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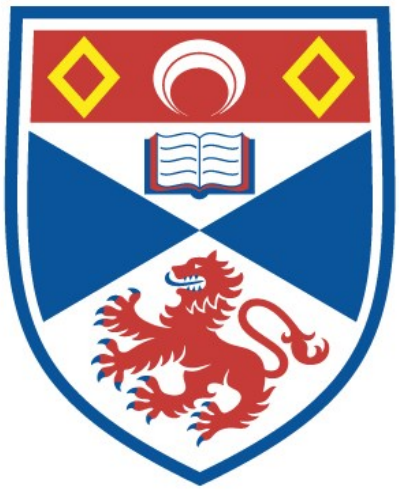

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The Effects of Thyroid Hormone on some Permeability and Electrical properties of Rat Small Intestine.

A Thesis prepared by Hugh MacAsKill Noble for presentation to the University of St. Andrews in application for the degree of Master of Science.

Supervisors Certificate.

I certify that Hugh MacAsKill Noble has spent the equivalent of nine terms at research work in the Wellcome Laboratories of Pharmacology, Gatty Marine Laboratory, The University, St. Andrews, under my supervision, that he has fulfilled the conditions of Ordinance No.51 (St. Andrews) and that he is qualified to submit this thesis for the Degree of Master of Science.

Declaration.

I hereby declare that the following thesis is based on the results of the investigation conducted by me, that the thesis is my own composition, and that it has not been previously presented for a higher degree.

The research was carried out at the Wellcome Laboratories of Pharmacology, Gatty Marine Laboratories, The University, St. Andrews, under the supervision of Dr. A.J. Matty.

Academic Record.

I obtained a B.Sc. (ord.) in pure science at Glasgow University in September 1959. I was admitted to the course for the degree of Master of Science in January 1962, at St. Andrews University and since that time I have been engaged on the research project under the supervision of Dr. A.J. Matty.

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INTRODUCTION.

The topic of research to be described was chosen for a number of reasons. First, it served to introduce someone, more familiar with Physics and Mathematics, to the mysteries of Biology: second, it gave a little scope for using some knowledge of the former, and third, it fitted in well with the type of research prevalent in the laboratory at the time. Other workers in the laboratory had already used rat intestine for studies of glucose, water and sodium movements under the influence of thyroxine so that the technique for eversion of the intestine was already familiar and easily passed on. Work had also been done on frog skin and toad bladder using the now popular short circuit current technique of Ussing and Zerhan (1951). It was, therefore, natural to try to extend and combine this work, in fact to 'short circuit' the small intestine of rat, as a means of determining the actively transported ions. It was hoped, in particular, to measure the effects of thyroxine on the active transport mechanisms. Negative results and the publication of Clarkson and Toole (1964) which described a very similar apparatus led to a change of emphasis on to calcium and phosphate tracer experiments which later developed into the main line of research.

The small intestine is not an ideal tissue for simple laboratory experimentation. The convolutions of the villi destroy the usefulness of many simple mathematical models of transport systems and it is a delicate tissue. The mucosa demand a plentiful supply of oxygen, a demand which is sometimes difficult to meet in the '<u>in vitro</u>' type of experiment. The thick layer of smooth muscle presents a diffusion barrier to many types of solute particles which may disguise subtle alterations in the active transport systems or permeability of the mucosal cell membrane. Nevertheless a number of highly ingenious techniques have been developed to study the important processes of absorption, each with disadvantages and a potential for giving misleading or ambiguous results. The truth can only be arrived at by a combination of many methods.

The everted sac or loop of intestine as a device for the study of intestinal processes was first described by Wilson and Wiseman in 1954. Since then it has proved to be both useful and popular with other researchers.

There are two basic variants of the method.

(i) The sac is incubated for a long period, say three hours, until the concentration differences of solutes between the inside and outside compartments has reached a steady state. The concen-

-tration difference is then determined. The advantages are that the length of segment is not critical and, provided the electro potential difference is also known, that it can give an unambiguous demonstration of the presence of an active transport mechanism. The disadvantage is that the concentration difference can be altered by either a variation in the active transport process or a simple change in the passive permeability of the tissue to the solute under consideration, and it is not always easy to draw a distinction between these two possibilities.

(ii) The sac is incubated for a short time and the flux is followed by means of tracers. If the activity of the unlabelled compartment does not rise much it can be assumed that none of the tracer is lost by reverse flux. This gives unidirectional flux rates from which it is easier to determine the contribution of active transport. The great disadvantage is that the length of segment is critical.

I have chosen the latter course because it has greater potentialities for detailed information but I am very conscious of the fact that not all of the attendant problems have been successfully resolved.

DEFINITIONS.

Absorption, Secretion: These terms will be reserved for discussing the results of *in vivo* experiments performed by others. They will refer respectively to movement from the lumen of the gut to the tissues of the body, and vice versa.
All the original experiments reported here were carried out *in vitro* using a method known as the "everted sac technique". In such experiments identical fluids bathe the segment of gut on its mucosal surface and on its serosal surface. These will be distinguished by the terms **Mucosal Fluid** and **Serosal Fluid**.

Influx: Is the unidirectional movement of material from the mucosal fluid to the serosal fluid. It is measured by noting the rate at which the material makes its appearance in the serosal fluid.

Outflux: Is the reverse of influx.

Mucosal Uptake: Is the quantity of material, derived wholly from the mucosal fluid, which is present in the segment of tissue at the end of the incubation.

Serosal Uptake: The meaning of this is obvious from the above.

Mucosal transfer: The Sum of influx and mucosal uptake. It is a measure of the material lost from the mucosal fluid.

Outward Serosal transfer: The sum of outflux and serosal uptake. It is a measure of the material lost from the serosal fluid.

Segment, A,B,C,D: The segments of small intestine used in the experiments. They are labelled alphabetically from the proximal end. They were taken from the distal end of each quarter and coincided with duodenum, jejunum and two samples of ileum.

Significance: In most cases the difference between the means of two groups was examined for significance by applying the "Student's t-test". This test assumes that the individual values are drawn from a parent population which is distributed according to a gaussian curve. Where samples were large enough this assumption was tested and was not found to be incorrect. A p-value < 0.05 is taken here as the level of significance. In a few instances the t-test was not applied if the lack of a significant difference was obvious. In these cases no p-value has been given. The abbreviation N.S. = "No Significance".

Review.

Biochemical Aspects of Thyroid Action.

It is not known if the many physiological effects of thyroid hormone are due to a single action at cellular or subcellular level, but since hyperthyroidism is associated with heat production and emaciation it is clear that the hormone exerts a strong influence on some process or processes of energy production. Experiments with *in vitro* preparations have demonstrated that a number of possibly related phenomena are associated with the addition of Thyroxine (T_4) or its analogues, notably Triiodo-L-thyronine (T_3).

Lardy and Feldott (1951) found that T4 depressed oxidative phosphorylation in a homogenate of rat liver or kidney and that enzyme preparations from hyperthyroid rats was less efficient at coupling phosphorylation than were preparations from normal rats. Bain (1954) wrote: "Thyroxine is, under certain circumstances, a selective inhibitor of phosphate uptake during processes of oxidative phosphorylation".

Glick and Bronk (1964) and Bronk (1965) have reported that they found impaired efficiency of phosphorylation in rat liver mitochondria in the presence of T4. Smith (1964) suggests that the effect of T4 is not to uncouple phosphorylation but to redirect it so that only one high energy phosphate pond is created where formerly three were created. On the other hand

Roodyn, Freeman and Tata (1965), working with thyroectomized rats, found that treatment of the rats with T3 prior to sacrifice, while enhancing the incorporation of amino acids into mitochondrial proteins had no observable effect on oxidative phosphorylation.

Lejsek and Simak (1964) examined the formation of TBA chromogen (an oxidative product of lipids) by liver tissue from rats which had undergone thyroxine and nutritional variations. They found that the combination of undernourishment and thyroxine injections inhibited the production of TBA chromogen. The metabolism of cholesterol in the presence of mitochondria from the livers of normal and T4 treated rats has also been studied (Mitropoulos and Myant 1965) and it was found that T4 may stimulate a rate limiting reaction leading to cleavage of the side chains of cholesterol.

A number of workers report stimulation of mitochondrial swelling in the presence of thyroxine. Glick and Bronk (i) (1964) who used rats and Greif and Alfano (i) (1964) who used dogfish liver. Glick and Bronk (ii) (1964) state that the swelling is not clearly associated with any other thyroxine effect. Bronk (1965) could not observe this swelling in conditions of phosphorylation but, contrary to other workers, found that swelling was induced by thyroxine in conjunction with A_5TP and $MgCl_2$ and at high levels of ATP the swelling

was followed by an increase in the ATP activity of the mitochondria.

It is not possible at this stage to draw many general inferences from the mass of combined detail of such reports. The observed effects are dependent to a great extent on the conditions of the experiment. There are discrepancies between the effects produced by adding T_4 or T_3 to an in vitro preparation and those produced by pretreating the animal with these hormones. For example, it is clear that the hormone interferes in some way with oxidative phosphorylation in vitro, yet, as mentioned above, Roodyn et al. could not detect this with pretreated tissue.

In the experiments which are to be described later, certain differences appeared between the intestinal absorption of normal and thyrotoxic rats. It follows then from the above that if this is due to interference of oxidative phosphorylation it should be detectable in an experiment where the hormone is applied directly to the bathing medium. This test has been applied. In such experiments there is a difficulty due to the limited time for which the tissue survives. It is widely recognised that T_3 produces the same effects as T_4 but that it does so more rapidly (TaTa 1962). T_3 was therefore used in place of T_4 in all experiments where time was limited.

Clinical aspects of hyperthyroidism and the metabolism of Calcium and Inorganic Phosphorus.

In 1929 Aub Bauer Heath and Ropes noted that the excretion of Calcium (both urinary and faecal) was abnormally great in patients with exophthalmic goitre and in those with hyperfunctioning thyroadenomata. The increase was 230% for increases in the basic metabolic rate of some 55%. The excretion of inorganic phosphate was likewise increased in such proportions as to lead the authors to suggest that the calcium was derived from tertiary calcium phosphate in bones. The high rate of calcium elimination was not reflected by any change in serum calcium levels but the authors were confident that the calcium derived from the body pool, since the diet had a very low concentration of calcium. Albright, Bauer and Aub (1931) extended this work by feeding thyroid extract to patients and noted an increase in urinary elimination of both calcium and phosphate, but an increase of faecal excretion of calcium only.

Further evidence that a disruption of calcium metabolism frequently accompanies thyrotoxicosis was supplied by Golden and Abbott in 1933. Of 110 cases examined by them 22% had osteoporosis and the actual incidence may well have been higher, since in 63 of these cases only a chest X-ray was available for diagnosis. In nine complete studies six patients had decalcification.

Desiccated thyroid was fed to rats by Pugsley and Anderson (1934) and an increase in calcium excretion was noted which gave rise to a negative calcium balance. This enhanced elimination was faecal rather than urinary and there was no appreciable effect on serum concentrations of the mineral. The lack of change in serum level clearly distinguished the phenomenon from the effects of Parathyroid hormone.

Note: There is some evidence of a change in serum levels being induced by Thyroid hormone.

(a) Tibbetts McLean and Aub. 1932 found that the hypocalcemia of hypoparathyroid patients could be corrected by administering thyroid hormone.

(b) Greenberg, Fraenkel-Conrat and Glendening 1947 recorded elevated plasma concentrations of inorganic phosphate in rats with experimentally induced thyretoxicosis.

(c) Rawson, 1953, found that treatment with T_4 or T_3 raised serum levels of phosphorus in myxedematous patients.

Confirmation of some of these results have been obtained with dogs (Logan, Christensen and Kirklin, 1942) (but no effect on phosphate metabolism) and with lactating cows (Owen 1948) (but apparent digestibility of inorganic phosphate increased during treatment).

Krane, Brownell, Stanbury and Corrigan (1956) carried out an experiment on human subjects which involved injecting Radio-calcium. The curve of specific activity against time for each fitted a series of exponential curves suggestive of discrete compartments. A change in thyroid function appeared to modify the size of these compartments with flow rates greatest in Hyperthyroid patients and least in myxedemic patients.

Recently Gennari (1964) has found evidence strongly suggesting impaired intestinal absorption of calcium in two hyperthyroid patients, one also with osteoporosis.

Similar observations were made for phosphate turnover in conditions of thyrotoxicosis by Greenberg Fraenkel-Conrat and Clendening (1947) who studied Kidney, Liver and muscle turnover rates and concluded that the hormone enhanced the rate of transfer of this ion through cell membranes.

Absorption of Calcium and Phosphorus

Nicolaysen (1943) showed that the rate of absorption of calcium in young rats is dependent on the calcium in the diet provided the skeleton is unsaturated. Deprivation of calcium does not increase the absorption rate in adult rats. He went on in 1951 to show that absorption of the mineral is dependent on the presence of Vitamin D. and that absorption *in vivo* takes place preferentially in the upper part of the small intestine. Similar results were obtained by Harrison and Harrison (1951). Further experiments by Nicolaysen and Eeg-Larsen (1953) demonstrated that while young rats were able to absorb a little calcium despite an absence of Vitamin D., humans and dogs were completely dependent on the vitamin which, in addition, promoted the absorption of inorganic phosphate. When Vitamin D. was fed to rachitic rats absorption of calcium citrate was rapidly restored, but the phosphate "cure" took longer.

In 1959 Rasmussen found that everted sacs of rat small intestine were able to transfer calcium ions from the mucosal surface to the serosal surface 'up' a concentration gradient. Parathyroidectomy three hours or more before isolation of the sacs diminished this ability to maintain a concentration gradient. Schachter and Rosen (1959) obtained similar results and found that the transfer mechanism had a maximum, dependent

on oxidative phosphorylation and was specific for calcium and magnesium in contrast to strontium and barium. Neither the 'active' transport of calcium nor the Vitamin D. effect could, in their opinions, be explained by accumulation of citrate and the formation of a calcium citrate complex. Harrison and Harrison (1960) reported that the "Vitamin D. effect", namely the promotion of calcium transfer in the small intestine, operated in all regions of the intestine and not merely in the proximal region where 'active transport' was maximal. Furthermore it still operated in the presence of various metabolic inhibitors. They concluded that Vitamin D. increased the permeability of the mucosa to the diffusion of calcium ions. Wasserman (1960) also found that active transport of calcium was mainly in the proximal small intestine and that the mechanism which discriminates calcium from strontium was dependent on metabolism.

Schachter, Dowdle and Schenker (1960), in two papers, reported that the bulk of the transported calcium was in ionic form, that the mechanism was specific to calcium (with no concentration of magnesium, strontium, barium or potassium), that young and pregnant rats had possessed a mechanism of transport more efficient than that of adult rats, that the calcium flux shows saturation kinetics, and that the accumulation of calcium by respiring slices of intestine was depressed by various metabolic inhibitors and by potassium.

It should be noted here that the term "active transport' has been used in this context to mean a mechanism of transport which is dependent on metabolic activity and has the ability to maintain a concentration difference between the cellular contents (or interior of a sac) and the external medium. There is, however, an electro-potential gradient across the cell membrane and the intestinal wall as a whole. The use of the phrase 'active transport' would, therefore, have dubious validity when considering ion movements, were it not for the fact that the electro-potential gradient (P.D.) is directed in the same way as the concentration gradient (serosal positive), and should therefore also oppose the accumulation of cations. This is not so with anions. Asano (1960) studied the transfer of both ions and decided that the electro-chemical gradient was sufficient to explain the movement of phosphate and confirmed the active transport of calcium which did not, however, make a great contribution to total flux. He added that the mechanism of transfer for phosphate was not simple free diffusion since a change in P.D. (brought about by replacing sodium with sucrose) produced a change in flux ratio, which was different from the ratio calculated under the assumption of simple free diffusion.

The absorption of inorganic phosphate <u>in vivo</u> was studied by McHardy and Parsons (1956). They found that

absorption increased with increasing pH, there being no optimum in the range pH 4.4 to 7.9. Absorption increased linearly with increasing concentration of phosphate in the lumen. It did not appear to be affected by the presence of glucose but there was a range of absorptive capacity with the highest values in the jejunum and the lowest in the ileum. The gradient of absorptive capacity was equal to the gradient of surface area of the villi. Harrison and Harrison (1961) found that inorganic phosphate was also concentrated by everted loop of small intestine but this does not constitute evidence for active transportation. They did show, however, that the movement of phosphate was more complex than simple diffusion. It was stimulated by Vitamin D. and requires the presence of calcium. The Vitamin D. effect was probably secondary to the influence of calcium. They also demonstrated that while glucose and inorganic phosphate required the presence of sodium ions, the transportation of calcium is enhanced by the removal of sodium. Williams, Bowser, Henderson and Uzgiries (1961) confirmed that the active transport of calcium is confined to the proximal region of the intestine in the presence of physiological amounts of Vitamin D. but found that excess vitamin could induce active transport in lower regions. Wasserman and Taylor (1963) have confirmed the non-essentiality of sodium ions to the transport of calcium.

Kimberg, Schachter and Schenker (1961) on the other hand found some evidence for active transport in the mid and lower gut under normal conditions, albeit considerably less than that to be found in the proximal region. The fraction of total calcium influx which is due to active transport was measured by Asano and found to be quite small. Solvent drag was also unimportant. Recently Harrison and Harrison (1965) by separating the intestinal mucosa from the underlying muscle layers have been able to demonstrate that the intestinal mucosa present a diffusion barrier to calcium and this barrier is lessened by Vitamin D. Rasmussen, Waldorf, Dziewiatkowski and Deluca (1963) also separated the intestinal villi from the muscle layer and studied the calcium uptake of these. They came to the conclusion that the accumulation by the villi is controlled by three processes:

1. Active uptake dependent on oxidative metabolism,
2. Passive uptake - a function of the concentration of calcium in the medium,
3. Release - controlled by temperature.

The ions calcium and magnesium appear to play a special role in membrane transport in general. It has been frequently reported that calcium in particular has an affinity for many membrane materials. Cent, Trounce and Walser (1964) have shown a maximum of 170 μ moles of

calcium ion can bind to one gramme of dry membrane (human erythrocyte), and devised a theoretical relationship to explain the dependence of ion bindings on ionic strength. Tidball (1964) has suggested that it is by binding on the membrane surface that calcium and magnesium can control the water permeability of rat small intestine. A similar phenomenon has been noticed in bull frog intestine (Forte and Nauss 1963), and even in artificial membranes, formed by depositing Phospho-Lipids on filter paper (Leitch and Tobias, 1964).

Hopkins and Sage (1964) have suggested that the 'uphill' entry of inorganic phosphate into human erythrocytes occurs via a rate limiting step at the cell surface by interaction with a few polycationic sites. On the other hand Borle, Meutmann and Neuman (1963) in the course of an investigation into the parathyroid control of phosphate movement in the intestine, came to the conclusion that there are many pathways for inorganic phosphate in this tissue, and they included in this the possibility of active transport.

Effects of Thyroid hormone on Intestinal absorption.

Althausen (1949) used an *in vivo* technique to make a direct study of calcium absorption. He placed an aliquot of calcium lactate into the Gastro-intestinal tract of anaesthetised rats by means of a stomach tube. The rats were sacrificed after some time and the quantity of the mineral remaining in the lumen was determined. As measured in this way the absorption of calcium lactate was slightly increased by injections of thyroxin (1 mg/Kg body wt.) for twelve days before experimentation.

Pfleger, Rummel and Jacobi (1958) used guinea pigs and one of the modern techniques for studying surviving segments of intestine *in vitro*. Similar injections of thyroxine produced an increase in the flux of radio-phosphorus through the intestinal wall.

As has been mentioned, the small intestine, particularly the duodenum in rats (ileum in guinea pigs) has the ability to maintain a concentration gradient of certain solutes across itself. Some workers have used this gradient or the ratio of concentrations as a measure of "active transport". This was the technique used by Kimberg, Schachter and Schenker (1961) to examine the effects of high and low concentrations of dietary calcium, on calcium absorption. In the course of this investigation they thyro-parathyroectomized some rats and found

that the concentration ratio produced by sacs of intestine from these animals was slightly below the control value, which confirms results obtained by Dowdle Schachter and Schenker (1960).

Other aspects of intestinal absorption and the influence of thyroid hormone on it have been studied. Halliday, Howard and Munro (1962) found that hyperthyroidism reduces the efficiency of the intestinal cellular mechanism for glucose transfer in in vitro experiments, but that in in vivo conditions this can be off-set by such factors as increased gastro-intestinal mobility and blood flow. They also found no change in water movement. Levin and Smyth (1963) found little effect in the hexose transfer mechanism except possibly in the lower ileum, but that there was associated with hyperthyroidism an increase in the rate of uptake of glucose. This they attributed to increased metabolism of glucose. They confirmed the lack of effect on water flux and added that there appears to be an increase in weight of intestinal tissue attributable directly or indirectly to thyroxine.

Bronk and Parsons (1964) made a study which involved thyroectomized rats. Intestinal sacs from these animals were able to accumulate galactose as efficiently as normal rats, but an injection of triiodo-L-thyronine

(T_3) two days before sacrifice increased this ability. They made two interesting hypotheses:

a) that there is some compensatory mechanism which comes into play when the thyroid is removed, so that injection of T_3 produces a double effect;

b) thyroid hormones have no direct effect, but T_3 has an indirect effect through some other gland.

Mandelstam (1964) measured the absorption of 3methy-D-glucose (3MG), an actively transported but non-utilised sugar, and of 6-deoxy-D-glucose (6DG) which is both actively transported and utilised. He used both in vivo and in vitro techniques and found that while the absorption of 3MG increase in vivo under the influence of thyroxine there was no significant difference in the active transportation of 3MG on 6DG, in vitro. This discrepancy he attributed to enhanced blood supply in the in vivo experiments. In 1962 Giordano, Foppiani and Romano examined the morphology of intestinal tissue from rats previously treated with thyroxine. They found that the villi became elongated with a granular infiltration behind the mucosal epithelium and that there were an increased number of cells undergoing mitosis. Moog (1961) found that an injection of thiourea at eleven days prevented the formation of alkaline phosphatase in the duodenum of chick embryos. There is in addition a failure to differentiate rapidly and

steadily and the chicks were three days overdue in hatching. Thyroxine injected at 13-15 days increases alkaline phosphatase levels to normal for eighteen days but has no effect in normal chicks.

Warburg manometric readings and measurements of the loss of phosphate from the bathing medium indicate that there is an inhibition of respiratory and phosphate estrifying processes respectively in the proximal segments of rat small intestine following treatment of the animal with thyroxine (Seshadri, in press). Matty and Seshadri (1965) have also noted a reduction in the flux of water and the non-utilized amino acid α - amino isobutyric acid (AIB) through the same tissue. The same workers noted a reduction in the uptake of glucose by thyrotoxic proximal intestine but were unable to confirm the findings of Levin and Smyth (1963) concerning the increase in weight of the tissue.

Although there is no direct proof of a link between glucokinase being involved in glucose absorption it may be relevant to note that Nishi Kawara (1958) has found that the administration of thyroid hormone to rats increased the glucokinase activity but not that of glucose-6-phosphatase in homogenates of intestinal mucosa.

Other hormones.

1. Parathyroid.

Some difference between the effects of parathyroid and thyroid hormones have already been mentioned. The profound influence which parathyroid hormone exerts on calcium metabolism has been known since 1909 and a good deal of work has been done on the subject. There is also evidence which suggests that it is equally potent in controlling phosphate. Geschwind (1961) has written an excellent review on this subject. It appears to be established that parathyroid hormone produces hypercalcemia and hypophosphatemia. It also increases the urinary elimination of both minerals. References to parathyroid influence on intestinal absorption are fairly recent and sparse. Moreover the results are somewhat at variance. Nicolaysen, Eeg-Larsen and Malm (1953) reported that although hyperparathyroid patients showed enhanced absorption of calcium, parathyroidectomy had no effect on the calcium absorption. Talmage and Elliot (1958) however found that two to four hours after parathyroidectomy of rats, the absorption of both calcium and strontium from ligated sections of small intestine was reduced by 50%. Rasmussen (1959) removed the parathyroid from rats and found that three or more hours later, sacs of intestine isolated from the animals had an abnormally poor ability to concentrate calcium ions.

Reaven, Schneider and Reaven (1959) studied the uptake of calcium-45 by voluntary intestinal muscle and came to the conclusion that parathyroid extract depressed uptake of calcium by this tissue and in general regulates the movement of calcium between intra and extracellular fluids. Eisenstein and Passavoy (1964) put forward the hypothesis that both parathyroid hormone and vitamin D act by inducing synthesis of new enzymes in bone through a DNA directed RNA process.

A qualification must be made to the interpretation of the above data on intestinal absorption. Toverud (1964) has stressed the importance of the calcium content of secreted digestive juices in such experiments. If, as he has shown, this content increases during a particular treatment and the transport mechanism has a maximum value, then this would result in an apparent decrease in the absorption ability of the intestine as measured by, say, radioisotope tracers.

Borle, Keutmann and Neuman (1963) perfused everted loops of rat intestine <u>in vitro</u> and found that parathyroid extract (PTE) without affecting the transmural electropotential difference or p.H. could increase the influx of inorganic phosphate by 70% and tissue uptake from mucosal surface by 30% and transfer to the serosal side by 30%. It did not affect uptake from the serosal surface. The PTE effect was eliminated by

iodoacetate, arsenite and dinitrophenol.

2. Growth hormone.

Pugsley and Anderson (1934) gave growth hormone to hypophysectomized rats and found that the calcium balance was restored to a positive one. Since then Hanna, Harrison, MacIntyre and Fraser (1961) have reported that the absorption of calcium by man is stimulated by growth hormone and Kimberg, Schachter and Schenker (1961) have found that intestinal sacs from hypophysectomozed rats are abnormally inefficient at maintaining a concentration gradient of calcium. More recently Finkelstein and Schachter (1962) treated hypophysectomized rats with growth hormone and found that this increased the transport of calcium through the intestine as measured <u>in vitro</u>, and absorption as measured <u>in vivo</u>.

3. Other anterior pituitary hormones have been studied but their effects are difficult to dissociate from those of their target glands.

4. Adrenals in General.

Kimberg Schachter and Schenker (1961) report that adrenalectomy appears to increase the ability of rat intestine to concentrate calcium. Gounelle and Raoul (1964) found that it depressed the absorption of calcium. It would appear that these contradictory results

are a function of very different experimental conditions.

5. Cortisone.

Cortisone is thought to have a depressant effect on the transport of calcium through rat intestine (Williams, Bowser Henderson and Uzgiries 1961). Harrison and Harrison (1960) describe its action as 'antagonising' the vitamin D effect.

6. Aldosterone. Is thought to have an effect on the transport of monovalent cations.

7. Serotonin. Two papers report that Serotonin (5H.T) stimulates absorption of calcium in man (Tessari and Poerini 1961) and in rat (Garattini, Grossi, Paoletti Paoletti and Poggi 1961).

8. Calcitonin.

References to this interesting hormone have begun to appear in the literature with increasing frequency. There is some doubt as to whether it has a thyroid or parathyroid origin. Indeed it may well be that two separate hormones exist. One with a parathyroid origin which lowers the plasma levels of calcium very rapidly in sheep (Care, Copp and Henze 1964) and another with thyroid origin in rats (Talmage, Neuenschwander and Kraintz 1965) with a longer lasting action.

Talmage et al. 1964 have suggested that this calcitonin is involved in the active transport of calcium.

An Interrelationship between the Thyroid and the Adrenal Cortex

Since it has been noted that cortisone has an effect on the intestinal absorption of calcium, any link between the thyroid and the adrenal cortex is of interest.

Straw (1964) has examined this possibility. He found that the weight of the adrenals increased linearly with the dose of thyroxine given to rats. Secretory activity per mg. of tissue was not altered for quartered adrenals stimulated to a maximum by ACTH. There was no evidence that the adrenals were in any way sensitized to ACTH but he postulated that thyroxine may stimulate the production of ACTH in response to stress. Martin and Mintz (1965) found that in hyperthyroid patients there was an "advance and exaggeration" of the normal circardian variation in adrenocortical response to exogenous ACTH. They suggest that there are two ways in which the thyroid can influence steroid secretion.

(i) indirectly through the peripheral metabolism of cortisol mediated by a feed back mechanism on the magnitude of ACTH secretion.

(ii) a direct effect on the central nervous system regulating ACTH release.

Note.

Estrogen

Engstrom and Markardt (1954) found a significant change in serum precipitable iodide (an index of circulating thyroxine) in men and women during the administration of estrogen. For this reason female rats were used in all experiments except where otherwise stated.

CHAPTER ONE

Experiment No.1

The movement and accumulation of calcium-45 and of phosphorus-32 in the small intestine of hyperthyroid rats.

Outline of the experiment and its aims.

In this experiment, everted sacs, taken from selected portions of rat small intestine, were incubated in a suitable physiological saline medium. The movement of calcium and inorganic phosphate was then observed by adding tracer quantities of radio-isotopes to either the external or internal medium. Half the total number of sacs were taken from animals which previously had been rendered hyperthyroid by injecting them with large doses of thyroxine, and the data from these were compared with those from the control group.

Materials and Methods.

Numbers and Dates of Experiment.

A set of similar experiments were carried out over a period of time on different groups of rats with slight variations of technique as appeared necessary to improve the significance of the results. They were as follows:

1. February 14 rats (7 treated; 7 control)
   Low specific activity calcium-45 and
   High specific activity phosphorus-32

2. May — 16 rats (8 treated; 8 control)
High specific activity calcium-45 and phosphorus-32

3. September — 16 rats (8 treated; 8 control)
phosphorus-32, influx only, using segments A and D

4. November — 12 rats (6 treated; 6 control)
calcium-45, outflux only, using segment A
Mucosal uptake using segment B

Selection of Animals.

The selection and treatment of all the animals used in the above experiments did not vary and was as follows:

From the colony maintained at the laboratory some young female rats were chosen. So far as possible these were all from one or two litters. They were then separated into "treated" and "control" groups according to a random selection from a pack of shuffled cards. They were fed ad libitum on a diet which was standard for the colony.

Treatment

A few milligrams of thyroxine[1] were dissolved in a drop of N/10 sodium hydroxide and then 0.9% saline was added to give a concentration

of 1 mg./ml. This was the solution which was injected into the treated group in sufficient quantity to give a dose of 1 mg. thyroxine/kg. body weight. Such an injection was given once per day for seven days and the experiment was carried out on the eighth day. The control group were given a similar injection of salt water containing a little sodium hydroxide.

Loss of Weight

The rats were also weighed each day. The results of a typical experiment given in Table 1 show that there was a general loss of weight in the treated group while the control group gained. This is taken as evidence that the treatment was effective in producing hyperthyroidism.

Bathing Medium

The medium used was Krebs-Ringer-Bicarbonate made up according to the directions given by Umbreit, Burris and Stauffer (1959) with chemicals of analar grade. Table 2 gives its composition. The ringer was made in quantity and was stored at 5°C. The pH was adjusted to between 7.1 and 7.2 just before use.

During preparation the tissue was kept emersed in this medium and at a low temperature (0° to 5°C) in an attempt to prevent anoxia, but during incubation, the temperature of the medium was 38°C + .5. and it was gassed continuously with 95/5% mixture of O_2 / CO_2.

Preparation of the everted sac[1]

The rats were killed by a blow on the head. The small intestine, including the duodenum, was then flushed out with cold ringer, excised, everted and quartered. A piece of gut from the distal end of each quarter was tied off at one end and cannulated at the other. The length of each sac was 5.6 cm. These were labelled alphabetically as mentioned in the Definition Page(VII). 0.5 ml. of warmed ringer was placed inside the sac which was then incubated in 5.0 ml. of similar fluid.

Depending on whether influx or outflux was to be measured, the mucosal or serosal fluid, respectively, was drawn from a pool of ringer which had been labelled previously with tracer quantities of calcium-45 and phosphorus-32. This ensured that the flux in each experiment initially had a source of uniform specific activity.

1. Wilson and Wiseman (1954)

After an incubation lasting one hour, the sac was removed and the excess external liquid was washed off rapidly with three changes of 0.9% saline. The sac was punctured and its contents flushed out, again with 0.9% saline. Finally the sac was removed from its cannula and was broken down by boiling in 2N. caustic soda.

This procedure provided three test tubes containing fluid.

1) Mucosal fluid (less a small and supposedly fixed quantity which adhered to the tissue when it was lifted out).
2) Diluted serosal fluid.
3) Tissue homogenate, (made up to 5.0 ml. with dilute hydrochloric acid).

The weights of these were measured accurately. It remained then to determine the total activity and hence the total quantity of the corresponding mineral in each tube.

Counting

A blood sugar pipette was used to take a sample from each tube.[1] This was evenly spread on an aluminium planchette and dried in a warm oven. The activity of the sample was measured with a thin end window G.M. tube.

1. For calibration of all pipettes etc., see Appendix No.4

A separation of the radiations could be made by inserting an aluminium screen,of suitable density, between the sample and the G.M. tube. Such a screen absorbs all of the radiation from the calcium and only about half of the radiation from the phosphorus. The detailed figures of the screening factors and corrections are given in Appendix 3.

From the sample activity and the weight of its parent solution the total activity could be calculated. The sum of the three totals should equal the total activity present at the outset of the incubation. The standard deviation of the actual figures from the expected was 7% and a 14% deviation was tolerated.

No correction was applied to allow for self absorption by the sample on the planchette since all samples were of the same volume. There is likely to be a small error in the tissue uptake figures, particularly in the case of calcium which emits weak beta rays, but these samples of homogenate were diluted to 5 ml. to give them a mass density similar to the other samples and minimise individual variation.

Isotope samples used

In the February experiments the sample of calcium-45 used was obtained from Amersham under the code number CES.1. This has a specific activity of 10-20mc per g. Ca. Calculation shows that this must have an appreciable effect on the concentration of calcium in the medium. In this case the increase is about 25%. To avoid such an increase in later experiments a sample (CES 2) with a higher specific activity was used (2-5 c. per g. Ca.). Any alteration to the calcium concentration caused by the addition of one ml. of this to 200 ml. of ringer-fluid is negligible as is also the case for the sample of phosphorus-32 (PBS I, carrier free, as orthophosphate).

It is interesting to examine the effect of the higher calcium concentration on the flux and uptake measurements. Table No.3 gives comparative figures for the two concentration levels. In the case of the CES 1. the figures were computed on the assumption that there was no concentration increase. A lack of any significant difference between the two groups implies, therefore, that any increase in concentration was accommodated by a proportional rise in flux or uptake. Only in the case of mucosal uptake does this condition not hold. Here the uptake appears to have increased even more than can be accounted for by assuming uptake to be proportional to concentration.

This may be due to a poorly standardized washing technique used in the early experiments.

Note: In the February experiments the mucosal surface was washed by drenching it with 0.9% saline from a wash bottle. Later the tissue was agitated for about two seconds in each of three beakers containing 0.9% saline. The extra flexing of the tissue probably scoured the spaces between the villi more thoroughly.

Results and Calculations

The flux and uptake values are given in Tables 4 to 11 and these data are presented as a histogram in Figures 1 to 8. The dimensions are μ.eq.hr.$^{-1}$ cm.$^{-2}$, with the area referring to the serosal surface.

The results have been calculated using a simple two chamber model with irreversible flux. This is an over-simplification, and the results should be regarded as a comparative measure only. In Appendix 1 the results are compared with those obtained when reversible flux is allowed and isotropic diffusion is assumed. The error, when typical figures are used, appears to be about 26%. Other factors, (such as the release of phosphate from the tissue to the bathing medium which was noted by Harrison and Harrison, 1961), are difficult to allow for, and for this reason it has not been thought necessary to use the more complicated 'reversible flux' equations.

The figures for mucosal uptake measured with the low grade sample CRS 1 have not been used in the calculations. To allow calculations and tests of significance to be performed on as large a group of figures as possible the corresponding values for influx, outflux and serosal uptake have been used without taking into account the undoubted increase in concentration. Such a procedure is justified by the finding noted above

that these values are proportional to concentration. (ie. the value quoted is that which would be true if there had been no alteration in concentration).

Schachter, Dowdle and Schenker (1960) reported that calcium influx in the region of the gut immediately distal to the pylorus has a maximum value of 0.2 μM $hr.^{-1}$ $cm.^{-2}$ which is reached when the ambient concentration of calcium is 0.4 μM $ml.^{-1}$ *

* Footnote: Harrison and Harrison (1965) working with a phosphate free ringer were unable to detect any evidence of saturation kinetics for calcium flux. In the experiments being reported here the ambient concentration of calcium had a minimum value of 2.6 μM. ml. At such a value a proportional increase in flux with rising concentration is not to be expected. Schachter et al found, however, that the presence of potassium ions depressed the calcium flux and excluded it from their medium. It is possible that the presence of potassium delays the approach to the maximum value until a much higher concentration is reached.

A consequence of using the simple model is that the comparison of influx to outflux has dubious validity, and it is not possible to test for active transport according to Ussing's criterion (Ussing, 1949). In spite of these reservations it is noted that the calculated results show fair agreement with those in the literature (appendix 2), and that influx is similar to outflux for both ions.

The general picture presented by the results is one of inhibition following treatment, the effect being most noticeable in the duodenum. Table No. 15 is an extract of the important data for the duodenum and Table No. 14 shows the ratio of calcium to phosphate for each measurement.

Detailed examination of the data is deferred until Chapter 5.

Fig 1 Influx of inorganic phosphate in μ.eq.hr. cm. for each segment(A,B,C,D). Control group (open column) compared to treated group (shaded).
The number above each column is the number of animals in the group for which the column represents the mean value.
An asterisk * indicates that there is a significant difference between the groups ($p < 0.05$)

Fig. 2 Mucosal uptake of inorganic phosphate in μ.eq.hr. cm.
The dotted line is the gradient of mucosal surface area according to Fisher and Parsons (1950).

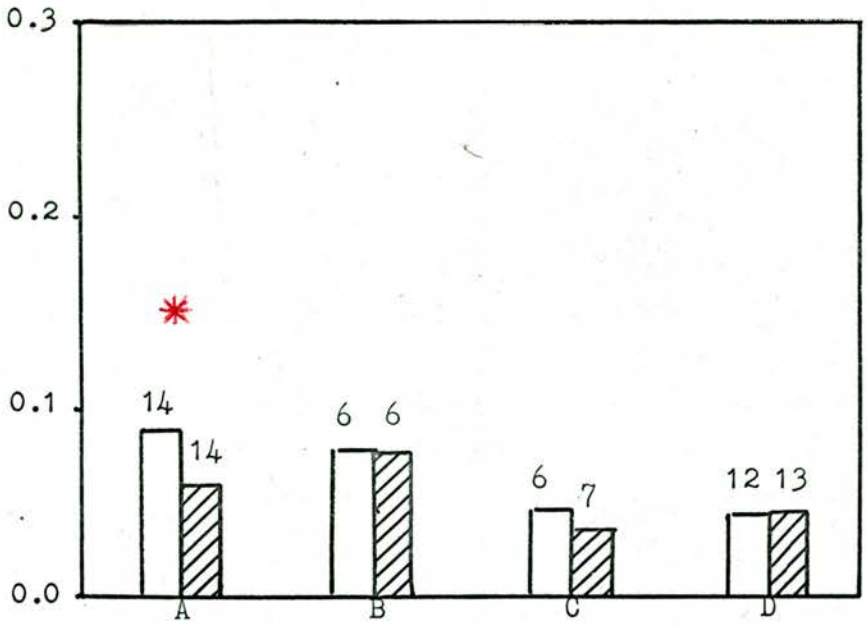


Fig. 1

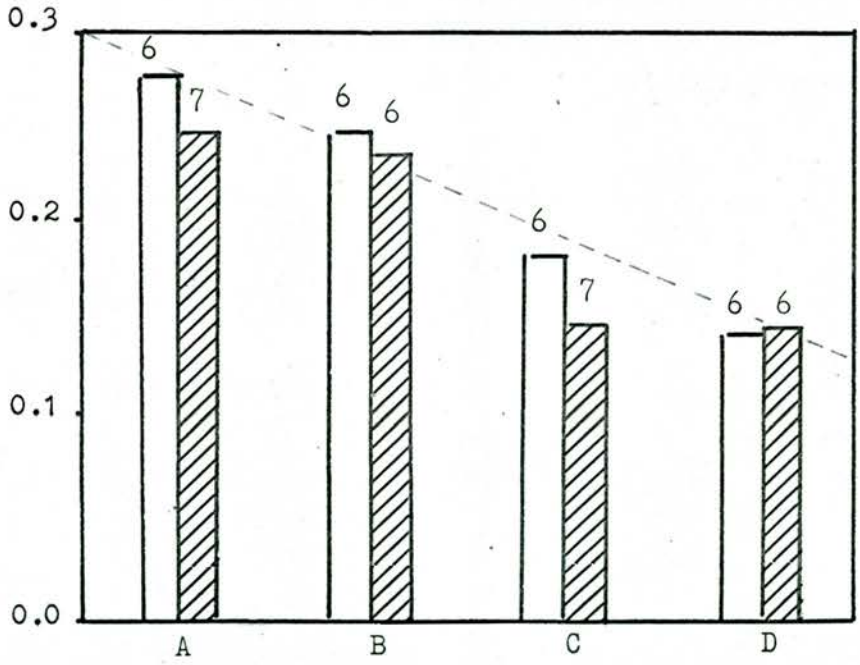


Fig. 2

Fig. 3 Outflux of inorganic phosphate in μ.eq.hr. cm.

Fig. 4 Serosal Uptake of inorganic phosphate in μ.eq.hr. cm.

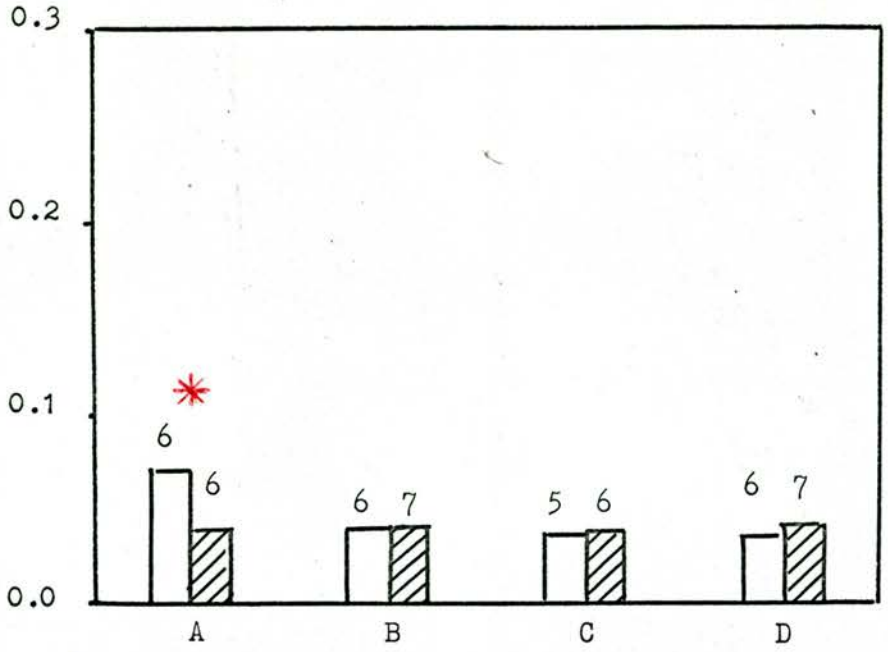


Fig. 3

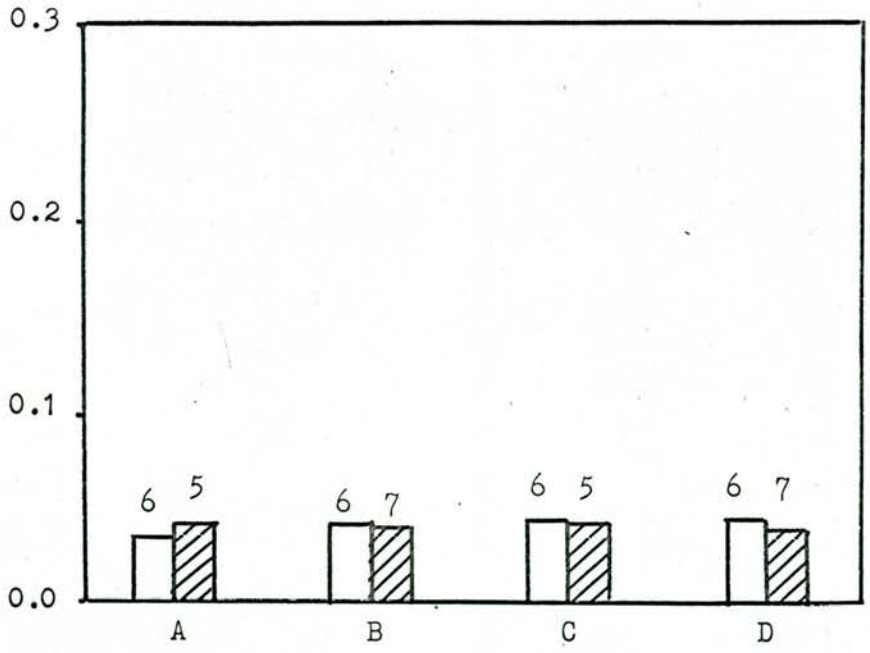


Fig. 4

Fig. 5 Influx of calcium in μ.eq. hr. cm.

Fig.6 Mucosal Uptake of calcium in μ.eq. hr. cm.

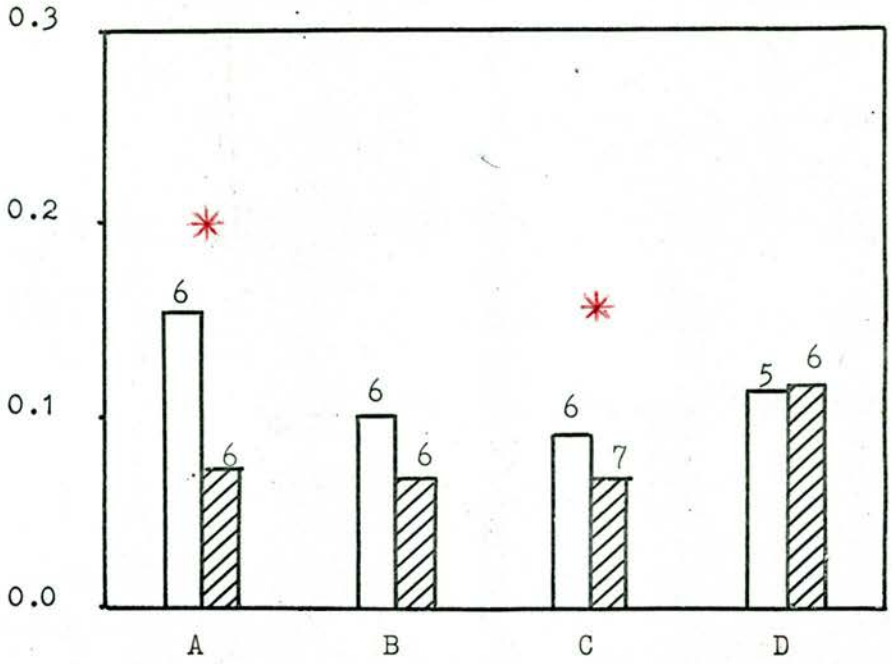


Fig. 5

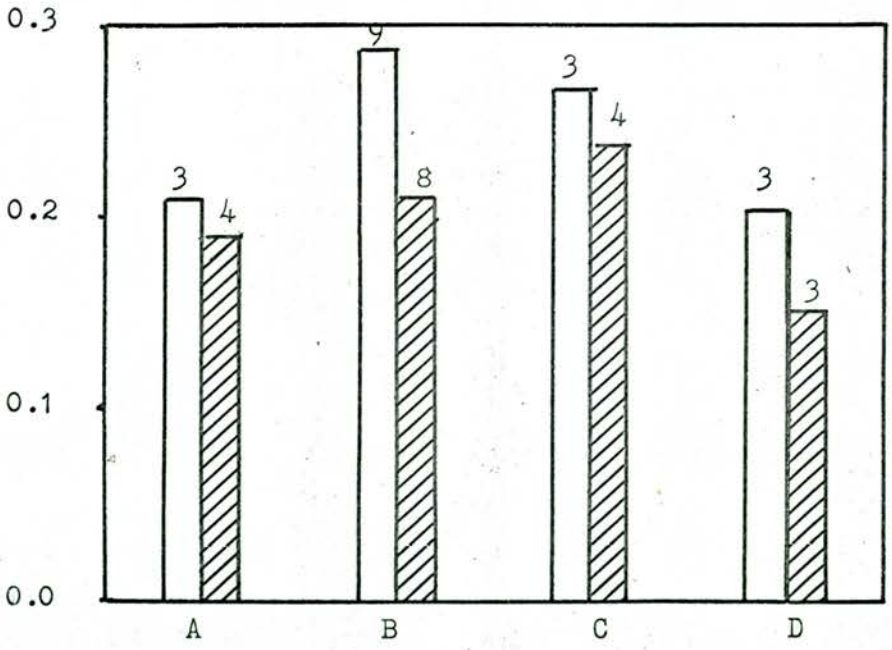


Fig. 6

Fig. 7 Outflux of calcium in μeq. hr. cm.

Fig. 8 Serosal Uptake of calcium in μ.eq. hr. cm.

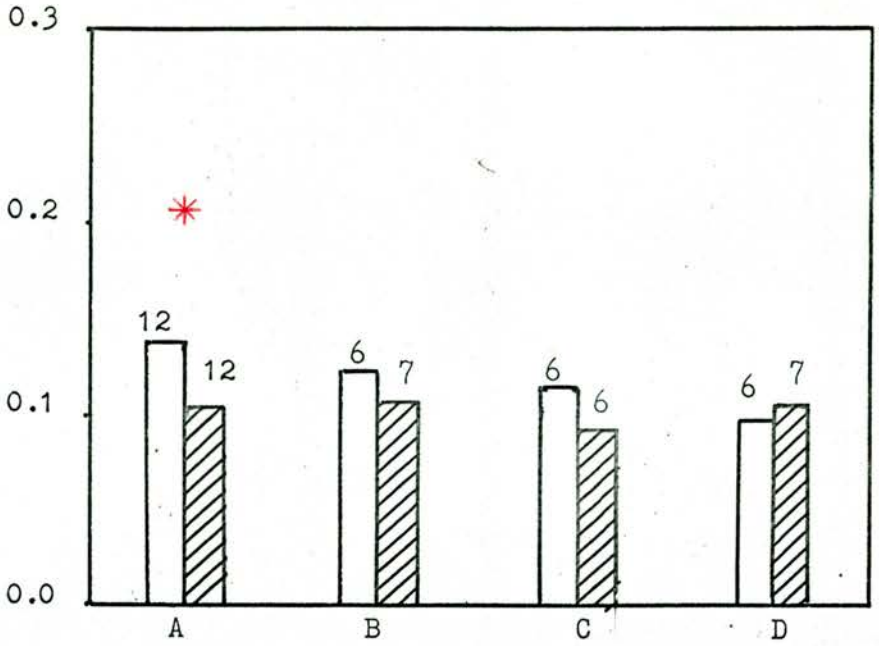


Fig. 7

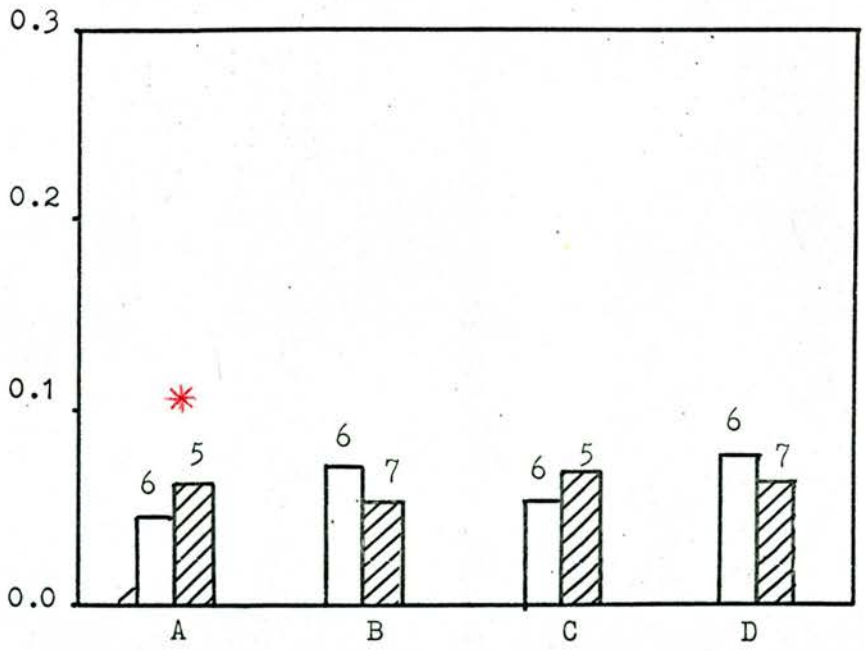


Fig. 8

CHAPTER TWO

The effect of Thyroxine on the transmural electro-potential gradient of rat small intestine and on the pH of the medium.

Experiment No.2

P.D. of the intestinal sacs.

There is a possibility that the transmural Electro-potential difference (P.D.) might be affected by the treatment described in Chapter One. The following experiment was carried out to check this point.

After the sacs of intestine had been selected and mounted as described in Chapter One, the remaining segments of intestine were formed into similar sacs and incubated in the same physiological ringer, at the same temperature as the original sacs. The P.D. generated by such a sac can be measured thus:

Polythene capillary tubes are filled with a conducting gel made of saturated potassium chloride solution and agar. The tubes (or electrodes) connect the serosal and mucosal fluids to the liquid phase of a pair of balanced calomel half cells. The metal phase of the cells are connected to the input terminals of an electronic voltmeter which has a very high input resistance.

In practice the four sacs from a single animal were incubated simultaneously in the same 250 ml. beaker. A single mucosal electrode served as reference and four others dipped separately into the four serosal solutions.

The output of the voltmeter was fed into a multi-point pen recorder and this recorder also drove a cam operated switch so that the voltmeter was connected to each of the four sacs synchronously with the pen recorder switching to its various channels.

Each curve so obtained was integrated over a 50 minute period beginning 10 minutes after the sacs were mounted. (The sacs were stored in cold ringer until all were ready. They were then placed in the heated incubating fluid simultaneously). The integrals represented an average P.D. over the period. These figures for treated and control animals (six in each group) were compared using the t-test. Table 16 presents the results and, as can be seen from the p-values, the differences, between groups for all segments are insignificant.

Experiment No.3

Inorganic phosphate in solution is actually a mixture of two ions, $PO_4^{=}$ and HPO_4^{-}. The proportions of these depends on the pH of the solution. At pH 7-1 the avarage valency is about -1.7. A change in the pH would change the effective valency and thus have a dramatic effect on any transfer process which depended on the ionic status of the phosphate.

McHardy and Parsons (1956) have found that the absorption of inorganic phosphate from the lumen of rat investine (in vivo) increases linearly with pH, there being no optimum in the range 4.4 to 7.9.

Hopkins and Sage (1964) have suggested that the first stage in the 'uphill' movement of phosphate ions into erythrocytes is an interaction with a few polycationic sites. It is quite possible that a similar mechanism operates in the intestinal mucosa.

Before looking for complicated mechanisms to explain changes in the movement of phosphate it is necessary to check the pH of the medium for variations. This was done.

At the outset of the experiment (November) the pH of the ringer was adjusted to about 7.1. The incubation was then carried out as described in Chapter One. At the end of the incubation period the pH of the mucosal fluids from segment A were determined, using a glass electrode.

Since the greatest difference between the flux rates of control and treated groups was noted in segment A it might be expected the pH differences (if these are the source of the effect) would be most noticeable in this segment. The results are shown in table No. 17
There is no significant difference between treated and control groups.

CHAPTER THREE.

The effect of Thyroxine and Triiodo-L-thyronine, added '*in vitro*'

Explanation

Effects, directly attributable to the presence of Thyroxine have been demonstrated in a number of 'in vitro' preparations. Bain (1954) found that the hormone "uncoupled phosphorylation" in a preparation of rat liver mitochondria. Lardy and Feldott (1951) found that thyroxine inhibited the respiration of ram sperm but reversed the inhibition due to the presence of calcium. They also found that T_4, added in vitro, depressed oxidative-phosphorylation in rat liver homogenates. In some experiments carried out by Barker (1956) thyroxine was able to 'protect' the mechanism of oxygen consumption during prolonged incubation of 5°C.

It is of interest to find out if the decrease in calcium flux or phosphate flux can be associated with any of these effects. If this is the case then the depression of flux rates should be capable of reproduction in a completely in vitro experiment. A number of attempts to do this have been made using a variety of techniques. As yet some of these techniques have not been fully explored. This Chapter is a description of one experiment and a progress report on others.

Experiment 4.

Nine normal female rats were sacrificed and sacs were made from their intestines in the way that has already been described. The sacs were then divided into two groups according to the scheme.

	GROUP 1	GROUP 2
Rat 1 segments:	A, C	B, D
Rat 2	B, D	A, C
Rat 3	A, B ... etc.	

Group One was incubated in normal Krebs Bicarbonate Ringer at 5°C, the other group in ringer containing Thyroxine at a concentration of 10^{-6}M. They remained thus for two hours and then were transferred to warm incubating Medium (with T_4 as appropriate). From this point on the experiment continued exactly as the tracer experiment of Chapter One. One small point of technique should be mentioned. When the sacs were first prepared they were intentionally longer than was required for the 'tracer' part of the procedure. After cold incubation the bottom of the sac was cut off. Most of the cold serosal fluid poured out but inevitably some remained adhering by surface tension to the lower section of the segment. The sac was then re-tied to the correct length and the excess cut off, taking with it the bulk of the unwanted fluid. The sac was refilled

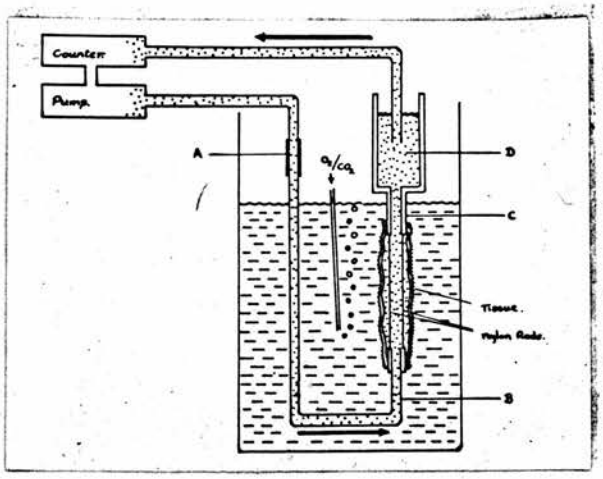


Fig. 9. Experimental Apparatus. Expt. 5. Ch. 3. Page 21.

with the appropriate fluid (with or without thyroxine).

Influx only was investigated in this way, the intention being to examine the other types of movement if a significant effect could be demonstrated.

Tables 14 - 15 present the results. The difference between the groups insignificant.

EXPERIMENT FIVE

Figure 9 is a diagram of the apparatus used in this experiment. The tubing was disconnected at A, and the appropriate section of gut was 'threaded' on to the tube. As it passed up, the leading end was tied at B, and the remainder was everted on to the 'cage' of nylon rods. The upper end was tied at C. The excess could then be cut away. The tube with the segment of gut was emersed in the bath E as shown, and coupled up to an external circulation which passed through a liquid flow G.M. Tube. The main body of the apparatus was kept in a constant temperature bath, while the external tubing was kept in an atmosphere of warm air by means of a small fan heater. The temperature of the circulating fluid was monitored by placing a thermocouple junction (thinly coated with "araldite") in the reservoir D.

The G.M. Tube (20th Century FM 6) was incapable of detecting the weak radiation of calcium-45 and

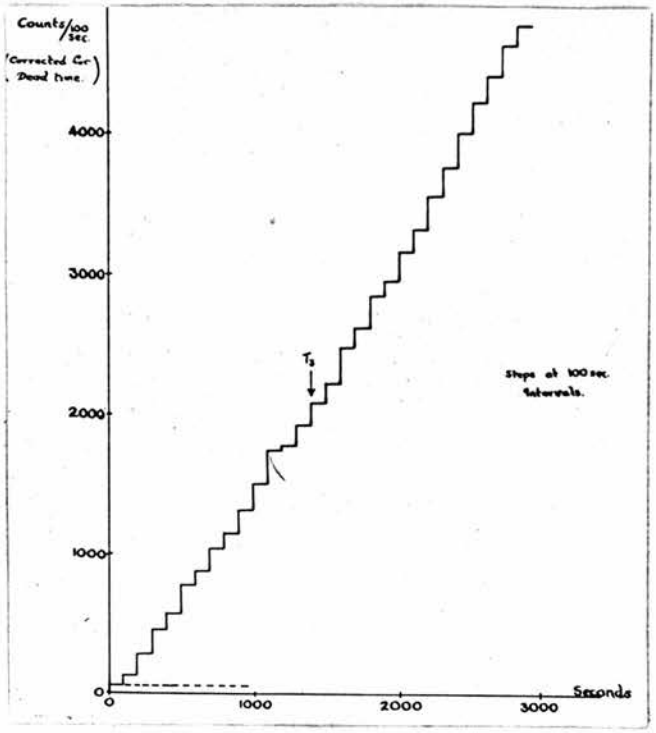


Fig. 10. Results Expt. 5. Ch.3. Ordinate has dimensions Counts per 100 seconds. corrected for the dead time of the GM tube quenching unit. The horisontal dashed line indicates the background count.

(P-32 influx)

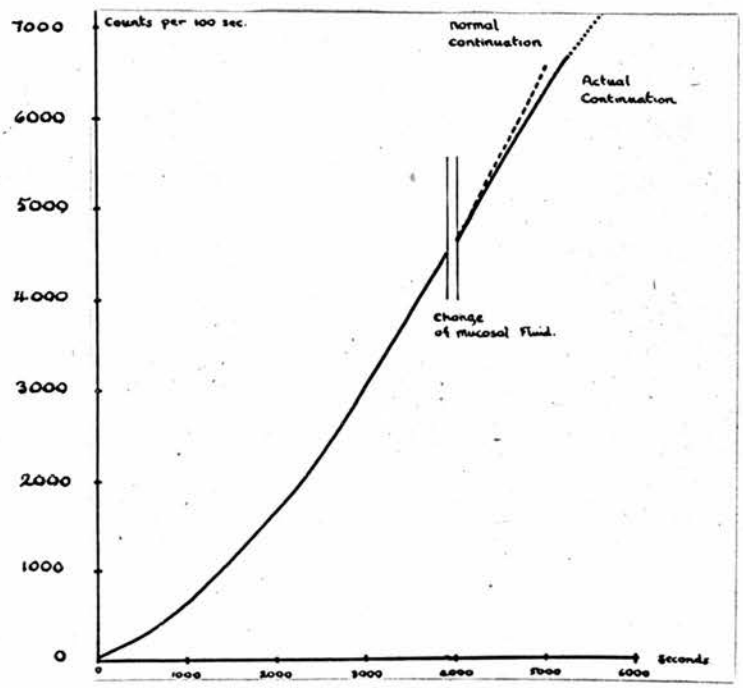


Fig. 11. Effect of changing the labelled for unlabelled mucosal fluid (P-32 influx)

consequently this experiment examined only the effect on phosphorus.

Each segment served as its own control. The tubing was lifted from the bath E and transferred to a similar bath in which the ringer had been labelled with phosphorus-32. Simultaneously the 'start' button was pressed on a scaler connected to the G.M. Tube. Successive counts were made lasting 100 seconds, and these, after adjustment for paralysis were plotted against time. After some twenty minutes the analogue of thyroxine, Triiodo-l-Thyronine (T_3), dissolved in alkaline sodium chloride solution, was added in sufficient quantity to the mucosal+ fluid to produce a concentration of 10^{-6}M. and counting was continued for a similar period.

Figure 10 shows a typical curve. Characteristically it is slightly concave upwards. This is due possibly to a gradual increase in the permeability of the membrane, or to a steady relaxation of the muscle layers of the intestinal segment. The same thing is featured in curves obtained from segments not treated by Thyroxine. There is no significant change in the form of the curve after the addition of the hormone within the duration of the experiment. Three experiments were carried out in this way and each gave the same null result.

+ Thyroxine is absorbed by intestinal mucosa see Chung and Middlesworth 1964.

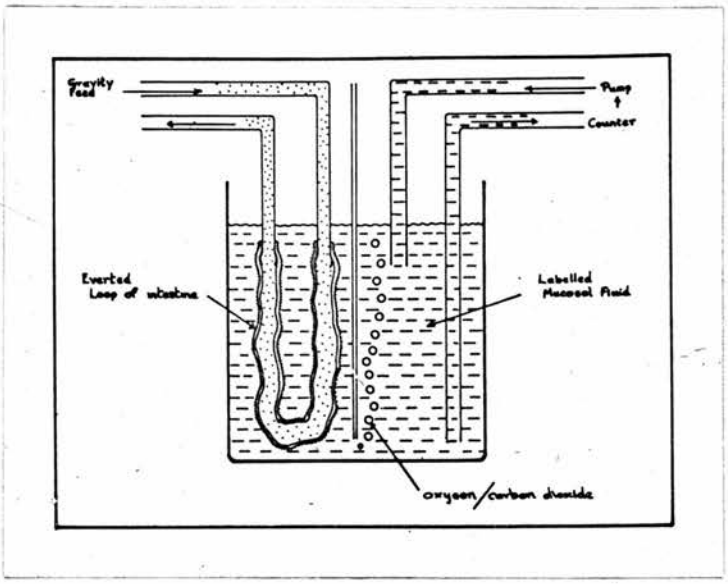


Fig. 12. Experimental Apparatus Expt.2 Ch.3.
Page 23.

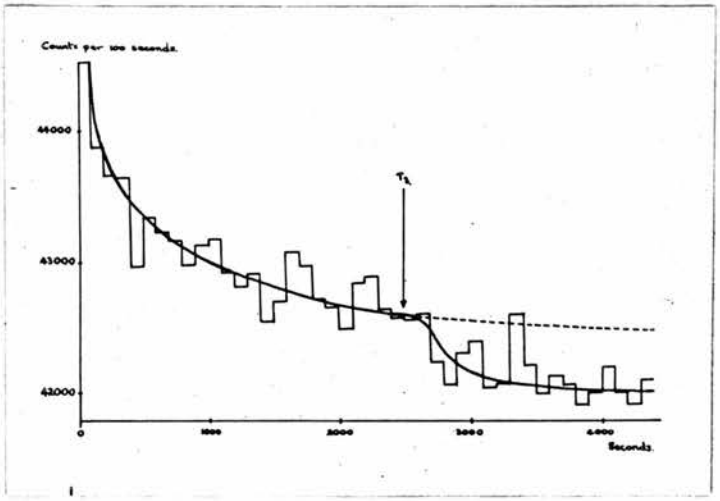


Fig. 13. Results. Expt.2. Ch.3. Activity of mucosal fluid (P-32). The sudden decrease after the addition of T3 is a dilution not a hormonal effect.

EXPERIMENT SIX

During one run of Experiment Two the tubing was lifted out of the bath containing labelled ringer and was placed in the original bath which contained no phosphorus-32. Nevertheless the rate of increase in the activity of the serosal fluid continued unchecked for several minutes (Figure 11) showing that there is a considerable time lag between change of conditions at the mucosal surface and the effect becoming obvious in the serosal fluid.

Figure 12 illustrates an apparatus designed to overcome this difficulty. With this the rate at which the labelled substance disappears from the mucosal fluid is measured. The single experimental run so far completed indicates the same lack of effect when thyroxine is added as found in Experiment Five. (The drop in specific activity (Figure 13 is exactly equal to the degree of dilution brought about by adding the ~~ringer~~ solution containing T_3 to the labelled ringer.

EXPERIMENT SEVEN

It was suspected that the muscle layers of the intestine constituted a considerable proportion of the ion barrier. Accordingly a piece of everted jejunum was laid on a piece of filter paper and the mucosal cells were 'shaved' off with a sharp scalpel. This proved to be the most satisfactory method of isolating the muscle layer. Several methods were tried and the results checked by examining histological cross-sections under a microscope.

The isolated muscle layer was then subjected to the experimental treatment which has just been described above for intact tissue.

After a suitable time had elapsed and sufficient readings taken to determine the influx in counts per second per 100 second interval, the tube was lifted from the reservoir D and dropped into the mucosal fluid which was then pumped through the G.M. Tube. An equivalent figure for the activity of the mucosal fluid was thus obtained.

From these figures the influx in μ eq.$hr^{-1}cm^{-2}$ could be calculated.

Only two such experiments were performed since exact measurements were not required. They gave consistent results, the value of influx being about $0.1\ \mu$ eq.$hr.^{-1}$ $cm.^{-2}$

CHAPTER FOUR.

The affect of Thyroxine on the Short Circuit Current of Rat small intestine.

THE SHORT CIRCUIT CURRENT OF RAT GUT.

In 1951 Ussing and Zerhan described the short circuit current technique for membranes, using Frog skin, and explained its utility as a measure of the actively transported ion current through the membrane.
It can be summarised as follows:
A membrane may generate an electro-potential difference across itself even when the solutions bathing both surfaces are identical. This is thought to be due to the transportation of ions preferentially in one direction, powered by some metabolically linked process. The measurement of the actively transported ion current is confused by the movement of other ions which are driven in particular directionsby the P.D. (this includes also the ions which are actively transported). It is, therefore, desirable that the P.D. be reduced to zero without altering the active ion current. This could be achieved, in theory, by connecting the two solutions on either side of the membrane by a perfect electrical conductor. The current flowing in the conductor would then be exactly equal to the active ion current. In practice, this can be achieved by using an external P.D. to reduce the effective resistance of the external circuit to zero. Two agar/gel electrodes are placed close to the two sides of the membrane. Two others,

some distance away, are used to pass a measurable current of even density through the membrane until the P.D. recorded by the first two electrodes equals zero. The external current then equals the active ion current and only actively transported ions will undergo net transportation from one chamber to the other.

The method has been applied to many membranes, but the application to rodent small intestine has proved troublesome. A number of attempts, meeting with varying success, have been described. The main obstacles are:

1. The potential difference generated across the membrane is small (in the range 4-10 mv.) and the Short Circuit Current (S.C.C.) is correspondingly small and difficult to measure accurately unless a large piece of tissue is used.
2. The tissue is cylindrical and this makes it difficult to ensure uniform current density. In addition the S.C.C. appears to vary slightly from one point to another.
3. Cutting the membrane so that it can be opened out into a flat sheet seems to damage the tissue irrepairably.

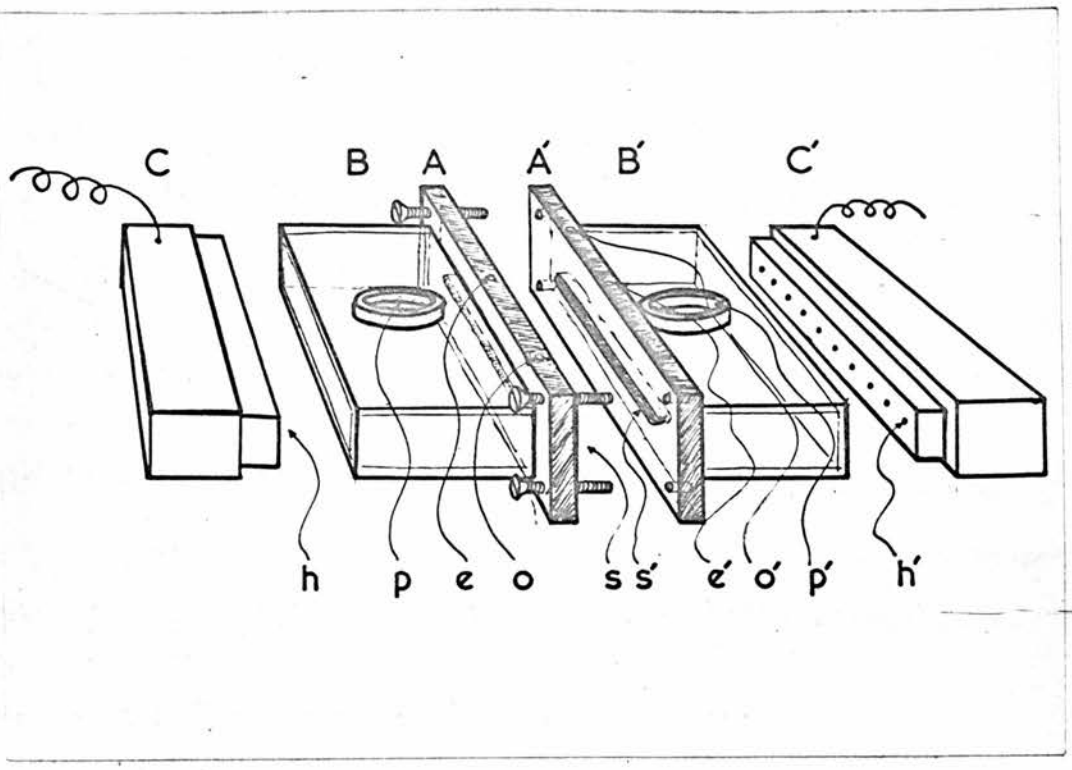


Fig. 14. Experimental Apparatus for measuring Short Circuit Current. "Flat sheet method". The slot SS' is 7cm. long by 0.5cm. wide. The compartments BB' hold 25 ml. fluid each.

Schults and Zalusky (1964) used the flat sheet method on Rabbit ileum. Their careful study of the connection between S.C.C. and Sodium flux suffered from the disadvantage that measurements had to be made on a falling base-line of P.D. Asano (1964) has used a similar method to investigate the effect of metabolic disturbances on the S.C.C.

More recently methods based on the everted sac have appeared, Clarkson and Toole (1964) have described their method in detail and it resembles in many ways one technique described here.

The remainder of this Chapter will describe two experiments, the first using a 'flat sheet' method and the second an 'everted sac' or 'cylindrical' method.

EXPERIMENT EIGHT

THE FLAT SHEET

Reference should be made to the 'exploded' diagram in Figure 14 . The Segment of gut is cut along the mesenteric border and opened out. The flat sheet so formed is laid across the slot S, S' so that it covers it completely. The two plates A,A' are screwed together. The tissue now separates the two chambers B,B' which can be filled with fluid (20 ml) through the ports P,P'. C,C' are chambers containing a silver/silver

chloride wire, which is parallel to the slot and lies in agar/KCl gel. These chambers communicate with the main compartments B,B' through a row of small holes H,H'.

The polythene/agar electrodes for measuring the P.D. enter through the holes E,E' and the gas mixture is introduced by means of the holes O,O'. Motor driven glass propellers stir the fluid through the ports.

There is a tendency for the tissue to behave like a piece of coiled spring steel which makes mounting practically impossible. It was found, however, that if the gut is distended with a head of about a metre of solution during the flushing out process this tendency disappears. (This does not seem to damage the tissue in any way. The same thing was done to intestines subsequently turned into everted sacs, and the P.D. which was generated lasted for at least two hours; nor could evidence of damage be seen in slides prepared from this tissue).

Rats were selected and treated as has been described in Chapter One. A segment from the geometrical centre of the small intestine was clamped between the chambers as above and the whole apparatus was kept in constant temperature bath at $38^{\circ}C + 0.5^{\circ}$. The P.D. and S.C.C. were monitored for about 30 minutes.

The reading at 20 minutes for each was taken as typical and subjected to a statistical test for comparison of the treated and control groups.

Table 16 presents the data. There is no significant difference between the groups.

Note: The portion of intestine from the jejunum-ileum region has the highest P.D. and therefore greatest current. Later, when the results quoted in Chapter One demonstrated the effect on the duodenum attention was directed towards this region.

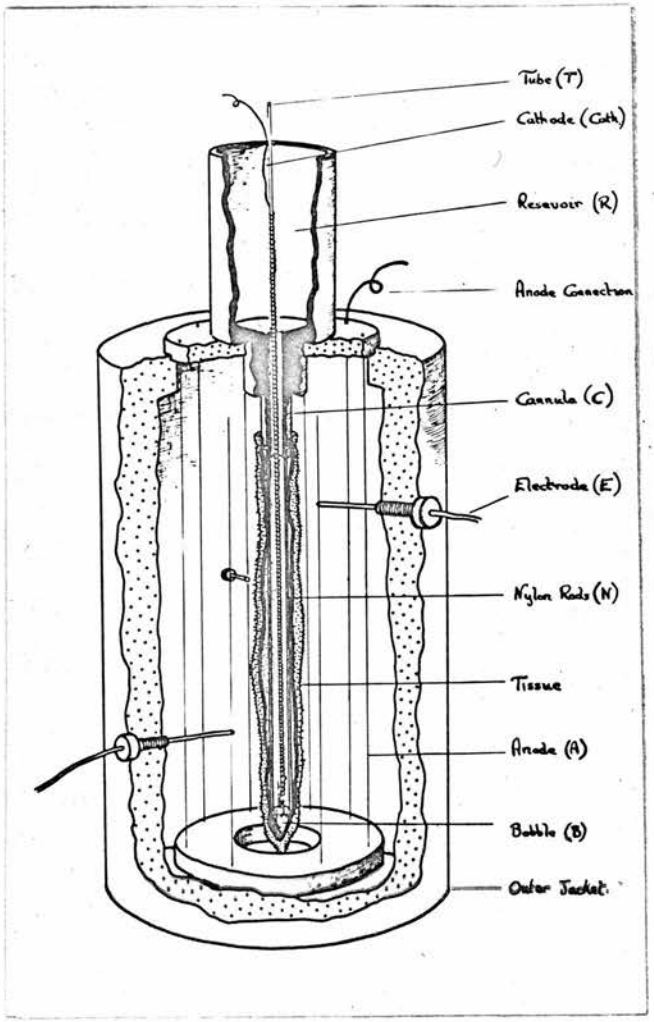


Fig. 15. Experimental Apparatus for measuring Short Circuit Current. "The cylindrical method"
The outer jacket holds 25 ml. fluid. The gut segment is 5.4 cm.long.

EXPERIMENT NINE.

THE CYLINDRICAL SYSTEM

Figure 15 is a cut away drawing of the apparatus. The sac of everted gut is tied over the 'cage' of four vertical nylon rods (N) between the cannula (C) and the perspex bobble (b). It is surrounded by another 'cage' of vertical silver/silver chloride wires (a) which form the anode of the S.C.C. circuit. The cathode (Cath.) is also silver/silver chloride and is wound helically round a glass capillary tube (t) which is introduced into the sac. The serosal fluid can be changed by forcing fresh ringer down the capillary tube and withdrawing the displaced solution from the reservoir (r). Electrodes for measuring the transmural P.D. are the polythene/agar gel type. The external electrodes (e) penetrate the outer jacket and terminate near the mucosal surface of the tissue. Their positions are fixed. The internal electrode takes the form of a very thin glass capillary. The very thin glass tip is necessary to allow it to pass down through the cannula. Its position is controlled from above. In practice it is clamped so that it can be quickly placed opposite any of the three fixed external electrodes.

O_2/CO_2 bubbles are introduced through a thin tube which pierces the base of the outer jacket.

Constant temperature is maintained by placing it in a water bath. It was found however that the stirring of the water bath disturbed the polythene electrodes and caused the P.D. recorded by them to oscillate. After the correct working temperature had been attained, therefore, the water bath heater/stirrer was switched off.

The poor thermal conductivity of the thick perspex outer jacket along with the large volume of water in the bath, ensured that the temperature of the bathing medium did not drop appreciably during the experiment. Figure 16 presents a trace of the temperature during a typical experiment as recorded by a thermocouple junction placed in the mucosal fluid.

Clarkson and Toole (1964) have demonstrated the fact that the S.C.C. can vary over quite small distances of the gut surface. For an accurate measure of S.C.C. therefore, it is necessary to take an average value based on a number of determinations made at different points. When measuring the effects of hormones and drugs, on the other hand, a determination at one point only is sufficient for a qualitative comparison. One requires to know only that the effect so measured is not an artifact. The agar/KCl electrodes are notoriously liable to defects. The extra electrodes serve the purpose of reducing this danger. After measuring the

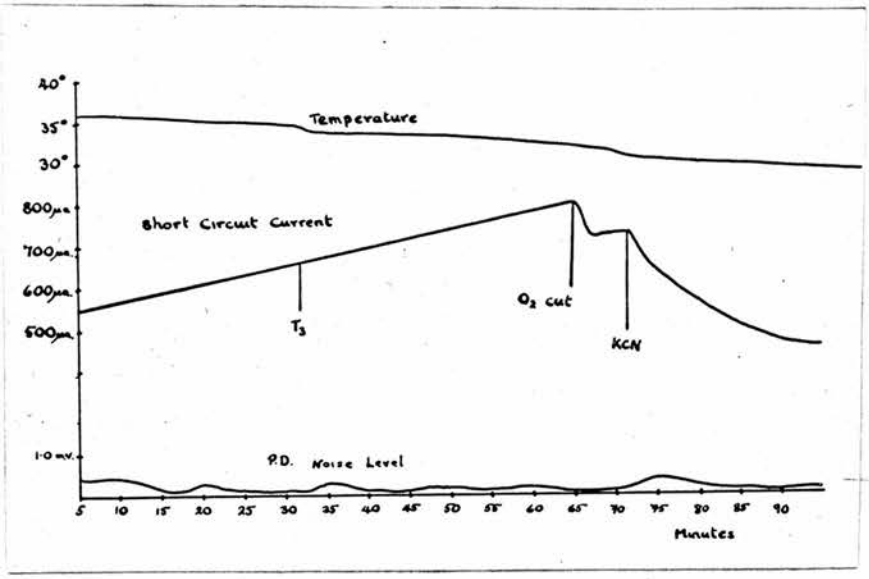


Fig. 16. Short circuit current, in microamperes, plotted against time. T3 was introduced, the oxygen supply cut off and Potassium Cyanide added at the times indicated.

Also included are the temperature and 'noise level' which is the maximum discrepancy between the the values for P.D. recorded by the three agar gel electrodes.

S.C.C. at the three points the experiment was run using only one as a null P.D. point. The P.D. of the others were monitored to ensure that they mirrored the behaviour of the first. When the hormone had been added this process was repeated. In Figure 16 the 'noise' level represents the maximum departure of the P.D. from zero as measured by the other electrodes.

As shown in Figure 16, the addition of T_3 made no appreciable difference to the short circuit current.

Note: Male rats were used.

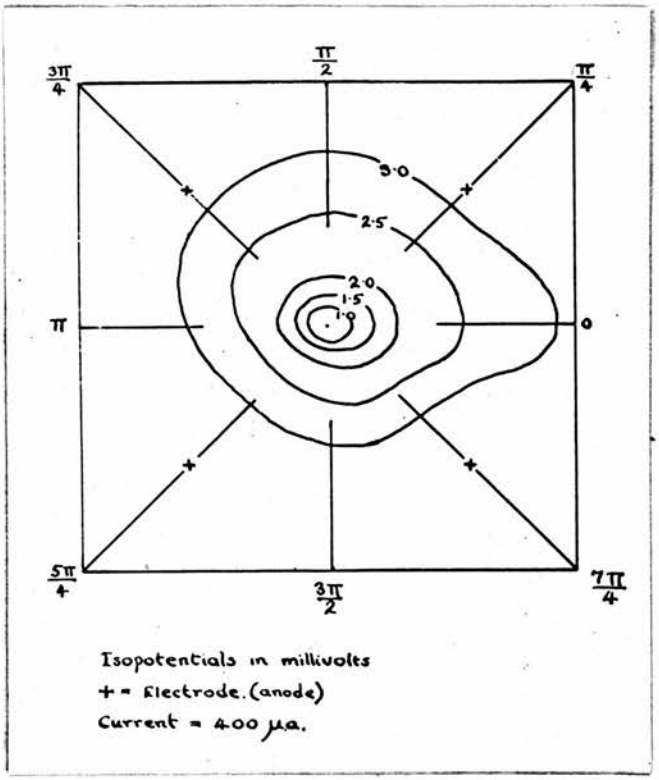


Fig. 17. Isopotentials drawn at 0.5 mv. intervals showing a roughly cylindrical pattern.

Some characteristics of Experimental Design

At the outset it was thought desirable to check whether the arrangement of the S.C.C. Anode (which was chosen for economy) would provide a current of sufficiently uniform density round the perimeter of the gut. To this end a subsidiary piece of apparatus was constructed. It consisted of a square glass tank with a heavy perspex lid. A system of concentric circles were drawn on the lid and a single vertical wire passed through the centre into the fluid in the tank. This became a cathode. The anode consisted of four vertical wires passing through the ends of two mutually perpendicular diameters to the largest circle. A number of holes drilled at various points on the system of circles served as a "jig" for glass agar/gel electrodes, which were used to map the field between the wires when a current was passed. Figure gives a typical result. In the actual apparatus the anode wires are considerably nearer the centre, but this is compensated by the addition of four more wires. The extra resistance of the tissue when it is in position will also tend to make the isopotentials approximate even more closely to circles.

This experiment also gives an indication of the true null point for S.C.C. measurements. This depends on the separation of the Agar/KCl electrodes and on the current.

It is that P.D. which is recorded when a particular current flows in the absence of the tissue, and will not be exactly equal to zero, because of the finite resistance of the solution and the separation of the electrodes.

The pattern of isopotentials shown in Figure 17 leads one to ask what effect small changes in the position of an electrode will have on the potential recorded by the electrode. Consider the current as flowing radially in a hollow cylinder bounded on the inside by the external surface of the cathode and on the outside by the cylindrical surface passing through the eight anode wires. The equations of ion flux and electro-potential in solutions (in the range of ohmic behaviour), are strictly analogous to the equations of heat flux and temperature in solids. The appropriate equations have been given by Carslaw and Jaeger.

(1959)

For steady state flux: $$\frac{d}{dr}\left(r.\frac{dv}{dr}\right) = 0 \quad \ldots . \quad (1)$$

Where: r = radius
r = a
r = b are boundaries (a < b)
v = electro-potential
v = v' (at r = a)
v = v" (at r = b)

integrating equation (1) $$\frac{dv}{dr} = \frac{A}{r} \quad \ldots \ldots \ldots \quad (2)$$

Where $A = \frac{v''}{\ln(b/a)}$, a constant

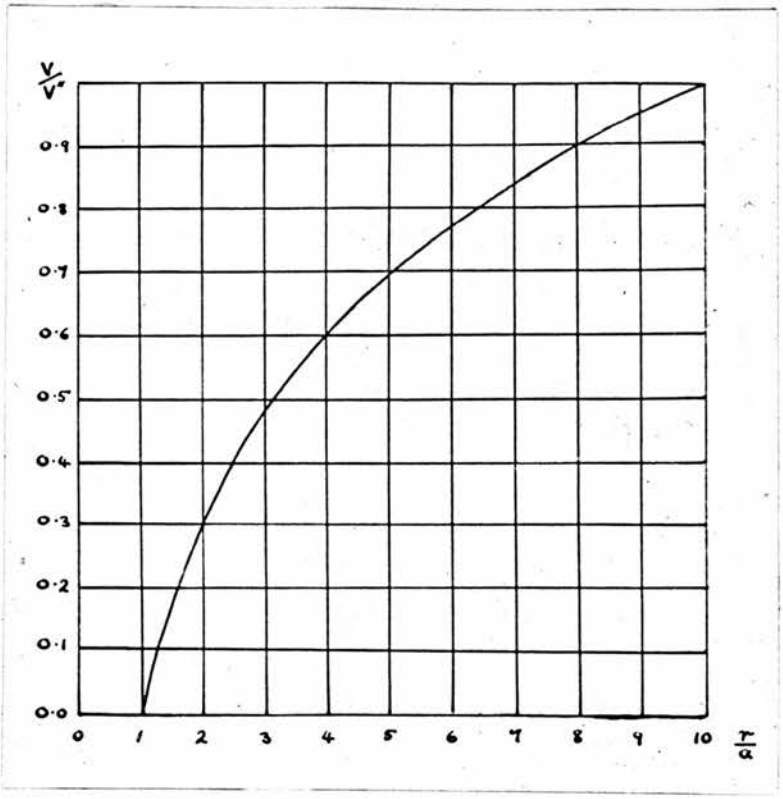


Fig.18. The curve $V/V'' = \text{Log}(r/a)$

where

r = radius
r = a is internal boundary

V = Potential at r
V" = Potential at external boundary (unit potential)

Therefore the gradient of electro-potential is inversely proportional to the radius. This confirms the casual observation from Figure that small changes in position are likely to be important for small radii.

The general solution to equation (1) is obtained by further integration

$$v = \frac{v' \ln(b/r) - v'' \ln(r/a)}{\ln(b/a)} \quad \ldots\ldots (3)$$

put $v' = 0$ $\quad v = \frac{\ln(r/a)}{\ln(b/a)}$

$v'' = 1$

$$= \frac{\log(r/a)}{\log(b/a)} \quad \ldots\ldots\ldots (4)$$

approximate dimensions of the apparatus

$$a = 1 \text{ mm.}$$
$$b = 10 \text{ mm.}$$

$$v = \log(r/a) \quad \ldots\ldots\ldots (5)$$

Figure 18 plots curve (5)

From this it can be seen that a displacement of 1 mm. of the serosal electrode can give rise to a variation in potential amounting to 0.3 v"

For a current of 400 ma. v" = 2mv(approx.). The variation would then be 0·6 mv. This difficulty is inherent in the cylindrical system and it is therefore necessary to position the serosal electrode accurately. Clarkson and Toole (1964) overcame this difficulty by securing three polythene electrodes to the Ag/AgCl wire; this must, however, occlude a sizable proportion of the wire. The apparatus described here is thought to be a reasonable compromise particularly if the serosal electrode is not moved in the critical period immediately following administration of the hormone.

CHAPTER FIVE

Discussion and Summary

Transfer of calcium under normal conditions.

The mechanism by which calcium is transferred through the wall of the small intestine is not clearly understood but it is widely accepted (see review) that an active transport system is involved and that this system, though contributing only a fraction of the total quantity of the mineral which is transferred, is most active in the proximal regions. *In vivo* measurements confirm that the duodenum is the most efficient site of absorption. It is therefore surprising to find that the 'mucosal transfer' of calcium (i.e. the sum of influx and mucosal uptake) which is a measure of the total quantity of material which was lost from the mucosal fluid, is fairly constant along the length of the intestine. (table No.12). Uptake, which is included in this figure, is a rather crude measurement giving little indication of how the material is distributed. It may lump in ions bound to the membrane surface or even an unstirred boundary layer of mucosal fluid adhering to the membrane surface. If the latter was the case, however, one would expect that the uptake figures for calcium and phosphorus would have a ratio similar to that of their concentrations in the mucosal fluid (approximately 2.5:1). The measured uptake ratio is, in fact, closer to 1:1 (Table No.14) and so it is thought likely that the bulk

of the uptake value is contributed by a region lying on or just within the mucosal cell membrane. From figures 5 and 6 it can be seen that influx has a negative correlation with uptake (control tissue) which implies that influx constitutes a 'leak' from the tissue pool.

To comply with the findings of Schachter et al (1960), that the flux has a maximum value, this leak must be carrier mediated.

Note: Harrison and Harrison (1965) have found a linear increase in calcium flux with increasing concentration, a result which would obviate the necessity for carrier mediation.

The transfer of inorganic phosphate under normal conditions

There is some disagreement about the manner of transfer of inorganic phosphate through the small intestine but it does appear to be a complex system or group of systems. Asano (1960) states that the P.D. is sufficient to explain the normal flux values but agrees that variations in the P.D. produces variations in flux ratios which do not comply with simple laws of free diffusion. Borle, Kautmann and Neuman (1963) have suggested that numerous systems are involved including active transport, while Harrison and Harrison (1961) are of the opinion that calcium assists its transport. In vivo measurements by McHardy and Parsons (1956) have suggested that the surface area of the intestinal villi has a rate limiting control over the absorption of inorganic phosphate and in the enquiry reported by this thesis it was found that the gradient of uptake along the length of the untreated tissue agrees well with gradient of surface area as estimated by Fisher and Parsons (1950). (See fig.1), nor do the uptake values for treated tissue depart significantly from this line.

Unlike calcium, the influx of phosphate is only a small fraction of mucosal uptake (tables 4 and 5).

This is probably due to its being utilised by metabolic processes within the mucosa. It is quite possible, therefore, for a small reduction in the rate of entry from the mucosal fluid to cause a significant reduction in influx without affecting uptake, the demands of metabolism having first priority for the available phosphate.

The similarity between the mucosal uptake and mucosal transfer of calcium and phosphate which is most noticeable in the proximal regions is very striking, particularly in view of the different concentrations of these ions in the bathing medium. The influx ratio is also surprisingly low in the duodenum and jejunum while outflux, outward serosal transfer ratios and also for influx in the ileum are roughly equal to the expected values. The step in the transmural P.D. must necessarily be located somewhere in the mucosal cells. This will tend to enhance inward phosphate movement and depress calcium inward movement. Outward movement from the serosal fluid will be relatively unaffected by the P.D. There does not appear, however, to be any obvious correlation between the P.D. along the intestine and the calcium/phosphate ratios. (Tables 14 and 16). What is probably being observed is the compound influence of active transport of calcium, the surface area restriction on phosphate, the availability of binding sites and the P.D.

Borle et al (1963) have already noted a great disparity between mucosal and serosal uptake. They quote a ratio of 3:1 mucosal to serosal. Tables 5 and 7 of this thesis give even greater ratios but this may be partly due to the limited pool of activity available on the serosal side, but in any case it is not thought to be necessary to invoke different mechanisms for influx and outflux to explain this as Borle et al are inclined to do. The formidable diffusion barrier of the muscle layer noted in chapter 3 seems adequate to produce such results if there is a fairly rapid exchange between the mucosa and the mucosal fluid. The similarity between influx values of intact tissue and of muscle layer alone, indicates that in the intact tissue the concentration of phosphate within the tissue is similar to that of the mucosal fluid since there seems no reason to suppose that net flux through the muscle layer is produced by anything other than a concentration gradient.

The Effect of Treatment on phosphate and calcium movement

Tissue from treated rats appears to be less efficient at transferring both calcium and phosphate in either direction, and this effect is mainly confined to the duodenum. Uptake is not significantly affected except for the serosal uptake of calcium (table 11). The symmetry of the effect on flux seems to rule out the possibility that the mechanism for the active transport of calcium has been affected in any way. It appears then that there is an additional barrier to passive movement, introduced by the treatment. Appendix 5 examines the effect of a decrease in the diffusion coefficient of a single phase membrane on the flux and uptake of material in it. It shows that the effect on flux may be much more dramatic than the effect on uptake. The addition of a second phase (corresponding to the muscle layer) would make little difference to inward flow if the diffusion coefficient for this was not greater than that of the first phase. For outward flow, however, the second phase would play an important role in the uptake of the composite membrane.

It is interesting to note that Harrison and Harrison (1965) found that the movement of calcium through the muscle layer to be much more rapid than through the

intact tissue, while in experiment 7 described in chapter 3 it was found that the flow of phosphate through the muscle was comparable with that through the intact tissue. These findings may explain the difference in the effect of the treatment on the serosal uptake of phosphate and calcium.

Some possible explanations of the observed effects will now be examined.

1. P.D. The results of Experiment No.2 suggest that there is no alteration in the transmural P.D. which can be attributed to the treatment.

2. p.H. The pH of the medium has a profound effect on the movement of phosphate (Chapter 2) but there is no indication that this undergoes any change due to treatment. (Experiment 3). There is still a possibility that the intracellular pH is affected.

3. Distension of the intestinal muscle.

During the mounting process it is obvious that the small intestine undergoes a steady change in length. (extension in warm solution contraction in cold). The duodenum was always the first segment to be mounted and it is therefore highly probable that this was carried out while it was in a different state of tension to that

prevailing during the subsequent mountings which followed at roughly four minute intervals. It follows that the true lengths of the intestinal segments are slightly different and this could explain differential absorption rates along the length of the gut and also explain the observed change in flux after treatment if it could be shown that the smooth muscles of normal and hyperthyroid rats relax at different rates. The first possibility seems unlikely in view of the good agreement with the surface area gradient already noted by McHardy and Parsons (1956) and the second also seems equally unlikely since one would expect in this case that uptake values would increase in proportion to flux.

4. A change in water flux

Asano (1960) has already observed that solvent drag does not make an important contribution to the flux of either calcium or phosphate. In addition the investigations of the thyroxine effects on water flux through rat intestine have found either no effect (Levin and Smyth 1963) or reduced flux (Matty and Seshadri (1965). A reduced influx of water might conceivably reduce the influx of solute particles but would have the reverse effect on outflux.

Having abandoned these simple explanations one is tempted to try and link the results with those of other workers.

Seshadri (in press) has recorded decreased accumulated of phosphate by slices of intestine from thyrotoxic rats and has attributed this to decreased esterification. Thus far in this discussion it has been assumed that the effects of thyroxine on calcium and phosphate are the same because of the similarity of percentage reduction etc. The added complication of the metabolism of phosphate cannot be ignored, however, and it is possible that the transfer of phosphate depends on metabolically assisted processes.

Levin and Smyth (1963) found that there was an increase in weight of the intestinal segments taken from thyrotoxic rats and Johnston Posey Patrick and Caputto (1958) found that there was an increased uptake of phosphate by the smooth muscle of such animals. Neither of these two effects were confined to the duodenum, however, and although they may contribute to the overall result they are unlikely to be decisive especially since there is no significant increase in mucosal uptake which might be expected to follow.

The morphological changes in the intestine which were observed by Giordano Foppiani and Romano (1962) to accompany thyrotoxicosis are significant

They noted that the villi are elongated and that there appears to be a granular infiltration behind the mucosal epithelium. There is a general increase in mast cells and they remark that the overall picture is one associated with the mineralising corticoids. This is an interesting statement. Williams et al (1961) found that cortisone depresses the transport of calcium through rat intestine, Harrison and Harrison (1960) said that it antagonises the vitamin D stimulatory effect. In the review, mention has been made of a connection between thyroxine and the adrenal cortex. This then is a feasible explanation of the effects observed in the tissue from pretreated animals and the lack of effect during in vitro experiments in which the hormone was added directly to the bathing medium. The "granular infiltration" of Giodano et al may be the morphological aspect of the increased diffusion barrier. A centrally positioned diffusion barrier could produce just those effects on uptake which were observed.

It is interesting to note that the inhibitory effects of thyroxine on the intestinal transfer of a number of materials (Seshadri (in press), and Matty and Seshadni (1965)) were also confined to the duodenum. There is a distinct possibility that these are further results of the same permeability barrier.

The biochemical activity of thyroxine has been reviewed earlier. These effects were observed *invitro*. If the effects observed in experiment one are a result of such biochemical activity it might be expected, therefore, that they would be capable of reproduction in an *in vitro* experiment. That the experiments described in chapter 3 failed to do so, either by adding the hormone into the medium during the experimental period or by prolonged incubation with the hormone at 5°C., has not completely exhausted this possibility, but has certainly made it more remote.

The short circuit current of rat small intestine is thought to be equivalent, to a great extent, to the current of actively transported sodium ions. (Clarkson and Toole 1964, 94% sodium: Barry Smyth and Wright 1963, approx. 70% sodium). The fact therefore that T_3 added to the bathing medium (concentration 10^{-6}M) was unable to affect the short circuit current (S.C.C.) is taken as evidence that, at least in the short term, the hormone has no influence over sodium flux. In addition there was no difference between the control and experimental group of pre-treated animals as far as transmural P.D. was concerned. The P.D. is closely related to the S.C.C. and, provided the ionic conductance remains constant, should be proportional to it. A steady P.D. implies that neither the S.C.C. nor the conductance have altered or that both have altered in such a way as to cancel out the opposing effects - an unlikely state of affairs.

It should be noted that the actively transported ion current is not a current obeying Ohms law. The P.D. may well be the result not the cause of the current.

In Chapter two it was shown that pretreatment of rats with thyroxine has no effect on the P.D. In view of the above, therefore, it is unlikely that the sodium active transport system or the resistance of the tissue to sodium or chloride ions is affected.

The failure generally to produce effects by the addition of Triiodo-L-thyronine to the bathing medium imples:

a) that the 'thyroxine' effect requires a considerable time to become apparent,

or b) thyroxine exerts its influence through some other process not available in the isolated tissue.

Summary

The results and deductions based on them may be itemized as follows.

1. The flux of calcium and phosphate is abnormally low in the proximal small intestine of thyrotoxic female rats.

2. No satisfactory explanation can be given for the fact that this affect is most noticeable in the duodenum.

3. Although it is thought that thyroxine is able to uncouple phosphorylation in certain '*in vitro*' preparations where the hormone has been added to the bathing medium, a reduction in flux of the minerals and of phosphate in particular could not be produced by adding the hormone to the bathing medium (T_3 10^{-6}M.).

   It is deduced that the reduction of flux is not a result of the uncoupling of phosphorylation.

4. No evidence has been found which suggests that the reduction of flux is a result of change in either the pH of the medium or in the transmural P.D.

5. The effect is symmetrical and affects both minerals one of which is not thought to be actively transported.

and it is deduced that the effect arises out of interference with passive movements rather than a dislocation of an active transport mechanism.

6. The distribution of uptake and flux suggests that the increased barrier to transfer is located in a central position.

7. It is suggested that the granular infiltration noted in the intestinal tissue of thyrotoxic rats is the morphological aspect of this barrier.

8. It is also suggested that the effect of thyroxine is mediated by the hormones of the adrenal cortex.

9. The Short Circuit Current (S.C.C.) of jejunum-ileum in thyrotoxic rats is normal.

The addition of T_3 at a concentration of 10^{-6}M. does not affect the S.C.C. of male rat duodenum.

The Short Circuit Current in rat intestine has been attributed to the active transport of sodium.

It is deduced, that sodium transfer is unaffected by the metabolic disturbances produced by thyroxine.

Tables

20. Short Circuit current in micro amps

TABLE 1

Weights of experimental animals before and after treatment (in grammes)

Data from a typical experiment.

	Initial	Final	Gain
Control group	265	267	2
	252	257	5
	243	246	3
	235	242	7
	254	260	6
	255	265	10
	273	280	7
Treated group	246	241	-5
	237	235	-2
	262	253	-9
	253	251	-2
	270	272	2
	237	238	1
	269	263	-6
Average gain - - - - -	(control	5.7	
	(treated	-3.1	
Standard deviation - -	(control	2.6	
	(treated	3.5	
Significance - - - - -	P < 0.001		

TABLE 2

Composition of Krebs-Bicarbonate-Ringer

Sodium	3.2956 gm/l	.....	0.143 M
Potassium	0.2310 gm/l	.....	0.0059 M
Calcium	0.1040 gm/l	.....	0.0025 M
Magnesium	0.0286 gm/l	.....	0.000125 M
Chlorine	4.5550 gm/l	.....	0.128 M
Sulphate	0.1146 gm/l	.....	0.001 M
Phosphate	0.1140 gm/l	.....	0.001 M
Bicarbonate	1.5250 gm/l	.....	0.025 M
Glucose	5.0 gm/l		

TABLE NO.3 The Effect of (Ca) on flux and uptake

Parameter	Influx		Outflux		Serosal Uptake		Mucosal Uptake	
Concentration	Low	High	Low	High	Low	High	Low	High
	152	150	125	168	47	41	207	439
	78	62	94	122	60	62	252	309
	71	126	132	110	89	53	213	326
	49	82	93	127	65	46	157	329
	81	93	134	90	62	51	271	451
	83	64	87	94	72	67	310	240
	133	132	108	89	89	66	200	315
	92	78	99	112	62	58	144	347
Mean difference	6.25		5		16		125.25	
Estimated S.d. of differences	55		10.9		9		34	
Significance	NS		NS		P >0.1 (NS)		P <0.01	

Explanation: The dimensions of the figures in the Table are 10^{-9} eq.$hr^{-1}cm^{-2}$. The figures in the body of the Table are the mean values of a specific group of values eg. the 1st figure (152) is the mean value of influx in segment A of untreated tissue. The other figure in the pair (150) is the mean value under the same conditions using the high concentration medium. The eight pairs for each parameter are then compared using the paired t-test.

TABLE NO. 4 P^{32} influx μeq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	14	14	6	6	6	7	12	13
	0.086	0.076	0.117	0.104	0.038	0.052	0.036	0.085
	0.045	0.037	0.096	0.033	0.041	0.029	0.030	0.031
	0.098	0.097	0.069	0.149	0.026	0.051	0.077	0.027
	0.056	0.047	0.067	0.075	0.030	0.027	0.045	0.042
	0.068	0.054	0.060	0.044	0.077	0.027	0.034	0.026
	0.079	0.057	0.047	0.055	0.038	0.013	0.033	0.023
	0.107	0.100				0.023	0.046	0.024
	0.117	0.042					0.029	0.052
	0.084	0.053					0.039	0.088
	0.190	0.059					0.046	0.036
	0.067	0.064					0.053	0.057
	0.054	0.033					0.031	0.031
	0.099	0.046						0.038
	0.064	0.055						
Mean	0.087	0.059	0.076	0.075	0.042	0.032	0.042	0.043
St. Dev.	0.034	0.018	0.023	0.043	0.016	0.013	0.012	0.021
P	< 0.02		N.S.		> 0.2 N.S.		N.S.	

TABLE 5 P 32 Uptake from Mucosal Fluid μ eq. hr^{-1} cm^{-2}

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	6	7	6	6	6	7	6	6
	0.217	0.261	0.299	0.249	0.143	0.240	0.158	0.046
	0.340	0.212	0.269	0.167	0.138	0.164	0.159	0.125
	0.253	0.206	0.233	0.196	0.124	0.105	0.195	0.154
	0.263	0.151	0.246	0.298	0.233	0.110	0.208	0.223
	0.374	0.292	0.222	0.262	0.290	0.125	0.130	0.169
	0.213	0.243	0.225	0.238	0.153	0.140	0.039	0.154
		0.355				0.140		
Mean	0.277	0.246	0.249	0.235	0.183	0.146	0.148	0.145
St. Dev.	0.059	0.060	0.027	0.043	0.051	0.044	0.056	0.054
P	> 0.4 (NS)		> 0.5 (NS)		> 0.2 (NS)		(NS)	

TABLE NO. 6 p^{32} Outflux μ eq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	6	6	6	7	5	6	6	7
	0.062	0.061	0.033	0.044	0.046	0.063	0.046	0.048
	0.086	0.030	0.051	0.035	0.054	0.043	0.044	0.039
	0.055	0.023	0.051	0.052	0.017	0.037	0.039	0.040
	0.053	0.044	0.032	0.042	0.045	0.021	0.029	0.052
	0.060	0.044	0.025	0.033	0.024	0.040	0.032	0.047
	0.047	0.034	0.037	0.046		0.025	0.028	0.034
				0.029				0.040
Mean	0.061	0.039	0.038	0.040	0.037	0.038	0.036	0.043
St. Dev.	0.010	0.013	0.010	0.015	0.015	0.014	0.009	0.005
P	<0.02			(NS)		(NS)	$P > 0.1$	(NS)

TABLE NO. 7 P^{32} Uptake from Serosal Fluid μ eq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	6	5	6	7	6	5	6	7
	0.026	0.052	0.034	0.043	0.045	0.037	0.033	0.033
	0.033	0.041	0.043	0.032	0.053	0.033	0.047	0.043
	0.047	0.035	0.035	0.028	0.052	0;049	0.039	0.032
	0.030	0.032	0.038	0.038	0.040	0.051	0.051	0.039
	0.031	0.033	0.049	0.041	0.032	0.035	0.049	0.034
	0.032		0.032	0.037	0.033		0.020	0.021
				0.040				0.039
Mean	0.033	0.039	0.039	0.037	0.043	0.039	0.040	0.034
St. Dev.	0.007	0.005	0.006	0.005	0.008	0.007	0.001	0.007
P		(NS)		(NS)		(NS)		(NS)

TABLE NO. 8 Ca^{45} Influx μ eq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	6	6	6	6	6	7	5	6
	0.129	0.060	0.144	0.080	0.092	0.081	0.151	0.090
	0.156	0.069	0.121	0.078	0.077	0.046	0.147	0.190
	0.165	0.058	0.112	0.089	0.110	0.064	0.097	0.119
	0.127	0.095	0.077	0.083	0.086	0.087	0.082	0.135
	0.274	0.083	0.075	0.023	0.095	0.043	0.075	0.066
	0.054	0.057	0.061	0.042	0.061	0.051		0.074
						0.069		
Mean	0.151	0.070	0.098	0.066	0.087	0.063	0.110	0.112
St. Dev.	0.065	0.016	0.031	0.024	0.014	0.016	0.034	0.043
Significance	$P < 0.01$		$0.05 < P < 0.1$ (NS)		$P < 0.05$			(NS)

TABLE NO. 9 Ca^{45} Mucosal Uptake μ eq. 1 $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	3	4	9	8	3	4	3	3
	0.209	0.224	0.214	0.264	0.371	0.205	0.221	0.048
	0.231	0.211	0.565	0.229	0.218	0.300	0.207	0.146
	0.181	0.137	0.209	0.253	0.224	0.207	0.173	0.239
		0.183	0.210	0.204		0.217		
			0.399	0.234				
			0.307	0.173				
			0.151	0.138				
			0.257	0.160				
			0.231					
Mean	0.207	0.189	0.283	0.207	0.271	0.232	0.200	0.144
St. Dev.	0.022	0.032	0.120	0.043	0.071	0.041	0.023	0.078
Significance		(NS)	P > 0.4	(NS)		(NS)		(NS)

TABLE NO. 10 Ca^{45} Outflux μ eq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	12	12	6	7	6	6	6	7
	0.165	0.143	0.115	0.119	0.065	0.076	0.091	0.127
	0.202	0.125	0.094	0.149	0.119	0.122	0.107	0.103
	0.138	0.097	0.121	0.112	0.087	0.083	0.070	0.106
	0.114	0.104	0.083	0.109	0.087	0.122	0.107	0.122
	0.135	0.047	0.127	0.062	0.081	0.078	0.109	0.077
	0.095	0.044	0.185	0.124	0.234	0.062	0.109	0.079
	0.034	0.108		0.077				0.117
	0.161	0.129						
	0.191	0.124						
	0.107	0.177						
	0.140	0.060						
	0.151	0.055						
Mean	0.137	0.101	0.121	0.107	0.112	0.091	0.099	0.104
St. Dev.	0.044	0.034	0.032	0.029	0.057	0.021	0.013	0.022
Significance	$P < 0.05$			(NS)		(NS)		(NS)

TABLE NO.11 Ca^{45} Serosal Uptake μeq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	6	5	6	7	6	5	6	7
	0.036	0.057	0.034	0.051	0.046	0.042	0.029	0.055
	0.044	0.059	0.070	0.043	0.055	0.090	0.069	0.046
	0.044	0.070	0.055	0.043	0.051	0.068	0.099	0.073
	0.061	0.046	0.083	0.061	0.062	0.080	0.102	0.065
	0.037	0.074	0.122	0.045	0.068	0.065	0.110	0.045
	0.043		0.062	0.088	0.056		0.056	0.044
				0.067				0.096
Mean	0.044	0.061	0.071	0.051	0.056	0.069	0.078	0.061
St. Dev.	0.010	0.011	0.027	0.029	0.021	0.016	0.028	0.018
Significance	$P < 0.05$		$P > 0.1$	(NS)		(NS)	$P > 0.2$	(NS)

TABLE NO. 12

Mucosal Transfer of Calcium and Phosphate.

(i) Calcium. figures = 1 x 10^{-9} eq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
influx	151	70	98	66	87	63	110	112
muc.upt.	207	189	283	207	271	232	200	144
muc.trans.	358	259	381	273	358	295	310	256
difference		-99		-108		-63		-54

(ii) Phosphate figures = 1 x 10^{-9} eq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
influx	87	59	76	75	42	32	42	43
muc.upt.	277	246	249	235	183	146	148	145
muc.trans.	364	305	325	310	225	178	190	188
difference		059		415		-47		-2

TABLE NO.13 Serosal outward transfer.

(i) Calcium. figures = 1 x 10^{-9} eq. $hr.^{-1}$ $cm.^{-2}$

Segments	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
outflux	137	101	121	107	112	91	99	104
ser.uptake	44	61	71	51	56	69	78	61
outward ser.trans.	181	166	192	158	168	160	177	165
difference		-15		-34		-8		-12

(ii) Phosphate figures = 1 x 10^{-9} eq. $hr.^{-1}$ $cm.^{-2}$

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
outflux	61	39	38	40	37	38	36	43
ser.uptake	33	39	39	37	43	39	40	34
outward ser.trans.	94	78	77	77	80	77	76	77
difference		-16		0		-3		+1

TABLE NO.14

Ratio calcium to phosphate for various measurements.

Segment	A		B		C		D	
Group	control	treated	control	treated	control	treated	control	treated
influx	1.77	1.19	1.29	0.88	2.11	1.97	2.61	2.60
muc.upt.	0.75	0.77	1.14	0.88	1.48	1.59	1.35	0.99
muc.trans.	0.98	0.85	1.17	0.88	1.59	1.66	1.63	1.36
outflux	2.25	2.59	3.18	2.70	3.03	2.40	2.75	2.42
Ser.upt.	1.33	1.56	1.82	1.38	1.30	1.77	1.95	1.79
out.ser.trans.	1.93	2.11	2.49	2.05	2.10	2.04	2.33	2.14

TABLE 15

Summary of Results for Duodenum only.

Calcium 1×10^{-9} eq. $hr.^{-1} cm.^{-2}$

	Control	treated	difference	significanc
influx	151	70	- 81	highly sign
muc.uptake	207	189	- 18	-
outflux	137	101	- 36	sign.
ser.uptake	44	61	+ 17	sign.

Phosphate 1×10^{-9} eq. $hr.^{-1} cm.^{-2}$

	Control	treated	difference	significanc
influx	87	59	- 28	sign.
muc.uptake	277	246	- 31	-
outflux	61	39	- 22	sign.
ser.uptake	33	39	+ 6	-

TABLE 16 P.D. of Rat's small intestine. Figures in millivolts.

Segment adjacent	A			B				C				D		
Group														
Number of Animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	2.85	5.66	9.60	10.16	8.81	10.30	2.73	3.27						
	6.27	2.84	8.17	12.97	9.27	13.15	6.53	5.35						
	2.60	5.11	4.33	8.23	9.94	8.20	1.80	8.40						
	5.80	6.13	10.62	9.10	9.42	9.46	6.59	6.42						
	6.74	5.60	7.68	6.00	10.11	11.62	4.82	2.00						
Mean	4.85	5.07	8.08	9.29	9.51	10.55	4.42	5.09						
St.Dev.	1.77	1.15	2.14	2.30	0.15	1.69	2.26	2.26						
Significance		NS		NS		NS		NS						

TABLE 17 pH Units - mucosal fluid - segment A

	control	treated
	5.7	5.1
	5.2	5.3
	5.2	4.9
	5.0	5.0
	5.1	5.2
	5.3	5.1
Average	5.25	5.1
St. Dev.	0.22	0.17

By inspection the difference between means is insignificant.

TABLE 18 Calcium 45 Influx after cold incubation with T_4 (Counts/sec.)

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals	4	5	4	5	5	4	4	4
	43.6	18.5	30.5	17.5	19.6	23.4	32.0	27.2
	21.1	34.6	19.4	44.1	34.1	24.7	28.6	20.8
	31.9	18.7	27.7	20.8	15.6	22.3	24.1	19.6
	29.0	28.1	28.1	42.0	31.1	24.4	36.8	19.8
		39.3		72.2	28.9			
Mean	31.4	27.4	26.4	39.3	25.9	23.7	26.2	21.9
St. Dev.	8.1	9.6	4.3	19.6	6.9	1.0	5.1	3.1
P	> 0.5	(NS)		(NS)		(NS)		(NS)

TABLE 19 Phosphorus 32 Influx after cold incubation with T_4 (Counts/sec.)

Segment	A		B		C		D	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Number of Animals								
	46.2	42.8	63.3	35.4	16.4	12.8	9.4	15.4
	19.3	88.4	26.0	21.7	8.7	6.7	13.6	10.0
	31.9	25.7	41.1	49.3	9.6	8.8	11.9	5.2
	38.4	39.4	37.0	36.4	10.1	8.8	18.8	6.7
		53.3		26.4	16.5			
Mean	34.0	49.9	41.9	33.8	12.3	9.3	13.4	9.3
St. Dev.	9.8	21.2	13.4	7.9	3.3	1.85	3.5	4.0
P		NS		NS		NS		

TABLE NO.20. Short Circuit Current in micro amps. 20 Minutes after death

Sex	Male		Female	
Group	Control	Treated	Control	Treated
Number of Animals	6	6	6	6
	275	170	190	80
	235	180	45	100
	90	100	95	90
	125	105	195	125
	65	195	225	105
	250	300	130	115
Mean	173	175	147	109
St. Dev.	83	67	63	16
Significance	NS		P > 0.2 N.S.	

APPENDICES

1. Reversible Flux in a 2 chamber system.

2. Flux Rates of control groups compared with values given in the literature.

3. Separation of Radiations from Calcium 45 and Phosphorus 32.

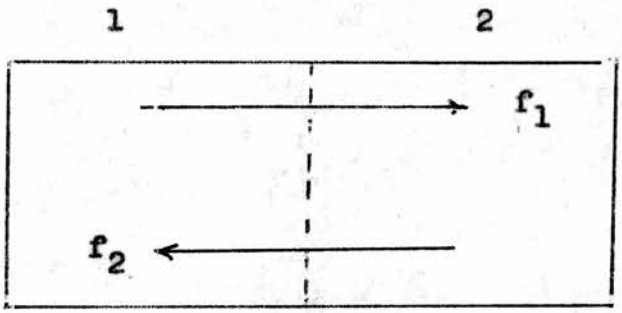
4. Calibration of Pipettes -

   5.0 ml. pipette

   1.0 ml. tuberculin

5. The effect on uptake compared with transfer in a simple one phase membrane as a result of a change in the diffusion constant.

APPENDIX 1 Reversible and Irreversible Flux in a Conservative Two Chamber System



Let Chamber 1 have a Large Volume (V_1) and a specific activity a_1 which will not alter appreciably since

$$a_1 V_1 \gg \text{loss due to } f_1$$

where f_1 = flux from 1 to 2

f_2 = flux from 2 to 1

f_1 is proportional to a_1 and therefore constant.

$$f_1 = \alpha_1 a_1 \quad \ldots\ldots\ldots\ldots (1)$$

similarly $$f_2 = \alpha_2 a_2 \quad \ldots\ldots\ldots\ldots (2)$$

CONDITION NO. 1 Reversible flux

Since we have no reason for invoking active transport from 1 → 2 (which would imply $\alpha_1 > \alpha_2$) and since we wish to be as pessimistic as possible:

$$\text{put} \quad \alpha_1 = \alpha_2 = \alpha \quad \ldots\ldots\ldots \quad (3)$$

Now the rate of increase of total activity of Chamber 2 $\frac{d(a_2V_2)}{dt}$ will equal the net influx

$$\frac{d(a_2V_2)}{dt} = f_1 - f_2$$

$$V_2 \frac{da_2}{dt} = f_1 - f_2 \text{ (if } V_2 \text{ const.)} \quad \ldots \quad (4)$$

$$= (a_1 - a_2)\alpha$$

$$\frac{da_2}{(a_1 - a_2)} = \frac{\alpha}{V_2} dt. \quad \ldots\ldots\ldots \quad (5)$$

The general solution to (5) is

$$-\log_e (a_1 - a_2) = \frac{\alpha}{V} t + K.$$ where K is an arbitary constant.

when $t = 0$; $a_2 = 0$: $-K = -\log_e(a_1)$

$$\log_e \frac{a_1}{a_1 - a_2} = \frac{\alpha}{V_2} t \quad \ldots\ldots\ldots\ldots\ldots (6)$$

CONDITION NO. 2 Irreversible Flux

$$\text{put} \quad f_1 = f^1 = \alpha^1 a_1$$

$$f_2 = 0$$

Eq. (4) becomes

$$V_2 \left(\frac{da_2}{dt}\right) = f^1 = \alpha^1 a_1$$

$$da_2 = \frac{\alpha^1}{V_2} \; a_1 . dt \quad \ldots\ldots\ldots\ldots (7)$$

The general solution is

$$(a_2)_{t=t} = \frac{\alpha^1}{V_2} \; a_1 \; t + K$$

if $t = o$; $a_2 = o$; $K = o$

$$(a_2)_{t=t} = \frac{\alpha^1}{V_2} \; a_1 t \quad \ldots\ldots\ldots\ldots (8)$$

Now comparing the flux measured according to equations (6) and (8)....

$$\frac{f}{f_1} = \frac{\alpha}{\alpha_1} = \frac{\log_e(a/a-a_1)}{(a_2/a_1)} \quad \text{.....(9)}$$

Taking typical values for influx of Phosphorus-32

Specific Activity $a_1 = 1261$ units

$a_2 = 482$ units

Substituting these values in Eq.(9) we get

$$\frac{f}{f^1} = 1.26$$

APPENDIX 2 Flux Rates for control groups compared to values published by other workers.

a) Influx of P^{32} (jejunum) Asano: 0.0976 eq.$hr.^{-1}$ $cm.^{-2}$

Noble: 0.087 eq.$hr.^{-1}$ $cm.^{-2}$

b) Influx of CA^{45} (duodenum) Schachter: 0.16 eq. $hr.^{-1}$ $cm.^{-2}$

Noble: 0.15 eq. $hr.^{-1}$ $cm.^{-2}$

APPENDIX 3 Separation of Radiations from Ca^{45} and P^{32} in a mixed sample.

An aluminium screen (area density 100mg)cm^2) was introduced between an end window G.M. tube and an arbitrary but pure sample of phosphorus-32. It was found that the screen absorbed 50% of the phosphorus-32 radiation. When the sample was replaced by a pure sample of calcium-45 it was found that all of the radiation was absorbed. The shielding factor is then equal to 2.

Two sets of counting apparatus were used for greater speed and in the second case the shielding factor was 1.6.

A single mixed sample was counted on both counters and a factor was obtained which converted the results on the second to equal the results of the first. With this factor all counts were then so converted for equal efficiency of count. Care was taken however to 'pair' the counting so that samples from the first control were counted on the same apparatus as the first treated animals. This was not thought however to give sufficient justification for a paired t-test to be used as a test of significance.

The phosphorus-32 counts were corrected for decay but this was not thought to be necessary for the calcium-45 count since all samples were counted within a few days.

APPENDIX 4

Calibration of pipettes and syringes

0.9% saline was measured out into a beaker with the various pipettes, etc., and the increase in weight of the beaker was measured accurately.

a) Blood Sugar Pipettes: used to take nominal 0.2 ml. samples for radio-active assay

average delivery of 24 pipettes = 0.19345 gm.

s.d. = 0.00283 gm.

The average of 0.193 was used in all calculations.

b) Tuberculin Syringes: used to put 0.5ml. ringer inside each sac.

(i) for radio-active solutions

average of 5 deliveries = 0.491 gm.

s.d. = 0.00127 gm.

(ii) for normal solutions

average of 5 deliveries = 0.521 gm

s.d. = 0.0024 gm

c) Bulb Pipettes: used for measuring out mucosal fluid

(i) Radio-active solutions

average of 5 deliveries = 5.006 gm

s.d. = 0.0017 gm

(ii) Normal solutions

average of 5 deliveries = 5.033 gm

s.d. = 0.00305 gm

APPENDIX 5 The effect of a change in diffusion coefficient on uptake and transfer in a simple single phase isotropic membrane.

Carslaw & Jaeger (1959) p.128 describe heat flux in a slab with one face in contact with a layer of perfect conduction on well stirred fluid.

i.e., the region $o < x < l$ with zero initial temperature

at $x = l$ solid is in contact with well stirred fluid of specific heat c^1 initially at zero temperature

$x = o$ is kept at temperature V for $t > o$.

This problem is exactly analogous to the problem under discussion with

Temperature V = the concentration

Specific heat C^1 = Volume of fluid

V = Temperature at $x = o$ = Specific Activity (or concentration) of source solution

Conduction (K) = Diffusion Coefficient

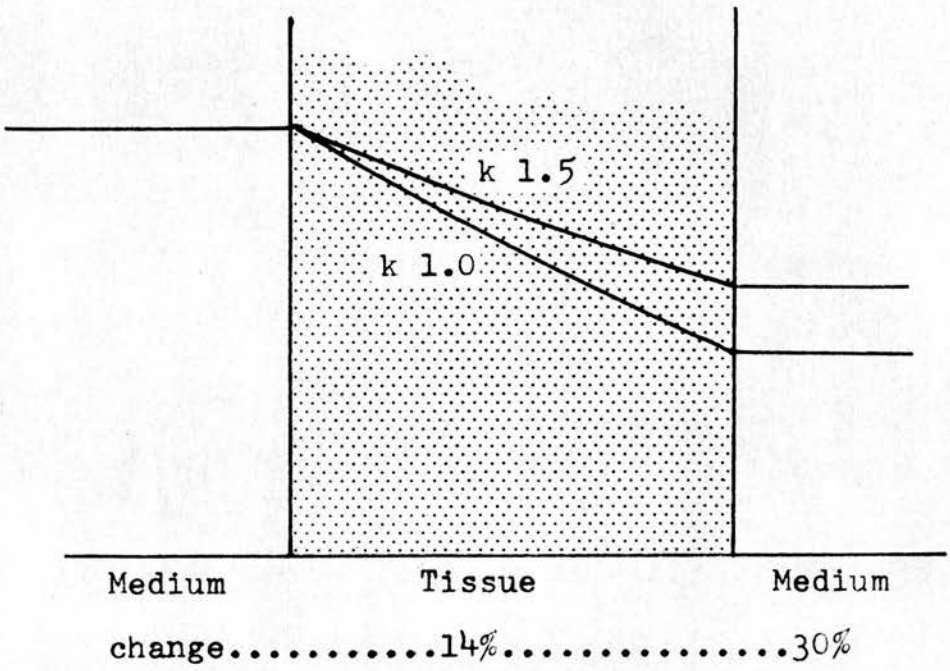


Fig. 19

Fig.19. Concentration of material within a uniform sheet of finite thickness, bathed on one side by a medium of constant concentration and on the other by a well stirred fluid of finite volume. Time (t) unit.

Two curves plotted for diffusion coefficient

k 1.0

k 1.5

these values were chosen arbitarily to illustrate the difference in total uptake (the area under the curves) between the membrane phase and the medium.

The equation given by Carslaw & Jaeger is

$$\frac{v}{V} = 1 - \sum_{n=1}^{\infty} \frac{2(\alpha_n^2 + h^2)\, e^{-k\alpha_n^2 t} \quad \sin \alpha_n x}{\alpha_n \left[l(\alpha_n^2 + h^2) \quad + h \right]} \quad \ldots\ldots(1)$$

where α_n are the roots of $h = \alpha \tan \alpha l$. . . . . .(2)

Fig.19 plots the curves obtained when Eq.(1) is solved using the first sin roots of (2) and putting K = 1 and 1.5.

The difference in tissue uptake is 14% while the difference in transfer = 30%

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