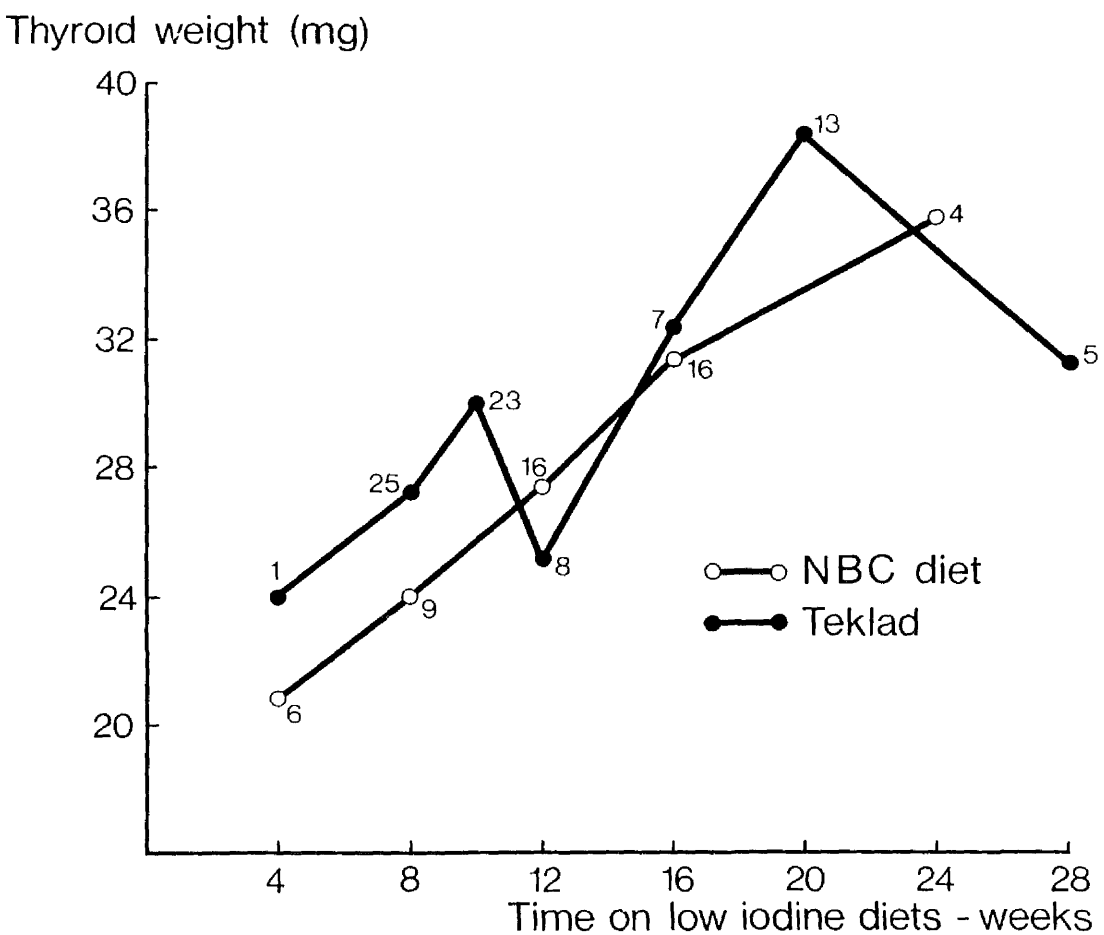
The body weights have increased from the initial weights of 120-150g to 300-430g depending on the length of time on the diets. In each experiment the rat body weights do not differ much for the different diets, although rats receiving the highest concentration of dietary calcium (3% $CaCO_3$ in total) tended to be smaller than the others since they did not eat as much of that diet.

The average thyroid weight at the beginning of each experiment was about 7-10 mg. The basic low iodine diet used in these experiments, obtained from NBC is only slightly goitrogenic. Even after 16 weeks on it, the thyroid only reached a weight of 32 mg which is a little more than twice the normal thyroid weight for this size of rat. The graph (fig. 3) shows the increase in thyroid weight with increasing time on NBC low iodine diet. After 4 weeks on the diet the rat thyroid weight has increased to 20.79 mg and this weight increases by about 3 mg/4 weeks on the diet till a weight of 35.92 mg is reached at 24 weeks on the diet.

In the first two experiments shown in table 2, the rats were kept on LID for 4 weeks before adding calcium to the diet and thus they would have thyroids of about 21 mg when their dietary regimes were changed.

FIGURE 3

Comparison of thyroid weights of rats on low iodine diets produced by NBC and Teklad


Thyroid weight (mg)
40
36
32
28
24
20
4
8
12
16
20
24
28
Time on low iodine diets - weeks
NBC diet
Teklad
1
25
23
8
7
13
5
6
9
16
16
4

After a further 8 weeks on LID the average thyroid weight increased to 28.84 mg whereas rats on diet X during this time had an average thyroid weight of 23.28 mg. Diet Z containing the highest concentration of $CaCO_3$ produced thyroids of similar weight to those produced by LID. After 12 weeks on the three diets (Experiment 2) the thyroid weights were the same for animals on LID and diet X and showed a small increase for those on diet Z.

In the next two experiments (Experiments 3 and 4) the initial period of feeding LID was increased to 8 weeks giving a thyroid weight of 24 mg before addition of the calcium. A further 4 weeks on the four diets of differing calcium contents produced thyroids of the same average weight for each diet. 8 weeks on LID then 8 weeks on the diets (Experiment 4) also produced thyroids of about the same weight although diet X produced a slightly larger goitre.

Comparing the thyroid weights of rats in Experiment 2 with those in Experiment 4 which have been on a low iodine intake for a total of 16 weeks, although the calcium intake is different, there is no difference in their thyroid weights. Similarly for rats in Experiment 1 and Experiment 3 which have been on a low iodine intake for 12 weeks, there is again no difference in the thyroid weights.

The calcium concentration of some of the glands was measured and the results are shown in table 2. The calcium concentration of the thyroids is lower than that found in normal glands (see p.77) but there is little difference between the differing calcium diets.

Since the NBC diet only produced a goitrogenic effect after a long period of time, it was decided in August 1975 to try the Remington low iodine diet produced by Teklad Test Diets which has the same formulation. Information from Teklad states that this diet should contain less than 50 µg I/kg diet. Riesco, Taurog and Larsen (1976) have compared the iodine concentrations and the effect on the rat thyroid of both the NBC Remington low iodine diet and the Teklad Remington low iodine diet. The NBC diet contained a much higher concentration of iodine, ranging from 70-123 µgI/kg diet and it produced a smaller goitre than the Teklad diet which contained about 22 µgI/kg diet when corn purchased in Ohio was used in its formulation.

As can be seen from fig. 3 this low iodine diet does initially produce a larger goitre than the NBC diet but after 12 weeks on the diets, similar weights of thyroids are produced by both diets.

Since this Remington diet already contains a substantial amount of $CaCO_3$ and since the preliminary experiments showed that addition of more calcium does not produce a further

increase in goitre size, in subsequent experiments the diet was obtained from Teklad Test Diets without the 1% $CaCO_3$ it normally contains. This calcium deficient low iodine diet is designated diet A.

2. Definitive feeding experiments.

Results of the thyroid weights after 8 and 10 weeks on the diets.

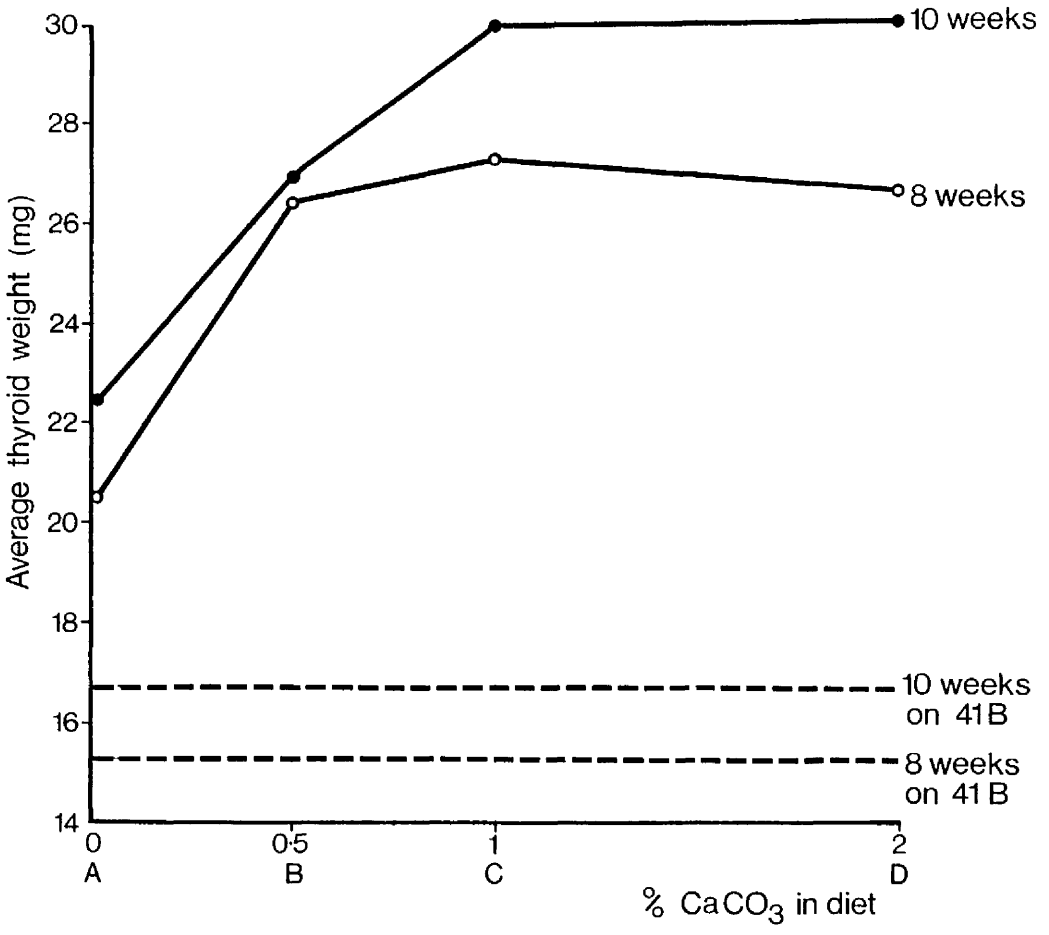
In all the following dietary experiments both the Remington low iodine diet (Diet C) and the calcium deficient low iodine diet (Diet A) were obtained from Teklad Test Diets.

In these experiments male Sprague-Dawley rats, initially weighing 120-150g were divided into five groups each containing 6-8 rats. One group was given diet 41B our normal stock diet. The second group was given diet A the basic calcium deficient, low iodine diet. The other three groups were given the calcium supplemented diets B, C or D. After 8 or 10 weeks on these dietary regimes the rats were sacrificed and the body weights were recorded. The thyroid glands were removed and weighed.

The rat body weights and the thyroid weights of the rats on diets for 8 weeks (Experiment 5) are shown in table 3. The thyroid weights are also shown on the graph (fig. 4).

FIGURE 4

Thyroid weights after 8 and 10 weeks on calcium diets


30
28
26
24
22
20
18
16
14
Average thyroid weight (mg)
10 weeks
8 weeks
10 weeks
on 41B
8 weeks
on 41B
0
A
0·5
B
1
C
2
D
% CaCO3 in diet

Diet	No. of Rats	Rat body weight (g)	Thyroid weight (mg)
41B	31	338	15.23
A	21	207	20.59
B	20	297	26.40
C	30	320	27.28
D	22	296	26.75

Experiment 5. Rats on diets for 8 weeks

Table 3

Control rats attain an average body weight of 338g after 8 weeks and this weight is also reached by animals on diet C. Rats on diet A are much smaller than those receiving calcium supplements. They also showed other signs of calcium deficiency - fur loss, nervousness, tetany after 7 to 10 weeks on this diet. Those on diet B have an average body weight of about 300 g which is the same weight as is reached by those rats receiving the maximum amount of calcium on diet D.

All the low iodine diets cause an increase in thyroid weight compared to the control group on 41B. After 8 weeks on the diets, rats receiving calcium supplements had larger thyroids than those on diet A. When the results are analysed statistically by Student's t test of significance, the differences between the thyroid weights

on diet A and diet C and also for diet A and diet D are significant at the 2% level. However there is no difference in the thyroid weights of rats on diets B, C or D.

The rat body weights and the thyroid weights of rats on the same diets as above for 10 weeks (Experiment 6) are shown in table 4 and in the graph (fig. 4).

Diet	No. of rats	Rat weight (g)	Thyroid weight (mg)
41B	31	386	16.66
A	30	238	22.44
B	32	335	26.91
C	29	359	30.05
D	32	339	30.25

For A and D, $P = 0.005$

A and C, $P = 0.0025$

A and B, $P < 0.01$

Experiment 6. Rats on diets for 10 weeks

Table 4

The normal rat body weight has now increased to 386g, an increase of nearly 50g in two weeks. Rats on diet A are the smallest having a body weight of 238g, an increase of about 30g in two weeks. The rat body weight increases with the amount of calcium in the diet up to the addition of 1% $CaCO_3$ (diet C). Rats on diet D are smaller than those on diet C probably because they did not each as much of this diet.

The dotted lines on the graph (fig. 4) represent the thyroid weights of the control rats on 41B for 8 and 10 weeks. There is a significant difference between the normal rat thyroid weight and the thyroid weight after 8 or 10 weeks on the low iodine diets. As can be seen from the graph feeding the diets for 10 weeks instead of 8 weeks increases the goitre weight except for rats on diet B whose thyroids remain about the same size. The effect of adding calcium is more clearly seen after 10 weeks and there is a gradual increase in thyroid weight with increasing concentration of $CaCO_3$ in the diet up to the addition of 1% $CaCO_3$ (that is diet C = Remington low iodine diet). As found before, the addition of more calcium does not result in a further increase in thyroid weight. Statistical analysis of these results shows that the effect of diet A is very significantly different from the other diets (at better than the 1% level).

3. The effect on thyroid weight of feeding diet C then the calcium diets.

In the next group of experiments all animals except the control group on 41B were fed the Remington low iodine diet (diet C) initially for various lengths of time before the administration of the different calcium diets A, B, C, and D. The results obtained for the rat body weights and their thyroid weights are shown in table 5.

Diet	No. of rats	Rat body weight (g)	Thyroid weight (mg)
Experiment 7.	Rats on diet C for 4 weeks + 4 weeks on diets		
41B	7	350	15.59
A	7	348	28.32
B	7	330	26.20
C	7	342	34.10
D	8	334	34.39
Experiment 8.	Rats on diet C for 4 weeks + 8 weeks on diets		
41B	8	390	14.71
A	7	270	22.79
B	7	385	33.52
C	8	378	25.24
D	7	373	28.63
Experiment 9.	Rats on diet C for 8 weeks + 8 weeks on diets		
41B	7	451	14.78
A	13	345	30.24
B	13	399	32.04
C	8	410	32.29
D	13	383	32.56
Experiment 10.	Rats on diet C for 8 weeks + 12 weeks on diets		
41B	7	480	17.42
A	7	364	30.08
B	7	399	28.41
C	6	456	31.03
D	6	456	38.91

Diet	No. of rats	Rat body weight (g)	Thyroid weight (mg)
Experiment 11.		Rats on diet C for 12 weeks + 8 weeks on diets	
41B	5	445	18.16
A	7	434	36.43
B	5	434	40.16
C	7	449	44.92
D	5	422	40.09
Experiment 12.		Rats on diet C for 16 weeks + 12 weeks on diet	
A	5	454	34.06
B	5	488	33.76
C	5	498	31.15
D	6	481	34.09

Table 5

Results of feeding diet C then the calcium diets

The rat body weights shown in table 5 for animals on the low iodine diets for a total of 8 weeks (Experiment 7) are the same as the control rats on 41B. The animals have not received diet A long enough for their low calcium intake to inhibit normal body growth.

After the initial 4 weeks on diet C before changing the diets, the rat thyroid weight would be about 24 mg. Therefore, a further 4 weeks on the different calcium diets increases the thyroid weight in each case. The smallest increase is obtained for diet B but this is not

different from that obtained with diet A. Rats on diet C and on diet D have the largest goitres in this group.

In Experiment 8 the time of administering the different calcium, low iodine diets was increased to 8 weeks. The control rats slightly increase in weight while their thyroids remain the same size. Rats on diet A are again retarded in body weight. In this group diet B causes the largest increase in thyroid weight. For the other diets, the thyroid size increases as the amount of dietary calcium increases.

In Experiments 9 and 10 diet C was given for 8 weeks initially before the administration of the calcium diets. For Experiment 9, after 16 weeks on the control diet, the rat body weight has reached 450g while the thyroid weight remains the same as before at about 15 mg. Rats on the low iodine diets are all smaller in weight than the controls with those on diet A being the smallest. The thyroid weights of rats on diets B, C and D are all identical, while those on diet A are very slightly smaller.

Comparing these results with those obtained by feeding the calcium diets for a further 4 weeks, that is animals on diet C initially for 8 weeks then 12 weeks on the calcium diets (Experiment 10) not much increase in thyroid weight has occurred although rat body weight has

increased. This time diet B caused the smallest goitre size but this is much the same as for diet A and diet C. Rats on diet D have the largest goitres.

Although the next group (Experiment 11) have been on a low iodine diet for the same length of time in total (20 weeks) as rats in Experiment 10, larger goitres are produced for each of the four diets. The rat body weights are the same for the control group and for each of the four experimental diets. Again diet A has the smallest average thyroid weight, diet B and diet D are the same, with diet C causing a slightly larger goitre.

In the last experiment in this group, no control rats were included. After 16 weeks on diet C plus 12 weeks on the four diets all the thyroids are the same size and they do not increase any more than after a total of 20 weeks on a low iodine diet. Rats on diet A are again smaller in body weight than the others.

Time on diets

On diet C	4 weeks	4 weeks	8 weeks	8 weeks	12 weeks	16 weeks
	+	+	+	+	+	+
On diet D or diet A	4 weeks	8 weeks	8 weeks	12 weeks	8 weeks	12 weeks
Difference in thyroid weight (mg)	6.07	5.84	2.06	7.88	3.66	0.03

Table 6
Difference between thyroid weights for rats on diet D and diet A (mg)

The results of Experiments 7-12 are summarised in table 6 which shows the difference in thyroid weights between rats on diet D and diet A. The animals have been on diet C for increasing lengths of time and then on either diet A or diet D. These results indicate that the longer the animals are on the 1% $CaCO_3$ low iodine diet initially the less is the effect of increasing the dietary calcium since the difference in thyroid weights decreases as the length of time on diet C increases.

4. Effect of diets on weanling rats

In few of the preceding experiments were very large goitres obtained despite changing the manufacturers of the diet used, or increasing the time on the diet. It was found that the animals used were excreting a large amount of iodine in urine (about 54 μmol/l). The diet that the breeders use to bring the animals up to the required weight of 120-150g does contain a large amount of iodine - 750 μgI/kg diet according to Spillers who manufacture the diet used. Thus it was decided to obtain weanlings to see if this affected the results of administering the low iodine diet.

The weanlings were about 60g in body weight initially. The increase in body weight, thyroid weight and the decrease in thyroid iodine concentration for one rat at each point on diet C for increasing times is shown in figure 5. The body weight increases linearly as the length of time on the diet increases. The thyroid weight trebles after 8 weeks on the low iodine diet. The thyroid iodine concentration (mgI/100g fresh thyroid weight) which is a measure of the iodine deficiency of the animal decreases from 30.7 mgI/100g after 2 weeks on the diet to 8.6 mgI/100g 6 weeks later.

In Experiments 13 and 14 (Table 7) the weanlings were kept on diet C for 8 weeks initially (except for the control group on 41B).

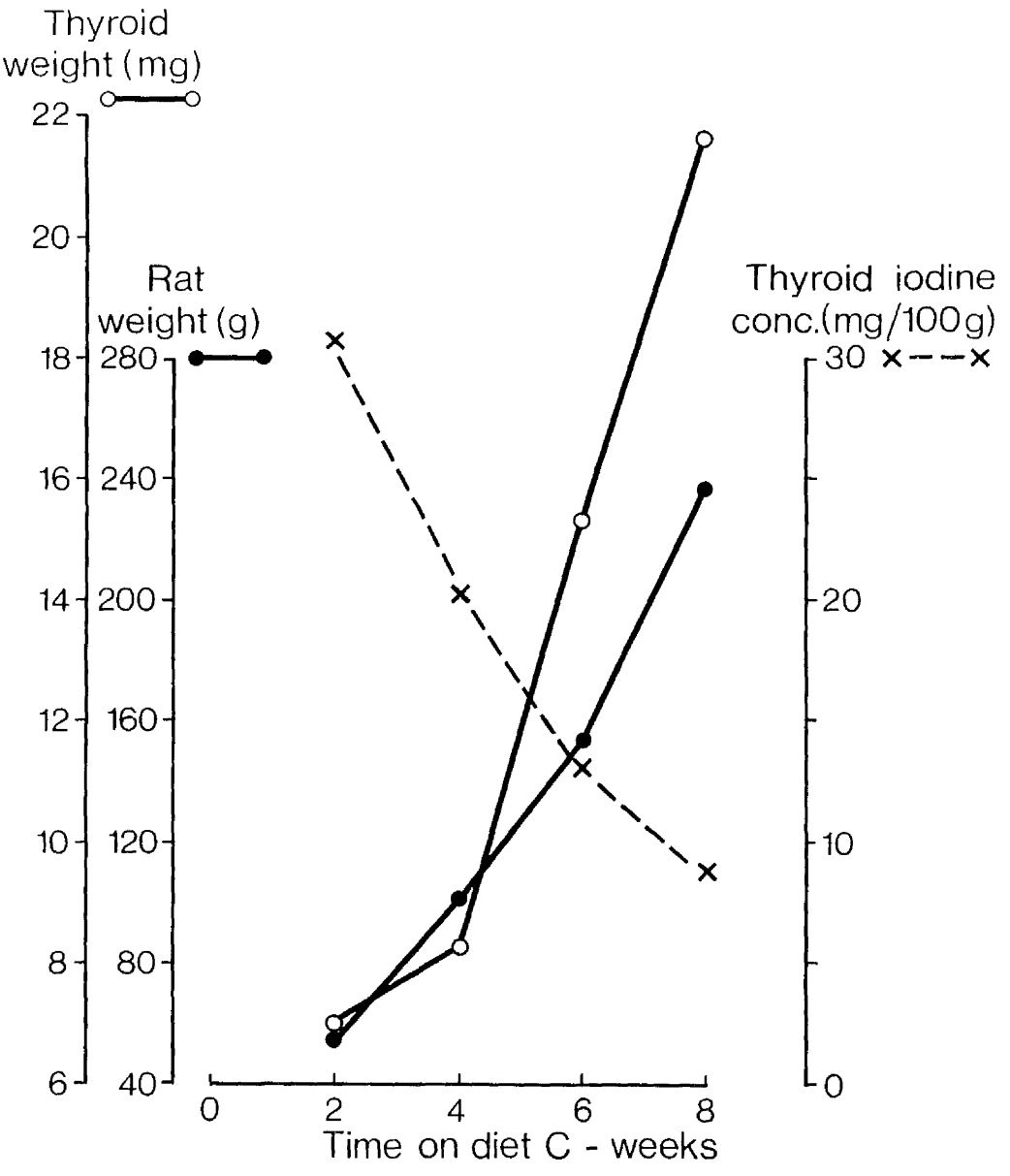
Diet	No. of rats	Body weight (g)	Thyroid weight (mg)
Experiment 13.	Rats on diet C for 8 weeks + 8 weeks on diets		
41B	6	380	17.75
A	8	281	21.55
B	7	323	25.74
C	7	341	25.20
D	7	326	25.70
Experiment 14.	Rats on diet C for 8 weeks + 12 weeks on diets		
41B	10	443	18.20
A	6	342	24.05
B	7	402	27.68
C	8	416	23.90
D	7	349	25.65

Table 7

Weanling rats on diet C then calcium diets

FIGURE 5

Production of goitre by Teklad low iodine diet


Thyroid weight (mg)
22
20
18
16
14
12
10
8
6
Rat weight (g)
280
240
200
160
120
80
40
Thyroid iodine conc.(mg/100g)
30
20
10
0
0
2
4
6
8
Time on diet C - weeks

After a total of 16 weeks on 41B the rats have reached a body weight of 380g which is the same weight as the older animals attained after 10 weeks on 41B. Their thyroids are also the same weight. Rats that have been on diet A are again the smallest in body weight. The other rats on the calcium containing diets are much the same weight as obtained previously for rats on these diets for 10 weeks (Experiment 6).

However, the thyroid weights for those rats on diets C and D are not as large as those previously obtained. The thyroid weights for animals on diets B, C and D are all the same but rats on diet A have significantly smaller thyroids.

After 8 weeks on diet C, then 12 weeks on the diets (Experiment 14) the rat body weights all increase, but the thyroid weights remain much the same. Although diet B has the largest thyroid size, it is not statistically different from the others.

5. The effect on thyroid weight of KI or $CaCl_2$ in the drinking water.

Weanling rats were also used for this experiment (15). 1.31 mg KI/l or 2% $CaCl_2.6(H_2O)$ was added to the water supply and rats given either diet A or diet C for 8 weeks. The results obtained for their body weights and thyroid weights are given in table 8.

Diet	No. of rats	Body weight (g)	Thyroid weight (mg)
A	5	152	14.92
A + KI	5	135	8.75
A + $CaCl_2$	6	226	17.58
C	6	247	15.67
C + KI	7	239	11.68
C + $CaCl_2$	6	192	15.54

Experiment 15. Rats on diets for 8 weeks

Table 8

As before rats on diet A with no calcium supplements do not gain as much weight. The animals receiving iodine supplements in their drinking water have glands significantly smaller than the others. The animals receiving calcium supplements either as $CaCl_2$ or $CaCO_3$ in the diet have larger thyroids than those on diet A but the difference is not statistically significant and the goitrous glands are less than twice the weight of those from rats on diet 41B.

6. The effect on thyroid weight of adding PTU or $KClO_4$ to the diets.

The effect of calcium in a diet containing a strongly goitrogenic substance was investigated by adding PTU or $KClO_4$ to diet A and to diet C. Weanling rats were also used in this experiment.

The results obtained after feeding these diets for 4 weeks (Experiment 16) are shown in table 9.

Diet	No. of rats	Rat body weight (g)	Thyroid weight (mg)
A	7	129	14.27
C	6	169	17.10
A + PTU	7	100	69.21
C + PTU	6	107	78.97
A + $KClO_4$	6	90	29.94
C + $KClO_4$	6	116	38.88

Experiment 16. Rats on diets for 4 weeks

Table 9

Animals receiving the goitrogenic substances PTU or $KClO_4$ are slightly smaller than those on the basic low iodine diets. Rats on diet A have significantly smaller thyroids than those on diet C (Student's t test gives $P < 0.005$) although the difference is small. Since there is a large variation in the thyroid weights for animals receiving PTU there is no statistical difference ($P = 0.15$) in their size for the rats on diet A or diet C. For animals on $KClO_4$, those receiving calcium have larger thyroids than those on the calcium deficient diet A ($P = 0.025$).

Since PTU is such a good goitrogenic agent, it may be masking any effect of the calcium and therefore the time of feeding the diets was reduced to 2 weeks (Experiment 17).

Diet	No. of rats	Rat body weight (g)	Thyroid weight (mg)
A + PTU	9	96	45.24
C + PTU	10	85	31.41
A + $KClO_4$	9	97	24.10
C + $KClO_4$	9	79	18.48

Experiment 17. Rats on diets for 2 weeks

Table 10

The rat body weights are the same for all the four diets in Experiment 17. The goitres obtained are less than half the weight of those found at 4 weeks on the same diet. In this experiment, however, calcium appears to have the opposite effect because rats on diet A + PTU have larger thyroids than those on diet C + PTU which receive 1% $CaCO_3$. Student's t test gives $P < 0.005$. The same result is found for animals receiving $KClO_4$ although the difference in thyroid weights is not so great. Rats on diet A + $KClO_4$ have larger thyroids than those on diet C + $KClO_4$. In this case Student's t test gives $P < 0.025$

Section 2

Results of the measurement of calcium concentrations of thyroid and serum

(1) Calcium concentrations of the thyroid and some other rat tissues

The results obtained for the calcium concentrations, measured as mg calcium/100g fresh weight, of rat kidney,

liver, muscle, heart, thyroid and serum from control rats on diet 41B are given in table 11.

	No. of analyses	Calcium concentration mg/100g	S.D.
Kidney	16	6.85	0.62
Liver	15	2.97	0.48
Muscle	18	4.27	0.49
Heart	18	2.59	0.34
Thyroid	18	26.4	5.3
Serum	27	12.88	0.68

Table 11

Concentration of calcium in various tissues and serum of control rats (serum concentration measured as mg/100 ml).

These figures show that the thyroid contains a greater concentration of calcium than the other tissues analysed. The thyroidal concentration of calcium is twice that of serum (measured as mg Ca/100 ml serum). Calculating the calcium as µg Ca/mg protein, the thyroid contains 2.48 µgCa/mg protein while the serum concentration is 1.19 µgCa/mg serum protein, again indicating that the thyroid concentration is twice that of serum.

The calcium concentration of sheep thyroid was also measured using the same method. The concentration of calcium in the sheep thyroid is not as high as that of the rat thyroid. The mean of 23 measurements was 16.4 ± 2.6 mg Ca/100g fresh thyroid weight.

(2) Effect of diet on thyroid and serum calcium concentrations

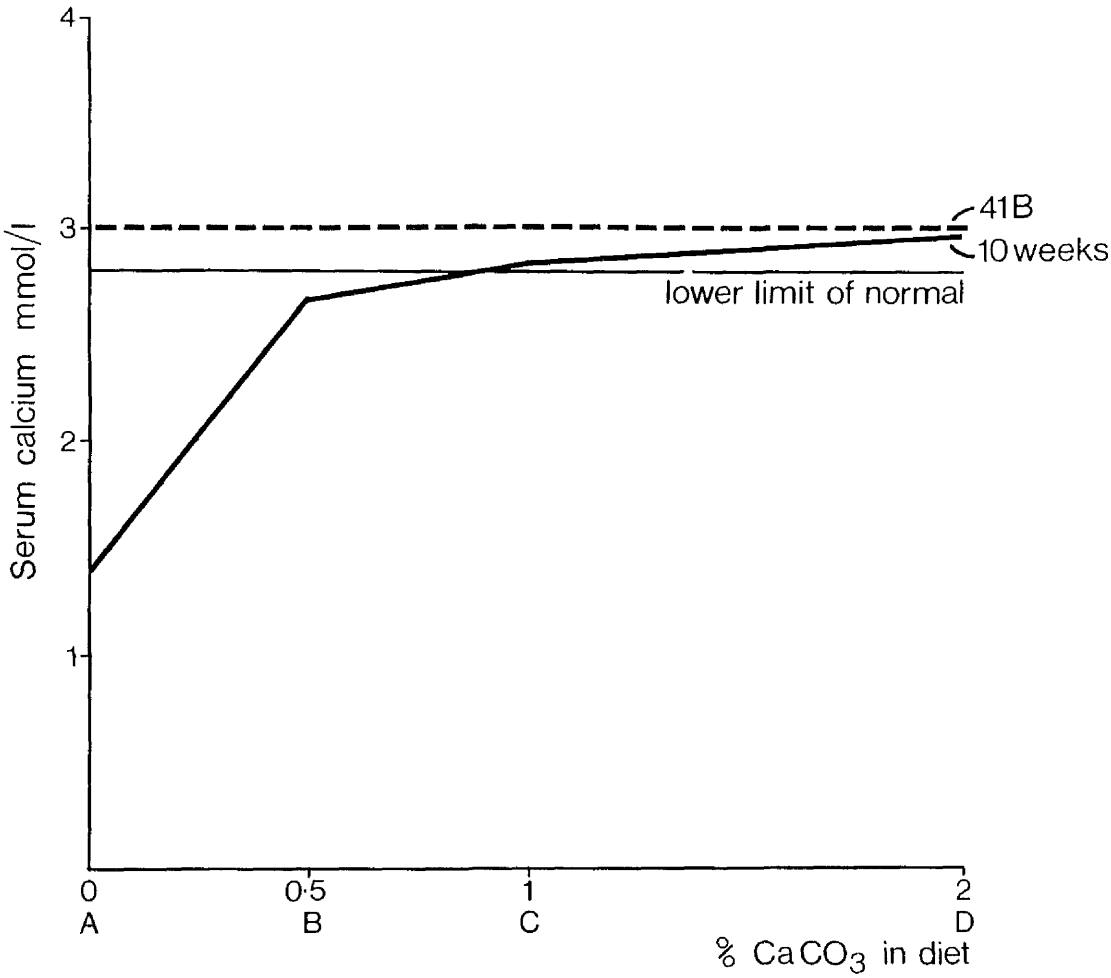
Most of the thyroids from rats in the dietary experiments were analysed for their calcium contents by the acid digest method described. Blood samples taken from the heart after death were removed from some animals in each experiment. The blood was allowed to clot and then it was centrifuged on a bench centrifuge for a few minutes. The serum was separated and the serum calcium was measured by A.A.S.

Figure 6 shows the serum calcium concentrations (mmol/l) measured after the animals had been fed the five diets (41B, diets A, B, C, D) for 10 weeks (Experiment 6). Sera from five rats on each diet were analysed. The upper dotted line represents the average serum calcium for rats on the control diet 41B for 10 weeks and the lower line is the lower limit of the normal range. Boelter and Greenberg (1941) give a normal rat serum calcium range as 2.1-3.0 mmol/l. Samples of sera from 27 control rats on diet 41B for various lengths of time were analysed and their normal range was 2.8-3.5 mmol/l with an average of 3.22 mmol/l.

Thus in Experiment 6 rats on 41B and on diet D for 10 weeks have serum calcium concentrations within this range. The calcium deficient diet A causes the serum calcium to fall to 1.40 mmol/l which is well below the normal limit.

FIGURE 6

The variation of serum calcium with the concentration of Ca CO_3 in the diets


4
3
2
1
Serum calcium mmol/l
41B
10 weeks
lower limit of normal
0
A
0·5
B
1
C
2
D
% $CaCO_3$ in diet

Several of the animals on this diet, therefore, developed tetany and died before the end of the experiment. They are not included in any of the results. The addition of 0.5% $CaCO_3$ in diet B was sufficient to prevent this, since it brings the serum calcium back to the lower limit of normal. Thus, it can be seen from the graph that the serum calcium increases with increased dietary calcium, but it does not rise above the higher limit of normal, even with the addition of 2% $CaCO_3$.

Table 12 shows the thyroid calcium concentrations measured in mg calcium/100g fresh thyroid weight for rats on the diets for 8 weeks (Experiment 5) and 10 weeks (Experiment 6). The figures in brackets after the results are the number of determinations carried out for each diet.

Diet	Thyroid calcium concentration mgCa/100g	Thyroid calcium concentration mgCa/100g
41B	24.5 (4)	23.5 (4)
A	19.4 (3)	18.9 (4)
B	16.1 (3)	23.9 (4)
C	16.8 (3)	22.6 (4)
D	18.5 (3)	22.6 (4)
	Experiment 5	Experiment 6

Table 12

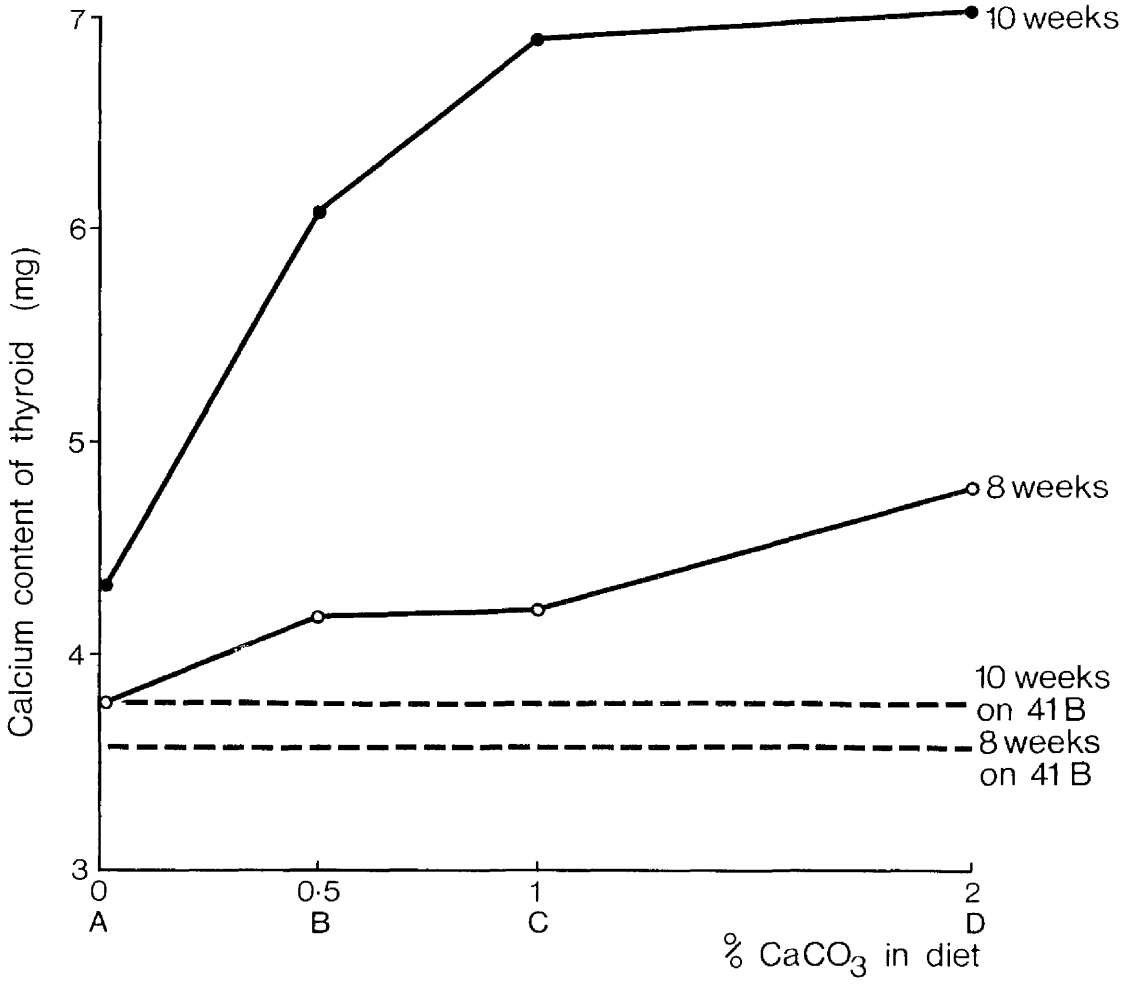
Results of thyroid calcium concentrations for rats in Experiments 5 and 6

For the rats in Experiment 5 which have been on a low iodine diet for 8 weeks, the thyroid calcium concentration is lower than the normal concentration found for animals on 41B. Since the serum calcium is low for rats on diet A their thyroid calcium concentration may be expected to be low but there is not any real difference between the thyroid calcium concentrations for the four different diets. For Experiment 6 the thyroid calcium concentration for diet A remains low while for the other diets there is a slight rise towards the normal level. The concentrations for diets B, C, and D are the same.

The total calcium content of these thyroids measured as μg/gland is shown in the graph (fig. 7). The dotted lines represent the thyroid calcium contents for control rats on 41B for 8 and 10 weeks. After 8 weeks on 41B (Experiment 5) the normal rat thyroid contains 3.58 μg calcium. The low iodine diets along with increasing the thyroid size have also caused the amount of thyroid calcium to be increased. The thyroid calcium content increases with increasing concentration of calcium in the diet although thyroids from rats on diets B and C have nearly the same calcium content. For rats in Experiment 6 which have been on the diets for 2 weeks longer, the calcium content of the thyroids has increased for every diet in comparison to those in Experiment 5. In this experiment there is a greater increase in thyroid calcium content with increasing dietary calcium.

FIGURE 7

Calcium content of thyroids from rats on diets for 8 and 10 weeks


Calcium content of thyroid (mg)
7
6
5
4
3
10 weeks
8 weeks
10 weeks on 41 B
8 weeks on 41 B
0
A
0·5
B
1
C
2
D
% $CaCO_3$ in diet

The serum calcium and thyroid calcium concentrations for rats on diet C initially and then on the calcium diets (Experiments 7-12) are shown in table 13. The figures in brackets are the numbers of determinations carried out.

Diet	Serum calcium (mmol/l)	Thyroid calcium concentration (mg/100g)
Experiment 7. Rats on diet C for 4 weeks + 4 weeks on diets		
41B	3.29 (1)	24.2 (1)
A	1.80 (1)	16.5 (1)
B	2.51 (1)	22.7 (1)
C	2.91 (1)	12.9 (1)
D	3.05 (1)	14.4 (1)
Experiment 8. Rats on diet C for 4 weeks + 8 weeks on diets		
41B	3.19 (1)	21.1 (1)
A	1.59 (1)	18.6 (1)
B	2.52 (1)	16.4 (1)
C	3.02 (1)	22.2 (1)
D	3.15 (1)	27.3 (1)
Experiment 9. Rats on diet C for 8 weeks + 8 weeks on diets		
41B	2.64 (1)	27.4 (1)
A	1.54 (1)	20.7 (1)
B	2.57 (1)	22.7 (1)
C	2.64 (1)	20.1 (1)
D	2.28 (1)	19.3 (1)

Diet	Serum calcium (mmol/l)	Thyroid calcium concentration (mg/100g)
Experiment 10. Rats on diet C for 8 weeks + 12 weeks on diets		
41B	2.97 (1)	36.2 (2)
A	1.42 (1)	19.6 (1)
B	3.07 (1)	25.5 (1)
C	3.09 (1)	24.2 (1)
D	2.97 (1)	21.2 (1)
Experiment 11. Rats on diet C for 12 weeks + 8 weeks on diets		
41B	3.26 (3)	36.2 (2)
A	2.52 (3)	26.7 (2)
B	3.21 (3)	31.5 (1)
C	3.28 (3)	18.6 (2)
D	3.39 (3)	25.1 (1)
Experiment 12. Rats on diet C for 16 weeks + 12 weeks on diets		
A	2.07 (3)	34.3 (1)
B	3.43 (3)	37.7 (1)
C	3.19 (3)	36.5 (1)
D	3.29 (3)	31.6 (1)

Table 13

Results of serum calcium and thyroid calcium concentrations for rats in Experiments 7-12

In every experiment the serum calcium for rats on diet A is lower than for the other diets and it is usually well below the lower limit of normal. In nearly every experiment the serum calcium increases with increased dietary calcium up to the normal range. For Experiments 11 and 12 diet A does not cause such a large decrease in serum calcium possibly because the animals are larger and more mature before the diet is started.

In all experiments (except for two groups of rats in Experiment 8) the low iodine diets cause a decrease in the thyroid calcium concentration. For rats in Experiment 7 the thyroid calcium concentration is about the same for diets A, C and D with diet B having a larger calcium concentration. For Experiment 8, the thyroid calcium concentration increases with increasing dietary calcium. For the other experiments in this group there does not seem to be any correlation between thyroid calcium concentration and dietary calcium content.

The total calcium contents in μg for these thyroids are given in table 14.

Diet	Total Thyroid calcium content (µg)					
	4 weeks on diet C +4 weeks on diets	4 weeks on diet C +8 weeks on diets	8 weeks on diet C +8 weeks on diets	8 weeks on diet C +12 weeks on diets	12 weeks on diet C +8 weeks on diets	16 weeks on diet C +12 weeks on diet
	Experiment 7	Experiment 8	Experiment 9	Experiment 10	Experiment 11	Experiment 12
41B	3.77	3.10	4.07	6.47	6.47	-
A	4.69	4.23	6.07	6.13	10.5	11.7
B	5.94	5.49	7.13	7.20	11.7	12.7
C	4.40	5.60	6.51	7.44	8.64	11.4
D	4.95	7.83	5.87	8.08	10.1	10.8

Table 14

Thyroid calcium content for rats in Experiments 7-12

The calcium content of the control thyroids increases from about 3 µg to 6 µg as the length of time on the diet increases from 8 weeks to 20 weeks. The thyroids of rats on the low iodine diets have a higher calcium content than the controls since they are larger in size. In most of the above group of experiments there is a tendency for the thyroid calcium content to increase with increasing dietary calcium.

Table 15 shows the serum calcium, thyroid calcium concentration and thyroid calcium content for the rats from Experiments 13 and 14 which started diet C when they were weanlings. In Experiment 13 the serum calcium for rats on diet A is again low and is the same as for the rats in Experiment 9 which have been on the same diets for the same length of time. The serum calcium rises into the normal range for rats on diet B. In the next experiment the serum calcium for rats on diet A is not as low as before but it is still below the lower limit of normal. The other animals have a serum calcium within the normal range.

Diet	Serum calcium (mmol/l)	Thyroid calcium concentration (mg/100g)	Total thyroid calcium content (µg/gland)
Experiment 13. Rats on diet C for 8 weeks + 8 weeks on diets			
41B	3.26 (2)	25.0 (1)	4.48
A	1.50 (3)	22.0 (1)	4.87
B	2.51 (3)	23.9 (1)	6.13
C	3.22 (3)	25.2 (1)	6.40
D	3.14 (2)	20.1 (1)	5.40

Diet	Serum calcium (mmol/l)	Thyroid calcium concentration (mg/100g)	Total thyroid calcium content (µg/gland)
Experiment 14. Rats on diet C for 8 weeks + 12 weeks on diets			
41B	3.36 (7)	35.2 (2)	6.31
A	2.49 (3)	29.0 (1)	6.96
B	3.44 (3)	24.6 (1)	6.80
C	3.35 (3)	29.4 (1)	7.33
D	3.13 (4)	30.6 (1)	7.93

Table 15

Results of serum calcium and thyroid calcium measurements for rats in Experiments 13 and 14

The thyroid calcium concentration is lower for most of the animals on the low iodine diets than for those on diet 41B. The thyroid calcium content again increases with increased dietary calcium and is much the same as for the rats in Experiments 9 and 10 which have been on the diets for the same length of time.

Table 16 shows the serum calcium, the thyroid calcium concentrations and the thyroid calcium content for rats in Experiment 15. These rats started on the diets when they were weanlings and remained on them for 8 weeks. Again, for rats on diet A, the serum calcium is very low. It increases slightly for rats on diet A plus KI supplemented drinking water and is within the normal range for all the animals receiving calcium supplements in the diet or in their drinking water.

Diet	Serum calcium (mmol/l)	Thyroid calcium concentration (mg/100g)	Thyroid calcium content (µg/gland)
Experiment 15. Rats on diets for 8 weeks			
A	1.38 (3)	17.5 (1)	2.60
A + KI	2.26 (1)	16.1 (1)	1.40
A + $CaCl_2$	3.15 (3)	19.0 (1)	3.36
C	2.83 (2)	16.6 (1)	2.72
C + KI	3.20 (2)	21.1 (1)	2.56
C + $CaCl_2$	3.04 (3)	18.7 (1)	2.96

Table 16

Results of serum calcium and thyroid calcium measurements for rats in Experiment 15

The thyroid calcium concentrations are lower than that found for rats on diet 41B. Rats on diet C + KI have the highest calcium concentration while rats on the other diets all have the same thyroid calcium concentrations. Rats on diet A + KI supplements which have the smallest thyroids have the lowest calcium content. Rats on diet A alone have the same calcium content as the calcium supplemented animals.

The effect of PTU and $KClO_4$ on the thyroid calcium is shown in table 17.

Diet	Serum calcium (mmol/l)	Thyroid calcium concentration (mg/100g)	Total thyroid calcium content (µg/gland)
Experiment 16. Rats on diets for 4 weeks			
A	2.62 (3)	25.1 (1)	3.67
A + PTU	1.76 (2)	8.62 (1)	6.33
A + $KClO_4$	1.48 (2)	9.57 (1)	2.87
C	3.22 (3)	21.4 (1)	3.67
C + PTU	3.24 (1)	10.0 (1)	7.93
C + $KClO_4$	3.32 (1)	14.6 (1)	5.67
Experiment 17. Rats on diets for 2 weeks			
A + PTU	3.00 (7)	10.6 (2)	4.93
A + $KClO_4$	2.96 (8)	13.0 (2)	3.16
C + PTU	3.20 (6)	12.8 (2)	4.00
C + $KClO_4$	3.21 (5)	13.8 (2)	2.67

Table 17

Results of serum calcium and thyroid calcium measurements for rats in Experiments 16 and 17

After 4 weeks on the three calcium deficient diets the serum calcium of the rats is low although for diet A without supplements it is only just below the lower limit of the normal range. After 2 weeks on these diets the serum calcium for the calcium deficient rats has not decreased below the normal range. The thyroid calcium concentration after 4 weeks on PTU or

$KClO_4$ is very much lower than that obtained after 4 weeks on either diet A or diet C alone. For rats on diet A supplemented with either PTU or $KClO_4$ the thyroid calcium concentration has decreased to less than half the calcium concentration of the glands from rats on diet A. Rats receiving $KClO_4$ supplements have a slightly higher concentration of thyroid calcium than those receiving PTU and those on the calcium supplemented diet C have a higher thyroid calcium concentration than those on the corresponding diet A. The results of Experiment 17 in which the PTU and $KClO_4$ diets were administered for 2 weeks show the same pattern.

The thyroid calcium content is the same after 4 weeks on either diet A or diet C. Rats receiving PTU supplements have nearly double this calcium content with those receiving calcium in diet C having a higher content of thyroid calcium than those rats on diet A. For rats on diet A + $KClO_4$ the thyroid calcium content has decreased while for those on diet C + $KClO_4$ it has increased although not as much as for the PTU supplemented diets. After 2 weeks on these diets the thyroid calcium content is about the same as is obtained for the low iodine diets. Again the $KClO_4$ diets have caused the thyroid calcium content to be less than that obtained for the PTU diets although in this experiment the calcium supplemented rats have a slightly lower thyroid calcium content.

Section 3

Effect of diets on thyroid iodine content and thyroid radioiodine uptake

Control rats which had been on diet 41B for various lengths of time, had a thyroid iodine concentration of 157 mg/100g fresh thyroid weight (mean of nine determinations). Their total thyroid iodine content varied from 23 µg iodine per gland to 49 µg per gland with a mean of 29 µg iodine per gland. The iodine concentration of normal sheep thyroid was also measured. The mean of nine determinations gave a concentration of 141 mg iodine/100g fresh weight which is in the same order of magnitude as the rat thyroid iodine concentration.

Table 18 shows the thyroid weights, thyroid iodine concentrations and total thyroid iodine contents for some rats from Experiment 6 which have been on the diets for 10 weeks.

Diet	Thyroid weight (mg)	Thyroid iodine concentration (mg/100g)	Thyroid iodine content (µg/gland)
41B	17.50 (6)	180.7	30.0
A	29.15 (4)	10.3	3.00
B	35.68 (5)	5.05	1.80
C	44.51 (5)	2.70	1.20
D	35.27 (5)	5.67	2.00

Experiment 6. Rats on diets for 10 weeks

Table 18

Results of thyroid iodine measurements for rats in Experiment 6.

Only one iodine estimation was carried out for each diet since all the glands were digested together. After 10 weeks on the low iodine diets the thyroid concentrations of iodine are less than 1/10th the normal concentration. Rats on diet A have the largest concentration and also the greatest thyroid content of iodine. The iodine concentration decreases from diet A to diet B to diet C, that is, it decreases as the calcium concentration of the diet increases. The iodine concentration also increases as the thyroid weight decreases and thus rats on diet D containing 2% $CaCO_3$ which have smaller thyroids than those on diet C containing 1% $CaCO_3$ have a larger concentration of iodine.

The same pattern is observed for rats in Experiment 11 in table 19.

Diet	Thyroid weight (mg)	Thyroid iodine concentration (mg/100g)	Thyroid iodine content (µg/gland)
Experiment 11. Rats on diet C for 12 weeks + 8 weeks on diets			
41B	18.16	105.0	19.0
A	36.43	11.6	4.15
B	40.16	3.49	1.40
C	44.92	2.75	1.14
D	40.07	3.00	1.20

Diet	Thyroid weight (mg)	Thyroid iodine concentration (mg/100g)	Thyroid iodine content (µg/gland)
Experiment 12. Rats on diet C for 16 weeks + 12 weeks on diets			
A	34.06	8.22	2.80
B	33.76	9.49	3.20
C	31.15	16.06	5.00
D	34.09	9.96	3.40

Table 19

Results of thyroid iodine measurements for rats in Experiments 11 and 12

Rats on diet A which have the smallest thyroids have a thyroid iodine concentration more than three times that found for the other low iodine diets. The largest glands are those from rats on diet C and although they do have the smallest iodine content and concentration there is little difference between them and the iodine contents or concentrations of the thyroids from rats on diets B and D.

In Experiment 12, rats on diets A, B and D which have about the same average thyroid weights have similar thyroid iodine concentrations and thyroid iodine contents. Those which have been on diet C for the whole period have a higher thyroid iodine content and a thyroid iodine concentration nearly double that of the other rats in this experiment.

Table 20 shows the thyroid weights, iodine concentrations and content of the thyroids of rats in Experiments 13 and 14

Diet	Thyroid weight (mg)	Thyroid iodine concentration (mg/100g)	Thyroid iodine content (µg/gland)
Experiment 13. Rats on diet C for 8 weeks + 8 weeks on diets			
41B	17.75	138.0	24.8
A	21.55	15.1	3.33
B	25.74	15.6	4.00
C	25.20	21.6	5.50
D	25.70	19.2	5.17
Experiment 14. Rats on diet C for 8 weeks + 12 weeks on diets			
41B	18.20	184.2	32.8
A	24.05	20.0	4.80
B	27.68	18.1	5.00
C	23.90	26.7	6.77
D	25.65	23.8	6.17

Table 20

Results of thyroid iodine measurements for rats in Experiments 13 and 14

In both experiments 13 and 14 the thyroid concentration of iodine is higher than in the previous experiments. Contrary to the previous experiments there is a tendency for the thyroid iodine concentration and content to increase with increasing dietary calcium.

Table 21 shows the results obtained for Experiment 15, in which the rats were fed the diets for 8 weeks.

Diet	Thyroid weight (mg)	Thyroid iodine concentration (mg/100g)	Total Thyroid Iodine content (µg/gland)
Experiment 15. Rats on diets for 8 weeks			
A	14.92	13.5	2.00
A + KI	8.75	63.4	5.50
A + $CaCl_2$	17.58	13.6	2.40
C	15.67	13.5	2.20
C + KI	11.68	47.8	5.80
C + $CaCl_2$	15.54	18.9	3.00

Table 21

Results of thyroid iodine measurements for rats in Experiment 15

As expected rats receiving iodine supplements have a high concentration of thyroid iodine and although those on diet A + KI have a higher concentration of iodine than those on diet C + KI, the total thyroid iodine content is the same for both these diets. The other thyroids have half this content of iodine and about a quarter the concentration of iodine as the iodine supplemented animals. The addition of calcium to the diet has made little difference to the thyroid iodine since rats on diet A, diet A + $CaCl_2$ and diet C have the same concentration and content of iodine in their thyroids.

The rats receiving the maximum amount of calcium, that is those on diet C + $CaCl_2$ have slightly more thyroid iodine.

The effect of PTU and $KClO_4$ administered for 2 and 4 weeks on the thyroid iodine is shown in table 22.

Diet	Thyroid weight (mg)	Thyroid iodine concentration (mg/100g)	Thyroid iodine content (µg/gland)
Experiment 16. Rats on diets for 4 weeks			
A	14.27	232.4	34.0
C	17.10	220.4	37.7
A + PTU	69.21	2.92	2.14
C + PTU	78.97	3.56	2.81
A + $KClO_4$	29.94	2.71	0.81
C + $KClO_4$	38.88	2.08	0.81
Experiment 17. Rats on diets for 2 weeks			
A + PTU	45.24	0.188	0.075
C + PTU	31.41	0.326	0.077
A + $KClO_4$	24.10	0.245	0.060
C + $KClO_4$	18.48	0.385	0.060

Table 22

Results of thyroid iodine measurements for rats in Experiments 16 and 17

Rats on diets A and C in Experiment 16 have a normal amount of iodine in their thyroids. The addition of PTU or $KClO_4$ to the diets has reduced the iodine concentration to about 1/100th the normal concentration. PTU only

affects the protein binding of iodine and not the trapping of iodine by the gland and thus the thyroids from rats receiving PTU contain more iodine than those on $KClO_4$. The presence of calcium in diet C has not affected the thyroid iodine in this experiment.

In Experiment 17 even less iodine is present in the thyroids. Although rats receiving PTU have a slightly greater thyroid content of iodine their concentration of iodine is slightly less than those on the corresponding $KClO_4$ diet. The addition of calcium to the diet has not affected the total iodine content of the thyroid since thyroids from rats on diet A + PTU and diet C + PTU all contain a mean of 0.08 µg iodine and those on diet A + $KClO_4$ and diet C + $KClO_4$ all contain 0.06 µg iodine. Rats receiving calcium have a greater thyroid concentration of iodine than those on the corresponding diet A.

Radioiodine uptakes

In some dietary experiments one or two rats on each diet were injected intra-peritoneally with 10µCi ^{125}I. They were sacrificed four hours later and the thyroids were removed, weighed and counted on a Packard gamma counter. ^{125}I uptake of the thyroid as a percentage of the injected dose was calculated. The results are shown in table 23 along with the thyroid weight for the injected rat. The numbers in brackets are the numbers of rats injected for each diet.

Diet	Thyroid weight (mg)	4 hour ^{125}I uptake (% dose)
Experiment 13.	Rats on diet C for 8 weeks + 8 weeks on diets	
41B	16.90 (1)	3.33
A	19.94 (2)	25.7
B	26.34 (1)	37.3
C	23.89 (1)	30.6
D	18.45 (1)	23.7
Experiment 14.	Rats on diet C for 8 weeks + 12 weeks on diets	
41B	19.91 (1)	15.3
A	24.35 (1)	33.4
B	27.72 (1)	39.1
C	20.65 (1)	33.6
D	24.13 (1)	40.1
Experiment 15.	Rats on diets for 8 weeks	
A	15.26 (1)	33.2
A + KI	9.03 (1)	3.72
A + $CaCl_2$	17.30 (1)	56.1
C	15.67 (1)	18.6
C + KI	10.50 (2)	4.07
C + $CaCl_2$	13.92 (1)	33.2
Experiment 6.	Rats on diets for 10 weeks	
		6 hour ^{125}I uptake (% dose)
41B	19.36 (2)	6.02
A	21.36 (2)	35.8
B	32.68 (2)	51.3
C	32.12 (2)	50.0
D	32.48 (2)	48.7

Table 23

Results of thyroid radioiodine uptakes

The percentage uptake for control rats on 41B and for those on diet A or diet C receiving iodine supplements is usually between 3-6%. In Experiment 13 the low iodine diets have increased the uptake of ^{125}I more than 8 times in comparison with the controls. The uptake parallels the weight of the thyroid with the largest gland on diet B having the greatest uptake. In Experiment 14 the control rat has a higher uptake than is usual for a normal thyroid but it is still half that obtained with the low iodine diets. All the low iodine diets cause a similar uptake in this experiment with little correlation between thyroid weight and ^{125}I uptake.

In Experiment 15 there is a correlation again between thyroid weight and radioiodine uptake with the rat on diet A + $CaCl_2$ which has the largest thyroid having a much greater uptake than the others. The presence of calcium has not had much effect on the ^{125}I uptake since rats on diet A have the same uptake as those on diet C + $CaCl_2$ and this uptake is greater than rats on diet C alone.

In Experiment 6, a six hour uptake was measured instead of a four hour uptake. Again, there is a correlation between thyroid weight and radioiodine uptake.

Rats on diet A with the smallest thyroids have uptakes about 6 times the control value and about 15% less than the other low iodine diets. Rats on diets B, C and D which have the same thyroid weight also have the same uptake.

Section 4

Effect of diets on serum T_4 and serum TSH

Serum T_4 and serum TSH were measured for some animals in each experiment.

Table 24 shows the results obtained for rats in Experiments 5 and 6 which have been on the diets for 8 and 10 weeks respectively. The numbers in brackets are the numbers of analyses carried out.

Diet	Thyroid weight (mg)	Serum T_4 (nmol/l)	Serum TSH (μU/ml)
Experiment 5. Rats on diets for 8 weeks			
41B	15.23	71.0 (1)	133.0 (1)
A	20.59	28.4 (1)	69.1 (1)
B	26.40	58.0 (1)	55.2 (1)
C	27.28	33.5 (2)	93.4 (2)
D	26.75	29.7 (1)	123.0 (1)
Experiment 6. Rats on diets for 10 weeks			
41B	16.66	63.5 (4)	80.1 (3)
A	22.44	29.7 (4)	55.4 (4)
B	26.91	31.0 (5)	46.0 (4)
C	30.05	30.0 (5)	308.6 (4)
D	30.25	33.8 (4)	119.2 (4)

Table 24

Results of serum T_4 and serum TSH measurements for rats in Experiments 5 and 6

As expected, the low iodine diets have caused a reduction of the serum T_4 compared to the level found for the control group. Eight weeks on a low iodine diet has decreased the T_4 from about 70 nmol/l to about 30 nmol/l. However, there is no difference in the T_4 for the differing calcium diets. Although the rat on diet B has a higher T_4 than the others it is probably not significant.

The same level of serum T_4 is found after a further two weeks on the diets as is seen for Experiment 6. Again all the calcium diets give the same serum T_4.

The serum T_4 and serum TSH for rats in Experiments 7-12 which have been on diet C for various periods of time before the administration of the calcium diets, are shown in table 25.

Diet	Thyroid weight (mg)	Serum T_4 (nmol/l)	Serum TSH (μU/ml)
Experiment 7. Rats on diet C for 4 weeks + 4 weeks on diets			
41B	15.59	59.2 (1)	68.8 (1)
A	28.32	37.3 (1)	93.0 (1)
B	26.20	28.3 (1)	99.1 (1)
C	34.10	34.7 (1)	70.5 (1)
D	34.39	25.7 (1)	-
Experiment 8. Rats on diet C for 4 weeks + 8 weeks on diets			
41B	14.71	63.1 (1)	82.9 (1)
A	22.79	43.8 (1)	83.7 (1)
B	33.52	20.6 (1)	247.4 (1)
C	25.24	51.5 (1)	79.3 (1)
D	28.63	23.2 (1)	79.3 (1)

Diet	Thyroid weight (mg)	Serum T_4 (nmol/l)	Serum TSH (μU/ml)
Experiment 9. Rats on diet C for 8 weeks + 8 weeks on diets			
41B	14.78	39.2 (1)	72.7 (1)
A	30.24	35.5 (1)	61.7 (1)
B	32.04	34.6 (1)	87.5 (1)
C	32.29	21.2 (1)	205.1 (1)
D	32.56	-	102.3 (1)
Experiment 10. Rats on diet C for 8 weeks + 12 weeks on diets			
41B	17.42	49.6 (1)	42.8 (1)
A	30.80	30.7 (1)	123.0 (1)
B	28.41	45.6 (1)	-
C	31.03	37.5 (1)	89.9 (1)
D	38.91	34.8 (1)	121.2 (1)
Experiment 11. Rats on diet C for 12 weeks + 8 weeks on diets			
41B	18.16	48.7 (1)	81.1 (2)
A	36.43	38.6 (3)	102.1 (3)
B	40.16	24.8 (3)	121.3 (3)
C	44.92	22.5 (3)	156.4 (3)
D	40.09	23.9 (3)	76.6 (3)
Experiment 12. Rats on diet C for 16 weeks + 12 weeks on diets			
A	34.06	40.9 (3)	54.5 (3)
B	33.76	31.8 (3)	31.2 (3)
C	31.15	43.6 (3)	61.5 (3)
D	34.09	49.7 (3)	39.5 (3)

Table 25

Results of serum T_4 and serum TSH measurements for rats in Experiments 7-12

Most of the rats on the low iodine diets have a serum T_4 in the hypothyroid range or near the lower limit of the normal range. For Experiments 9 and 11 the T_4 tends to decrease with increasing dietary calcium. In the other experiments in this group there does not seem to be any consistent pattern and usually the T_4 is much the same for all four calcium diets.

Table 26 shows the T_4 and TSH for rats in Experiments 13 and 14.

Diet	Thyroid weight (mg)	Serum T_4 (nmol/l)	Serum TSH (μU/ml)
Experiment 13.	Rats on diet C for 8 weeks + 8 weeks on diets		
41B	17.75	45.4 (3)	68.6 (1)
A	21.55	53.0 (3)	38.9 (3)
B	25.74	30.6 (3)	47.1 (2)
C	25.20	44.4 (3)	48.1 (3)
D	25.70	47.4 (3)	64.1 (3)
Experiment 14.	Rats on diet C for 8 weeks + 12 weeks on diets		
41B	18.20	48.7 (10)	98.2 (9)
A	24.05	46.0 (3)	83.7 (3)
B	27.68	41.8 (3)	70.1 (3)
C	23.90	52.1 (3)	54.0 (3)
D	25.65	41.6 (4)	54.3 (3)

Table 26

Results of serum T_4 and serum TSH measurements for rats in Experiments 13 and 14

Rats in Experiments 13 and 14 were weanlings when they started the low iodine dietary regime. Their serum T_4 has not decreased as much as would be expected from previous results. Even after 20 weeks on diet C it is still in the middle of the normal range. The results in table 26 do not show any difference between the T_4 for the differing calcium diets.

The T_4 and TSH results for Experiment 15 are shown in table 27.

Diet	Thyroid weight (mg)	Serum T_4 (nmol/l)	Serum TSH (μU/ml)
Experiment 15. Rats on diets for 8 weeks			
A	14.92	49.9 (2)	70.3 (2)
A + KI	8.75	53.2 (2)	112.8 (2)
A + $CaCl_2$	17.58	49.3 (3)	62.4 (3)
C	15.67	57.1 (2)	59.7 (3)
C + KI	11.68	60.1 (3)	67.8 (3)
C + $CaCl_2$	15.54	49.1 (3)	67.4 (3)

Table 27

Results of serum T_4 and serum TSH measurements for rats in Experiment 15

Rats receiving iodine supplements do have a serum T_4 higher than the others but the low iodine diets have not suppressed the T_4 level very much after 8 weeks.

As can be seen from table 28 the administration of PTU or $KClO_4$ in either diet for 2 or 4 weeks decreases the serum T_4 below the detectable limit of the assay.

Diet	Thyroid weight (mg)	Serum T_4 (nmol/l)	Serum TSH (μU/ml)
Experiment 16. Rats on diets for 4 weeks			
A	14.27	35.3 (2)	49.2 (2)
C	17.10	62.3 (3)	54.3 (3)
A + PTU	69.21	<6.6 (2)	461.6 (2)
C + PTU	78.97	<6.6 (2)	628.2 (2)
A + $KClO_4$	29.94	<6.6 (1)	289.8 (2)
C + $KClO_4$	38.88	<6.6 (1)	343.0 (2)
Experiment 17. Rats on diets for 2 weeks			
A + PTU	45.24	<6.6 (3)	325.9 (3)
C + PTU	31.41	<6.6 (1)	510.2 (2)
A + $KClO_4$	24.10	<6.6 (3)	397.7 (4)
C + $KClO_4$	18.48	10.2 (1)	487.8 (2)

Table 28

Results of serum T_4 and serum TSH measurements for rats in Experiments 16 and 17

A different pattern is observed for the serum TSH. The administration of a low iodine diet would be expected to increase the serum TSH but since the normal range is so broad many of low iodine diet results fall into the upper region of the normal range. In some experiments no increase in TSH levels is found at all.

For animals in Experiment 5 (table 24) the TSH is high for the control rat and thus the low iodine diets

appear to have lowered the TSH. For the four calcium diets the TSH increases with increased thyroid weight and thus also with increased dietary calcium. The same pattern is observed for rats in Experiment 6. Rats on diet A or diet B for 10 weeks have a serum TSH within the normal range. Those on diet C have a mean serum TSH elevated to 308.6 μU/ml which is about three times the normal average TSH. The TSH for rats on diet D which received the highest concentration of dietary calcium is not as high although it is still in the upper region of the normal range and it is twice that of rats on diet A.

This is also found for rats in Experiment 9. Their serum TSH shown in table 25, increases from a normal value for rats on diet A to a value twice that of normal for rats on diet C. Rats on diet D again have a lower TSH than those on diet C but their TSH is higher than that of the control animals on 41B. Also, for Experiment 11 the TSH increases with increased dietary calcium from diet A to diet C with rats on diet D having a TSH within the normal range. In these two experiments there is some correlation of serum T_4 and TSH in that the TSH increases as the T_4 decreases. In the other experiments in this group the rats have a normal or slightly elevated TSH but there does not seem to be any correlation between

TSH levels and either thyroid weight, T_4 or dietary calcium.

For rats in Experiments 13, 14 and 15 (Tables 26 and 27) the TSH is within the normal range for all the different diets.

Table 28 gives the TSH concentrations for rats on the PTU and $KClO_4$ diets. In Experiment 16 rats on diet A and diet C have a normal TSH while those receiving PTU and $KClO_4$ have a very much elevated result. PTU has caused a greater increase in TSH than has $KClO_4$. The addition of calcium to the diet has also caused a slight increase in TSH. When these diets are administered for two weeks, the TSH is again elevated to about the same level. Again the addition of calcium causes a higher concentration of TSH in the serum with both the PTU and $KClO_4$ diets.

Chapter 4

Effect of calcium on protein binding of ^{125}I in the thyroid and on urinary excretion of iodine

Section 1

Discharge of ^{125}I from rat thyroids in vivo

To determine if calcium in the diet interferes with the binding of ^{125}I to thyroglobulin, rats on the five test diets for 10 weeks were first injected intra-peritoneally with 10 μCi ^{125}I. Three hours later half the number of rats on each diet were injected intra-peritoneally with 50 mg sodium thiocyanate (Na SCN) in 0.2 ml saline. Three hours after this, all the animals were sacrificed and the thyroids removed. The final 6 hour thyroidal uptake of ^{125}I was measured by counting the glands in the gamma counter.

The uptake results and the percentage of ^{125}I discharged by Na SCN are shown in table 29.

Diet	Thyroid weight (mg)	6 hr ^{125}I uptake % dose	Thyroid weight (mg)	6 hr ^{125}I uptake % dose	% ^{125}I discharged
			Rats injected with 50 mg Na SCN at 3 hr.		
41B	19.36 (2)	6.02	17.06 (3)	4.35	27.7
A	21.36 (2)	35.8	19.04 (2)	31.8	11.2
B	32.68 (2)	51.3	27.30 (3)	31.3	40.0
C	32.12 (2)	50.0	28.28 (2)	35.6	28.8
D	32.48 (2)	48.7	30.21 (3)	32.1	34.1

Table 29

Uptake of ^{125}I by rat thyroids and discharge by Na SCN

All the low iodine diets have caused an increase in ^{125}I uptake but this increase is less for the rats on the low calcium diet A. Na SCN discharges the iodine which is not protein bound thus the total uptake decreases after injection of Na SCN for each of the five diets. For diet A only 11% of the iodine taken up by the gland is dischargeable while for the other diets at least three times this amount is discharged by Na SCN.

Section 2

Urinary excretion of iodine

Two or three rats on each of the five test diets were put into metabolic cages for collection of urine. The urine was collected for the first 48 hours after the diets were started and then after 6, 8 and 10 weeks on the diets. The volume of the collection was measured and the iodine was determined by the Technicon Auto Analyzer technique. The results obtained for the total excretion of iodine are shown in table 30. The numbers of animals on each diet are given in the brackets.

Diet	Total iodine excretion in 48 hours (µg)			
	2 days on diets	6 weeks on diets	8 weeks on diets	10 weeks on diets
41B	100 (2)	29.7 (1)	58.7 (1)	53.6 (2)
A	23.3 (3)	5.47(4)	3.56(4)	3.41(3)
B	19.4 (2)	8.23(2)	3.12(2)	3.67(2)
C	16.4 (3)	3.49(3)	2.30(3)	2.64(3)
D	15.8 (2)	1.93(2)	3.66(2)	3.71(2)

Table 30

Urinary excretion of iodine

Even after only 2 days on the diets the animals on the low iodine diets show a decreased urinary excretion of iodine compared to the control rats on diet 41B. The amount of iodine excreted decreases as the length of time on the diets increases. After two days on the diets rats on diet A have excreted more iodine than those on the other low iodine diets. After 6 weeks on the diets rats on diets A and B are excreting more iodine than the rats on diet C and D but after a further two or four weeks on the diets all the animals on the low iodine diets excrete the same amount of urinary iodine.

Chapter 5

Section 1

In vivo uptake of ^{45}Ca by various rat tissues

In each experiment 10 male Sprague-Dawley rats of body weight 120-150g were injected intra-peritoneally with 100 µCi ^{45}Ca as $^{45}Ca\ Cl_2$ in 0.2 ml 0.9% saline. During the experimental period, the rats were maintained on diet 41B and distilled water. After the prescribed length of time, ranging from 5 minutes to 34 hours, two were sacrificed and the thyroid, one kidney, a piece of liver and a blood sample were removed from each. The kidney and liver were briefly washed free of blood in 5 ml cold PBS, sliced and homogenised in 5 ml PBS using a TRI-R STIR-R homogeniser fitted with a glass homogenising tube with a teflon pestle. The thyroid was homogenised in 2 ml PBS. The homogenates were then spun in an MSE refrigerated centrifuge at 20,000g for 10 minutes. The blood samples were allowed to clot and then spun on a bench centrifuge to collect the serum.

Since a highly coloured sample can cause quenching effects when counted by liquid scintillation, it was necessary to bleach the supernatants of the homogenised tissues before counting. Two 0.5 ml aliquots of each tissue supernatant were bleached with 0.2 ml propan-2-ol and 0.2 ml hydrogen peroxide and counted after the addition of 10 ml Dimilume in a Packard liquid scintillation counter.

0.1 ml aliquots of the serum were prepared and counted in the same manner .

The protein content of the tissue supernatants and of the serum was measured by the Lowry method. The uptake results were calculated as counts/min/mg protein for the soluble protein of each tissue and for the serum and this was plotted against time of uptake. The results were also expressed as a percentage of the uptake at one hour.

The results of the uptake of radioactive ^{45}Ca by rat thyroid, liver, kidney and serum are shown in figures 8, 9.

Figure 8 shows the time course of uptake over the first four hours after injection. The same pattern of uptake is shown by all three tissues although the thyroid uptake rises initially. The highest uptake per mg protein is found for the thyroid. Its uptake reaches an equilibrium after two hours and the amount of radioactivity in the thyroid falls very slowly over the next two hours. Although the uptake of the kidney at five minutes is the same as that of the thyroid it decreases sharply to also reach an equilibrium after two hours. The liver which initially takes up less ^{45}Ca than either the kidney or the thyroid reaches an equilibrium after one hour.

FIGURE 8

In vivo uptake of ^{45}Ca after i.p. injection

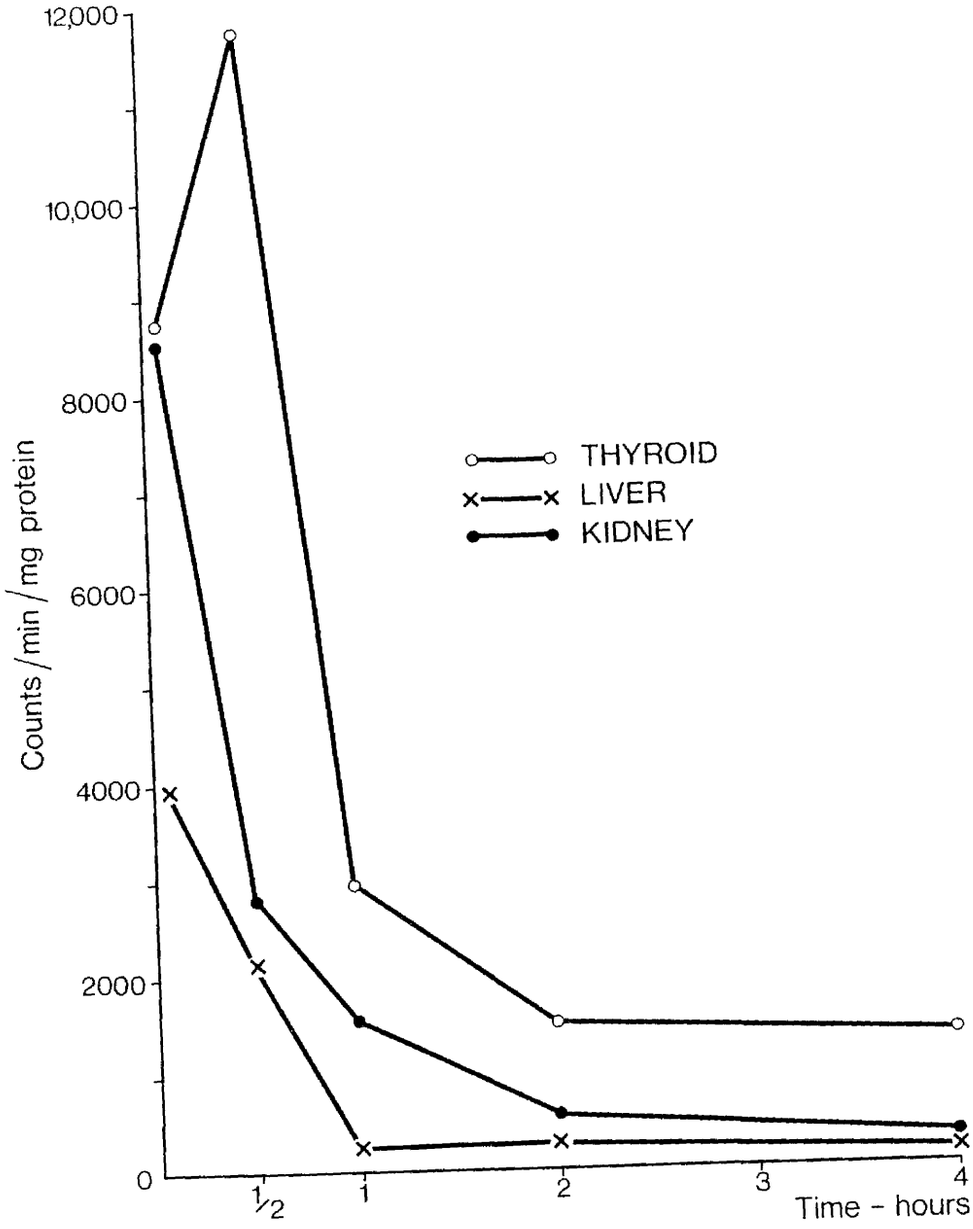

12,000
10,000
8000
6000
4000
2000
0
Counts / min / mg protein
THYROID
LIVER
KIDNEY
½
1
2
3
4
Time - hours

Figure 9 shows the uptake of the thyroid, liver, kidney and serum after a longer time period. The numbers in brackets are the numbers of rats sacrificed for each point. The serum at a one hour uptake contains a large concentration of ^{45}Ca. This concentration is twice that of the thyroid, five times that of the kidney and twenty times that of the liver. The serum level drops very steeply over the next five hours and continues to fall over the next two days.

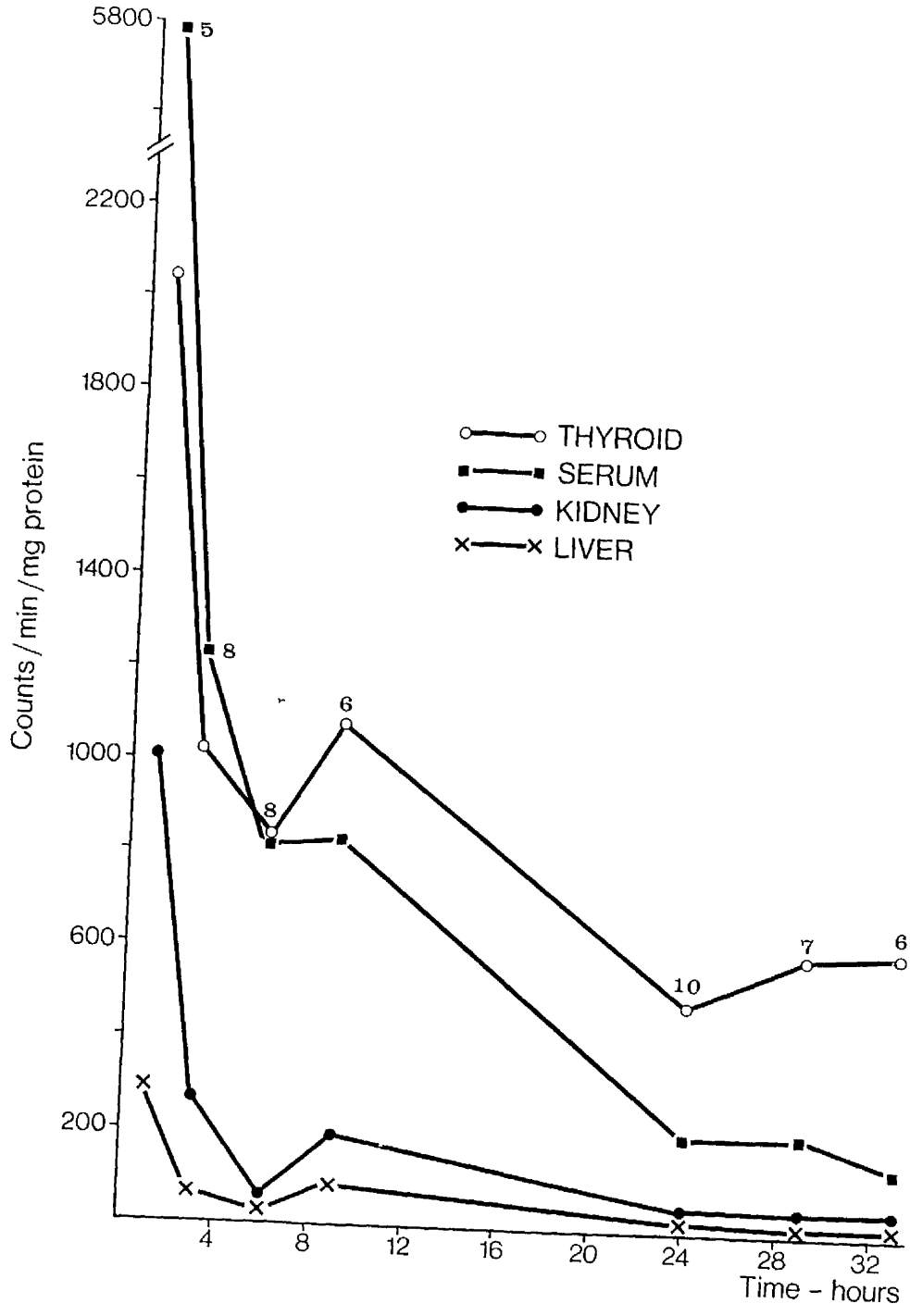
At one hour uptake, the thyroid has the next largest concentration of ^{45}Ca. Its uptake drops steeply in the same way as the serum uptake over the next five hours. At a six hour uptake the thyroid takes up as much radioactivity per mg protein as the serum. After this time the thyroid uptake remains higher than that of the serum, kidney and liver. Thirty three hours after the injection the thyroid uptake is more than four times that of the serum.

In figure 9 it can be seen that the uptake of the kidney and liver parallel each other and that the liver always takes up less ^{45}Ca than the kidney or the thyroid. Two days after injection there is not much ^{45}Ca left in the liver or kidney.

The results expressed as a percentage of the uptake at one hour are shown in figure 10. Each point represents

FIGURE 9

In vivo uptake of ^{45}Ca after i.p. injection

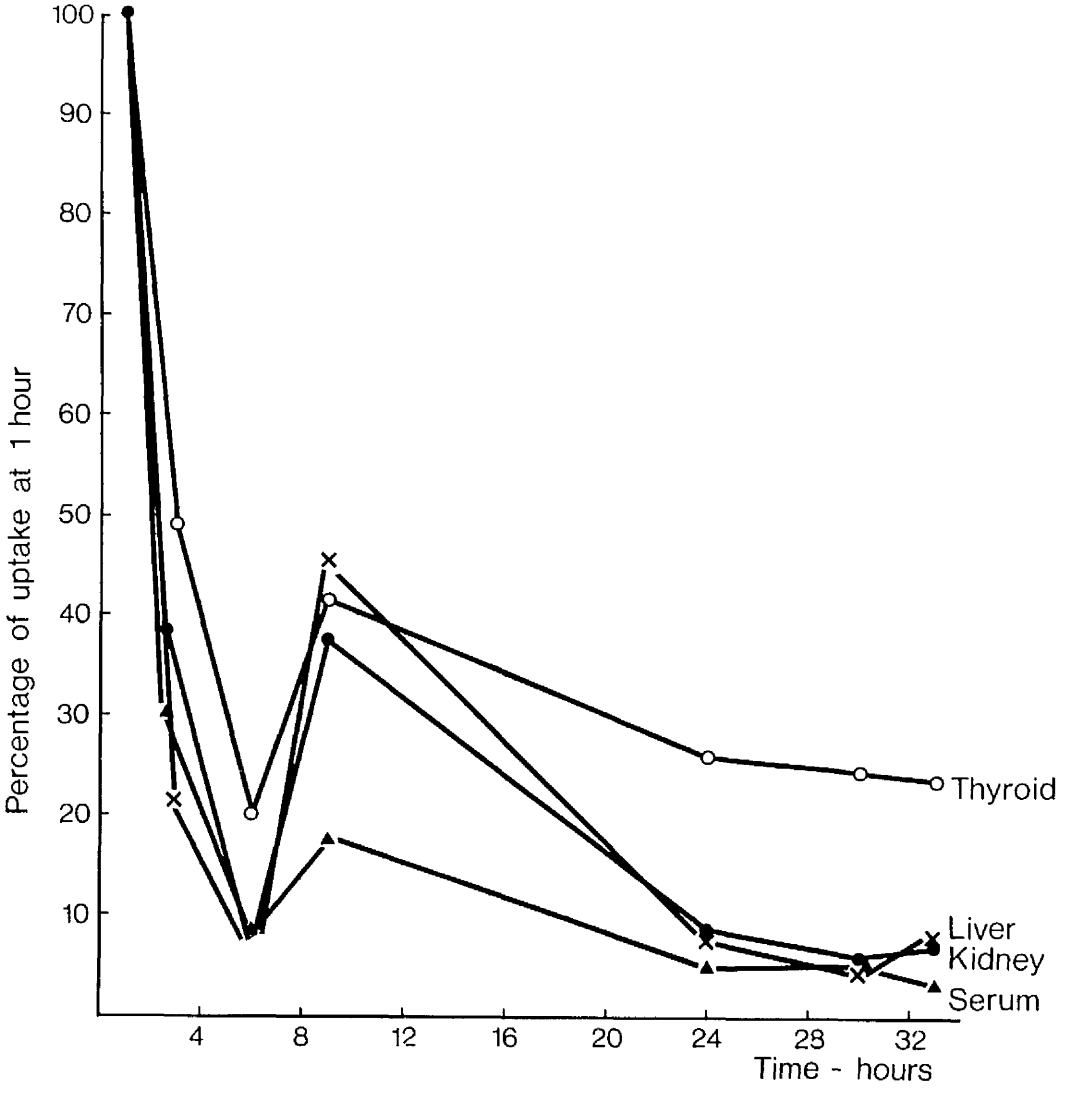

5800
2200
1800
1400
1000
600
200
Counts / min / mg protein
5
8
8
6
10
7
6
THYROID
SERUM
KIDNEY
LIVER
4
8
12
16
20
24
28
32
Time - hours

the average of two experiments each consisting of two animals. Three hours after injection the thyroid contains 50% of the radioactivity it took up at one hour. The amount of ^{45}Ca in the other tissues and the serum has decreased further. Six hours after injection the thyroid level has fallen to 20% while that of the liver, kidney and serum is below 10%. There is a rise in the percentage of ^{45}Ca in the tissues at nine hours with the thyroid, liver and kidney all containing about 40% of the original concentration. The serum percentage also rises at 9 hours but to a lesser extent. Twenty-four hours after injection, the serum, liver and kidney uptake has fallen to below 10% and it remains there over the next nine hours. The thyroid uptake, however, only decreases to 25% and it decreases very slowly over the next nine hours. Therefore, 33 hours after injection, the thyroid still contains 23% of the radioactivity present at one hour while the uptake of the serum and the other tissues has decreased to 5%

Two rats were put into a metabolic cage for collection of urine after the injection of 100 μCi ^{45}Ca. A sample from one rat was counted after two hours. Very little radioactivity was excreted (<0.001%). The other rat was left overnight before the urine was counted. After 21 hours the volume collected was 3.3 ml and this contained 0.12% of the injected counts.

FIGURE 10

^{45}Ca uptake results expressed as a percentage of uptake at 1 hour


100
90
80
70
60
50
40
30
20
10
Percentage of uptake at 1 hour
4
8
12
16
20
24
28
32
Time - hours
Thyroid
Liver
Kidney
Serum

Section 2

In vivo uptake of ^{45}Ca by rats on low iodine diets

Two rats which had been on the Remington low iodine diet (diet C) for 4 weeks and one control rat on 41B were injected intraperitoneally with 100 μCi ^{45}Ca. The ^{45}Ca uptake of the serum, kidney, liver and thyroid of each rat was measured after three hours as described previously. The results were calculated as counts/min/mg protein and then expressed as a ratio of the serum uptake. Table 31 gives the ratios obtained for these three animals.

Diet	Kidney uptake / serum uptake	Liver uptake / serum uptake	Thyroid uptake / serum uptake
41B (1)	0.142	0.0321	0.399
C (2)	0.130	0.0543	0.579

Table 31

^{45}Ca uptake of tissues from rats on diet 41B and diet C

The feeding of a low iodine diet does not affect the uptake of ^{45}Ca by the rat kidney but it has increased the uptake of both the liver and the thyroid in this experiment.

The time of feeding the low iodine diets was increased to 10 weeks. Two rats on each diet were maintained on diets 41B, A, B, C or D and after 10 weeks they were injected intraperitoneally with 100 μCi ^{45}Ca and sacrificed three hours later.

The uptakes were again expressed as a ratio of the serum uptake and are shown in table 32.

Diet	kidney uptake / serum uptake	liver uptake / serum uptake	thyroid uptake / serum uptake
41B (2)	0.159	0.0347	0.346
A (2)	0.134	0.1026	0.581
B (2)	0.130	0.0662	0.408
C (2)	0.186	0.0638	0.448
D (2)	0.156	0.0668	0.548

Table 32

^{45}Ca uptake of tissues from rats on diets 41B, A, B, C and D

The ^{45}Ca uptake of the kidney remains at the same level irrespective of the dietary regime. The liver uptake has doubled for all the rats on the low iodine diets. The thyroid uptake has also increased for rats on the low iodine diets but not as much as the increase in the liver uptake. The calcium content of the diet does not seem to have much effect on the ^{45}Ca uptake of any of the tissues.

Section 3

Radioactivity in the insoluble pellets of rat tissue homogenates

In section 1 of this chapter the changes in ^{45}Ca uptake in the soluble fraction only of the various tissues were considered. In this section the labelling of the insoluble fraction is discussed.

The pellets obtained after the tissues were homogenised and centrifuged were counted separately. These pellets consist of cellular debris, whole cells and any material that was not totally homogenised. Large pellets were obtained from the liver and kidney homogenates and they contained a high number of counts. The kidney pellets and liver pellets contained about 50% of the total counts. The thyroid pellet was small and normally contained about 13% of the total thyroid counts.

Over the first four hours of uptake (figure 11) the counts in the pellets follow the same pattern as those in the supernatants. Since there was more pelleted material from the liver it has the highest initial count rate but this falls off steeply to about the same level as the kidney pellet. The kidney pellet radioactivity decreases steeply to reach an equilibrium at two hours. The thyroid pellet has the lowest count rate since it was the smallest but the rate of decrease in radioactivity is not so great. Similar results are obtained over a longer period as is shown in figure 12. Again the thyroid pellet contains less counts than the liver or kidney pellets but over 33 hours the amount of radioactivity only falls by half, whereas for the liver and the kidney it decreases nearly 100-fold.

FIGURE 11

In vivo uptake-radioactivity in pellets

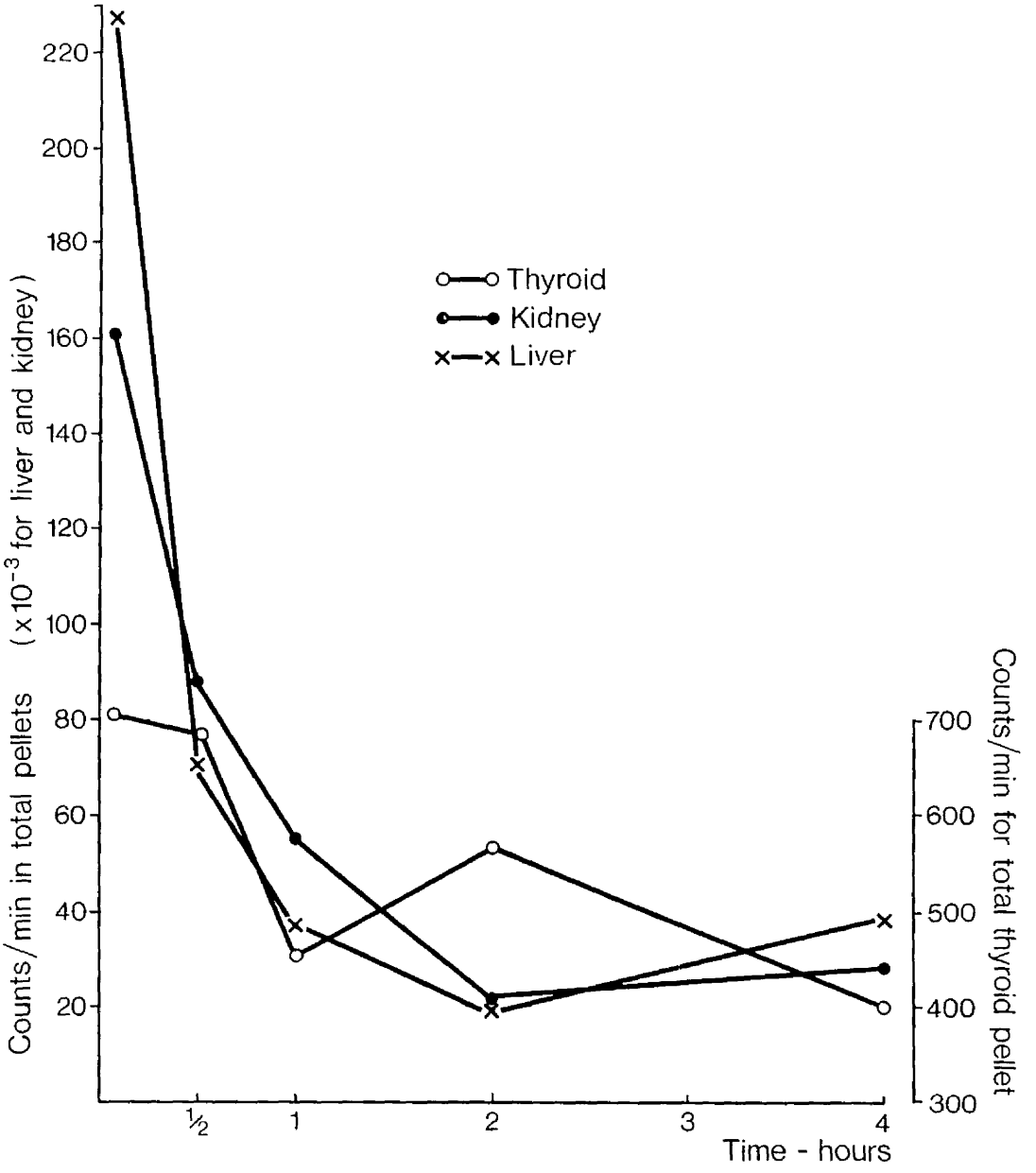
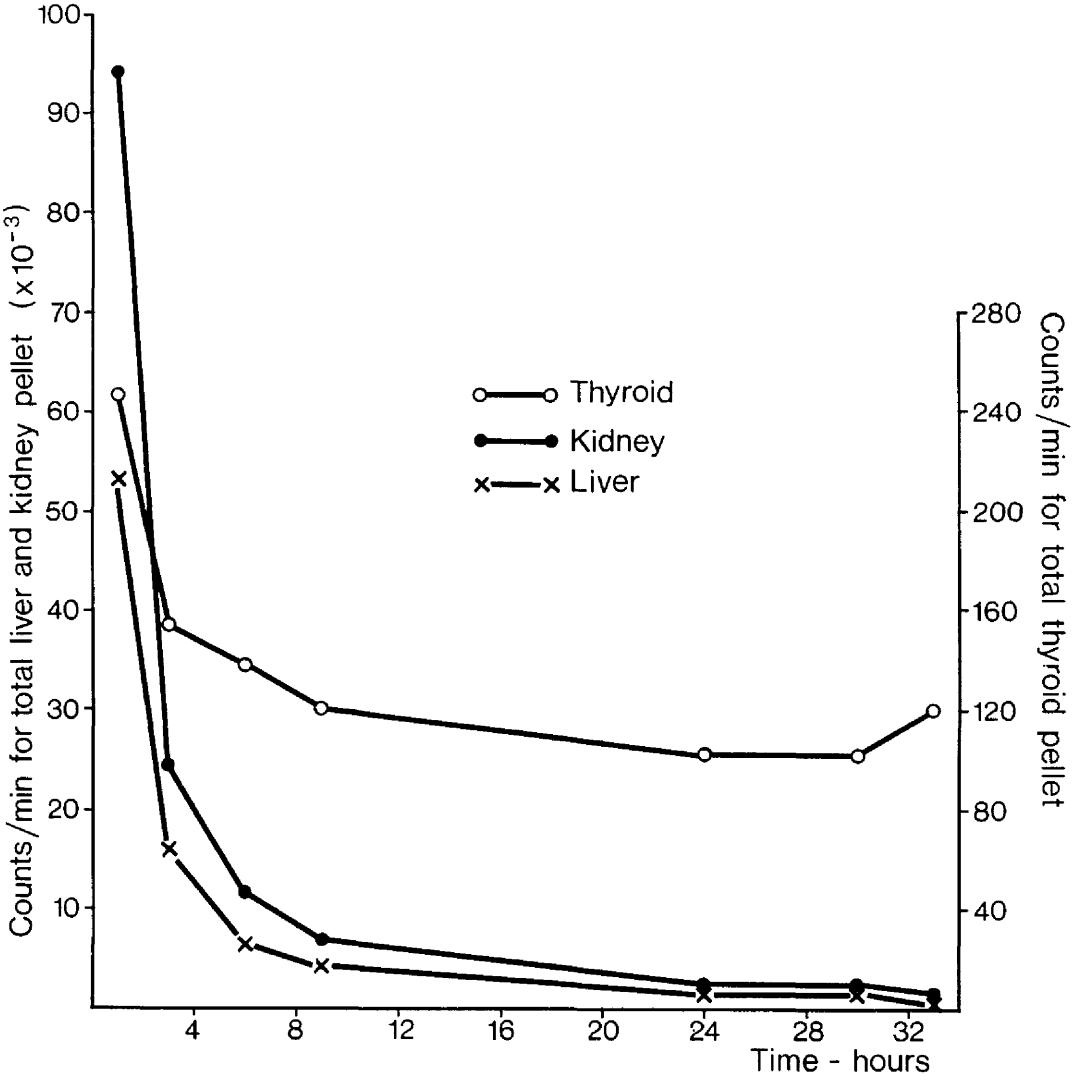

Thyroid
Kidney
Liver
Counts/min in total pellets (x10⁻³ for liver and kidney)
Counts/min for total thyroid pellet
Time - hours
220
200
180
160
140
120
100
80
60
40
20
700
600
500
400
300
½
1
2
3
4

FIGURE 12

In vivo uptake-radioactivity in pellets


Counts/min for total liver and kidney pellet (x10⁻³)
Counts/min for total thyroid pellet
Thyroid
Kidney
Liver
Time - hours
100
90
80
70
60
50
40
30
20
10
280
240
200
160
120
80
40
4
8
12
16
20
24
28
32

Section 4

In vitro uptake of ^{45}Ca by sheep thyroid slices

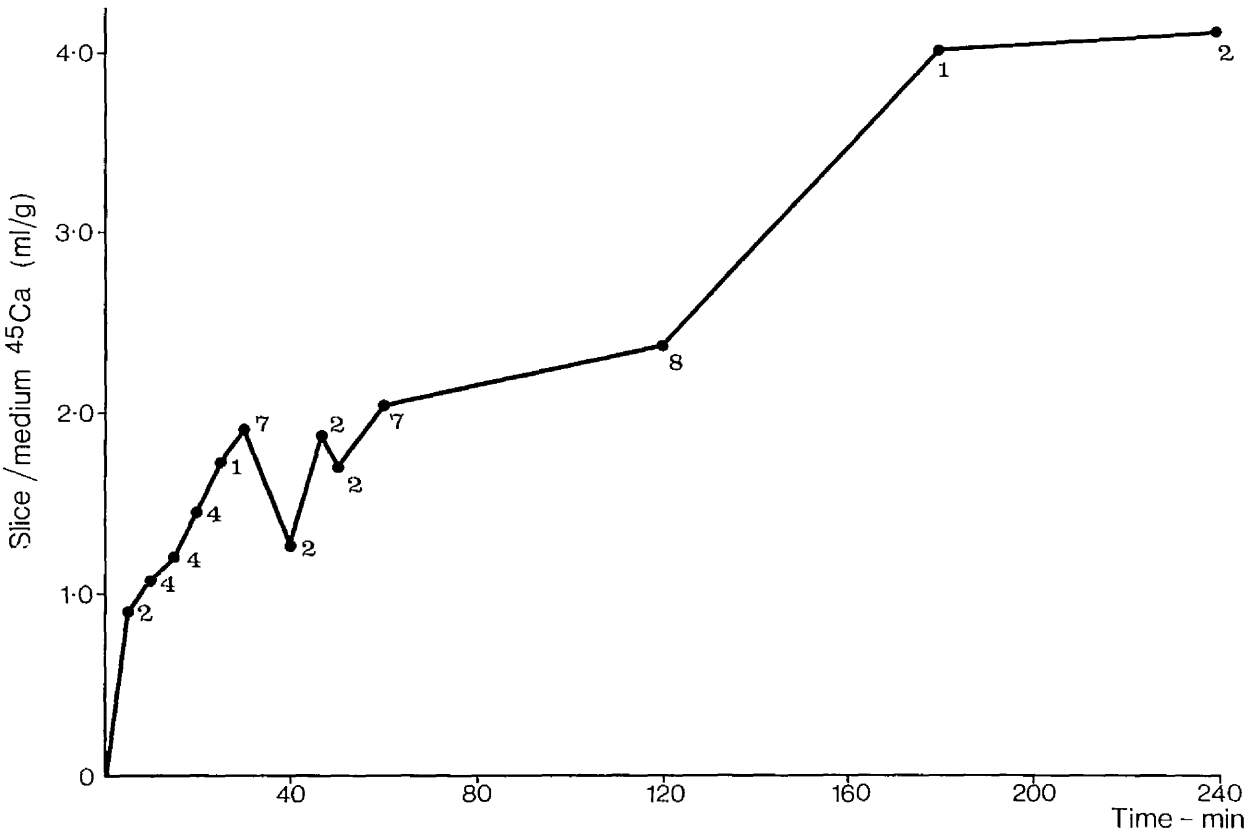
The time course of the uptake of ^{45}Ca by sheep thyroid slices incubated in vitro in a buffered media (as described on page 56) was determined. The results are expressed as concentration gradients between the tissue (supernatant and pellet) and the incubation medium and thus calculated as slice/medium (S/M) $^{45}Ca = \frac{\text{counts per min. in tissue/g of tissue}}{\text{counts per min./ml of medium}}$, as suggested by Freinkel and Ingbar (1955) for the accumulation of ^{131}I by sheep thyroid slices.

The slices were weighed before and after incubation and the everage percentage tissue recovery in 44 incubations was 97%. The final wet weight of the tissue was used to calculate S/M ^{45}Ca ratios.

Figure 13 shows the concentration gradients obtained after incubations for times ranging from 5 minutes to 4 hours. The numbers in brackets represent the number of incubations averaged for each point. The radioactivity is rapidly accumulated by the slices. An equilibrium is reached within one hour of incubation and at this time a concentration gradient of 2.1 ml/g is achieved. Not much change in the S/M ^{45}Ca ratio takes place over the next hour of incubation to give a S/M ^{45}Ca of 2.4 ml/g at a 2 hour uptake. Only three incubations were carried

FIGURE 13

In vitro uptake of ^{45}Ca by sheep thyroid slices


4·0
3·0
2·0
1·0
0
Slice /medium ^{45}Ca (ml/g)
40
80
120
160
200
240
Time - min
2
4
4
4
1
7
2
2
7
8
1
2

out for longer than two hours but the S/M ^{45}Ca ratio has again increased to 4.1 ml/g after four hours incubation.

In one incubation experiment with thyroid slices the percentage of ^{45}Ca taken up by the mitochondrial fraction of thyroid cells was measured. After a 2½ hour incubation of sheep thyroid slices with ^{45}Ca, the tissue was homogenised in Krebs No. 2 buffer containing 0.7 mM Ca^{2+} ions and then centrifuged at 900 g on the MSE for ten minutes at 4°C. This pellet which consists of nuclei, whole cells and cellular debris contains about 5% of the total counts in the homogenate after washing with 2 ml Krebs No. 2 buffer (+ 0.7 mM Ca^{2+} ions). The supernatant was then spun at 9,000 g for ten minutes giving a pellet containing the mitochondrial fraction. After washing with the same buffer, this pellet contains about 8% of the total counts. In the method used to determine the time course of the uptake of ^{45}Ca, the labelled tissue was homogenised then centrifuged at 20,000 g. An average of 12% (39 incubations) of the total tissue counts remain in the pellet. Thus, most of the tissue ^{45}Ca always remains in the supernatant obtained when the homogenised thyroid slices are centrifuged.

When the slices were heated for five minutes at 85° the S/M ^{45}Ca ratio was 2.9 ml/g (average of three incubations).

Identical incubations were also carried out with ^{125}I in the incubation medium instead of ^{45}Ca. The concentration gradients (S/M ratios) for this isotope are shown in fig. 14. The uptake of the sheep thyroid slices was measured over one hour of incubation. The thyroid takes up ^{125}I very rapidly and the S/M ratio for ^{125}I is over 40 ml/g after one hour of incubation. The pellet obtained after centrifugation of the thyroid homogenate contains 4.3% of the total tissue ^{125}I. When the slices were heated at 85° for five minutes the S/M ^{125}I ratio was 0.74 ml/g.

Section 5

Incubations with ^{45}Ca or ^{125}I in the presence of TSH

Sheep thyroid slices were incubated in the same manner as above.

TSH (10 mU/ml) was added to half the flasks containing the slices before the addition of ^{45}Ca. The time course of the uptake of ^{45}Ca in the presence and absence of TSH is shown in fig. 15. Over the first hour of incubation there is no difference in uptake of ^{45}Ca for the two sets of incubations. For the incubations with TSH equilibrium is reached after one hour and at this time S/M ^{45}Ca ratio is 2.6 ml/g. The S/M ^{45}Ca ratio remains at the same level over the next two hours of incubation. For the slices incubated without TSH

FIGURE 14

In vitro uptake of ^{125}I by sheep thyroid slices

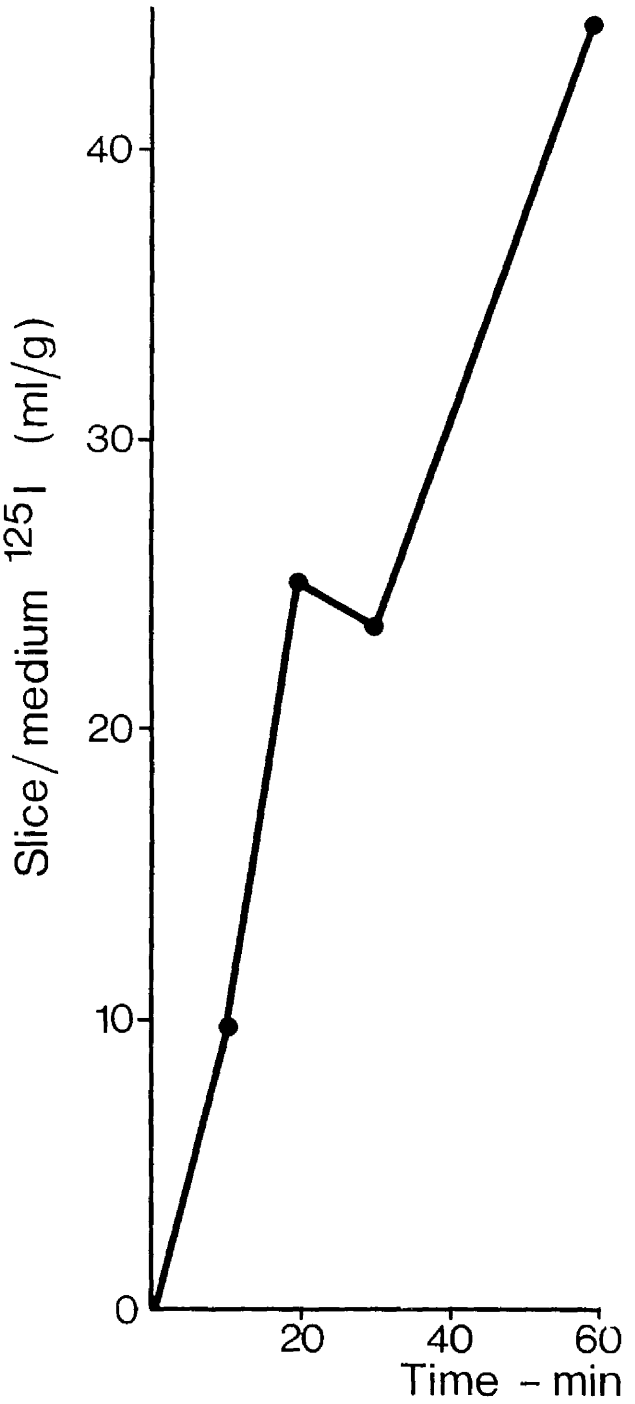
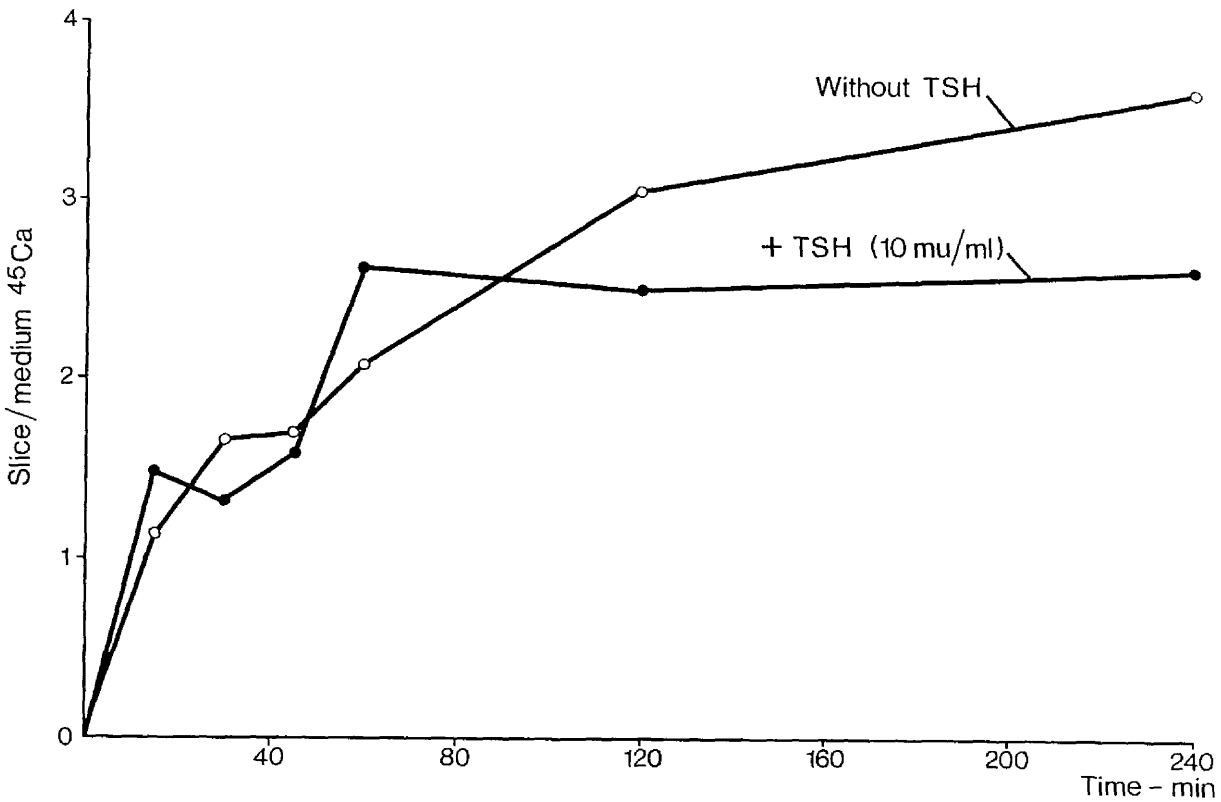

40
30
20
10
0
Slice/medium ^{125}I (ml/g)
20
40
60
Time - min

FIGURE 15

Uptake of ^{45}Ca by sheep thyroid slices - incubations with TSH


Without TSH
+ TSH (10 mu/ml)
Slice/medium 45Ca
0
1
2
3
4
40
80
120
160
200
240
Time - min

the S/M ^{45}Ca ratio at one hour incubation is 2.1 ml/g which is the same as was found in the previous experiments and is slightly less than the S/M ^{45}Ca ratio for the slices incubated in the presence of TSH. However, the S/M ^{45}Ca ratio increases over the next three hours of incubation to reach a ratio of 3.6 ml/g after four hours incubation. Thus after four hours incubation the slices incubated without TSH have taken up more radioactive calcium than those incubated with TSH.

10 mU/ml TSH was also added to thyroid slices incubated with ^{125}I. After counting the radioactivity in the slices, they were homogenised and centrifuged. The supernatants were precipitated with saturated ammonium sulphate solution to measure the proportion of ^{125}I that was protein-bound. The results of these incubations are shown in table 33.

Incubation	Time	S/M ^{125}I ratio	% ^{125}I protein-bound
Control	30 min	9.5	24.8
+ 10mU/ml TSH	30 min	9.3	28.2
Control	4 hour	57.4	72.7
+ 10mU/ml TSH	4 hour	122.6	74.9

Table 33

Thyroid slice incubations with ^{125}I and TSH

TSH has no effect on the uptake of ^{125}I when the incubation time is 30 minutes but it stimulates the uptake when the incubation is carried out for four hours. In both cases slightly more ^{125}I is protein-bound when TSH is included in the incubation medium.

Section 6

Kinetics of calcium efflux from ^{45}Ca labelled tissue

Sheep thyroid slices were prepared for incubation as before. For an uptake incubation about 300 mg tissue was incubated in 10 ml Krebs No. 2 buffer with 20 µCi ^{45}Ca for 2 hours at 37° in an oxygen atmosphere. The medium was then poured off and the slices briefly washed in cold buffer. For a discharge incubation the slices loaded with ^{45}Ca were divided into four clean flasks containing 2 ml fresh buffer and reincubated for various times.

When a third release incubation was carried out, the uptake incubation was carried out with 1 g tissue in 20 ml Krebs No. 2 buffer and 100 µCi ^{45}Ca for 2 hours. The second (discharge) incubation of 45 minutes took place in 40 ml Krebs No. 2 buffer containing 0.7 mM calcium ions. The slices were then incubated in separate flasks containing 2 ml Krebs No. 2 buffer + 0.7 mM Ca^{2+}. Between each incubation the slices were washed briefly with the calcium-containing buffer.

At the end of the incubation period the slices were removed from the medium and prepared for counting as before.

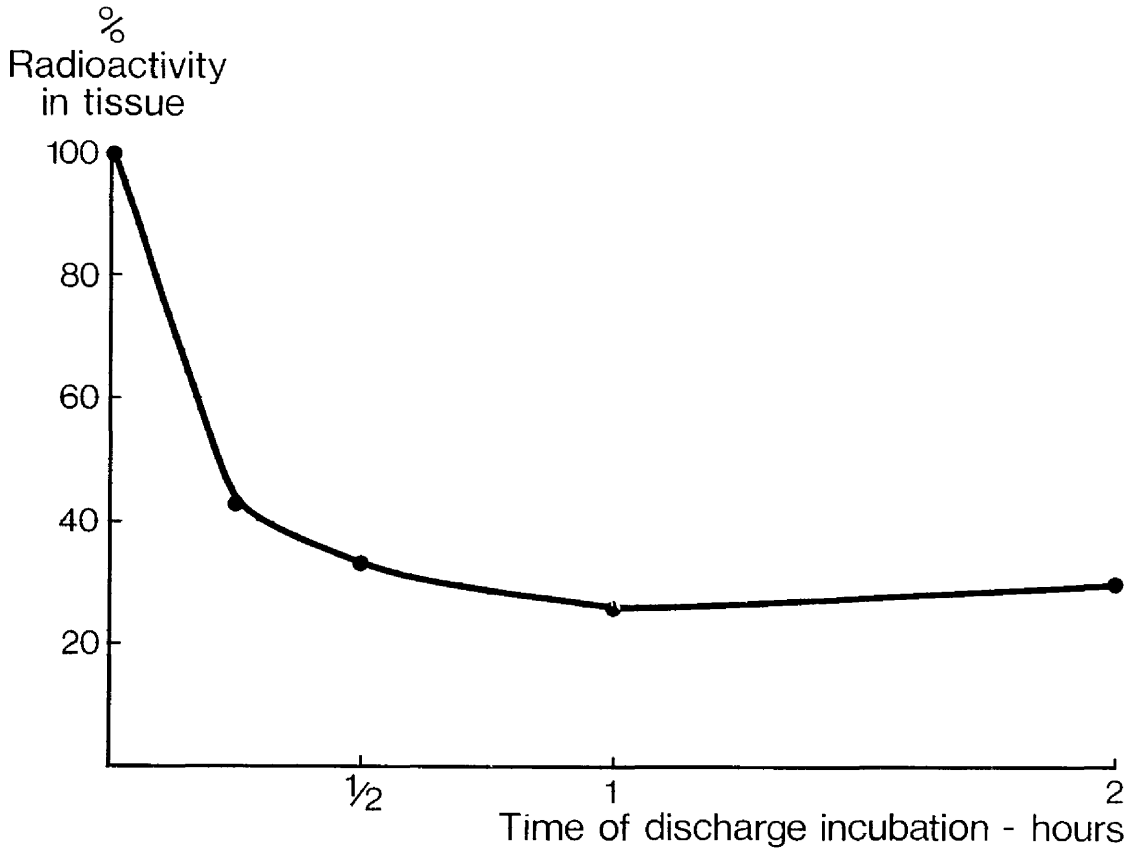
Figure 16 shows the discharge of ^{45}Ca from the sheep thyroid slices after an uptake incubation with ^{45}Ca for 2 hours. The results are expressed as a percentage of ^{45}Ca in the tissue at the start of the discharge incubation. As demonstrated by the previous uptake experiments, after a two hour uptake, the exchange of ^{45}Ca between the slices and the medium will have reached an equilibrium.

The graph shows that just under 60% of the radioactivity taken up by the tissue is rapidly washed out in the first 15 minutes of incubation in the fresh buffer medium. After this, the ^{45}Ca in the tissue falls more slowly and reaches an equilibrium after about an hour incubation. After a 2 hour uptake and a 2 hour discharge about 30% of the radioactivity taken up by the slices still remains in the tissue. Thus the calcium seems to be taken up into at least two different compartments. The radioactivity is rapidly washed out from the first compartment while it is more slowly removed from the second.

In order to study the kinetics of the removal of ^{45}Ca from the second compartment the tissue slices were loaded with ^{45}Ca for 2 hours. The ^{45}Ca was washed out of the first compartment by a discharge incubation for

FIGURE 16

Kinetics of ^{45}Ca discharge after uptake of 2 hours at 37°C


%
Radioactivity
in tissue
100
80
60
40
20
½
1
2
Time of discharge incubation - hours

45 minutes and then a third release incubation was carried out for various times.

After the discharge incubation about 32% of the initial radioactivity remains in the slices. This level falls slowly over the next two hours of incubation (fig. 17). After a two hour release incubation about 10% of the radioactivity still remains in the slices.

1 IU/ml salmon calcitonin was added to the discharge medium in one experiment. The kinetics of this discharge are shown in fig. 18 along with control incubations. There is no difference in the discharge patterns for the slices incubated with calcitonin in comparison to the controls. The radioactive calcium is washed out from the tissue at the same rate and after an incubation of two hours the same amount of radioactivity remains in the slices.

1.5 IU/ml calcitonin was added to the medium of the release incubation in two experiments. The time course of the efflux of calcium for these experiments is shown in figure 19. Initially at 15 minute incubation the calcitonin seems to retard the release of ^{45}Ca but after 30 minutes incubation the percentage of ^{45}Ca remaining in the tissue is the same for both incubations.

10 mU/ml bovine TSH was added to the release incubation in two experiments to see if TSH had any effect on the release of ^{45}Ca from the thyroid slices.

FIGURE 17

Kinetics of ^{45}Ca release after uptake of 2 hours followed by discharge of 45 minutes

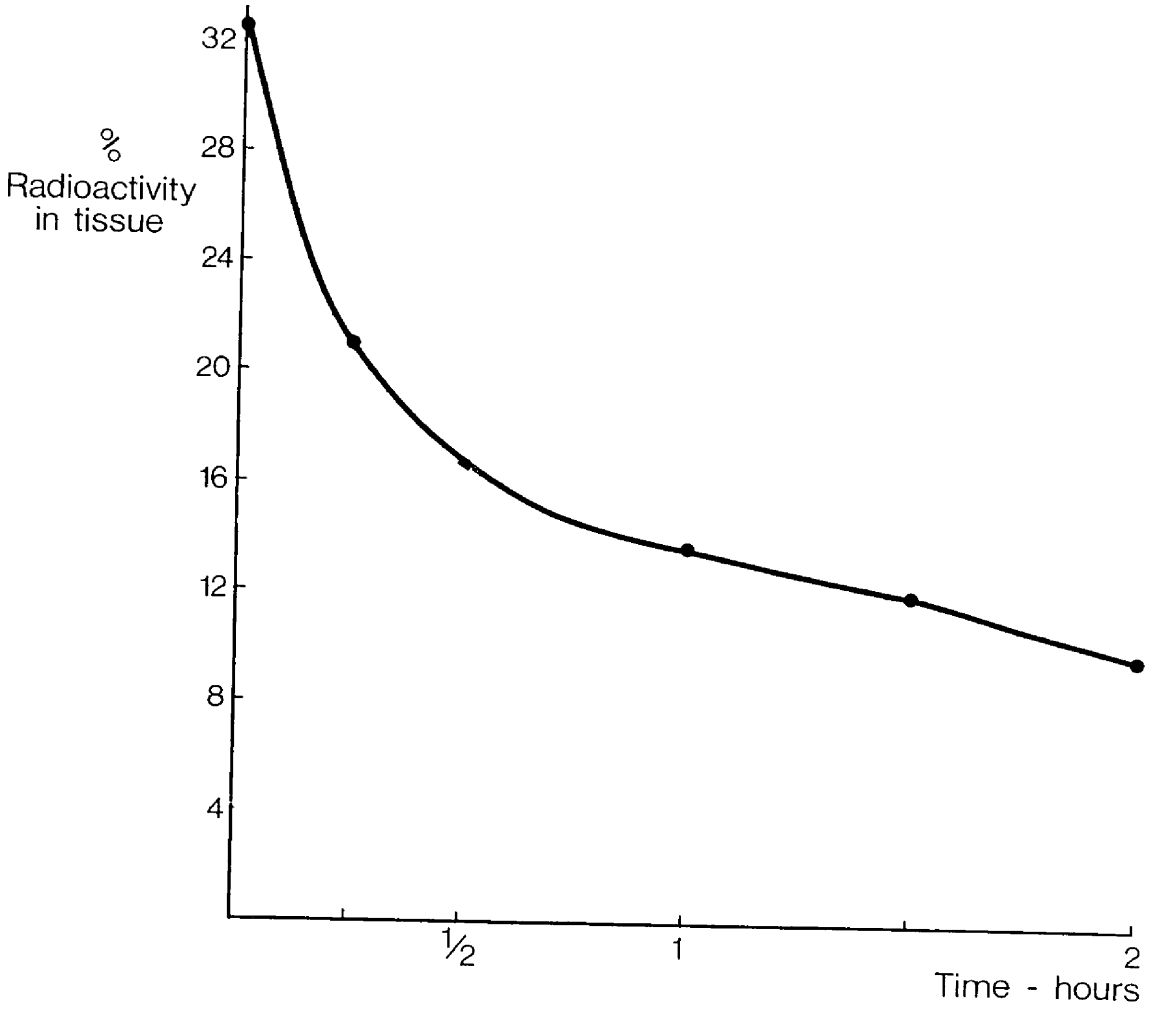

%
Radioactivity
in tissue
32
28
24
20
16
12
8
4
½
1
2
Time - hours

FIGURE 18

Kinetics of ^{45}Ca discharge with calcitonin in the medium

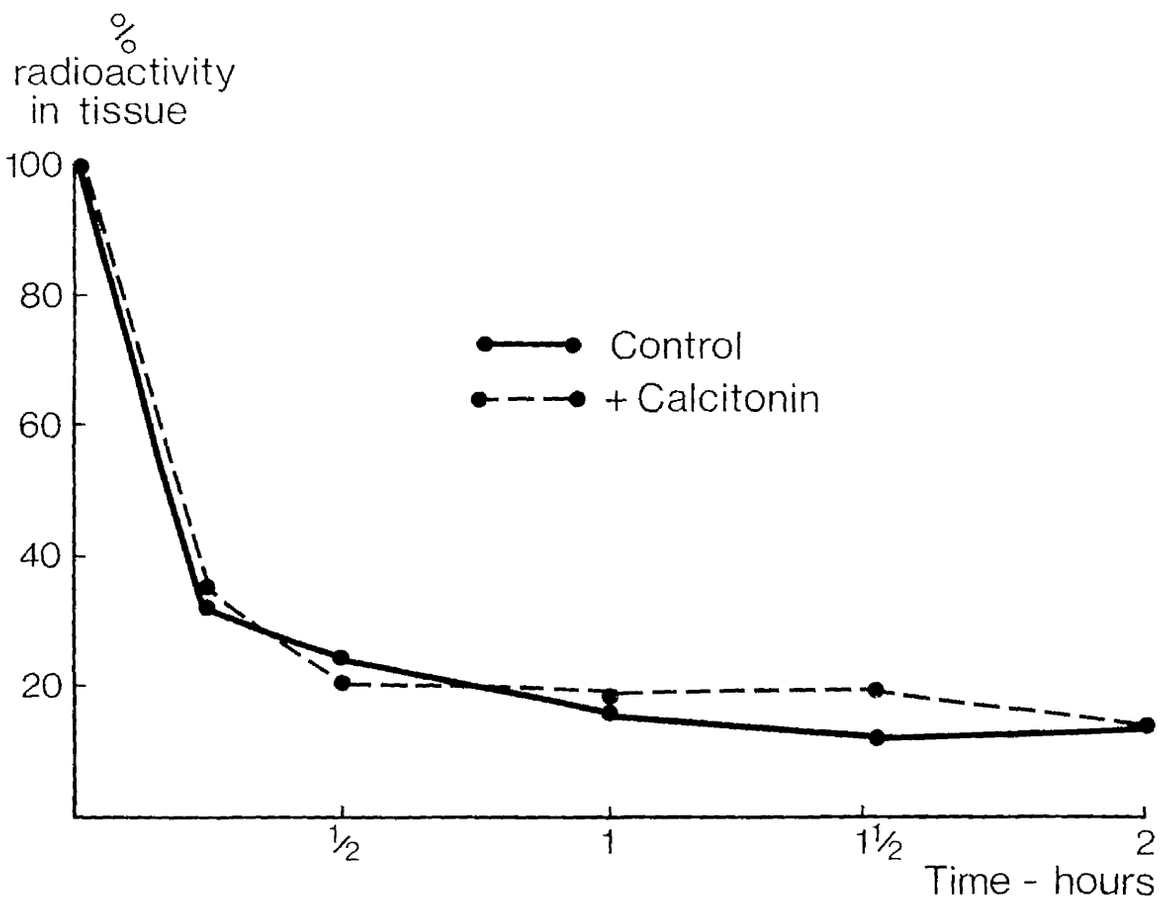
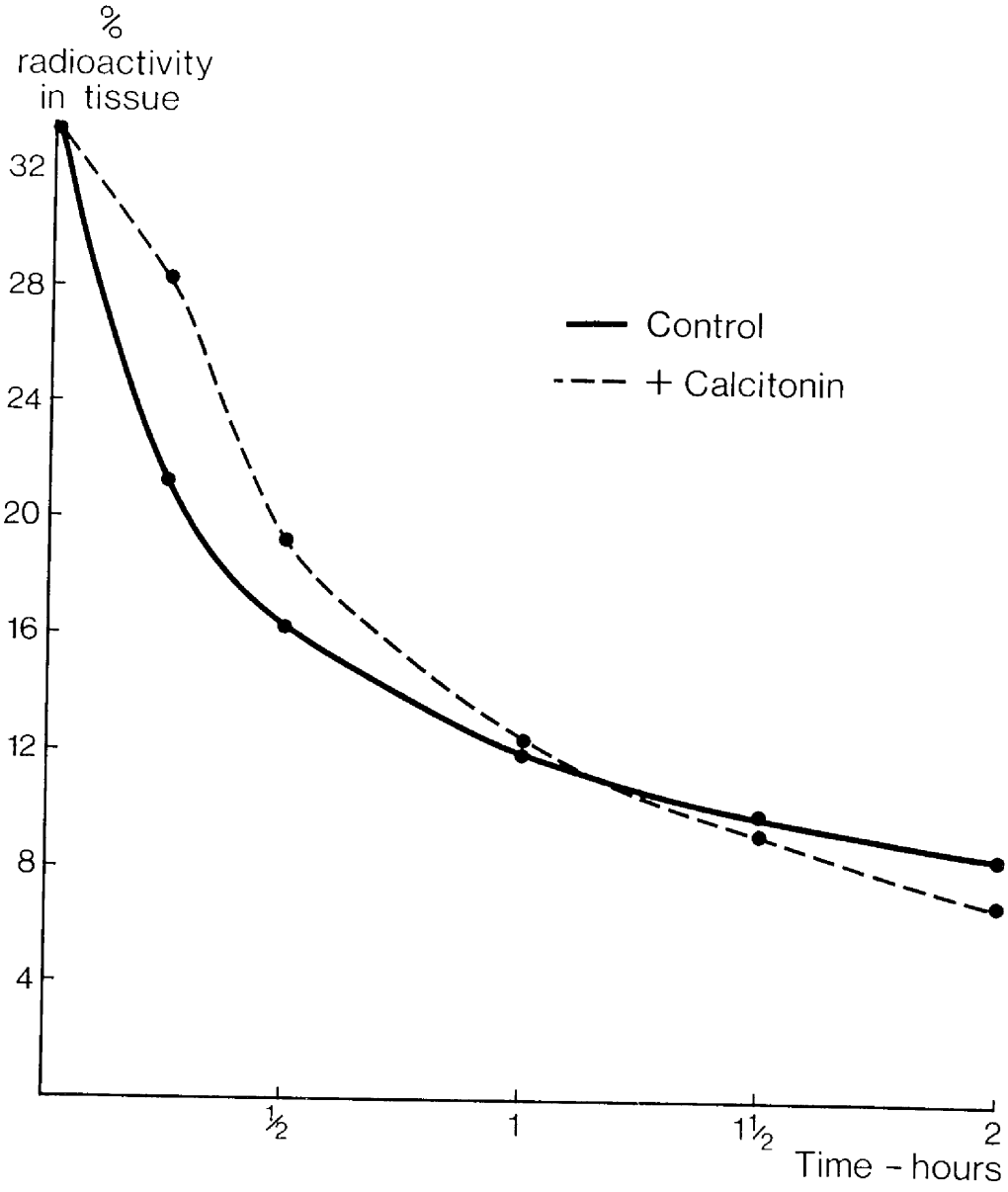

% radioactivity in tissue
100
80
60
40
20
Control
+ Calcitonin
½
1
1½
2
Time - hours

FIGURE 19

Kinetics of ^{45}Ca release with calcitonin in the medium


%
radioactivity
in tissue
32
28
24
20
16
12
8
4
Control
+ Calcitonin
½
1
1½
2
Time - hours

The time course of the efflux of ^{45}Ca in the presence of TSH is shown in fig. 20. TSH seems to stimulate the release of calcium from the second compartment. After a release incubation of 15 minutes 16% of the radioactivity is left in the slices incubated with TSH while 22% remains in the control slices. The same pattern of release is followed by both the control incubation and the TSH incubation. After 2 hours incubation about the same amount of radioactivity (9%) remains in the slices in both cases.

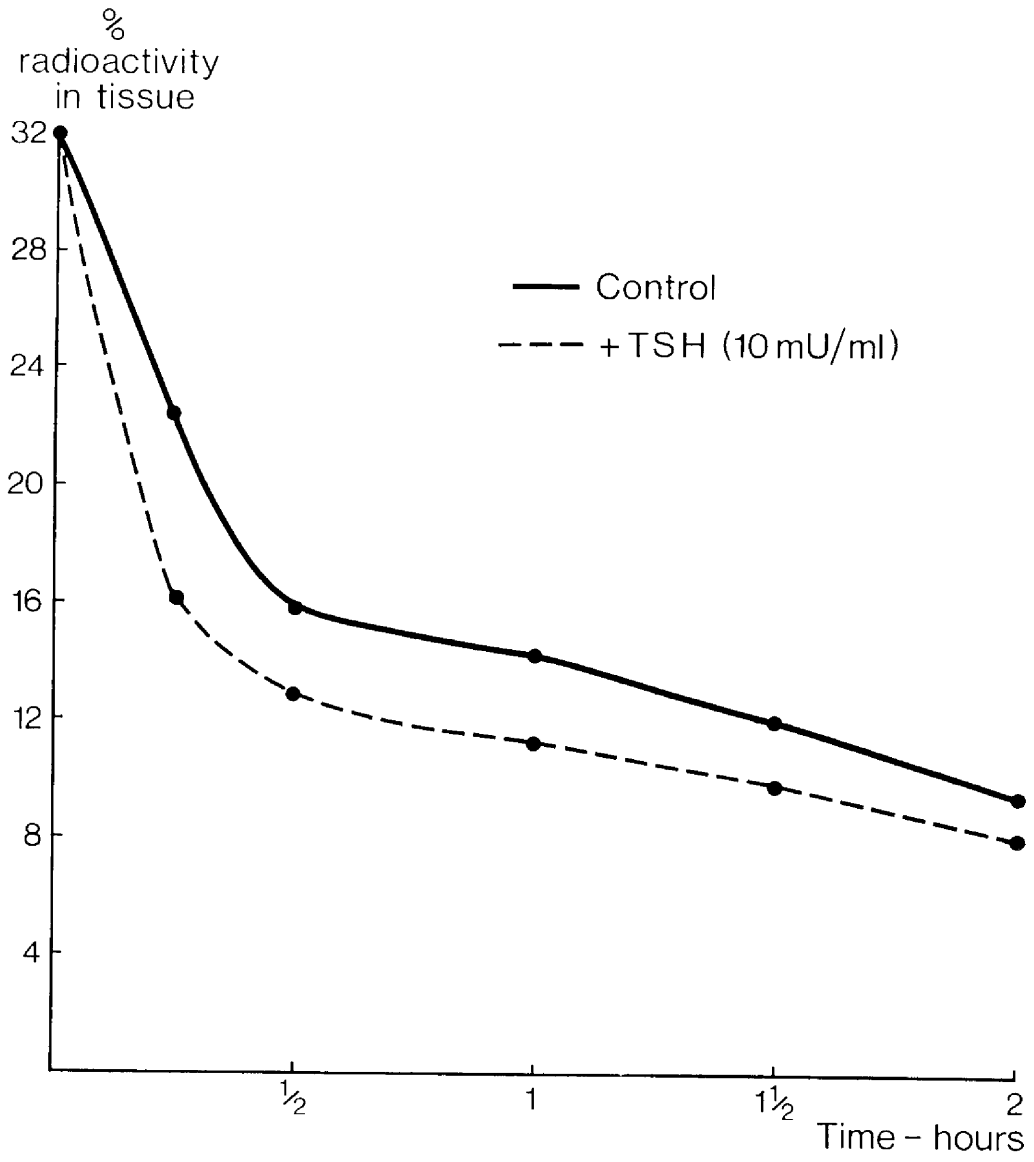
Section 7

Uptake of ^{125}I by thyroid slices in presence and absence of calcium ions

The same basic incubation procedure was again carried out with some modifications. PBS was used as the incubation medium instead of Krebs No. 2 buffer to avoid any effect of the divalent ions contained in the Krebs buffer. Before the addition of the isotope, 0.3 ml PBS containing different concentrations of calcium ions were added to each flask. After a further 15 minutes incubation 2.5 µCi ^{125}I was added and the incubation continued for one hour. The medium was then poured off. The slices were washed in 2 ml cold PBS, blotted on filter paper and counted in the gamma counter.

FIGURE 20

Kinetics of ^{45}Ca release with TSH in the medium


%
radioactivity
in tissue
32
28
24
20
16
12
8
4
Control
+TSH (10 mU/ml)
½
1
1½
2
Time – hours

The slices were homogenised in 2 ml PBS and centrifuged at 20,000 g for 10 minutes. 2 ml saturated ammonium sulphate solution were added to the supernatant to precipitate the protein. After being left to stand for one hour in the refrigerator this solution was again spun at the same speed and the supernatant was removed for counting. The protein precipitate was also counted.

Concentration gradients for ^{125}I (S/M ratios) were calculated in the same way as for the uptake incubations with ^{45}Ca. The percentage of ^{125}I that was protein bound was also calculated. The results of one experiment are shown in table 34.

Medium	^{125}I S/M	% ^{125}I protein bound
PBS	12.6	57.6
PBS + 0.7 mM Ca^{2+}	16.6	52.6
PBS + 1.4 mM Ca^{2+}	11.0	43.1
PBS + 2.1 mM Ca^{2+}	12.8	60.8

Table 34

^{125}I uptake of sheep thyroid slices in the presence of Ca^{2+} ions

The S/M ratio for ^{125}I after one hour incubation is very much depressed when the PBS buffer system containing only sodium chloride and sodium phosphate buffer is used instead of the Krebs No. 2 buffer which gave a concentration gradient of 40 ml/g after a one hour incubation in comparison to about 10 ml/g obtained in this experiment. This was

also found by Freinkel and Ingbar (1955).

The presence of calcium ions in the medium has made no difference to the uptake of ^{125}I by the slices. This experiment was repeated three times and in each case, although the ^{125}I S/M ratio varied from experiment to experiment, the same order of magnitude of ratio was found in each for the four different incubations. The percentage of ^{125}I that was protein bound was also the same for each of the four different incubations.

Chapter 6

Protein-binding experiments

Several experiments were carried out to try to determine if any of the calcium contained in the thyroid is protein-bound.

The soluble protein fraction was eluted from the gland by leaving thin slices of sheep or human thyroid in 0.9% saline (pH 7.4) at a concentration of 1g tissue/2ml saline overnight at 4°C. This procedure allows the protein to be eluted without destroying the cells or releasing proteases from them. The saline containing the soluble protein fraction was removed with a pipette. The remaining tissue was homogenised in fresh saline and centrifuged at 20,000g. The calcium in the eluted fraction, in the supernatant from the remaining tissue and in the total pellet was measured by A.A.S. after digestion with chloric acid. The protein present in the first two fractions was measured by the Lowry method.

About 60% of the total tissue calcium is eluted from the slices with the soluble protein fraction which also constitutes about 60% of the total tissue protein.

5 ml of the soluble protein fraction from sheep thyroid was dialysed against 100 ml saline overnight at 4°C. The dialysis was repeated using 2 ml of the soluble protein fraction from human thyroid. In both cases the calcium was found to dialyse out of the dialysis membrane.

1 ml of the human thyroid protein was precipitated with 1 ml 20% trichloroacetic acid (TCA). The protein was sedimented by centrifugation at 20,000g for 10 minutes. The supernatant was poured off and its calcium concentration measured by A.A.S. The pellet was washed twice with 5% TCA. The calcium concentrations of the pellet containing the protein and the two wash solutions were also measured. Most of the calcium was contained in the first TCA supernatant. The final pellet after being washed twice contained less than 2% of the total calcium.

4 ml of the soluble protein fraction from sheep thyroid was put through an ultrafiltration membrane and the calcium concentration of the ultrafiltrate was measured. About 70% of the calcium was ultrafiltrable. Altering the pH of the medium by adding a few drops of dilute hydrochloric acid to give pH 5.8 or dilute sodium hydroxide togive pH 7.9 made no difference to the amount of calcium which passed through the membrane.

Gel filtration was also used to try to show protein-binding of calcium in the thyroid. A Sephadex G200 column (Pharmacia) of dimensions 2.5 cm x 34 cm was prepared and connected to a fraction collector and a LKB Uvicord to give a protein trace at an optical density of 280 nm. 0.9% saline was used as eluant. 1 ml of the soluble protein fraction containing a few drops of a concentrated sucrose solution was applied to the top of the column.

A typical protein trace from this column is shown in fig. 21. Thyroglobulin which has a molecular weight of 660,000 appears at the void volume of the column as a single peak. A small amount of calcium - 12% is eluted with this peak but most of the calcium is eluted later.

A Sepharose 6B (Pharmacia) column of bed dimensions 1.5 cm x 77 cm was also used. This agarose gel separates the thyroid proteins into several peaks as is shown in fig. 22. The main peak contains 19-S thyroglobulin with a small amount of 27-S thyroglobulin running as a shoulder before it. The peaks eluted later contain albumin and smaller fragments of thyroglobulin. Using this column even less of the tissue calcium elutes with the main protein peak and the main calcium peak comes after all the proteins are eluted from the column.

Protein-binding experiments were also carried out on thyroid tissue which had been labelled in vitro or in vivo with ^{45}Ca.

Paper electrophoresis of the supernatants from some of the incubated sheep thyroid slices was run in a tris maleate buffer pH 8.6 overnight at 120 V. An unlabelled serum sample and a sample of ^{45}Ca were run concurrently. The electrophoresis strips were dried and scanned for

FIGURE 21

Thyroid protein and calcium eluted from a Sephadex G200 column

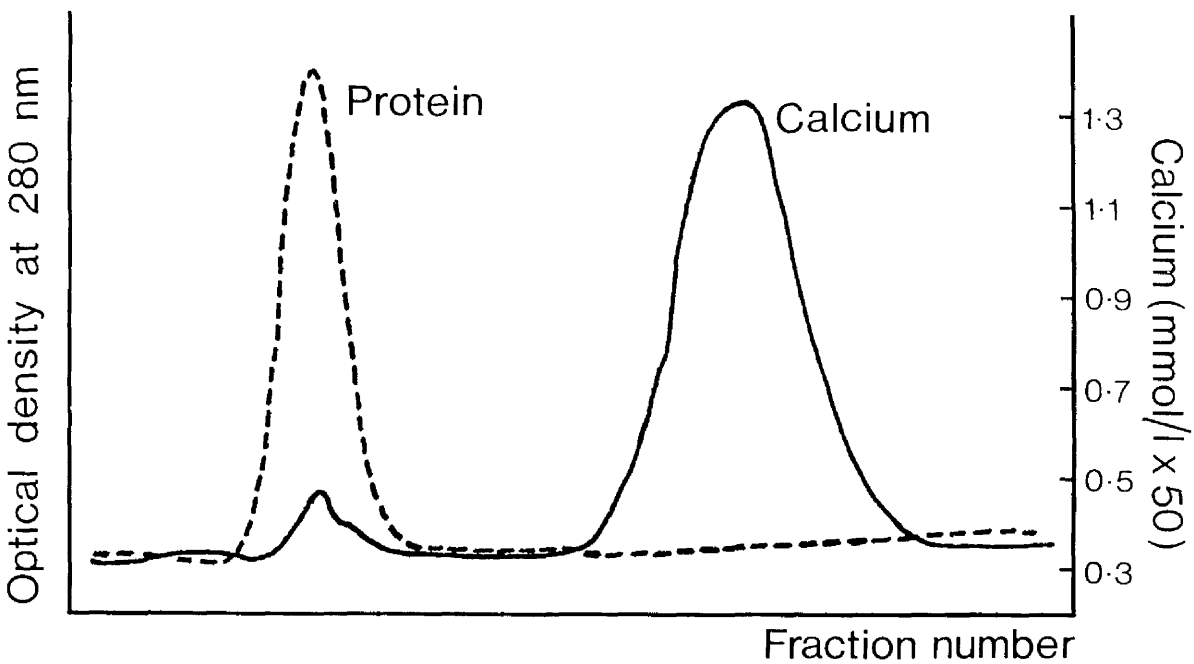
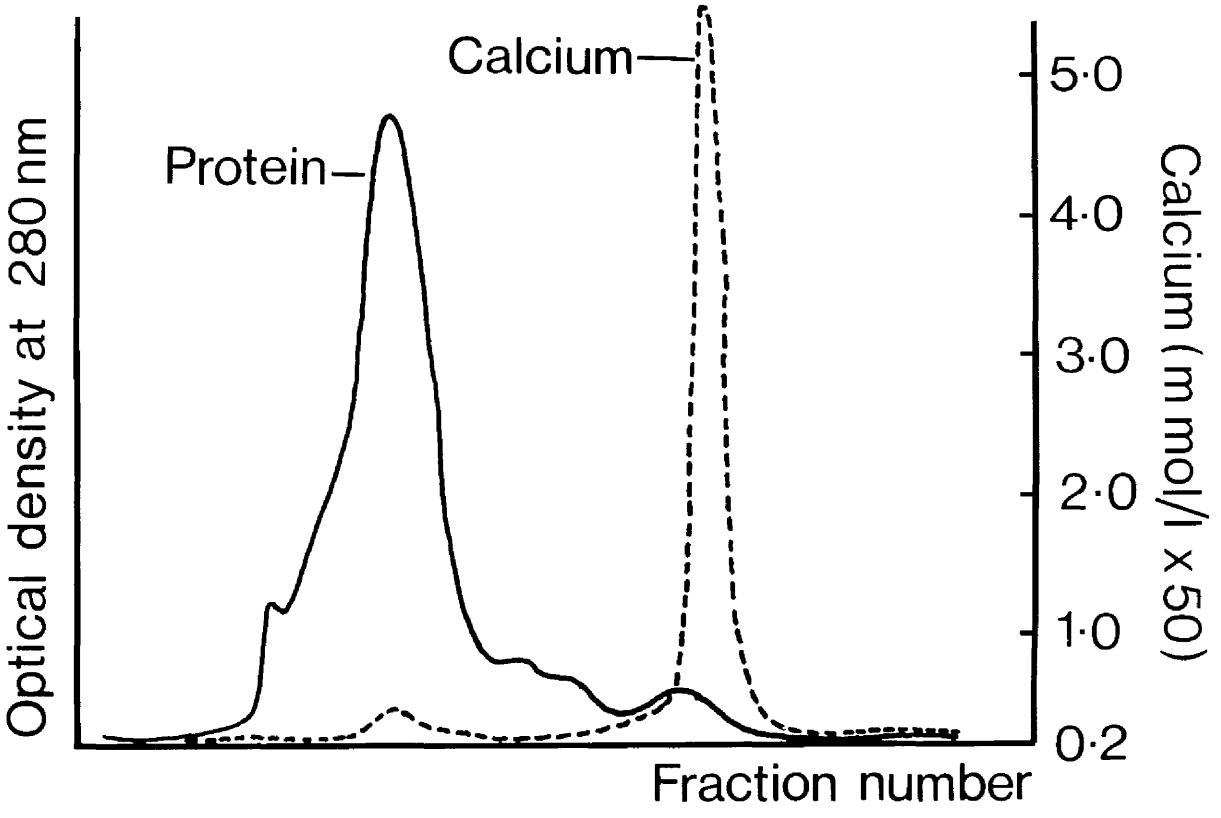

Optical density at 280 nm
Protein
Calcium
1·3
1·1
0·9
0·7
0·5
0·3
Calcium (mmol/l x 50)
Fraction number

FIGURE 22

Thyroid protein and calcium eluted from a Sepharose 6B column


Calcium
Protein
Optical density at 280 nm
5·0
4·0
3·0
2·0
1·0
0·2
Calcium (m mol/l x 50)
Fraction number

radioactivity on a Packard Chromatogram scanner. They were then stained with bromophenol blue, washed with 5% acetic acid and fixed with sodium acetate/acetic acid solution to show the position of the proteins.

The electrophoresis strips of the thyroid show the presence of an inter-α-protein, that is thyroglobulin and free radioactive calcium only (fig. 23). Even after an incubation of 90 minutes with ^{45}Ca, there does not appear to be any labelling of the protein. Electrophoresis was also carried out on samples of the supernatants from the rat tissues which had been labelled in vivo. Thyroid, liver, kidney and serum samples were applied to the electrophoresis strips. Again only free ^{45}Ca ions are present.

The technique of autoradiography was also used to try to show the location of ^{45}Ca in the rat thyroid after i.p. injection. Rats were sacrificed three hours after the injection of 100 μCi ^{45}Ca. The thyroid, one kidney and a piece of liver were removed. The autoradiographs were prepared by the Department of Pathology, Glasgow Royal Infirmary. If the tissues were put into 10% formol saline after removal from the animal, no radioactivity was found in the autoradiographs since it is washed out by this fixative. The tissues were instead frozen and sliced to prepare the autoradiographs. Using this technique some ^{45}Ca was found in the colloid of the thyroids.

A. Electrophoresis of serum stained with bromophenol blue.

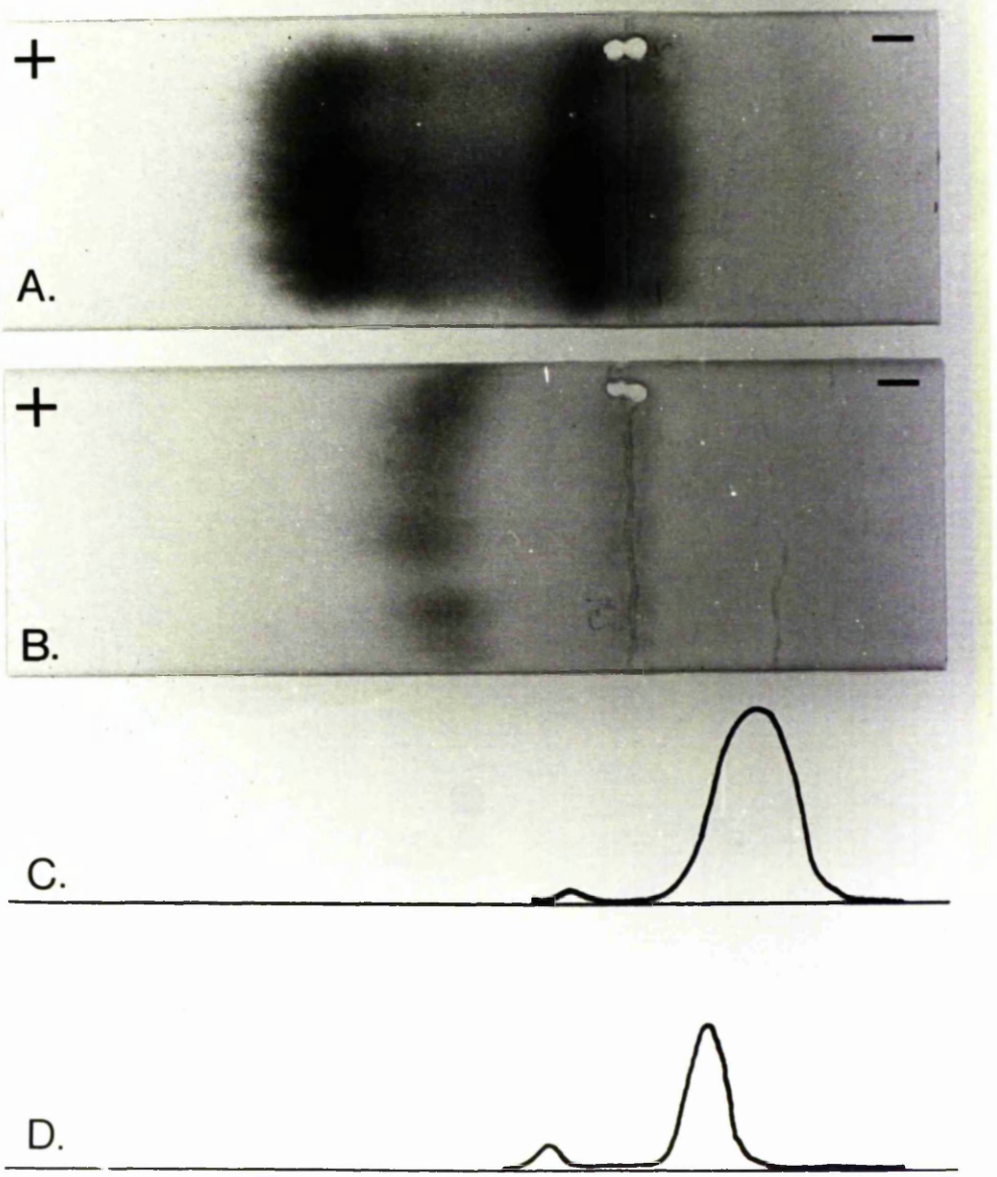
B. Electrophoresis of thyroid proteins.

C. Scan of electrophoresis of free ^{45}Ca ions

D. Scan of electrophoresis of thyroid proteins from slices incubated with ^{45}Ca.

FIGURE 23

Electrophoresis of soluble thyroid protein from slices incubated in vitro with ^{45}Ca


+
−
A.
+
−
B.
C.
D.

The protein in the supernatant of sheep thyroid slices labelled with ^{45}Ca in vitro was precipitated with saturated ammonium sulphate solution, $(NH_4)_2SO_4$, and with 20% TCA. About 9% of the total counts remain in the protein precipitate. This experiment was also carried out using the supernatant from the tissue that had been subjected to an uptake incubation followed by a discharge incubation which removes the extracellular, unbound calcium. After an uptake of 2 hours followed by a discharge of 15 or 30 minutes, 12% of the tissue calcium precipitates with the protein. If a longer discharge incubation of 1 or 2 hours is carried out, a slightly greater percentage of the tissue calcium seems to be protein-bound (about 16%).

Chapter 7

Discussion

Under the experimental conditions described for the definitive feeding experiments (Chapter 3, section 1 (2)) the addition of calcium to a basic calcium deficient, low iodine diet increased the size of the goitre in rats maintained on the diets for 8 or 10 weeks. The weight of the thyroid increased linearly with increasing calcium content of the diet up to the addition of 1% $CaCO_3$. Adding more calcium to the diet did not cause a further increase in thyroid weight. This was also demonstrated in the initial dietary experiments (Chapter 3, section 1 (1)) in which $CaCO_3$ was added to a diet already containing a substantial amount. The lack of effect of diets with more than 1% $CaCO_3$ may be explained by the fact that the animals did not eat as much of the diet with the highest calcium content. Therefore they would not ingest any more calcium than those on a diet with a lower calcium content.

Thus, the results of the rat feeding experiments obtained in this work would support the hypothesis that there is a connection between the incidence of goitre and a high concentration of calcium in the soil and water which has been suggested by surveys in several parts of the world (Boussingault (1831) in Colombia, Stott et al (1931) and Stott (1932) in India, Murray et al (1948) in Britain and Nwokolo and Ekejiuba (1974) in Zambia.

Previous rat feeding experiments have given conflicting results. Levine et al (1933) and Sharpless et al (1943) added excess calcium to a diet already containing a substantial amount of calcium and did not obtain an additional goitrogenic effect from the calcium-rich-diets. The initial feeding experiments (Chapter 3, section 1 (1)) agree with this and show that adding more calcium to a diet that already contains a substantial amount does not increase the goitre weight.

The calcium deficient, low iodine diet used in the definitive feeding experiments is the same basic diet as that used by Gandra and Coniglio (1961). In Experiment 6, this basic diet produced an increase of 5.8 mg in the thyroid weight in comparison to the controls fed diet 41B. The addition of 1% Ca CO_3 gave a thyroid weight increase of 13.4 mg. Gandra and Coniglio (1961) obtained an increase of 7.1 mg for the basic diet and 18.2 mg when they supplemented the diet with 3% Ca CO_3 for 30 days and then with 6% Ca CO_3 for a further 10 days. A similar goitrogenic effect of calcium was also obtained by Taylor (1954) although his basic diet has a different formulation (see table 1). Supplementing his basic diet with 2% Ca CO_3 produced an increase of 4.7 mg in thyroid weight compared to animals on the basic diet.

Therefore, when compared to a calcium deficient low iodine diet, the addition of 1% Ca CO_3 causes an increase in goitre size. Under these conditions calcium behaves as a goitrogenic substance.

In order to examine more closely the goitrogenic effect of calcium, the serum T_4 and serum TSH which had not been measured before by any of the authors mentioned above were assayed. As would be expected, animals on all the low iodine diets had low serum T_4 levels compared to the control rats. The calcium content of the diet had no effect on the serum T_4 or if there was any effect it was too small to be detected. On the other hand, the dietary calcium had a noticeable effect on the serum TSH. In experiments 5 and 6 the serum TSH increased with increasing dietary calcium. The rats on diets A and B had a normal serum TSH even though they were on a low iodine intake and had the same low level of serum T_4 as the animals receiving more calcium. The increase in TSH parallels the increase in the thyroid weight and thus in these experiments the goitrogenic effect of calcium proceeds through a TSH mechanism of thyroid stimulation.

Triantaphyllidis, Dugas du Villard and Guichard (1977) observed an increase in serum T_4 in rats fed a diet rich in calcium compared to that found in rats fed a calcium deficient diet. The serum and pituitary concentrations of TSH were unaffected by dietary calcium content. They showed that calcium enhanced iodine entry and concentration in the thyroid gland in vivo. They

supplemented the animals' drinking water with KI and therefore since the rats were not iodine deficient it is difficult to compare their results with those obtained here. They do not state the weight of the thyroid glands to show if the rats on the calcium deficient diet were more goitrous than those on the calcium rich diet as would be suggested by their lower serum T_4, nor do they give a normal range for the rat serum T_4.

The iodine deficiency of the thyroids was demonstrated by measurement of the radioiodine uptake and the concentration of stable iodine within the glands. The iodine concentration of the thyroids was much depressed by the low iodine diets. In two experiments (Experiments 6 and 11) rats on diet A had the largest content and highest concentration of thyroid iodine suggesting that calcium may be interfering with either the trapping or storage of iodine within the gland. However, trapping of iodine does not appear to be affected since in the ^{125}I uptake experiments the uptake increased with increasing thyroid weight and therefore with increasing dietary calcium. Taylor (1954) also found an increased radioiodine uptake of the thyroid when calcium was added to the diet whereas Gandra and Coniglio (1961) showed the opposite effect since the rats on their calcium deficient low iodine diet had a greater radioiodine uptake than those receiving calcium.

In human non-goitrous subjects taking calcium tablets for 6 weeks, it has been shown (Boyle et al (1966)) that the thyroidal plasma ^{132}I clearance and the thyroid radioiodine uptake fell. Since calcium did not seem to be interfering in the trapping of iodide by the thyroid, they suggested that the calcium may be interfering with the intestinal absorption of the isotope. Taylor (1954) showed that this is not the case in rats as the absorption of radioiodine by the rat gut was unaffected even after many weeks of calcium administration.

Measurement of the serum calcium showed that rats on diet A were very hypocalcaemic. The animals on the other diets had serum calcium within the normal range. Elevated serum calcium levels were not found even for the rats receiving the highest amount of dietary calcium.

In nearly every experiment (Experiments 5-11) the thyroid calcium concentration was lower for the rats on the low iodine diets than for those on the control diet 41B. This would be expected for rats on diet A since they had a low serum calcium but raising the serum calcium by increasing the dietary content of calcium did not increase the thyroid calcium concentration. This contrasts with the results of Kaellis and Goldsmith (1965).

Supplementing the Remington low iodine diet with 2% $CaCO_3$ raised both the serum calcium and the thyroid calcium concentration in their rats. They only carried out two analyses and they used a titration method which would be less accurate than the spectroscopic method used in this work.

The total calcium content of the thyroids increased with increasing calcium content of the diet. The thyroids of rats on the low iodine diets contained more calcium than those on the control diet 41B.

Thus Experiments 5 and 6 show that calcium has a slight but definite goitrogenic effect in rats on a diet which is relatively iodine deficient. The presence of calcium is required to raise the serum TSH but dietary calcium has no effect on the total serum T_4. Iodine is still trapped by the gland but calcium may be interfering with iodine binding.

To make rats more sensitive to this mild goitrogenic effect of calcium, the Remington low iodine diet was administered for several weeks before feeding the different calcium diets (Experiments 7-12). However, this regime gave the opposite effect. As the rats were made more goitrous initially the less was the effect of either raising or lowering their calcium intake. As can be seen

in table 6 (p . 70) the difference between the thyroid weights of rats on diet D (with 2% Ca CO_3) or diet A (calcium deficient diet) declined as the length of time on diet C initially increased. In Experiments 7-12, the serum calcium was low for rats on diet C followed by diet A. The thyroid calcium concentrations were lower for the animals on the low iodine diets than for the controls. The radioiodine uptake varied with the thyroid weight. The serum T_4 was low but although in some experiments the TSH increased with increasing thyroid weight it was often within the normal range.

Therefore if a goitre is already present it appears that the calcium intake has little effect on it. Thus calcium would seem to exert its goitrogenic effect at the beginning of the process of goitrogenesis. As a larger goitre is formed, the less is the effect of calcium on its growth.

In Experiments 5-12 rats of body weight 120-150 g were obtained and the diet used to bring them to this weight was high in iodine content which could explain why a long period of time on a low iodine diet was necessary to cause formation of a goitre. Therefore weanlings were purchased and raised on the low iodine diet to the same weight as the other rats and then placed on the calcium diets. These rats seemed less susceptible

to the effects of a low iodine diet and their thyroid weights did not increase as much as would be expected. (Experiments 13 and 14). Their thyroids had a greater concentration of iodine than in the previous experiments and their serum T_4 and their serum TSH were both within the normal ranges. In Experiment 13 rats on diet A had smaller goitres than the other animals but the addition of calcium did not have much effect in these two experiments.

In Experiment 15, the calcium supplements were added to the drinking water rather than to the food. The calcium salt used was $CaCl_2$ since $Ca\ CO_3$ is insoluble in water. Weanlings were also used in this experiment and, therefore, small goitres were obtained causing the differences in the thyroid weights between the dietary groups to be too small to detect any effect of calcium. Sharpless and Anthony (1943) have shown that an excess of chloride can decrease the thyroid iodine concentration although it does not cause goitre (except when calcium chloride and vitamin D were administered).

When two goitrogenic substances are administered they might be expected to have an additive effect and produce a larger goitre but Alexander and Wolff (1965) have shown that when both PTU and perchlorate were added to the rat diet a smaller goitre resulted than with PTU alone.

Therefore, perchlorate can act both as a goitrogenic and an antigoitrogenic substance.

The effect of 1% Ca CO_3 and either PTU or $KClO_4$ was investigated in Experiments 16 and 17. Feeding PTU for 4 weeks (Experiment 16) caused very large goitres and no effect of calcium was seen. However, reduction of the time on the diet as in Experiment 17 indicated that addition of calcium to the diet can have an antigoitrogenic effect in that the PTU goitre was reduced in size when PTU and 1% Ca CO_3 was administered. The same antigoitrogenic effect but to a lesser extent was also found when perchlorate and calcium were administered for a short period of time. By increasing the time of administering perchlorate and calcium, the goitrogenic effect of calcium again becomes apparent.

The administration of PTU or $KClO_4$ reduced the thyroid calcium concentration with PTU causing a greater decrease than $KClO_4$. The thyroid iodine concentration was also reduced. The serum T_4 was below the detectable limit of the assay while the serum TSH was elevated. Even although the thyroids of rats on diet A were larger than those on diet C in Experiment 17 the serum TSH was lower since calcium is required for the release of TSH by the pituitary.

Therefore, under certain conditions calcium can behave as an antigoitrogenic substance and can reduce the size of a goitre produced by PTU or $KClO_4$ when fed to rats for a short period of time. Increasing the time of feeding the diet decreases the antigoitrogenic effect of calcium.

Several hypotheses have been advanced to explain the goitrogenic effect of calcium. Taylor's (1954) hypothesis that calcium inhibits the synthesis of T_4 in the thyroid was extended by Kaellis and Goldsmith (1965) who suggested that calcium competes with iodine for the binding sites on thyroglobulin. The finding of Robison et al (1971) that there is a considerable amount of calcium in the colloid might support this theory.

If calcium is preventing binding of iodide to thyroglobulin then radioactive iodine taken up by the gland should be dischargeable by thiocyanate. In Chapter 4, section 1, it was shown that when calcium was added to the diet a greater percentage of the ^{125}I taken up by the gland may be discharged by thiocyanate. Thus, to some extent, calcium is inhibiting the binding of iodide to thyroglobulin. Since not all of the ^{125}I is discharged this inhibition is only partial.

Chapter 6 records work which was carried out to show if there is any protein binding of calcium in the

thyroid. Comparing the concentration of stable calcium in the thyroid to that in kidney, liver, heart, muscle and serum, the thyroid concentration is high and it is about twice that of serum. In vivo ^{45}Ca uptake experiments also showed that the thyroid has a greater capacity for taking up and retaining radioactive calcium for a longer period of time than the liver or the kidney. Therefore, it seems reasonable to suggest that at least part of the thyroid calcium is bound in the thyroid gland. However, the protein-binding experiments showed little if any binding of calcium to soluble thyroid proteins. The tissue calcium when extracted with saline along with the soluble proteins was dialysable, non-precipitable with TCA or $(NH_4)_2SO_4$ and passed through an ultrafiltration membrane. Gel filtration on Sephadex G200 showed that about 12% of the calcium eluted with the protein peak which consists of thyroglobulin.

Thyroid tissue which had been labelled in vivo or in vitro with ^{45}Ca was also analysed for protein binding by electrophoresis, autoradiography and precipitation. Some ^{45}Ca was found in the colloid of the thyroid by autoradiography but the other techniques indicated only the presence of free Ca^{2+} ions.

Thus, no evidence was found for protein binding by these methods but by extracting the soluble protein fraction from the thyroid slices with saline any weakly

ionic binding of calcium may be disturbed and, therefore, in vivo calcium binding to thyroglobulin may still be taking place.

Taylor (1954) also suggested that calcium may act by increasing iodide clearance by the kidney. Simpson (1947) found that the excretion of iodine by rats was increased and the iodine concentration of the thyroids was decreased by feeding a high percentage of $CaCO_3$ in the diet. Bhatt (1977) showed that the renal clearance of ^{131}I increased and its uptake by the thyroid decreased in patients given $CaCl_2$. This may have been an effect of the chloride rather than of calcium since increased intake of sodium chloride also increases clearance of iodide. Malamos and Koutras (1962) were unable to demonstrate an increase in renal iodide clearance by oral or intravenous administration of calcium to man. Thompson (1936) showed that in rats on diets containing the same amount of iodine (either low, adequate or high iodine contents), the total blood iodine level was lower when the diet was supplemented with calcium than in controls fed a low calcium diet. This may have been due to increased urinary excretion of iodine but this was not measured in these experiments.

Chapter 4, section 2, compared the amount of iodine excreted by rats on the different calcium diets. The excretion of iodine did not appear to be affected by

the amount of calcium in the diet although at the beginning of the experiment, rats on diet A which is calcium deficient excreted slightly more iodine than the others. Therefore, in animals fed the diets used here, calcium does not appear to increase the renal clearance of iodine.

On the control diet 41B, the rat thyroid took up more radioactive ^{45}Ca per mg protein than either the liver or kidney three hours after i.p. injection. The effect of a low iodine diet on this uptake was investigated (Chapter 5, section 2) and it was shown that after 4 or 10 weeks on the diets the thyroid uptake of ^{45}Ca increased. The uptake of the kidney was not altered but the liver uptake also increased. The difference in the amount of dietary calcium had no effect on the uptakes of either the kidney, liver or thyroid. Hachiya et al (1976) also showed an increased uptake of ^{45}Ca in mouse thyroid when the animals were fed a low iodine diet.

In vitro incubations of sheep thyroid slices show that the thyroid is capable of accumulating radioactive calcium from the surrounding medium. The concentration gradient for ^{45}Ca was 2.1 ml/g after a one hour incubation. The concentration gradient for ^{125}I which is actively transported by the thyroid was twenty times that of calcium after a one hour incubation. If the calcium was

confined to the extracellular space and passive diffusion occurred the concentration gradient would be about 0.4 ml/g (Rodesch et al., 1976). Some of the calcium taken up must, therefore, be bound in the tissue. Boiling the slices did not affect the concentration gradient for calcium and, therefore, an enzymic process is not involved.

Since a low-iodine diet increased the in vivo uptake of ^{45}Ca the effect of TSH at 10 mU/ml on in vitro incubations of thyroid slices with either ^{45}Ca or ^{125}I was examined. TSH did not stimulate the uptake of ^{45}Ca by the thyroid during the first two hours of incubation. Continuing the incubation up to four hours, the concentration gradient for incubation with TSH was depressed. This is in contrast to the results obtained by Hachiya et al (1976) who found increasing uptake of ^{45}Ca by mouse thyroids with increasing amounts of TSH in the medium.

Incubations with TSH and ^{125}I in the medium were also carried out. However, as Kerkof, Raghupathy and Chaikoff (1964) observed, TSH sometimes stimulates the uptake of ^{125}I by sheep thyroid slices and at other times it has no effect. TSH had no effect on the concentration gradient for ^{125}I after a 30 minute incubation although it did stimulate protein-binding,

while after a 4 hour incubation more ^{125}I was taken up by the slices incubated with TSH. It is possible that the effect of TSH on uptake of ^{45}Ca by thyroid slices is also irregular.

The kinetics of the release of ^{45}Ca by slices loaded with ^{45}Ca was studied in two experiments. The results indicate that the calcium is taken up into at least two compartments. The first from which the calcium is rapidly washed out is probably the extracellular space. Metabolic inhibitors such as sodium fluoride, dinitrophenol, iodoacetate, antimycin A were shown by Rodesch et al (1976) to have no effect on the efflux from this compartment. In Chapter 5, section 6, calcitonin which is produced in the thyroid in response to a rise in serum calcium did not affect the efflux of ^{45}Ca from this compartment.

The radioactivity is washed out slowly from the second compartment which Rodesch et al (1976) have suggested is mitochondrial since antimycin A, a specific inhibitor of mitochondrial respiration stimulated the efflux of ^{45}Ca during a release incubation. As is shown in Chapter 5, section 6, TSH also increased the efflux from this compartment but again calcitonin had little effect.

Since the in vivo experiments suggested that calcium may interfere with the binding of iodine to thyroglobulin in vitro incubation studies with sheep thyroid slices and ^{125}I were carried out with and without calcium ions in the medium. The results in Chapter 6, section 7, show no change in the ^{125}I concentration gradients or in the percentage of ^{125}I that was protein-bound, when the concentration of calcium in the medium was changed. Willems et al (1971) have shown a requirement of calcium ions for the organification of iodine. In the incubation experiments carried out here there was probably sufficient calcium present in the tissue to allow protein-binding of ^{125}I even in the absence of any calcium in the surrounding medium. The extracellular calcium did not prevent uptake of ^{125}I by the slices as was shown by the in vivo uptake measurements but nor did it interfere with binding of ^{125}I.

Other metal ions also have an effect on thyroid metabolism. Elevated levels of either calcium or magnesium inhibited TSH-stimulated release of ^{131}I from mouse thyroids in vitro (Williams 1972) although they were not required for thyroid hormone secretion. The influence of magnesium on the uptake of ^{125}I by the thyroid was investigated by Heaton and Humphray (1974). They found that excess magnesium caused accumulation of ^{125}I by the gland and that magnesium deficiency inhibited

uptake. This was thought to be a general influence of magnesium status on iodide transport, rather than a specific action on the thyroid gland since the same effect was observed in other soft tissues.

The antithyroid properties of lithium were described by Mannisto, Leppaluoto and Virkkunen (1973) who showed that massive doses in short term experiments decreased the thyroid uptake of ^{131}I, decreased the serum TSH level and decreased the synthesis of T_3 and T_4.

Calcium acts in a complex manner on the metabolism of iodine in the thyroid. Calcium ions are required for cAMP mediated stimulation of iodide organification and also for glucose oxidation but the accumulation of colloidal droplets and hormone secretion which are also stimulated by TSH, do not require calcium ions. However, high concentrations of calcium (higher than $10^{-4}M$) can inhibit the TSH induced hormone secretion in dog thyroid slices in vitro (Van Sande et al (1976)). The action of TRH on the pituitary also requires the presence of Ca^{2+} ions to allow secretion of TSH (Shrey, Brown and Ekins (1976)). Thus the intracellular level of Ca^{2+} ions has an important effect on thyroid metabolism. Altering the amount of calcium available would cause complex changes in these processes.

The results presented in this work show that the administration of excess calcium in a low iodine diet

intensifies the effect of the diet. The mechanism of this goitrogenic action is still not clear. Since calcium does not prevent trapping of iodine by the gland it does not behave like perchlorate. To a small extent, excess calcium inhibits protein binding of iodide but in vitro binding studies showed little evidence of calcium binding to thyroglobulin.

The relationship between calcium and iodine in thyroid metabolism is complex. Optimum conditions for the synthesis and release of the thyroid hormones require a certain balance in their concentrations. Usually in a normal individual there is sufficient iodine available to maintain the equilibrium level of thyroid hormones in the serum even if the calcium intake is altered but when the available iodine is at a critically low level then excess calcium acts as a goitrogen by interfering with hormone production.

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