CALCIUM METABOLISM IN THE CHICK WITH SPECIAL REFERENCE TO VITAMIN D_3

by

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TABLE OF CONTENTS

SUMMARY

The process of calcium transport across the small intestine of the chick was studied by *in vivo* and *in vitro* techniques. Some characteristics of *in vitro* transport differed from the natural process. The site of calcium absorption was determined with rachitic and vitamin D_3-treated chicks. Rachitic chicks given 100 I.U. vitamin D_3 by mouth 16 hours previously showed a marked improvement in calcium absorption and similar amounts of calcium were absorbed along the whole of the small intestine *in vivo*. With everted gut sacs, however, the distal one-third of the small intestine transported much more calcium than the duodenal and middle sections.

On an equal weight basis, vitamin D_2 had little activity in the chick, but dihydrotachysterol series 2 and dihydrotachysterol series 3 were almost as active as vitamin D_3 for calcium transport. An immediate effect of vitamin D_3 was not seen either *in vivo* or *in vitro*. A minimum time period of 4 hours was required. It was postulated, that the delay was caused by the formation of an active calcium carrier together with its subsequent accumulation at the site of calcium transport.

The influence of certain adrenal and synthetic steroids on calcium absorption was tested in rachitic and vitamin D_3-treated chicks. Long-term administration of cortisol or 11-deoxycorticosterone interfered with the enhancement of calcium transport generally brought about by vitamin D_3. The steroids had no effect on the absorption by rachitic chicks. The inhibitory effect was not a direct antagonism of vitamin D_3 action, as cortisol, Δ^1-cortisol and 11-deoxycorticosterone enhanced

the effect of vitamin D_3 when the steroids were injected intracardially 1 hour before the test.

The observations with long-term administration of cortisol and 11-deoxycorticosterone could be correlated with interference of adrenal function. Evidence from the use of 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane, a specific inhibitor of the adrenal cortex, indicated the importance of the adrenal gland in calcium metabolism. This substance, when fed for 3 days prior to the test, eliminated calcium transport usually brought about by vitamin D_3.

When the mechanism of calcium transport was investigated, the evidence suggested that calcium transport under the influence of vitamin D_3 was an active process. From studies with metabolic inhibitors, it was deduced that the *in vitro* energy for the process was derived mainly from glycolysis, with oxidative phosphorylation contributing to only a small extent. Energy for calcium absorption *in vivo* did not appear dependent upon either of these sources. A scheme was postulated to explain active calcium transport. A carrier mechanism was suggested.

A number of tissues and tissue fluids from vitamin D_3-treated chicks were examined for the presence of active carrier material. However, the isolation and identification of such material was not successful. A calcium complex was prepared from cholesterol, but this complex could not be identified as the calcium chelate of ketone[250] nor was it found to be biologically active in the rachitic chick. The proposed nature of the calcium carrier, believed to be derived from vitamin D_3 by a transformation in the adrenal cortex, was discussed.

STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in any University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except when due reference is made to such material in the text.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
AT-10_2	dihydrotachysterol series 2
AT-10_3	dihydrotachysterol series 3
c.p.m.	counts per minute
o,p-DDD	1-(2-chlorophenol)-1-(4-chlorophenol)-2,2-dichlore ethane
DOC	11-deoxycorticosterone
EDTA	ethylene diamine tetra acetic acid
IR	infra-red
I.U.	international unit
UV	ultra-violet

INTRODUCTION

INTRODUCTION

Calcium is quantitatively an important inorganic element in the body, being one of the principal mineral constituents of the skeleton. Mitchell, Hamilton, Steggerda and Bean (1945) have shown that 99% of the total body calcium is contained in the skeleton and teeth. Although there are many factors involved which can influence calcium absorption, Nicolaysen, Eeg-Larsen and Malm (1953) have described vitamin D as "the most dominating single factor concerned in the regulation of the absorption of calcium".

A deficiency of vitamin D causes the disease known as rickets. This disease is characterised by an inadequate calcification of bony structures together with a number of marked biochemical abnormalities. Until the early part of this century, although the disease had become well defined from a pathological standpoint, little progress had been made in the isolation of the antirachitic substances present in the natural products commonly used for remedial purposes. Monographs by Korenchevsky (1922) and Hess (1929) summarise the knowledge gained from early metabolic studies on the aetiology of rickets.

In 1921, the now classical investigations by Mellanby demonstrated that rickets could be produced experimentally in puppies and subsequently cured by the feeding of cod-liver oil. McCollum, Simmonds, Shipley and Park (1921) were among the first to induce rickets in rats and in 1922, McCollum, Simmonds, Becker and Shipley found that the antirachitic properties of cod-liver oil were quite distinct from the vitamin A antixerophthalmic properties which were also present.

They later termed this substance vitamin D (McCollum, Simmonds, Becker and Shipley, 1925).

Irradiation of foodstuffs was also known to produce antirachitic substances and, in 1931, vitamin D_2 was isolated in a pure state as an irradiation product of ergosterol (Askew, Bruce, Callow, Philpot and Webster, 1931: Windaus, 1931 a, b). Vitamin D_2 has since been crystallised from cod-liver oil (Brockmann and Busse,1938). Following the isolation of vitamin D_2 came the subsequent realisation that this vitamin was not the main component of cod-liver oil but that another form of the vitamin existed with potent antirachitic properties. Eventually Schenck (1937) managed to crystallise this compound, now known as vitamin D_3, following irradiation of the vitamin precursor, 7-dehydrocholesterol.

Both these vitamins possess an equal antirachitic potency (40.000 I.U./mg.) for rats and humans (Smith, Glynn, Wilkinson and Peevers, 1943). In chicks, although the potency of vitamin D_3 is the same as in humans, vitamin D_2 is much less potent (Bills, 1935). The history and chemistry of the vitamin D group have recently been adequately reviewed by Bills (1954).

Use of rats and chicks in calcium studies.

The majority of investigators have preferred to use rats in their attempts to study disturbances in calcium metabolism. These animals are not always ideal subjects for work of this nature, as recently pointed out by Gershoff and Hegsted (1956). Clearly defined rickets in rats can only be produced by altering the levels of calcium and phosphorus in a vitamin D-deficient diet. The response to vitamin D may then differ according to the type of diet employed (Reed, Struck and Steck, 1939),

especially if these diets are severely unbalanced with regard to their Ca:P ratio (Day and McCollum, 1939; Schneider and Steenbock, 1939). Under these conditions, it has even been suggested that a vitamin D deficiency is not necessarily a prerequisite to disturbed calcification (Coleman, Becks, Kohl and Copp, 1950). Even without the abnormalities of the calcium and phosphorus content of the diet, however, Harrison, Harrison and Park (1958) claim that a vitamin D deficiency does produce biochemical abnormalities in the rat which can readily be corrected by the feeding of vitamin D.

In contrast to rats, chicks deficient in vitamin D can become rachitic at the usual dietary calcium and phosphorus levels, and they are extremely responsive to vitamin D treatment. Migicovsky and Emslie (1947) demonstrated that vitamin D could still influence calcium absorption, despite variations in the dietary Ca:P ratios. However, early investigations indicated that an alteration of these ratios could contol the severity of the rickets produced (Bethke, Kennard, Kick and Zinzalion, 1929; Hart, Scott, Kline and Halpin, 1930; Nowotarski and Bird, 1943).

The nature of the vitamin D effect

The action of vitamin D in increasing calcium absorption from the intestine was established during early investigations by Telfer (1926-27). Since then, numerous workers have studied this aspect. Harris and Innes (1931) were the first to postulate that the action of vitamin D was to increase the net absorption of calcium and phosphorus from the intestine.

Nicolaysen (1937 a, b) suggested that vitamin D acted specifically on calcium absorption and that the movement of phosphorus was thereby

indirectly affected. Clinical observations in man further confirm these findings (Albright and Sulkowitch, 1938). These studies were mainly in the nature of balance experiments, as there were no means of distinguishing between absorption from and excretion into the digestive tract.

With the availability of radioactive calcium and the introduction of tracer techniques came an era of rapid progress. Greenberg (1945) using Ca^{45} lactate solutions demonstrated that vitamin D not only increased the intestinal absorption of calcium but also appeared to exert a direct effect on the mineralisation of bones in rachitic rats. Migicovsky and Emslie (1949-50) were unable to substantiate these findings in chicks. They studied the effects of oral and intramuscular administration of $Ca^{45}Cl_2$ to rachitic and non-rachitic chicks and concluded that, although vitamin D given by mouth affected the absorption and excretion of calcium, there was no evidence to suggest a direct effect on the mineralisation of bone when the isotope was injected intramuscularly. Although criticism has been levelled at the assessment of these results (Underwood, Fisch and Hodge, 1951), Migicovsky and Nielson (1951) confirmed these earlier findings and further suggested that the amount of radioactivity appearing in the tibia after a certain time interval was an accurate estimation of the absorption rate. This estimate was regarded as a sensitive criterion of the response to vitamin D treatment.

The nature of the vitamin D effect has been further elucidated by Keane, Collins and Gillis (1956) who measured the amount of radioactivity present in the bones of rachitic and of vitamin D-treated chicks following an intramuscular injection of $Ca^{45}Cl_2$. Deposition of the isotope

occurred in the bones of the vitamin D deficient chicks to approximately the same extent as in normal chicks. This finding confirmed the earlier work of Migicovsky and Nielson (1951). The data gained from their experiments suggested that once the calcium had been absorbed from the intestine into the blood-stream, then vitamin D was no longer greatly significant in the actual mechanism of calcification.

Resorption of bone salt

Experiments with rats have shown that the effect of vitamin D on serum calcium levels persists under conditions where any appreciable influence of the vitamin on the intestinal absorption is excluded (Carlsson, 1952; 1954 a, b; Nicolaysen and Eeg-Larsen, 1956). Mellanby (1949) in a study in dogs found that 20 I.U. daily were sufficient to promote the maximal absorption of calcium, but that much larger doses were required to produce a normal bone structure.

Vitamin D, contrary to the opinion of Migicovsky and Emslie (1949), can also promote the resorption of calcium from the skeleton with its subsequent appearance in the blood (Lindquist, 1952). Carlsson and Lindquist (1955 a) found that, if the dietary intake of vitamin D was adequate to permit maximum calcium absorption from the gut, then increasing the dosage of vitamin D could still cause a rise in serum calcium. Studies by Bauer, Carlsson and Lindquist (1955 a, b) led to the proposal that this bone resorption effect was of primary importance in the phenomenon of bone-salt accretion.

More recent investigations by Migicovsky (1957), however, raise doubts as to whether the process of resorption is actually under the direct influence of vitamin D, as previously suggested by Bauer et al. (1955).

Migicovsky found that the net effect in the presence of vitamin D was always a gain of calcium. He has suggested that if vitamin D does exert a direct effect on bone, then it is more likely to be associated with the rate of calcium accretion. So far, there has been no conclusive evidence to show that vitamin D has a specific effect on the calcification mechanism.

Effectiveness of vitamin D with time

Lindquist (1952) has studied the effectiveness of a physiological dose of vitamin D in relation to the time required for its influence on calcium absorption to be observed. Using rats which had been well dosed with vitamin D he concluded that the minimum time required before observing an effect of vitamin D was 4 hours between the administration of vitamin D and of the radioactive calcium. A maximum effect was not obtained under 60 hours. Patrick and Schweitzer (1955) claim to have seen an effect in chicks 4 hours after supplementation with vitamin D, while Migicovsky (1957) believes that only 2-4 hours are required. Investigations so far, have failed to demonstrate an immediate effect of the vitamin on calcium absorption.

Site of absorption

Although there is general agreement concerning the effect of the vitamin in increasing calcium absorption, no conclusive evidence has yet been put forward to indicate a specific site for this absorption. In 1939, Reed, Struck and Steck suggested that calcium was mainly absorbed from the upper intestinal tract. Harrison and Harrison (1951) found that calcium uptake from the proximal two-thirds portion of the small intestine was most rapid within 4 hours, but that this absorption was not under

the influence of vitamin D. The major portion of the unabsorbed calcium was located in the distal one-third of the small intestine after 4 hours and absorption of calcium from this region was observed only in rachitic rats receiving vitamin D_3. From the data available, it appeared that the vitamin only exerted its effect under conditions in which the calcium was poorly soluble. The conclusion was therefore reached that the site of action was the distal portion of the small intestine.

The problem was further investigated by Nicolaysen (1951) who followed the uptake of radioactive calcium from isolated segments of rat small intestine *in vivo* over a period of 24 hours. Results from these experiments pointed to the upper half of the small intestine as the principal site for calcium absorption. However, the physiological conditions under which these investigations were carried out would not seem conducive to an accurate appraisal of the situation. Underwood, Fisch and Hodge (1951), Carlsson (1954) and Lindquist (1952) also consider vitamin D to influence the absorption of calcium from the upper part of the small intestine. Using *in vivo* loops of chicken duodenum, Gershoff and Hegsted (1956) clearly present evidence for the influence of vitamin D in this section of the intestine. As yet, there has been no real evidence to establish the hypothesis that vitamin D increases the solubility of calcium salts. It is plainly evident that a more detailed examination of the small intestine is still required to indicate more specifically the site of calcium absorption. Such investigations would also considerably advance the limited knowledge available on the mode of action of vitamin D.

Calcium adaptation

An interesting feature of calcium absorption is the efficiency with which rats can adapt themselves to the variable ratios of calcium and phosphorus in the diet. Rottensten (1938) found that calcium deprivation during the period of growth established a condition whereby calcium absorption was rapidly speeded up when the rats were returned to a high calcium diet. These findings were confirmed by Nicolaysen (1943) but he was unable to show a response with vitamin D-deficient rats, which led him to postulate the existence of an endogenous factor. This factor, or factors, in association with vitamin D, was thought to control the absorption of calcium in young animals, although the primary regulating factor was still considered to be vitamin D. The efficiency of calcium absorption was found to vary according to the needs of the body (Nicolaysen, Eeg-Larsen and Malm, 1953; Malm, Nicolaysen and Skjelkvale, 1955), so that when the skeletal mineral stores were depleted, a higher percentage of a given amount of calcium was absorbed. The adaptation phenomenon does not function without vitamin D. The question of adaptation in rats and in man and the role of the endogenous factor have been discussed by Nicolaysen and Eeg-Larsen (1953; 1956).

At present the nature of the proposed endogenous factor is rather obscure and, indeed, recent evidence leaves some doubt as to its actual existence. Henry and Kon (1953) studied the effect of vitamin D on calcium retention in the rat over 2 years. During this period, groups of rats were reared on high (0.8%) and low (0.25%) calcium diets. These workers reported that there were no effects on the calcium metabolism of the rat which were attributable to vitamin D, and thus were unable to

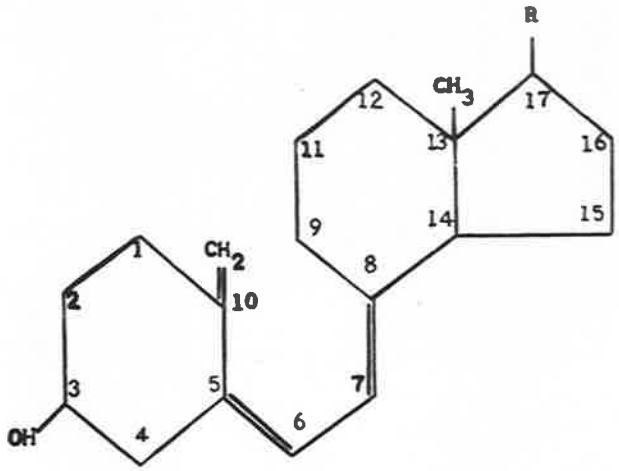
support the hypothesis proposed by Nicolaysen (1943).

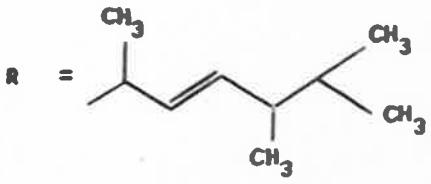
Following the giving of an oral dose of $Ca^{45}Cl_2$ to chicks, Migicovsky and Jamieson (1955) found that vitamin D influenced the rate at which the isotope appeared in the blood and bone. Keane, Collins and Gillis (1956) have confirmed this report. Further, the presence of the vitamin enabled the chicks to adapt their capacity to absorb calcium to the different dietary intakes of calcium. These workers also could find no direct evidence for the presence of an endogenous factor.

Dihydrotachysterol

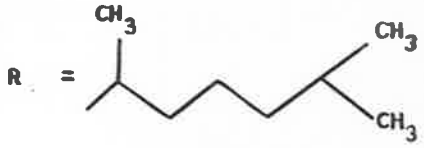
A number of compounds related to the vitamin D group have been isolated and tested for their antirachitic properties (Bills, 1954). Perhaps the most useful of these is dihydrotachysterol (AT-10), a substance which differs structurally from vitamin D in that a methyl group replaces the methylene group at C_{10} position of the original molecule (Figure 1).

Since it was first introduced by Holtz (1933), AT-10 has been widely used as a therapeutic agent in the control of hypocalcaemic tetany in cases of hypoparathyroidism. The plasma calcium concentration is rapidly raised as a result of an increased intestinal absorption in conjunction with a general mobilisation of bone calcium. AT-10 is known to cure rickets in rats and chicks (Correll and Wise, 1942) and it has also been used to treat phosphaturic rickets in man (Saville, Nassim, Stevenson and Mulligan, 1955). Although AT-10 is related to vitamin D in its chemical, antirachitic and calcaemic properties, the dose of AT-10 required to cure rickets is very much greater than the normal dose of vitamin D and the therapeutic range seems to be much narrower





VITAMIN D_2



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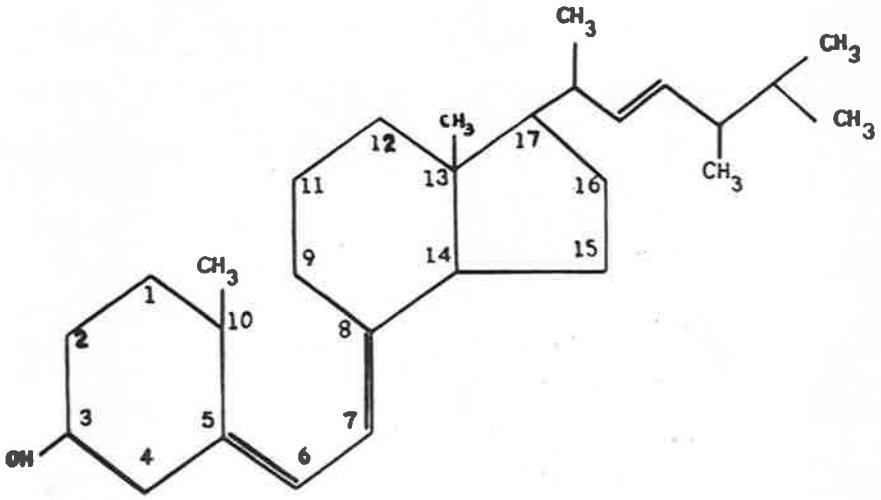


$AT-10_2$

FIGURE 1. FORMULAE FOR VITAMIN D AND $AT-10_2$

(Shohl, Fan and Farber, 1939; Shohl and Farber, 1941; Carlsson and Lindquist, 1955 b).

Clinical studies by Albright, Bloomberg, Drake and Sulkowitch (1938) indicated that the primary action of AT-10 was on phosphorus excretion rather than on calcium absorption, which is in direct contrast to the action of vitamin D. These workers have postulated that the differing ratios observed, i.e. $\frac{\text{action on calcium absorption}}{\text{action on phosphorus excretion}}$ could explain why vitamin D is a much more potent antirachitic agent than AT-10. The effect of vitamin D on calcium absorption is much slower than that of AT-10, which may also explain why AT-10 sometimes appears to be more efficient in increasing serum calcium than vitamin D.

Albright and Reifenstein (1948) have recently discussed the modes of action of vitamin D and AT-10 based mainly on clinical evidence. It is apparent, however, that the mechanism of action of AT-10 on calcium absorption is still obscure. Further investigations with this steroid in experimental animals might significantly advance our knowledge on the mode of action of vitamin D.

Ketone$_{250}$

The existence of an antirachitic compound known as ketone$_{250}$ has recently been reported (Raoul, 1958). This compound may be prepared synthetically from cholesterol, vitamin D_2, vitamin D_3 or dihydrotachysterol as starting material. Ketone$_{250}$ has also been isolated from fish liver and vegetable matter. Two forms of the ketone are said to exist: ketone$_{250}$ series 2 (side chain like vitamin D_2) and ketone$_{250}$ series 3 (side chain like vitamin D_3). Although this new substance has only one-tenth the antirachitic potency of vitamin D_3, as demonstrated

from chick assays, the enol form of ketone$_{250}$ can be stabilised in the presence of calcium. The calcium enolate possesses an antirachitic potency approaching vitamin D_3 itself. Hence the formation of such a compound has encouraged speculation as to its natural role in calcium transport.

There are many factors involved in regulating the dynamic equilibrium of the body calcium which are beyond the scope of these present investigations. Nevertheless, an introduction of this nature must be considered incomplete without some reference to the relationship existing between vitamin D and citric acid, and the parathyroid glands.

Vitamin D and citric acid

The role of citric acid in promoting calcium absorption has received much attention since this substance can form a soluble, poorly-ionised complex with calcium. Many workers have shown that orally administered citrate can bring about an increased calcification of the metaphyses (Hamilton and Dewar, 1937; Shohl and Butler, 1939; Yendt and Howard, 1955; Harrison and Harrison, 1952 a). The mechanism of this effect remains obscure. Yendt and Howard (1955) believe that the citrate assists by complexing with the calcium, thus reducing the amount of unabsorbable calcium phosphate being formed and allowing a greater absorption of phosphate from the gut. Recent studies by Schreier and Schnepf (1956) using both rachitic and vitamin D-treated rats demonstrate that orally administered citrate can increase the absorption of calcium.

Dickens (1941) found that over two-thirds of the body citrate was present in the bone salt. In rickets there is a decrease in the citric acid content of the bone tissue (Dickens, 1941; Nicolaysen and Nordbø, 1943; Waasjo and Eeg-Larsen, 1951). Vitamin D increases the citric acid level in blood, bone, kidney, heart and small intestines when given to vitamin D-deficient rats (Steenbock and Bellin, 1953).

A direct effect of vitamin D on citrate metabolism is indicated by recent experiments carried out in Steenbock's laboratory. In vitro the utilisation of citrate was found to be considerably reduced in kidney homogenate and kidney mitochondria preparations obtained from vitamin D-treated rats in contrast to the deficient animals (De Luca, Gran and Steenbock, 1957; De Luca and Steenbock, 1957). Freeman and Chang (1950) demonstrated that parallel changes in plasma calcium and citric acid can be produced by giving vitamin D in large doses to normal and parathyroidectomised dogs. Harrison and Harrison (1952 b) and Harrison, Harrison and Park (1958) pointed out, however, that following vitamin D-treatment the changes in the citrate content of the blood do not necessarily correlate with changes in the calcium content. Carlsson and Hollunger (1954) concluded that the dietary level of calcium played an important role in influencing the vitamin D effect on citrate metabolism in rats.

The relationship of vitamin D and citric acid is still uncertain. A number of reviews endeavour to sum up our present knowledge from the controversial reports pertaining to this aspect of calcium metabolism (McLean and Urist, 1955; Bourne, 1956; Neuman and Neuman, 1958).

Parathyroid gland and vitamin D

An important correlation exists between the calcium levels in the body fluids and the activity of the parathyroid glands. The relationship was first investigated by MacCallum and Voegtlin (1909) who showed that the serum calcium fell following parathyroidectomy, but that such changes could be prevented or alleviated either by the administration of calcium salts or by the injection of crude extract from the parathyroid glands. There has been much discussion concerning the primary site of action as the hormone produces two distinct effects, viz. an increased phosphate excretion via the kidney, and an increased mobilisation from the bone. The present weight of evidence would suggest that the main action of the parathyroid secretion in regulating calcium metabolism is directed towards the mobilisation of calcium from the bone. Bartter (1954) has reviewed these controversial findings. More recent theories advanced suggest that the two effects may not be due to the same parathyroid hormone but are caused by two different factors (Kenny and Munson, 1959).

There appears to be no conclusive evidence that the parathyroid glands exert a direct influence on calcium absorption from the intestine. Evidence recently put forward by Harrison, Harrison and Park (1958) reveals that vitamin D-deficient rats show no response to parathyroid extract unless the vitamin is administered prior to the giving of the hormone. As a result of these findings, it is suggested that vitamin D is required for the action of the parathyroid secretion. The role of the parathyroid glands in relation to the homeostasis of blood calcium has been the subject of many reviews (Howard and Connor, 1954; McLean

and Urist, 1955; Bourne, 1956; Copp, 1957; Howard, 1957; Neuman and Neuman, 1958).

The work presented in this thesis outlines attempts to define more clearly the mode of action of vitamin D in regulating calcium absorption from the intestine. Initially, methods are described for studying more effectively the calcium absorption phenomena both by *in vivo* and *in vitro* techniques. Several factors influencing calcium uptake are considered and, in particular, the mechanism of action for the active transport of calcium has been closely examined. The role of the adrenal glands in relation to calcium metabolism has also been investigated and experimental evidence is presented to indicate a possible site for the transformation of vitamin D_3 to a more active form of calcium carrier. Further studies related to the nature of such a proposed 'active' factor are discussed.

CHAPTER 1

MATERIALS AND METHODS

CHAPTER 1

MATERIALS AND METHODS

This chapter deals with those methods and materials used throughout these present investigations. It is therefore not intended as a summary of all the materials and methods referred to in this thesis as these will be dealt with separately in the relevant chapters. There are, however, one or two exceptions to this arbitrary division, for example, it has been found more convenient to group together methods relevant to P^{32} and Na^{22} studies in this general methods section.

Management of chicks

White Leghorn cockerels were obtained locally on the day of hatching and were immediately housed in electrically heated tier-brooders in an air-conditioned room from which sunlight was excluded. The previous history of these chicks assured a minimum carry-over of vitamin D_3 in the egg, and thus enabled a more rapid depletion for the early production of rickets. Chicks were raised by feeding the following rachitogenic ration for a period of approximately four to five weeks.

COMPOSITION OF DIET

Constituent	% in diet
Ground wheat	26
Ground maize	30
Soya meal	13.5
Casein (N.B.C. vitamin free)	5
Dried yeast	3
Dried lucerne	6
Salt	1
Wood charcoal	0.5
Manganese sulphate (4 H_2O)	20 mg./100 g. diet
Calcium phosphate (B.D.H. precipitated)	2
Vitamin A	2000-3000 I.U./450 g. diet
Vitamin E	13 I.U./450 g. diet
Arachis oil	1

The vitamins, dissolved in the arachis oil, were triturated with the calcium phosphate before addition to the remainder of the diet. Unless stated otherwise, the chicks were allowed access to food and water at all times.

After 18 days the birds showed a decline in body weight and within 5 weeks rickets was plainly evident. The chicks were weak and unable to support themselves on legs which had become splayed and deformed. Figure 2 illustrates the condition of a rachitic chick as compared with a chick of the same age given an adequate vitamin D_3 supplement from birth. The degree of rickets was assessed from the ash content of the dried fat-free tibia. Typical figures for rachitic

FIGURE 2

ILLUSTRATION OF A RACHITIC AND A NORMAL CHICK

The chick on the left in the photograph was fed a rachitogenic ration for 4 weeks and shows the typical symptoms of leg weakness. The chick on the right was the same age and was raised from birth on the same ration but fortified to include adequate vitamin D_3.

chicks approximately 28 days old were 26–30% bone ash, whereas chicks raised to the same age on a diet supplemented with vitamin D_3 (250 I.U. vitamin D_3/450 g. diet) gave a bone ash content from 40–44%. For experimental purposes, the chicks were selected as groups of approximately equal weight and size from the 26th - 35th day of hatching.

Controls

Two control groups of chicks were included in all the experiments performed, viz. rachitic and vitamin D_3-treated groups. The vitamin D_3 control group received 100 I.U. (2.5 µg) vitamin D_3 in arachis oil by stomach tube 16 hours prior to the experiment. This procedure has been abbreviated in the text to (100 I.U. - 16 hours). The rachitic control group received the vehicle alone in the manner described for each individual experiment. However, preliminary tests using undosed rachitic and rachitic chicks dosed orally with arachis oil 16 hours previously indicated that this vehicle alone had no significant effect on calcium absorption in these birds.

Bone-ash procedure

On the completion of the experiment the chicks were killed by wringing their necks and the left tibia was removed. The bone was scraped clean of all flesh with a scalpel and allowed to soak overnight in absolute ethanol. The following morning, the tibia was soxhlet-extracted for 8 hours using a mixture of petroleum ether (40°–60°) and absolute ethanol (2:1, v/v) before drying to a constant weight in a hot-air oven (100° C). The weight of the dried fat-free tibia was noted. Using an electrically heated muffle furnace at 800° C, the tibia was ashed in a silica crucible for 8 hours, allowed to cool and the weight

of ashed tibia expressed as a percentage of the weight of the dried fat-free bone.

Chemicals

All chemicals and solvents used were of A.R. quality whenever possible. All solutions were prepared with glass-distilled water.

Chemical estimation of calcium in intestinal loops

(a) Destruction of organic material. Intestinal loops, removed from the body at the completion of the experiment, were destroyed by the method of wet-oxidation, essentially as described by Reitz, Smith and Plumee (1960). 18.0 ml. nitric acid (15.8 N) were added to a 100 ml. conical flask containing the loop and digested slowly on a hot plate for 4 hours. At the end of this period, 2.0 ml. sulphuric acid (36 N) were carefully added and the mixture rapidly boiled until charring appeared. Oxidation was completed by the dropwise addition of a mixture containing 72% perchloric acid and 15.8 N nitric acid (2:1, v/v) and the mixture was then heated to fuming for 15 minutes until only sulphuric acid and a white residue remained. When cool, the contents of the flask were taken up in 10 ml. distilled water, followed by the addition of 3.0 ml. hydrochloric acid (10 N) and finally made to a constant volume (20 ml.) with distilled water.

(b) Precipitation of calcium. 5.0 ml. samples of the digest prepared as above were pipetted into 15.0 ml. centrifuge tubes together with 2.0 ml. saturated ammonium oxalate solution. Following the addition of 2 drops of methyl red indicator solution (0.05%), the mixture was just neutralised with ammonia solution (s.g. 0.88) and allowed to stand overnight to ensure complete precipitation of the calcium oxalate.

The calcium oxalate was sedimented by low speed centrifugation (2.500 X g) and the supernatant carefully aspirated to avoid any undue losses of the precipitate. After washing twice in the same manner with 0.5 ml. warm ammonia solution (1 Vol. ammonia s.g. 0.88 : 99 Vols. water), the calcium oxalate was taken up in a few drops of 4 N nitric acid with gentle warming, and the calcium content estimated by the EDTA-calcium titration method.

(c) EDTA-calcium titration. The titration was carried out essentially as described by Herrmann(1958). The assay mixture consisted of 0.5 ml. calcium sample, 1.4 ml. EDTA (0.025 M), 0.3 ml. 2 N KOH containing 1% KCN, 1.0 ml. calcein indicator solution* and 1 drop n-octyl alcohol (to prevent foaming). Titration was carried out using a standard calcium chloride solution (1 mg./ml.) and during this procedure the mixture was continually aerated to ensure complete mixing. The end-point as viewed under an ultraviolet light was sharply defined by the presence of light green fluorescence. With each experiment the EDTA solution was standardised against the standard calcium chloride solution. The amount of calcium present in the sample was calculated by difference, after taking into consideration the value of the control tubes.

A reagent blank together with a series of tubes containing varying known amounts of calcium chloride was carried through the entire procedure. These controls not only served to check for any possible calcium contamination, but also enabled the efficiency of the procedure to be assessed. Recoveries of not less than 98% were estimated with this method.

* Calcein indicator solution was prepared by dissolving 8 mg. calcein in 0.2 ml. 1 N KOH and diluting to 100 cc.

Surgical Procedures

The chick was anaesthetised with ether and then fastened to an operating board on the left side with the right leg pulled well forward to expose the right abdominal wall (Figure 3). Feathers were cleared from the surgical area and an incision approximately 1" long was made through the wall so that the required section of the intestine could be brought to the outside. The loop was immediately placed on a cotton-wool pad soaked in warm saline. For the isolation of the duodenal loop, one ligature was tied just below the gizzard and another tie made 1 cm. above the entry of the bile duct. With distal loops, a section of small intestine was measured upwards from the bottom of the caecum to, in most cases, a few cm. past the yolk sac, and, as described above, two ligatures were tied at these extremities. The length of intestine normally used was 12.5 cm.

The ligatured segments were partially severed below the upper and above the lower ligatures, and glass cannulae inserted. Each loop was flushed through with physiological saline at 37° C to remove its contents and the loops drained by blowing a volume of air through with a hand syringe. The cannulae were then either removed or tied into place, depending on the nature of the experiment.

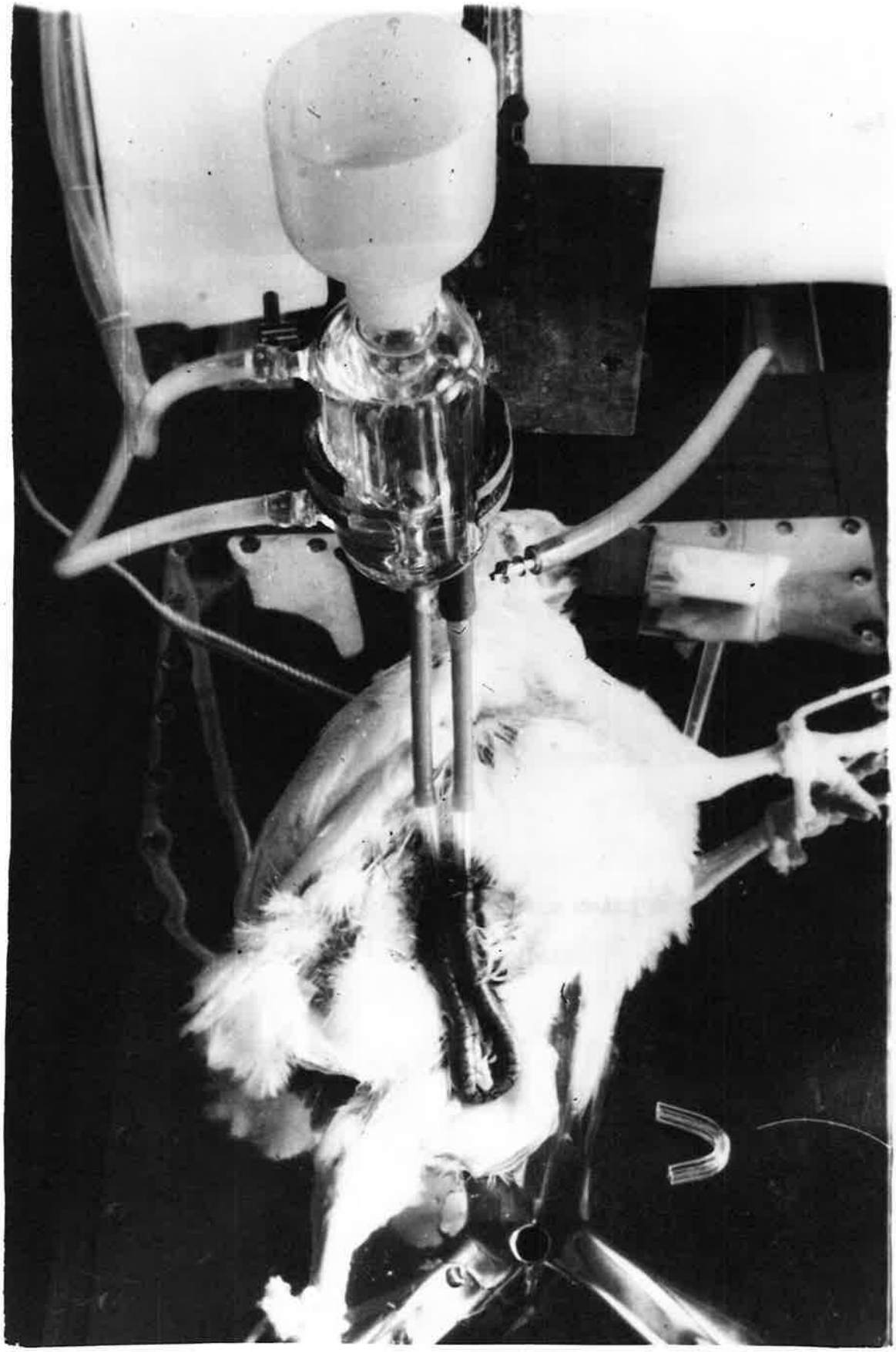
METHOD A.

In vivo isolated loop technique. After removing the cannulae, the lower end of the loop was tied off and the test solution of $Ca^{45}Cl_2$ (0.5 ml.) inserted through the upper end of the loop by means of a 22-gauge blunt-ended needle fitted to a 1.0 ml. graduated tuberculin syringe. A ligature held the needle in place and was pulled tight as the needle was

FIGURE 3

THE APPARATUS FOR PERFUSING ISOLATED INTESTINAL LOOPS IN VIVO (METHOD B)

The figure illustrates the operating position of a chick with the required portion of the intestine brought to the outside through an incision in the right abdominal wall. After inserting cannulae into the isolated loop, the perfusion apparatus was positioned over the bird and the outlets of the vessel then connected to the cannulae. The perfusion apparatus consists of an inner glass vessel containing the perfusion fluid surrounded by an outer glass jacket through which water at 38° C was continuously circulated. Flow of the perfusion fluid was maintained by carbogen gas injected through the rubber connection with a 24 gauge hypodermic needle. The polythene funnel attached to the perfusion apparatus prevented any contamination from radioactive material when frothing occurred.



withdrawn, thus preventing any loss of the radioactive solution. Unless otherwise stated, each loop received 0.5 ml. $Ca^{45}Cl_2$ solution containing 4 mg. Ca^{40} and 8.0 µc. Ca^{45} at a pH 6.0. The isolated loop was immediately replaced in the body cavity and the opening closed with two or three sutures.

Within a few minutes of the operation the chicks were sufficiently recovered and blood samples were thereafter removed at varying time intervals from a wing vein. When the intestine and bones were to be analysed for calcium the birds were allowed to live for 2 hours before removing the respective tissue. At the end of this period, the loops appeared perfectly normal, revealing no sign of any fluid distention.

METHOD B.

Perfusion of isolated loops in vivo. The in vivo transport of calcium across the intestinal membrane was studied using the apparatus described by Holdsworth and Coates (1961). Chicks were given an intramuscular injection (0.15 ml.) of sodium nembutal* (60 mg./ml.) 15 minutes prior to commencing the surgical operation and thereafter, anaesthesia was maintained with the aid of a little ether. After rinsing and draining the contents of the exposed loop, the glass cannulae were tied into place and connected with short pieces of rubber tubing to the perfusion apparatus (Figure 3). Care was taken to ensure that the connections did not pull or cause any constriction of the loop at the entry of the cannulae. Before commencing the operation, the perfusion fluid was

* An aqueous solution of sodium pentobarbital was a product of Abbott Laboratories, North Chicago, Illinois, U.S.A.

placed in the vessel to allow the contents to equilibrate with the water temperature (38° C). The circulating fluid consisted of 10 ml. Krebs-Ringer-buffer solution (Umbreit, Burris and Stauffer, 1949) to which had been added glucose (0.22 M), Ca^{40} (2 mg./ml.) and Ca^{45} (1.2 µc./ml.). Flow of fluid was prevented with screw clips until the cannulae were connected.

The fluid was circulated by means of carbogen gas (95% O_2, 5% CO_2) injected through a rubber connection with a 24-gauge hypodermic needle, which also provided adequate oxygenation of the tissue. On occasions the loop was returned to the body cavity but successful circulation was not always achieved in this position. It was preferable, therefore, to leave the loop outside the cavity where the tissue and surrounding area could be kept moist with saline pads. There was no indication of any harmful effect on calcium transport with this procedure. Perfusion was normally carried out for 45 minutes and blood samples were taken from a wing vein at intervals.

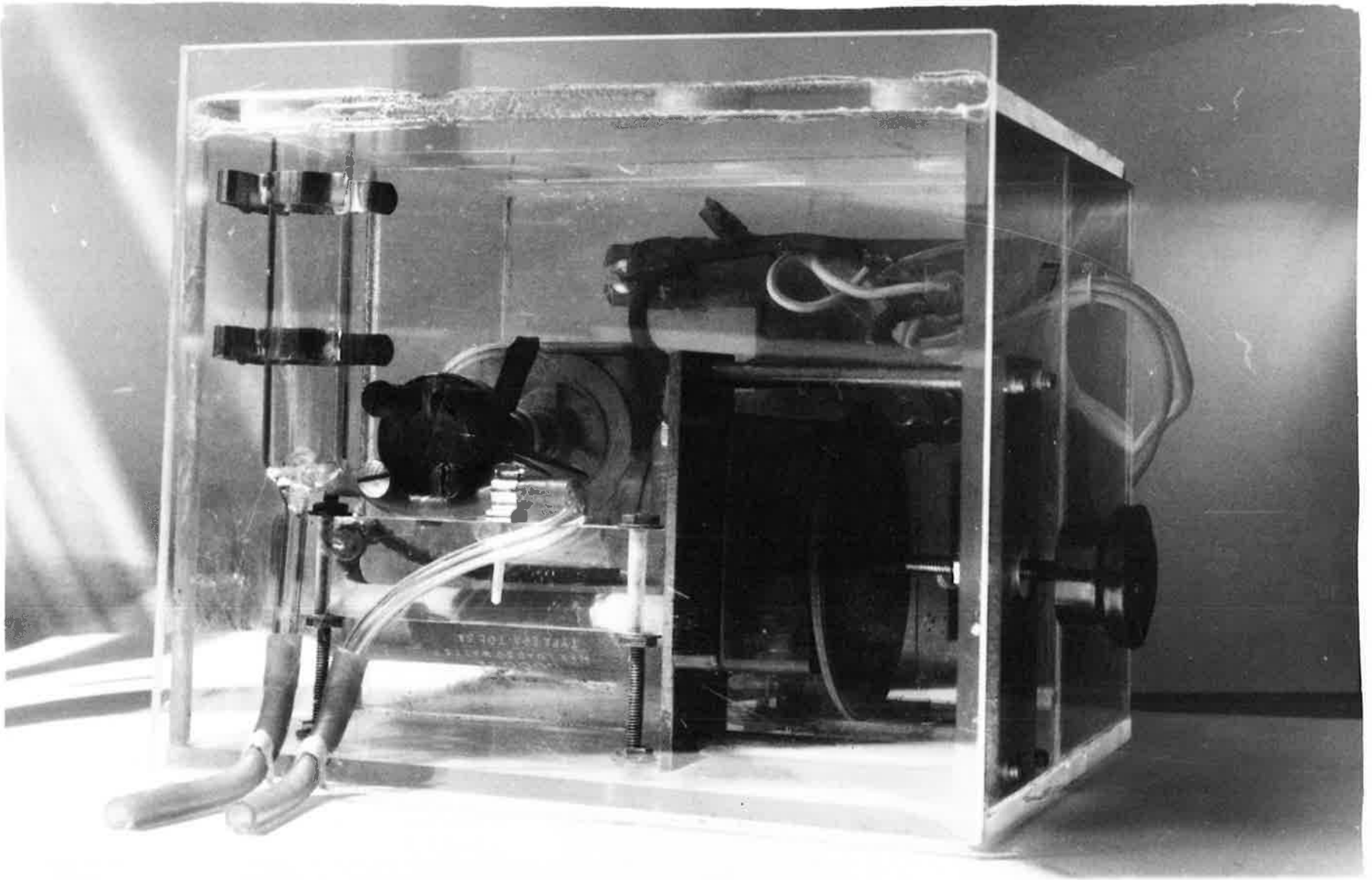
METHOD C.

Perfusion of isolated loops in vivo. Perfusion of in vivo isolated segments of intestine was made possible by means of a small peristaltic pump connected to the loop through glass cannulae (Figure 4). The pump and reservoir were enclosed within a thermostatically controlled chamber maintained at 38° C ± 1°. The connections from the pump and reservoir to the cannulae were of necessity kept short to reduce losses in temperature and also to restrict the volume of the circulating fluid. A synchronous motor ensured a constant rate of circulation and this rate could be varied through a rheostat.

FIGURE 4

THE APPARATUS FOR PERFUSING ISOLATED INTESTINAL LOOPS IN VIVO (METHOD C)

A peristaltic pump was used to perfuse isolated loops of small intestine *in vivo*. The pump and glass vessel containing the perfusion fluid are enclosed within a thermostatically controlled polythene chamber maintained at 38° C. A small heater was placed beneath the pump and fan blades attached to the pump ensured constant circulation of the air within this chamber. After inserting the cannulae into the prepared loop they were then connected to the pump and reservoir.



Before commencing the surgical operation, the outlets from the apparatus were clipped off and perfusion fluid was placed in the reservoir to enable it to reach the required temperature before circulating the fluid in the bird. When calcium absorption was studied by means of this apparatus the perfusion fluid consisted of 10 ml. of Krebs-Ringer solution containing 2 mg. Ca^{40}/ml. and 2 µc. Ca^{45}/ml. During similar experiments with Na^{22}, the perfusion fluid contained 10 ml. 0.15 M NaCl to which had been added glucose (0.22 m.) and Na^{22} (0.2 µc./ml.).

The intestinal loop was prepared with the cannulae tied in place, and, after connecting them to the apparatus, the perfusion was started. Blood samples were taken at intervals from a wing vein, and, at the completion of the test period, the apparatus and gut were drained thoroughly into a graduated cylinder.

METHOD D.

In vitro everted intestinal sac techniques. The apparatus described by Salomon and Johnson (1959) was modified to study transport of calcium across surviving everted segments of chick small intestine. Details of the apparatus are given in Figure 5. Following the surgical operation, the segment was severed at the ligatures and rapidly transferred to a bath containing Krebs-Ringer-bicarbonate-buffer prepared in the absence of added calcium to contain glucose at a concentration of 0.005 M. This solution was continually aerated with carbogen gas and the temperature of the bath was maintained at 38° C. The lumen was carefully stripped of its mesenteric attachments and the loop everted by means of a glass rod. The everted segment was then tied into place on the capillary supports with sutures and immediately placed in 30 ml.

FIGURE 5

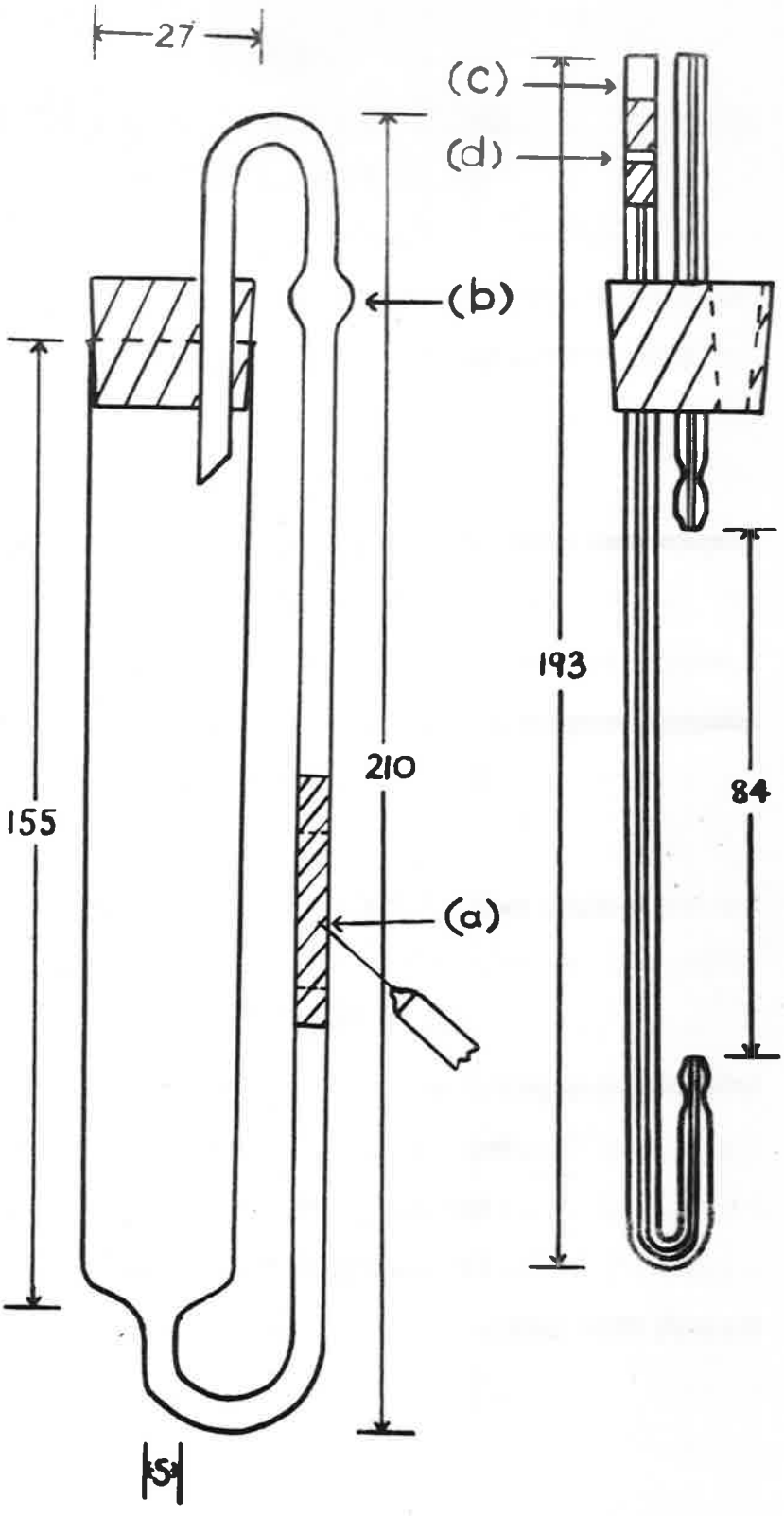
APPARATUS FOR PERFUSION IN VITRO OF ISOLATED SEGMENTS OF CHICK SMALL INTESTINE (METHOD D)

Numbers represent dimensions in mm.

The fluid bathing the mucosa tissue was called the mucosal fluid and that filling the serosa-lined lumen of the everted loop, the serosal fluid.

The outer apparatus (left) was partially immersed in a constant temperature water bath and contained 30 ml. mucosal fluid. Carbogen gas, injected through the soft rubber connection (a), permitted a continuous flow of the mucosal fluid and also ensured adequate oxygenation of the circulating fluid. The gassing tube had a glass bulb (b) which enabled frothing to be reduced to a minimum.

The suspending rack (right) was made of 5 mm. O.D. capillary tubing and was held in place by a two-holed notched rubber stopper. The notch in the stopper allowed the insertion of the gassing tube and also permitted the escape of gas. In operation, a short piece of soft rubber tubing closed at one end with a glass rod (c) was slipped on to the end of the straight (continuous) capillary. A small air space (d) permitted the serosal fluid to be injected through this rubber connection with a hypodermic syringe. Similarly the serosal fluid was withdrawn by the reverse process.


27
(c)
(d)
(b)
193
210
155
84
(a)
5

mucosal medium consisting of Krebs-Ringer buffer with added glucose (0.005 M), Ca^{40} (0.2 M) and Ca^{45} (3 µc./ml.). Carbogen gas was used to circulate the outer mucosal fluid. 1.0 ml. of a solution of Krebs-Ringer buffer containing glucose (0.005 M) and Ca^{40} (0.2 M) was injected into the loop and bathed the serosal surface. Oxygenation of the serosal fluid was not considered necessary as Salomon and Johnson (1959) have suggested that such a procedure makes little difference to the result.

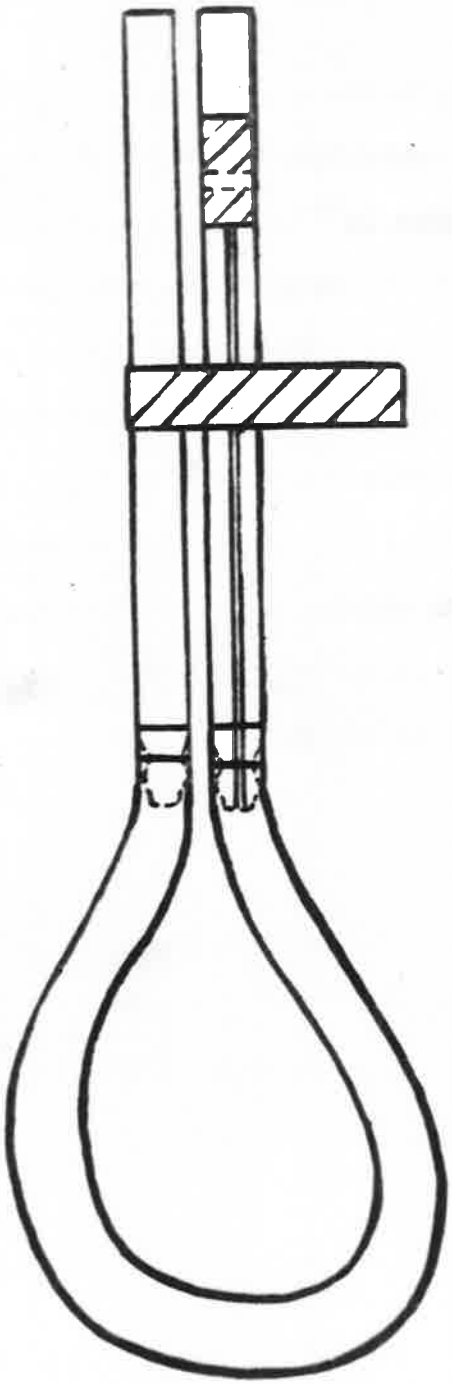
Transport of calcium was estimated by the appearance of Ca^{45} in the serosal fluid. At varying time intervals this fluid was completely removed and immediately replaced with fresh solution. Total transport over a period of 60 minutes was calculated as the sum of the radioactivity present in the individual samples. When the incubation was complete, the segments were removed, blotted with absorbent tissue, and finally placed in a desiccator until dry. The average dry weight of tissue from each group was determined.

METHOD E.

In vitro everted intestinal sac techniques. Everted sacs of small intestine were prepared essentially as described by Harrison and Harrison (1960). After removing the washed portion of intestine from the body, the segment was placed in an ice-cold solution of 0.13 M sodium chloride and 0.02 M potassium chloride and everted by means of a glass rod. Each end of the everted loop was tied over a short length of glass capillary and the two supports were fastened together with adhesive tape (Figure 6). Fluid contained within the loop and bathing the serosal surface is called serosal fluid and that in contact with the

FIGURE 6

The diagram illustrates an everted gut sac preparatory to placing in a conical flask containing 5.0 ml. of Krebs-Ringer-bicarbonate-glucose solution with added Ca^{45} (0.25 µc; 2.125×10^{-3} M). The preparation was tied with sutures to glass supports which were then held together with adhesive tape. A rubber connection sealed at one end with a glass rod was slipped on to the straight continuous capillary and 1.0 ml. of Krebs-Ringer-bicarbonate-glucose solution containing Ca^{40} (2.25×10^{-3} M) was injected through the rubber with a hypodermic syringe thus filling the inside of the everted sac. After placing the loop in the conical flask, the glass tubing was held firmly in place by a plug of cotton wool in the neck of the flask.



mucosal tissue, the mucosal fluid. Serosal incubation medium (1.0 ml.) was forced into the loop by injection through the connection rubber with a hypodermic syringe. This solution consisted of Krebs-Ringer-bicarbonate buffer with glucose and Ca^{40} added in final concentrations of 0.02 M and 2.25 X 10^{-3} M, respectively. The everted sac was transferred to a 30 ml. conical flask and immediately immersed in 5.0 ml. of Krebs-Ringer-bicarbonate buffer containing glucose (0.02 M) and Ca^{45} (2.125 X 10^{-3} M, 0.25 µc.). The preparation was incubated with shaking for 1 hour at 38° C in a metabolic shaker bath and was continually gassed with carbogen.

At the end of the incubation period, the sacs were removed, rinsed externally with cold 0.9% sodium chloride solution and then blotted dry with absorbent tissue. The serosal fluid was drained into a weighed container and rinsed through with exactly 1.0 ml. 0.9% sodium chloride solution, followed by several volumes of air forced through with a syringe so that complete emptying was obtained. The volume of solution recovered was measured by weighing. The effective length of tissue used was 12.5 cm. long. Accumulation of Ca^{45} in the serosal fluid was recorded as total counts accumulated/hour, i.e. c.p.m./0.1 ml. X total volume.

Vitamin D and related compounds

Crystalline vitamins D_2, D_3, and dihydrotachysterol$_2$(AT-10_2) were obtained from L. Light & Co. Ltd. (Colnbrook, England). Pure crystalline samples of dihydrotachysterol series 2 (AT-10_2) and dihydrotachysterol series 3 (AT-10_3) were kindly provided by Dr. Westerhof of Philips-Roxane (Amsterdam). For oral dosing, the compounds were

dissolved in arachis oil and 0.15 ml. was administered by stomach tube. When the compounds were given by intracardial injection, the appropriate quantity of the substance was completely dissolved in a small volume of absolute ethanol and diluted to volume with propylene glycol, the final concentration of ethanol being less than 0.5%. Each chick then received 0.15 - 0.20 ml. of the solution. The crystalline compounds and the solutions were stored at 4° C in air-tight containers.

Calcium chloride

The $Ca^{45}Cl_2$ was obtained from the Radiochemical Centre, Amersham, England, and was diluted for use with unlabelled calcium chloride to give a specific activity of approximately 2 µc./mg. calcium. As each solution usually lasted six months or more, the activity of the standard solution was counted at the same time as the experimental material and the necessary corrections made in order to standardise, as much as possible, the amount of radioactivity present. The calcium was invariably in the form of the chloride. Unlabelled calcium chloride was prepared by dissolving the appropriate amount of $CaCO_3$ (previously dried for 1 hour at 110° C) in 2 N HCl, evaporating to dryness and finally making to volume with distilled water. The pH was adjusted to 6.0 with sodium hydroxide.

Sodium^{22} chloride

Na^{22} was obtained from the Radiochemical Centre, Amersham, England, and diluted for use with unlabelled NaCl (0.15 M) to contain 2 µc./ml.

$P^{32}O_4$

The carrier-free orthophosphate from the Radiochemical Centre,

Amersham, England, was diluted with sodium phosphate to give 10 µc./mg. PO_4.

Radioactive counting procedures

Ca^{45}

(a) Plasma. (i) Blood was collected from a wing vein into a heparinised syringe and the plasma prepared by centrifugation. Virtually all of the calcium present in the blood is present in the plasma fraction (Irving, 1957). 0.1 ml. plasma was spread on a 1 cm^2 aluminium planchet together with one drop of 1% detergent solution to facilitate spreading. The mixture was allowed to dry and the radioactivity present measured by means of a G.E.C. EHM_2 end-window counter. The efficiency of this machine was 5-10%.

(ii) Plasma (0.2 ml.), obtained as described in the preceding paragraph, was mixed with 0.3 ml. 0.5 N perchloric acid and allowed to stand for 5 minutes. The protein was removed by centrifugation and 0.25 ml. supernatant taken for scintillation counting in an Ekco N664A counter. The sample was added directly to 5.0 ml. scintillator phosphor, as developed by Bray (1960). Quenching measurements were carried out using an internal standard of $Ca^{45}Cl_2$. The efficiency of counting as determined from a standard $Ca^{45}Cl_2$ solution was 64%. Perchloric acid extracts of plasma gave approximately 15% quenching.

(b) Tissue Fluid. (i) Serosal fluid (0.1 ml.), obtained during *in vitro* studies with everted sacs (Method E), was added directly to 5.0 ml. of Bray's scintillator phosphor and the $Ca^{45}Cl_2$ was estimated by scintillation counting.

(ii) Serosal fluid (0.1 ml.), obtained as under *in vitro* sac technique (Method D), was plated directly on 1 cm^2 aluminium planchets and counted at infinite thinness using a GEC-EHM_2 end-window counter.

(c) Mucosal tissue. The everted sacs were cut into very small pieces and retained in centrifuge tubes together with 2.0 ml. 5 N perchloric acid. After overnight extraction, the tubes were heated at 80° C for 2 hours, glass stoppers preventing any losses during this period. Precipitated protein was collected by centrifugation and samples of the acid extract removed to estimate radioactivity by scintillation counting. The quenching effect due to the perchloric acid was taken into account for the final evaluation. Accumulation of Ca^{45} in mucosal tissue was measured as total counts accumulated/hour i.e. c.p.m./0.1 ml. X total volume. The weight of tissue was not taken into account.

(d) Bone. Ashed tibia was dissolved in 10 ml. 2 N HCl and the calcium precipitated as the oxalate by the addition of 4 ml. saturated ammonium oxalate solution followed by neutralisation to methyl red indicator. The mixture was allowed to stand 8 hours before centrifugation. After washing the material in the same manner twice with dilute ammonia solution (1.5%) and twice with absolute ethanol, the calcium oxalate was finally dried and weighed.

For counting, the calcium oxalate was spread on 1 cm^2 polythene planchets and counted at infinite thickness using a GEC-EHM_2 mica end-window counter. The amount of material required to count at infinite thickness was determined from a self-absorption curve.

$P^{32}O_4$

Blood. For the counting of P^{32} in blood, the material was collected from a wing vein into a heparinised syringe. Whole blood (0.1 ml.), together with a drop of 1% detergent solution, was evenly spread on a 1 cm^2 aluminium planchet. When dry, the samples were counted with a GEC-EHM_2 end-window counter.

Sodium^{22}

Blood. Whole blood (0.4 ml.), collected as previously described from a wing vein, was pipetted directly into a deep-well polythene scintillation tube and the Na^{22} counted in an Ekco N597 NaI crystal scintillation counter.

CHAPTER 2

VITAMIN D, DIHYDROTACHYSTEROL AND OTHER FACTORS INFLUENCING CALCIUM TRANSPORT

Introduction

Part I: The influence of vitamin D_3 on calcium absorption as studied by various *in vivo* and *in vitro* techniques.

Part II: Factors affecting calcium transport and the specificity of vitamin D_3.

Part III: The influence of vitamin D_2 and dihydrotachysterol on calcium transport.

Discussion

Summary

CHAPTER 2.

VITAMIN D, DIHYDROTACHYSTEROL AND OTHER FACTORS INFLUENCING CALCIUM TRANSPORT.

INTRODUCTION

Prior to examining the mechanism of calcium transport in the small intestine of chicks it was first necessary to establish test systems, *in vivo* and *in vitro* capable of demonstrating changes affecting this process. Part 1 reports early attempts to study calcium transport under conditions described in the previous chapter. Calcium absorption in rachitic chicks was studied before and 16 hours after giving an oral dose of 2.5 µg. vitamin D_3. The experience gained from these experiments was an invaluable guide to more detailed investigations on calcium transport.

In Part II, the influence of several factors on the absorption of calcium under natural conditions is described. Attempts were made to locate the site of the maximum absorption of this ion, as previous studies with rats have produced no conclusive evidence in this regard. Nicolaysen (1951) has suggested that calcium is mainly absorbed from the upper portion of the small intestine, under the influence of vitamin D. Studies by Harrison and Harrison (1951), however, do not support this view. These authors believe that vitamin D is concerned only with the uptake of calcium from the distal region.

Using both *in vivo* and *in vitro* techniques it has now been possible to compare more thoroughly the response of various segments of chick small intestine to vitamin D_3 treatment. The influence of vitamin D_3 on the transport of ions other than calcium has also been investigated.

The effects of vitamin D_2 and dihydrotachysterol on calcium absorption are considered in Part III. Vitamin D_2 is reported to have little antirachitic activity in the chick (Bills, 1935) whereas in rats and humans, vitamin D_2 is equally as potent as vitamin D_3 (Smith, Glynn, Wilkinson and Peevers, 1943). The effectiveness of vitamin D_2 has been reinvestigated by the calcium absorption test, using isolated duodenal loops *in vivo*. Similarly, the influence of AT-10, a compound used therapeutically to produce a rapid increase in the level of serum calcium has been compared to vitamin D_3 for its effectiveness in the chick.

PART I

THE INFLUENCE OF VITAMIN D_3 ON CALCIUM ABSORPTION AS STUDIED BY VARIOUS *IN VIVO* AND *IN VITRO* TECHNIQUES

MATERIALS AND METHODS

Calcium absorption from either duodenal or distal portions of the small intestine was studied in rachitic chicks by methods previously described in Chapter 1. The level of Ca^{45} in the plasma or the accumulation of Ca^{45} in the serosal fluid of everted gut sacs was measured after chicks had been given an oral dose of 100 I.U. vitamin D_3 16 hours before the calcium test. These values were compared to the values obtained from similar measurements made with the rachitic control group.

During the early part of these present investigations the duodenal loop was extensively used as Holdsworth (personal communication) had suggested that there was no portion of the small intestine more suited for studies of this nature. However, more recent observations in this laboratory, which have been described elsewhere in this chapter, indicated that the distal loop was a more sensitive system for studying calcium absorption after vitamin D_3-treatment. Nevertheless, there is no experimental evidence to suggest that the effects observed from the use of the distal loop are not reproducible with duodenal segments.

RESULTS

Calcium absorption from in vivo isolated intestinal loops.

Calcium absorption was measured in duodenal loops prepared from rachitic and vitamin D_3-treated chicks (Method A). The influence of vitamin D_3 on this process was observed by following plasma Ca^{45} levels at varying time intervals. These results have been summarised in Figure 7. A striking increase in the amount of calcium present in the plasma can be demonstrated with vitamin D_3-treated chicks. A maximum D_3/rachitic ratio of 3.7/1 was observed within 30 minutes.

On occasions variations from this ratio have been observed and these are due mainly to the age and severity of rickets in the chick: the maximum ratio at 30 minutes obtained with duodenal loops using this method was 5.5/1 and the minimum 3/1. When calcium absorption was allowed to proceed for longer periods there was a slow decline in the amount of radioactivity present in the plasma until at the end of 2 hours levels comparable to those measured from the rachitic group were recorded for the vitamin D_3-treated birds.

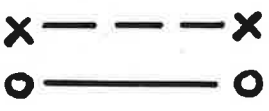
Calcium absorption from in vivo perfused intestinal loops.

(a) Figure 8 compares plasma Ca^{45} levels when duodenal loops from rachitic and vitamin D_3-treated chicks were perfused 'in situ' (Method B). Following vitamin D_3-treatment the rachitic chicks showed a three-fold increase in plasma Ca^{45} concentration. These findings confirm the results obtained with *in vivo* isolated duodenal loops. Previously, Coates and Holdsworth (1961) had been unable to demonstrate any effect of vitamin D_3 on calcium absorption when duodenal segments were perfused in a similar manner.

FIGURE 7

PLASMA Ca^{45} LEVELS DURING THE ABSORPTION OF Ca^{45} FROM ISOLATED DUODENAL LOOPS IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops of small intestine (Method A) from rachitic and vitamin D_3-treated chicks. Each point is the mean of 22 birds.



rachitic

vitamin D_3-treated

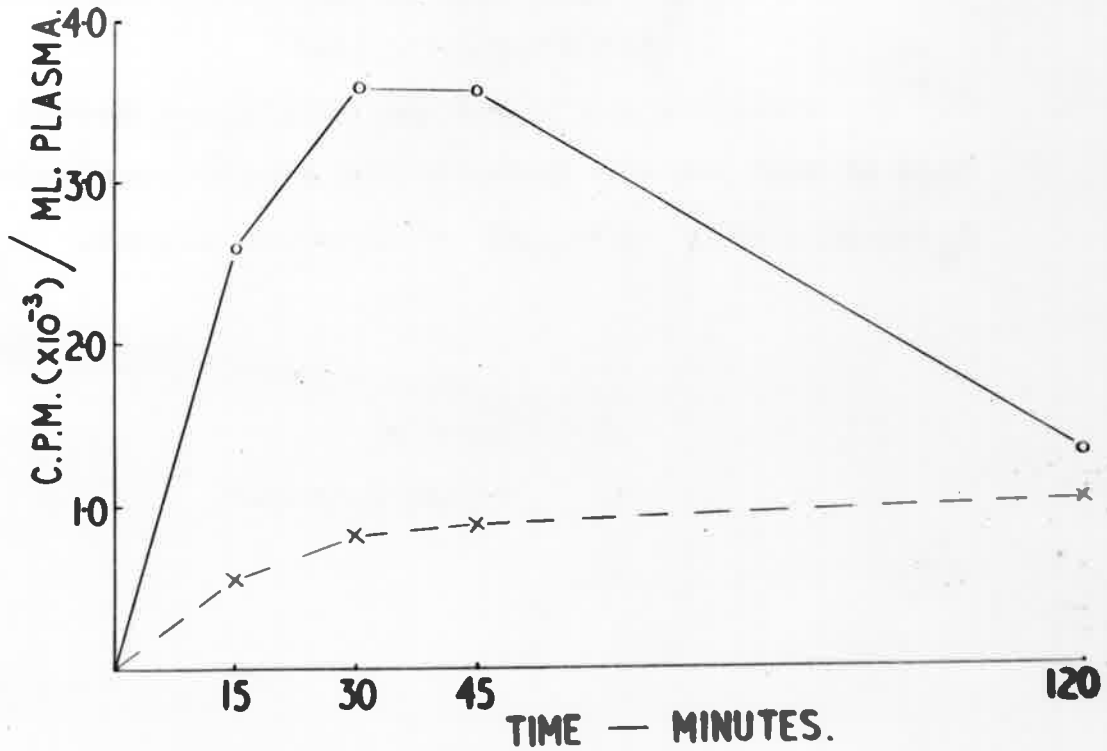
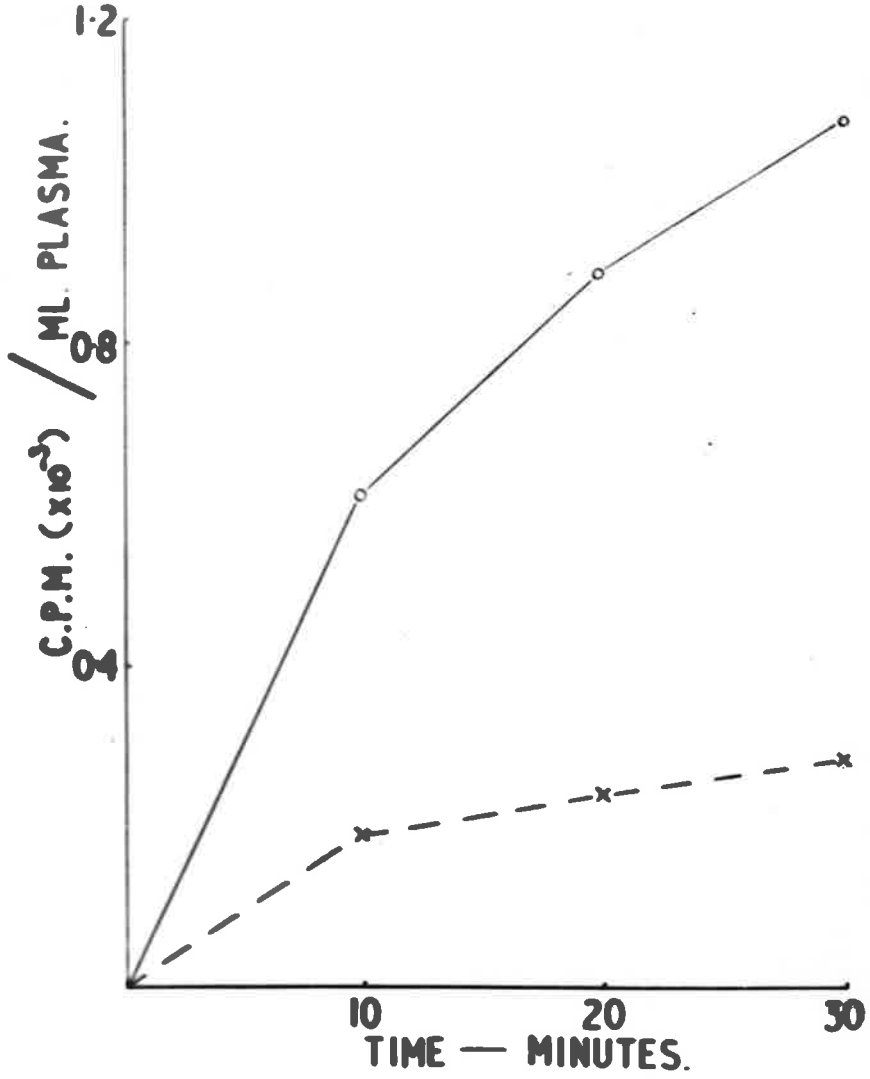

C.P.M. (x10⁻³) / ML. PLASMA.
40
30
20
10
15
30
45
120
TIME — MINUTES.

FIGURE 8

PLASMA Ca^{45} LEVELS DURING IN VIVO PERFUSION OF DUODENAL LOOPS

Ca^{45} was measured in plasma during perfusion of duodenal loops *in vivo* (Method B) from rachitic and vitamin D_3-treated chicks.


1·2
·8
·4
C.P.M. (x10⁻³) / ML. PLASMA.
10
20
30
TIME — MINUTES.

(b) Because of circulation difficulties often encountered with the perfusion apparatus described by Holdsworth and Coates (1961) an alternative method for perfusing the intestine was sought. When fluid containing Ca^{45} was circulated through isolated segments by means of a peristaltic pump more consistent results were obtained. The influence of vitamin D_3 on the uptake of Ca^{45} was studied with perfused distal loops of rachitic chicks. Ca^{45} present in plasma was measured by scintillation counting (p.27, (a) ii). The data summarised in Figure 9 indicate that there was a marked increase in radioactivity in the plasma from the vitamin D_3-treated group. The ratio D_3/rachitic was 8/1 after 30 minutes. As similar amounts of water were transported by both groups, the results obtained were not influenced by this factor.

Calcium absorption from in vitro everted intestinal sacs

(a) Isolated everted segments of duodenum were prepared from rachitic and vitamin D_3-treated chicks and the transport of calcium across the mucosal membrane was studies in vitro (Method D). The appearance of Ca^{45} in the serosal fluid was considered a measure of calcium transport from the mucosal to the serosal surface. It is evident from Figure 10 that duodenal sacs from chicks given vitamin D_3 showed a greater ability to transfer calcium to the serosal surface than similar sacs from the rachitic group. The rate of calcium transport after 20 minutes appeared relatively constant with time. Results obtained with this method were often difficult to interpret due to the small differences observed between the two groups. Studies with in vivo preparations had indicated a much greater improvement in calcium absorption after vitamin D_3 treatment.

(b) More reproducible results and a greater sensitivity of the rachitic chick to vitamin D_3 treatment were observed when everted sacs of

FIGURE 9

PLASMA Ca^{45} LEVELS DURING PERFUSION OF DISTAL LOOPS IN VIVO

Ca^{45} was measured in plasma during *in vivo* perfusion (Method C) of distal loops from rachitic and vitamin D_3-treated chicks. The number of birds is given in parenthesis.

rachitic (7)

vitamin D_3-treated (15)

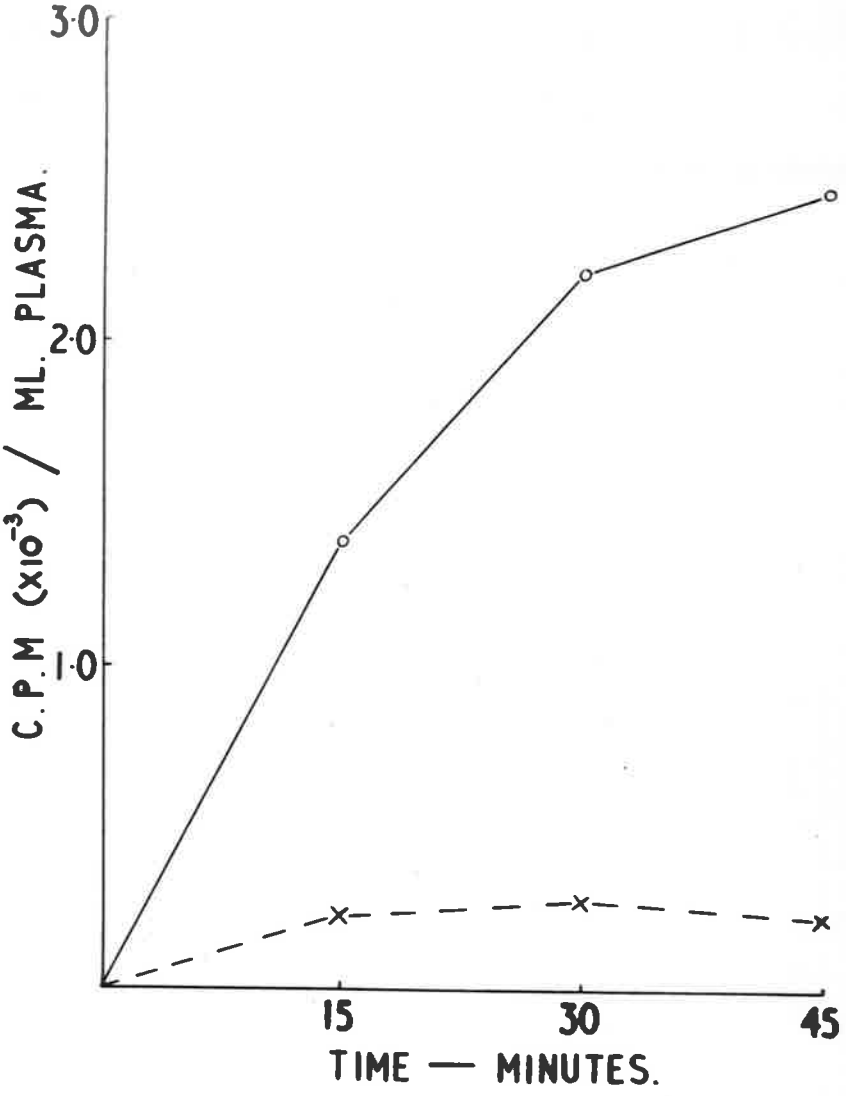

3·0
2·0
1·0
C.P.M (x10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

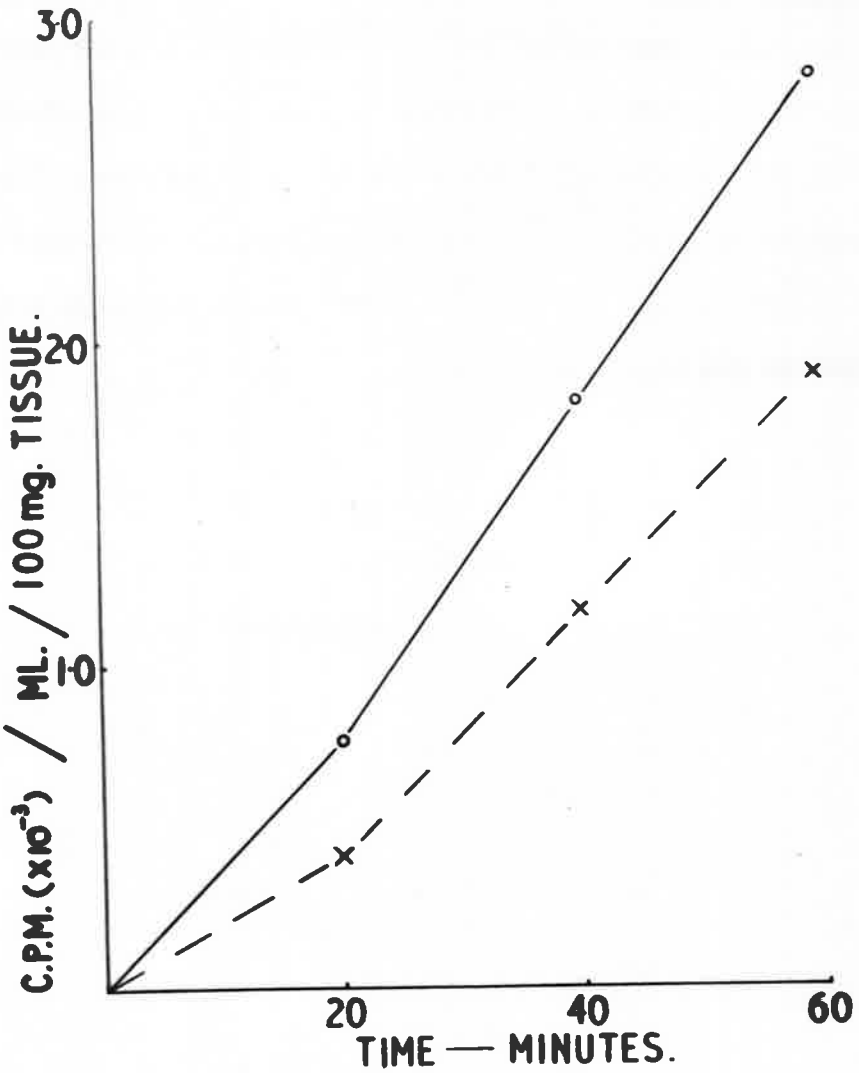
FIGURE 10

THE EFFECT OF VITAMIN D_3 ON CALCIUM TRANSPORT IN VITRO

Calcium transport was studied with everted duodenal sacs prepared from rachitic and vitamin D_3-treated chicks (Method D). Accumulation of Ca^{45} in the serosal fluid was measured at intervals. The results are expressed as total counts i.e. the radioactivity present in the individual sample plus the radioactivity of the preceding sample(s). Each point is the means of 9 birds.

rachitic

vitamin D_3-treated

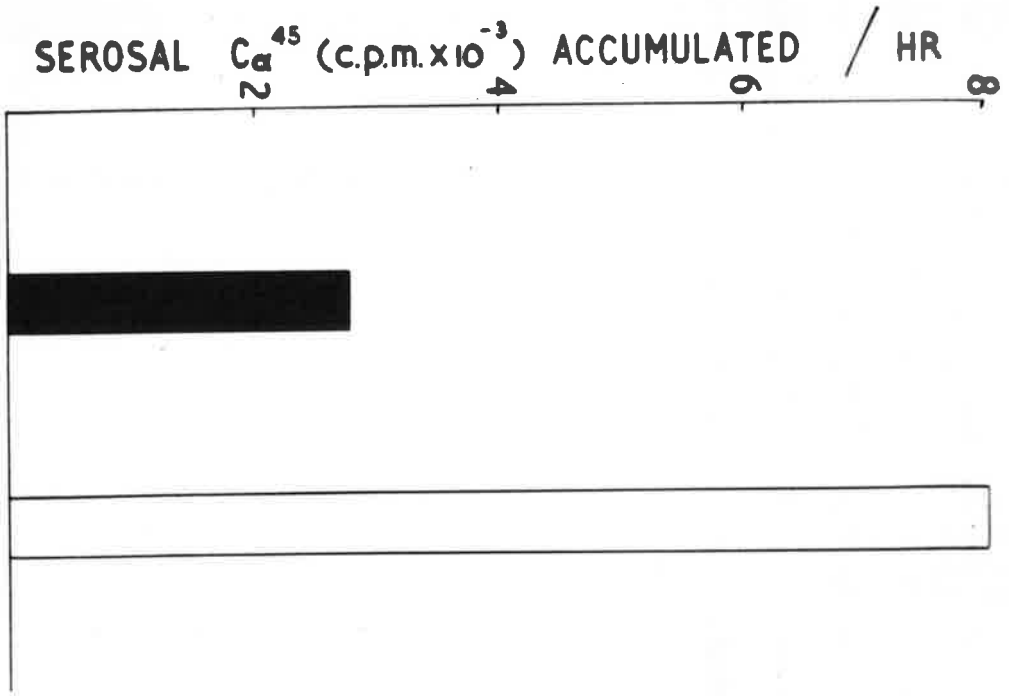

30
20
10
C.P.M. (x10⁻³) / ML. / 100 mg. TISSUE.
20
40
60
TIME — MINUTES.

distal small intestine were prepared and incubated in the manner described by Harrison and Harrison (1960) (Method E). Accumulation of Ca^{45} into the serosal fluid was measured after 1 hour. As demonstrated from the histograms (Figure 11) a ratio D_3/rachitic of 2.9/1 was observed when rachitic chicks were dosed with vitamin D_3. In the many hundreds of chicks examined by this method the minimum D_3/rachitic ratio observed using distal loops following 16 hours pretreatment with 100 I.U. vitamin D_3 was 2.4/1.

FIGURE 11

THE EFFECT OF VITAMIN D_3 ON CALCIUM TRANSPORT IN VITRO

Accumulation of Ca^{45} was measured in the serosal fluid of everted distal sacs of intestine from rachitic and vitamin D_3-treated chicks (Method E). Each histogram is the mean value from 15 sacs. The blocked histogram represents Ca^{45} in the serosal fluid from the rachitic preparations and the blank histogram that from the vitamin D_3-treated preparations.


SEROSAL Ca^{45} (c.p.m. x 10^{-3}) ACCUMULATED / HR
2
4
6
8

PART II

FACTORS AFFECTING CALCIUM TRANSPORT AND THE SPECIFICITY OF VITAMIN D_3

MATERIALS AND METHODS

Emulsified preparation of vitamin D_3. 2.5 mg. crystalline vitamin D_3 were suspended in 10 ml. water containing 0.05% Tween 80. The mixture was shaken vigorously for 20 seconds in a high speed mechanical disintegrator described by Nossal (1953) using Ballotini Number 12 glass beads. An amount of suspension equivalent to 2,000 I.U. vitamin D_3 was withdrawn into a hypodermic syringe followed by the test dose of $Ca^{45}Cl_2$ and the mixture expelled into the loop. A solution of water and Tween 80 prepared as above but containing no vitamin D_3, was placed in loops of the rachitic control group in the same manner.

Colloidal aqueous suspension of vitamin D_3. 12.5 mg. crystalline vitamin D_3 were dissolved in a minimum of absolute ethanol and the final volume adjusted to 1.0 ml. with propylene glycol. Immediately before use, the vitamin solution was rapidly injected into 4.0 ml. water, using a 24-gauge needle attached to a hypodermic syringe, and then thoroughly mixed. Of this solution, each chick received by stomach tube an amount equivalent to 10,000 I.U. vitamin D_3. Rachitic controls received the vehicle alone.

Administration of $Ca^{45}Cl_2$ by stomach tube. A solution (0.5 ml.) containing 2 mg. Ca^{40} and 2 µc Ca^{45} was given to each chick.

Riboflavin solution. A 1% solution of riboflavin in propylene glycol was administered by stomach tube.

Citrate buffer. Citric acid (0.2M) was adjusted to pH 4.0 or pH 6.0 with sodium hydroxide.

Amino acids. Solutions of L-lysine and L-arginine hydrochloride (50 mg./ml.) were adjusted to pH 6.0 with sodium hydroxide before use. $Ca^{45}Cl_2$ (0.5 ml.) followed by 0.2 ml. of the amino acid test solution was withdrawn into a syringe and the mixture inserted into isolated duodenal loops.

RESULTS

Site of absorption of calcium

(a) In vivo. Segments of small intestine from rachitic and vitamin D_3-treated chicks were prepared by the in vivo isolated loop technique (Method A) to locate the site of maximum absorption of calcium. The duodenal or distal loop was prepared in each chick to contain 4.0 mg. Ca^{45}. Figure 12 presents the results obtained in a typical experiment by following the plasma Ca^{45} levels at varying time intervals after giving the test dose. A marked increase in circulating calcium occurred from either loop under the influence of vitamin D_3. However a greater ratio (D_3/rachitic) was obtained with distal loops (7/1) as compared with duodenal loops (3/1).

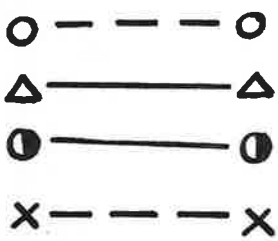
Further evidence that this plasma Ca^{45} level was a reliable indication of the absorption rate is shown by the measurement of calcium disappearance from the loop and its subsequent deposition or exchange in bone after 2 hours (Table 1). Vitamin D_3 increased the uptake from duodenal loops threefold and this calcium was deposited in bone. An even greater (sevenfold) increase in uptake occurred from distal loops. However the total amount of calcium absorbed by distal loops of vitamin D_3-treated chicks after 2 hours was only slightly greater than the amount absorbed from duodenal loops of vitamin D-treated chicks. The difference observed between the ratio of these sites appears to be due to the low absorption from the rachitic distal loops.

Some criticism could perhaps be levelled at the manner in which these results have been compared e.g. the isolated loops were prepared in separate birds. To minimise this, a double-loop technique was used in

FIGURE 12

PLASMA Ca^{45} LEVELS DURING ABSORPTION FROM DUODENAL AND DISTAL LOOPS

Ca^{45} was measured in plasma after preparing in vivo loops of small intestine from rachitic and vitamin D_3-treated birds. Each point is the mean of 4 birds in a typical experiment.



vitamin D_3-treated distal loops

vitamin D_3-treated duodenal loops

rachitic duodenal loops

rachitic distal loops

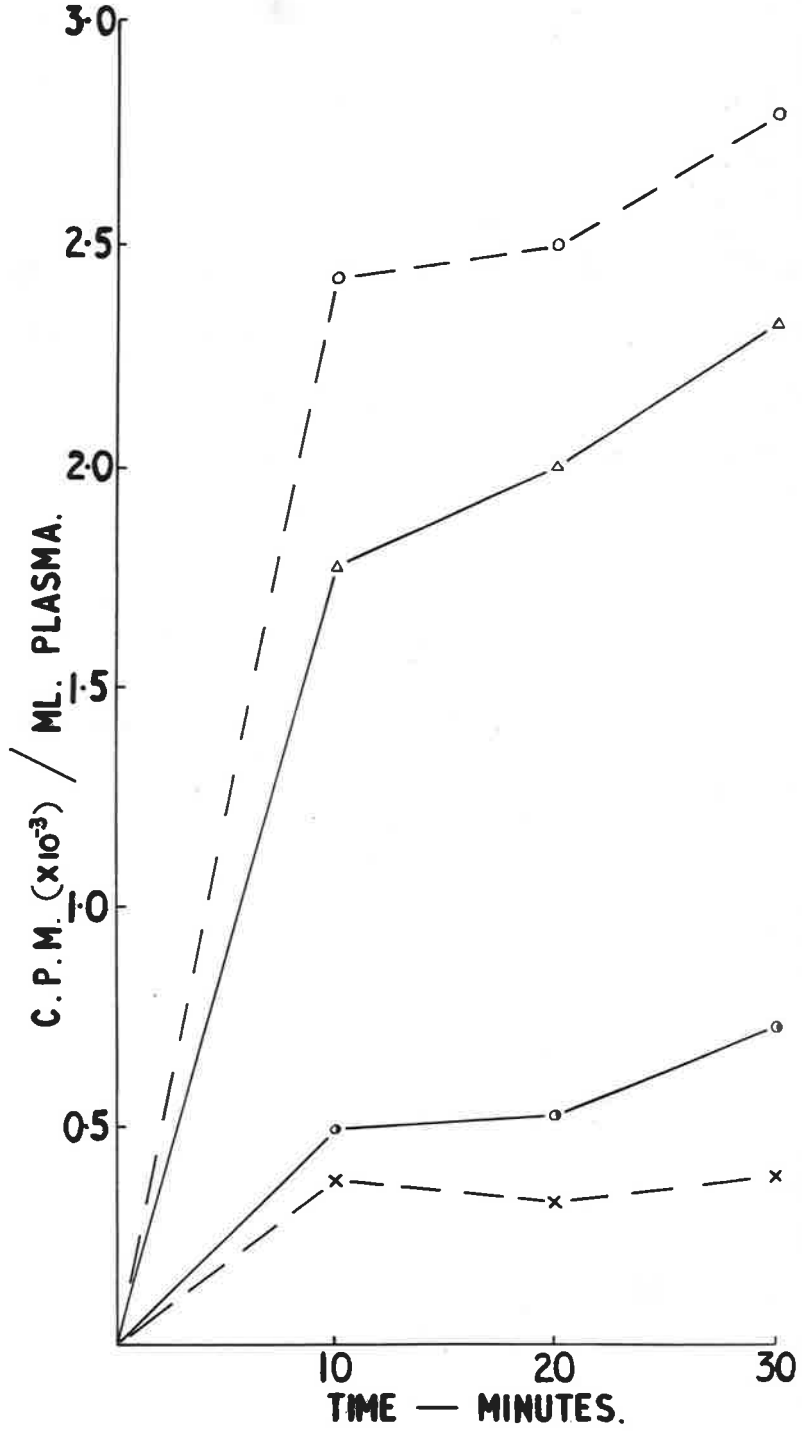

3·0
2·5
2·0
1·5
1·0
0·5
C. P. M. (x10⁻³) / ML. PLASMA.
10
20
30
TIME — MINUTES.

TABLE 1

THE EFFECT OF VITAMIN D_3 ON CALCIUM TRANSPORT FROM DIFFERENT PORTIONS OF THE SMALL INTESTINE

Treated birds were given 100 I.U. vitamin D_3 16 hours prior to the calcium test. Uptake and deposition of calcium in bone was measured after 2 hours from *in vivo* loops containing 4 mg. Ca^{45}. The residual calcium in the loop was determined and % calcium uptake is the amount absorbed expressed as a percentage of the initial calcium present. Each value represents the mean (number of birds is in parenthesis) ± the standard error of the mean.

Segment	Rachitic		Vitamin D_3-treated	
	% calcium uptake	Bone Ca^{45} c.p.m. X 10^{-4}/ ashed tibia	% calcium uptake	Bone Ca^{45} c.p.m. X 10^{-4}/ ashed tibia
Duodenal	16 ± 3.5 (20)	10.65 ± 3.08 (8)	48 ± 3.9 (20)	27.78 ± 4.12 (8)
Distal	8 ± 2.1 (12)	5.12 ± 2.39 (8)	56 ± 6.5 (12)	25.00 ± 3.15 (8)

which both the duodenal and distal segments were isolated in individual chicks from each group. Unlabelled calcium chloride was placed into each loop and at the end of 2 hours the amount of residual calcium was estimated in the manner described. The results obtained confirmed the values and conclusions established from the radioactive studies (Table 2).

(b) *In vitro*. Experiments similar to the *in vivo* studies but using isolated everted sacs (Method E) added support to the observations made *in vivo*. The whole of the small intestine was rinsed through with warm saline and then divided into three equal segments, duodenal, middle and distal. The effective length used during the incubation was 12.5 cm. Accumulation of Ca^{45} into both the mucosal tissue and serosal fluid was measured after 1 hour's incubation. The greatest amount of transport occurred with distal segments of small intestine from vitamin D_3-treated chicks (Figure 13) with lesser amounts in the middle and duodenal segments. The results expressed as Ca^{45} accumulated in the serosal fluid, show that the D_3/rachitic ratio was 7/1 with distal loops.

When the method of Harrison and Harrison (1960) was used for assessing total transport i.e. the sum of Ca^{45} in the serosal fluid plus the mucosal tissue Ca^{45}, then the ratios were 4/1 distal, 2.4/1 middle and 2.4/1 for duodenal loops. It is interesting to note that similar amounts of calcium were accumulated by mucosal tissue in all parts of the small intestine of the vitamin D_3-treated chicks. The absolute amount of calcium transported by the distal segment was 35 µg. Ca/hr./g. for rachitic gut and 150 Ca/hr./g. with vitamin D_3-treated chicks.

TABLE 2

CALCIUM ABSORPTION FROM DUODENAL AND DISTAL LOOPS IN VIVO

Duodenal and distal loops of small intestine in each chick were prepared and 4 mg. calcium was placed in each loop. Residual calcium was estimated after 2 hours. Each value represents the mean of 4 birds.

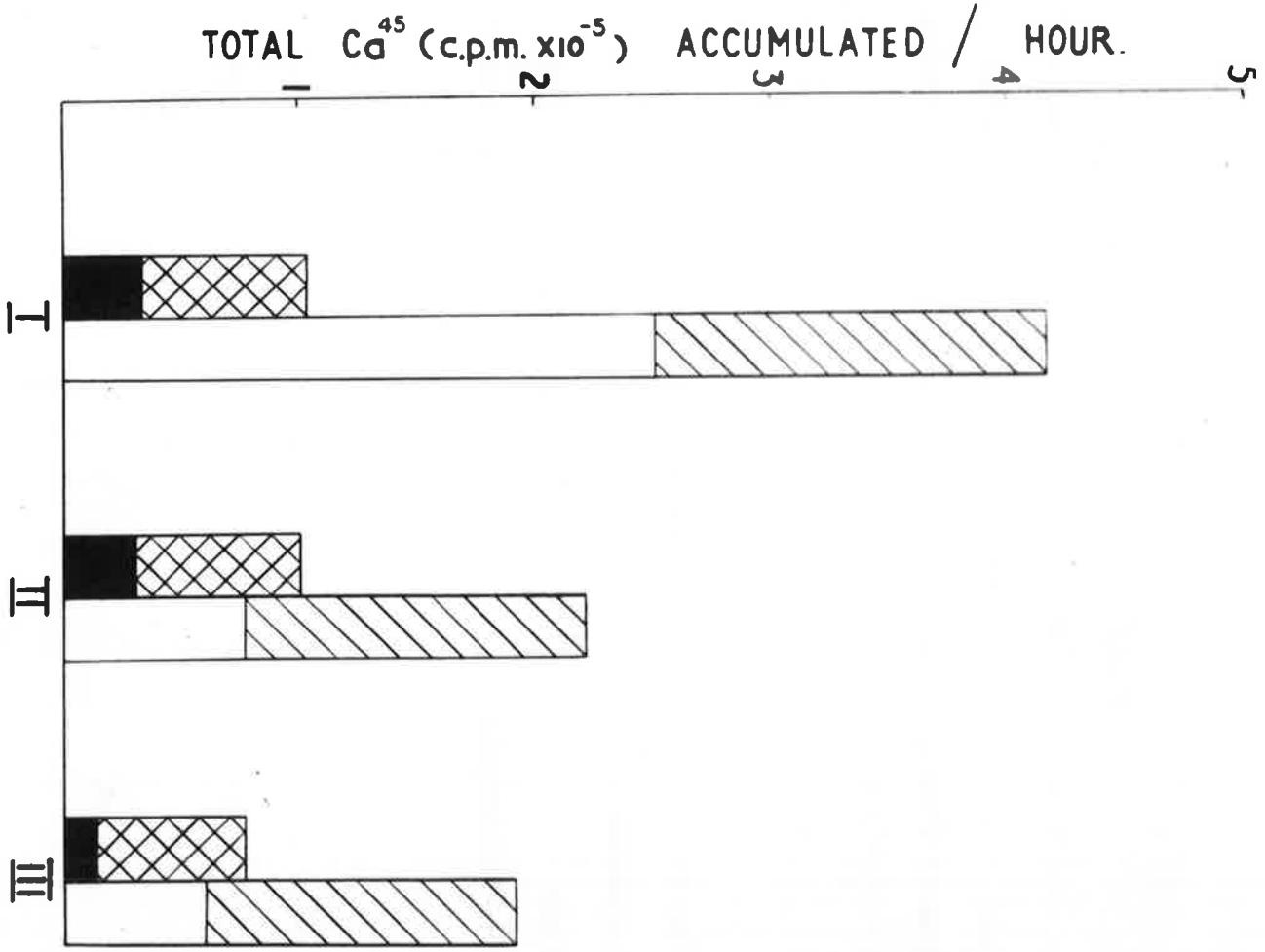
Segment of intestine	Calcium absorbed (mg.) Rachitic	D_3-treated
Duodenal	0.65 ± 0.13	1.98 ± 0.21
Distal	0.29 ± 0.07	2.05 ± 0.11

FIGURE 13

THE SITE OF CALCIUM ABSORPTION IN VITRO

Accumulation of Ca^{45} into mucosal tissue and serosal fluid of everted small intestine was measured after 1 hour. Each histogram is the mean of 8 sacs. Histograms I, II and III are distal, middle and proximal sacs respectively.

Blocked	rachitic, serosal fluid
Cross-hatched	rachitic, mucosal tissue
Blank	vitamin D_3-treated, serosal fluid
Diagonal hatched	vitamin D_3-treated, mucosal tissue


TOTAL Ca45 (c.p.m. x10-5) ACCUMULATED / HOUR.
1
2
3
4
5
I
II
III

The influence of vitamin D_3 in relation to the time of administration

Several investigators have endeavoured to show immediate or short-term effects on calcium metabolism following vitamin D administration to rats or chicks (Migicovsky, 1957; Coates and Holdsworth, 1961; Harrison and Harrison, 1960; Dowdle, Schachter and Schenker, 1960; Schachter, Kimberg and Schenker, 1961). These attempts have been extended in this present study. In an initial experiment vitamin D was studied for an immediate effect on calcium absorption by inserting an emulsified form of the vitamin together with $Ca^{45}Cl_2$ directly into duodenal loops at the commencement of the test. Table 3 indicates that the absorption of calcium as measured by plasma Ca^{45} levels was unaffected when the vitamin was given in this form. Even 5 hours after an oral dose of 2.5 µg vitamin D_3 there was only a slight increase in the level of Ca^{45} in the plasma. A marked increase in plasma radioactivity was observed following 16 hours pretreatment with vitamin D_3.

The lack of effect of vitamin D_3 within 5 hours of its administration could be the result of two factors (a) the amount of vitamin D_3 given (100 I.U.) was not sufficient to promote a significant increase in calcium absorption (b) the mode of administration. These factors were investigated in the following experiments.

A large dose of 10,000 I.U. vitamin D_3 was administered either (a) by stomach tube as a colloidal aqueous suspension or (b) intracardially in propylene glycol 1 hour before giving 2 mg. Ca^{45} by stomach tube. Ca^{45} was counted in the left tibia 2 hours after giving the Ca^{45}. In a further experiment, vitamin D_3 was given as above but 2 hours before the Ca^{45} and the tibia was then removed after a further 3 hours. The results of this

TABLE 3

PLASMA Ca^{45} LEVELS AT VARIOUS TIME INTERVALS AFTER VITAMIN D_3 ADMINISTRATION

Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops. Vitamin D_3 (100 I.U.) dissolved in arachis oil was administered by stomach tube 5 hours and 16 hours before inserting the $Ca^{45}Cl_2$ test solution in the loops. An emulsified form of vitamin D_3 (2,000 I.U.) was added directly to the loop together with the $Ca^{45}Cl_2$, when attempts were made to show an immediate effect with the vitamin. Each value is the mean of 4 birds.

Treatment	Time of treatment (hours)	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins) 15	30	45
-	-	0.58	0.75	0.70
D_3	0	0.57	0.70	0.75
D_3	5	0.60	0.90	0.80
D_3	16	1.30	2.75	2.38

latter experiment are shown in Table 4 and in spite of the highly unphysiological amount of vitamin D_3 in circulation, no significant increase in bone counts was detected.

Some doubt arose as to whether the time lapse between the oral administration of vitamin D_3 and the insertion of the Ca^{45} solution into intestinal segments was sufficient to actually allow the vitamin to penetrate the intestinal tract. In order to test this hypothesis, preliminary experiments were carried out using a solution of riboflavin as a fluorescent marker. 2 hours after oral dosing with riboflavin, the whole of the small intestine was split lengthwise and examined for fluorescence under an ultraviolet lamp. Very little riboflavin could be detected in the upper or lower parts of the small intestine although considerable quantities were present in the gizzard. Although this may be considered a crude test for determining the rate of movement of vitamin D_3 down the intestine, the evidence does suggest that administration of vitamin D_3 by stomach tube could lead to a delay in the localisation of the vitamin at its site of absorption in the intestine. Kodicek (1960) has suggested that the main site of absorption of vitamin D in the rat is the lower portion of the small intestine.

To eliminate this difficulty, 1,000 I.U. vitamin D_3 in propylene glycol were injected through the intestinal wall of the duodenum. After 2 or 4 hours, Ca^{45} was placed in isolated distal loops *in vivo*. The increases in plasma Ca^{45} levels obtained are shown in Figure 14 and indicate no effect of vitamin D_3 after 2 hours but a small increase following the 4 hour pretreatment with the vitamin.

Further experiments were designed in an attempt to show an

TABLE 4

THE INFLUENCE OF VITAMIN D_3 ON THE DEPOSITION OF Ca^{45} INTO BONE IN RELATION TO THE TIME AND ROUTE OF ADMINISTRATION

Vitamin D_3 was administered 2 hours before giving 2 mg $Ca^{45}Cl_2$ by stomach tube. Tibia was removed 3 hours later.

Route of administration	No. of birds	Amount D_3	Bone Ca^{45} c.p.m. X 10^{-4}/ashed tibia
-	4	0	3.5 ± 1.24
Intracardial	4	10,000 I.U.	4.2 ± 0.50
Oral	4	10,000 I.U.	4.1 ± 0.85

FIGURE 14

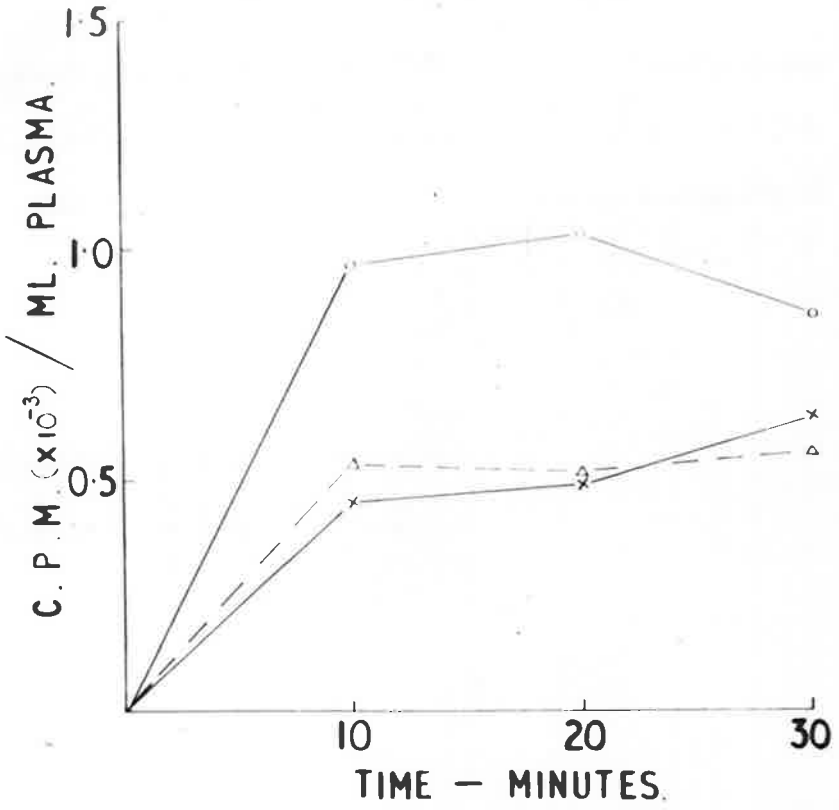
THE EFFECTIVENESS OF SHORT-TERM ADMINISTRATION OF VITAMIN D_3 ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* distal loops from rachitic or vitamin D_3-treated chicks. Vitamin D_3 1,000 I.U. in propylene glycol was injected into the duodenum. Each point is the mean of 4 birds.

4 hours after giving vitamin D_3

2 hours after giving vitamin D_3

rachitic control


1·5
1·0
0·5
C. P. M. (x10⁻³) / ML. PLASMA.
10
20
30
TIME — MINUTES.

immediate effect on calcium uptake. However, when vitamin D_3 (10,000 I.U.) in propylene glycol was added to $Ca^{45}Cl_2$ present in distal loops *in vivo* or to the mucosal fluid *in vitro* (Method E), there was no observable increase in calcium transport.

The influence of pH on calcium transport

Since the pH of the intestinal fluids might exert an influence on the solubility, and therefore indirectly on the absorption of calcium the pH of the duodenal and distal loop contents was measured in both rachitic and vitamin D_3-treated birds. During the surgical procedure, 0.1 ml. fluid was withdrawn by syringe from the respective loops and the pH measured with a Beckman single-drop electrode. The pH was found to be 6.9 increasing to 7.2 in the lower regions of the small intestine thus confirming the recent findings by Coates and Holdsworth (1961). There was no difference in pH following vitamin D_3-treatment.

The solubility of some calcium salts varies markedly with the pH of the environment. It would appear likely that a more acid medium would promote better absorption of calcium. Initially, attempts were made to study the influence of this factor on calcium absorption from preparations of intestine *in vivo* in the presence of phosphate buffer. However, difficulty was experienced with this medium as calcium phosphate precipitated after a short interval. Instead, 0.2 M citrate buffer together with 4 mg. calcium was inserted into *in vivo* duodenal loops. Coates and Holdsworth (1961) have demonstrated that citrate has no effect on the *in vivo* absorption of calcium in chicks. Figure 15 shows that there was no difference in plasma Ca^{45} levels when the loops contained medium at pH 4.0 or pH 6.0.

FIGURE 15

THE INFLUENCE OF THE pH OF THE MEDIUM ON THE UPTAKE OF CALCIUM FROM DUODENAL LOOPS

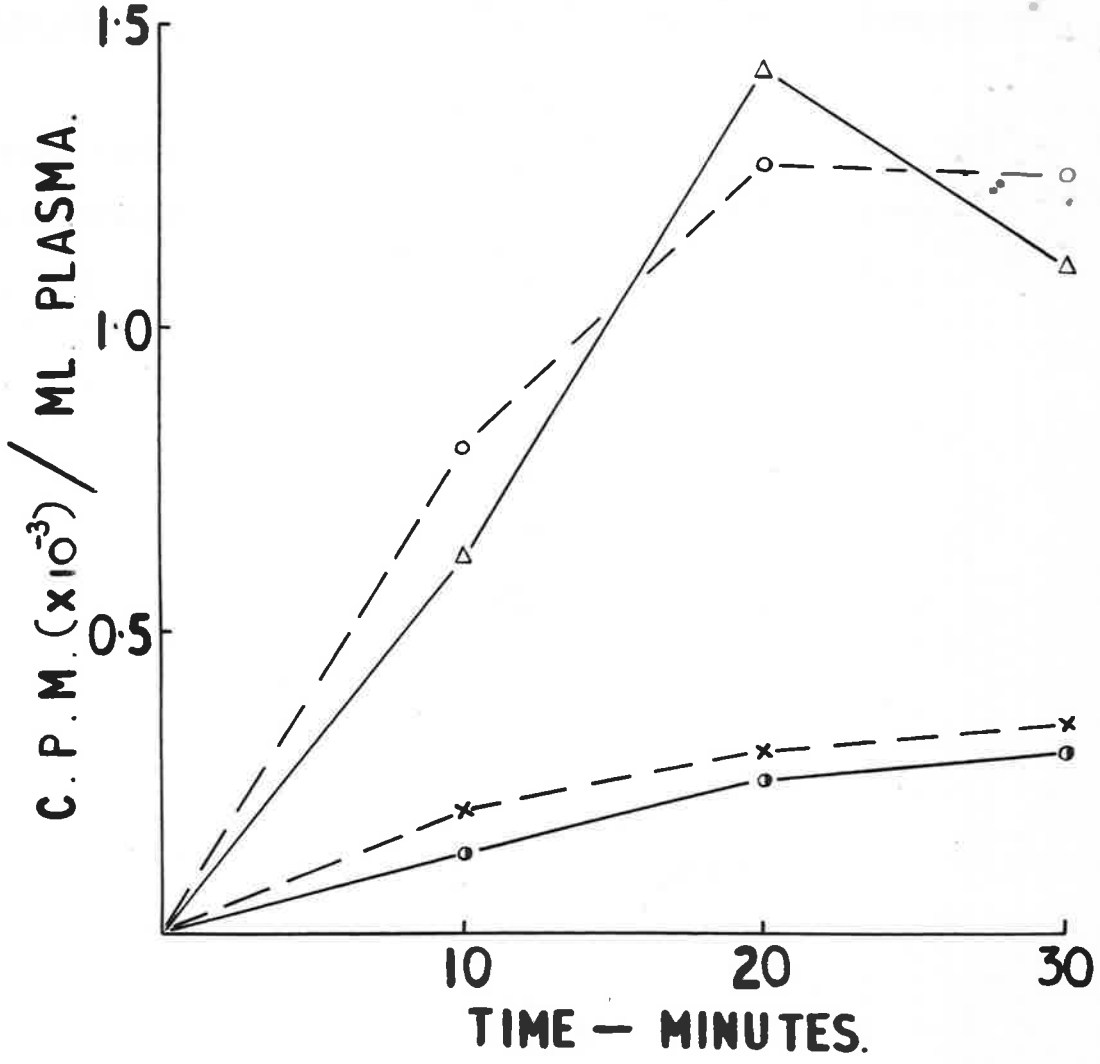
Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops from rachitic and vitamin D_3-treated chicks. Loops contained 4 mg. Ca^{45} in 0.2 M citrate buffer. Each point is the mean of 4 birds.

O— — — O vitamin D_3-treated pH 6.0

Δ——— Δ vitamin D_3-treated pH 4.0

×— —— × rachitic pH 6.0

◑——— ◑ rachitic pH 4.0


1·5
1·0
0·5
C. P. M. (x10⁻³) / ML. PLASMA.
10
20
30
TIME — MINUTES.

The influence of amino acids on calcium absorption

Wasserman, Comar and Nold (1956) have examined the effect of various amino acids on calcium absorption in the rat and have shown that lysine and arginine can promote a two-fold increase in mineral absorption. The influence of lysine appeared independent of the vitamin D effect as the combined effect of lysine and vitamin D could be attributed to the sum of the effects of the individual components. Further investigations on the effect of amino acids on calcium absorption revealed an interesting species difference (Wasserman, Comar, Schooley and Lengemann, 1957). Orally administered lysine and arginine did not influence the absorption of calcium when tested in the rachitic chick.

In view of these interesting findings, calcium absorption from *in vivo* duodenal loops of rachitic chicks was studied in the presence of 10 mg. L-lysine or 10 mg. L-arginine. Table 5 summarises the results of this experiment. There was no increase in the level of plasma Ca^{45} after administering these amino acids.

The effect of vitamin D_3 on the transport of other ions.

(a) Phosphate transport. The medium introduced into the duodenal loops *in vivo* was modified to include 1 mg. $P^{32}O_4$ (10 μc. P^{32}) and unlabelled calcium and the absorption of the phosphate ion was followed by counting the total P^{32} in whole blood. The results from rachitic and vitamin D_3-treated chicks are compared in Table 6. A small increase in the amount of P^{32} in the blood was observed. The residual P^{32} in the loop at the conclusion of the experiment was also counted, but no significant effect of vitamin D_3 could be detected. Normal values for calcium absorption were recorded during this experiment, being 16% for the rachitic

TABLE 5

THE INFLUENCE OF AMINO ACIDS ON CALCIUM ABSORPTION

Ca^{45} was measured in plasma after preparing duodenal loops *in vivo* from rachitic and vitamin D_3-treated chicks. 10 mg. amino acid was inserted together with $Ca^{45}Cl_2$ into loops prepared in rachitic chicks. Each value represents the mean of 6 birds.

Treatment	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins)		
	15	30	45
-	0.48	0.67	0.70
D_3	1.46	1.96	1.75
L-lysine	0.55	0.82	0.75
L-arginine	0.60	0.65	0.69

TABLE 6

THE INFLUENCE OF VITAMIN D_3 ON PHOSPHATE UPTAKE

The P^{32} content of whole blood was measured at intervals after inserting 1 mg. $P^{32}O_4$ into *in vivo* duodenal loops of rachitic and vitamin D_3-treated birds.

Treatment	No. of birds	P^{32} c.p.m. X 10^{-3}/ml. blood TIME (mins)			
		10	20	30	120
-	6	3.47	2.69	1.95	2.64
D_3	6	3.78	4.31	2.40	3.08

duodenal loop and 48% with loops from the vitamin D_3-treated group.

(b) Sodium transport. Attempts were made to study the absorption of sodium by the in vivo isolated loop technique (Method A). When however 1.0 ml. isotonic saline (labelled with Na^{22}) was placed into duodenal loops, absorption took place so rapidly that it was impossible to detect differences in uptake or to obtain reproducible results. For this reason duodenal loops were perfused in vivo from a reservoir containing 10 ml. isotonic saline containing Na^{22} (Method C). The data in Table 7 show that the transport of sodium was not affected by vitamin D_3. When Na^{22} was measured in the perfusion fluid after 45 minutes it was estimated that approximately 35% of the initial Na^{22} had been absorbed.

TABLE 7

THE INFLUENCE OF VITAMIN D_3 ON SODIUM TRANSPORT

The Na^{22} content of whole blood was measured after perfusing duodenal loops of rachitic and vitamin D_3 treated chicks with $Na^{22}Cl_2$ *in vivo*.

Treatment	No. of birds	Na^{22} c.p.m. X 10^{-3}/ml. blood TIME (mins)		
		10	20	45
-	12	1.12	1.94	6.04
D_3	12	1.30	2.02	6.11

PART III

THE INFLUENCE OF VITAMIN D_2 AND DIHYDROTACHYSTEROL ON CALCIUM TRANSPORT.

RESULTS

The influence of vitamin D_2 on calcium absorption was studied after giving a test dose of $Ca^{45}Cl_2$ to *in vivo* isolated duodenal loops from small intestine of rachitic and vitamin D-treated birds. The rise in level of circulating Ca^{45} in the blood subsequent to vitamin D-treatment is shown in Figure 16 and indicates that on an equal weight basis, vitamin D_2 in comparison to vitamin D_3 had only a slight effect on calcium uptake. Only with large unphysiological doses of vitamin D_2 could the plasma level of Ca^{45} be raised to a value similar to that obtained with 100 I.U. vitamin D_3.

The apparent lack of any striking increase in calcium uptake following treatment with 2.5 µg. vitamin D_2 would suggest that the relative inertness of this substance was associated with the structure of the side-chain. If this hypothesis were correct, then vitamin D_2 might well act as an antagonist to the functioning of vitamin D_3. When chicks received 250 µg. vitamin D_2 and 2.5 µg. vitamin D_3 together in oil 16 hours prior to the test, there was no significant alteration in the level of plasma Ca^{45} from that obtained with the group receiving the vitamin D_3-treatment alone.

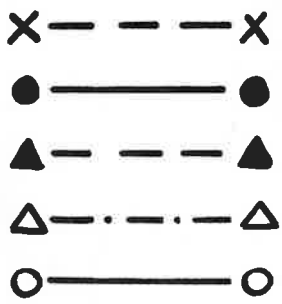
The influence of dihydrotachysterol on calcium absorption

Albright, Bloomberg, Drake and Sulkowitch (1938) have demonstrated an increased calcium absorption from the gut and an increased phosphorus excretion in the urine when patients suffering from hypoparathyroidism were treated with AT-10. In this respect, AT-10 is considered to have the same fundamental action as vitamin D. However,

FIGURE 16

THE INFLUENCE OF VITAMIN D_2 ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops. Each point is the mean of 4 birds in a typical experiment.



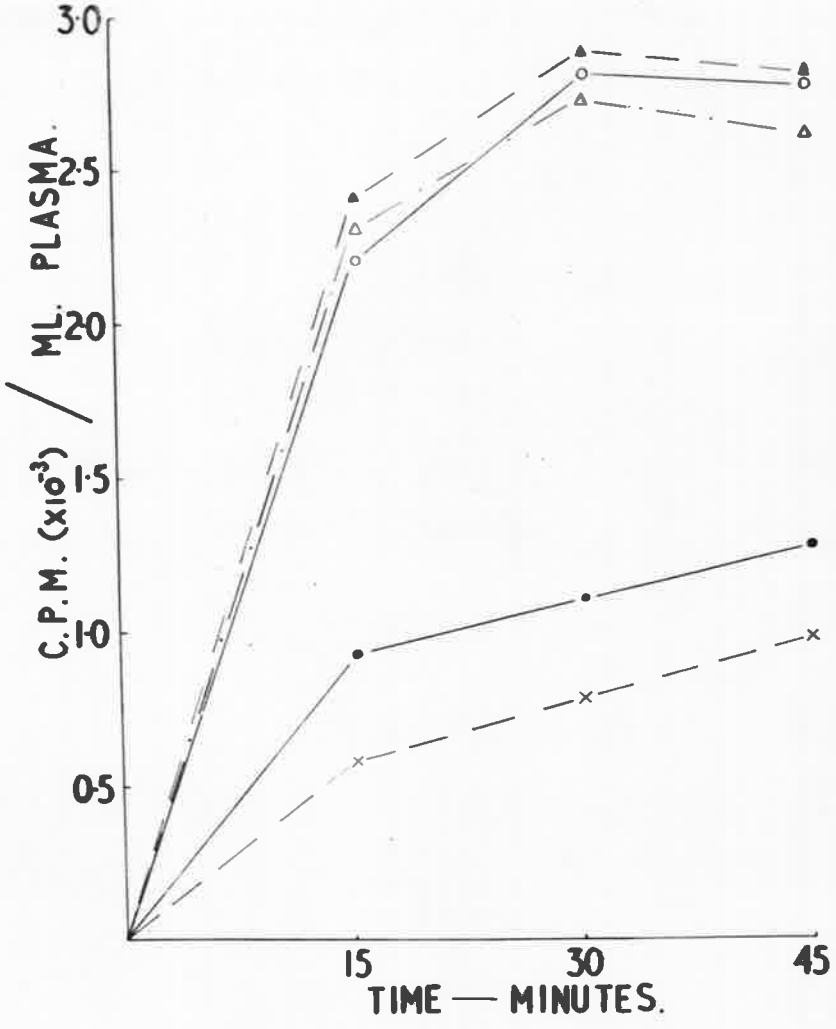
rachitic

vitamin D_2 (2.5 µg.)

vitamin D_2 (2.5 mg.)

vitamin D_2 (250 µg.), vitamin D_3 (2.5 µg.)

vitamin D_3 (2.5 µg.)


3·0
2·5
2·0
1·5
1·0
0·5
C.P.M. (x10³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

AT-10 in contrast to vitamin D, had very little antirachitic potency.

When AT-10_2 (2.5 µg) was given orally to rachitic chicks 16 hours before the experiment there was a marked increase in the plasma Ca^{45} level (Figure 17). The response to AT-10_2 however was not as great as with vitamin D_3 when comparison was made on an equal weight basis. When the dose level of AT-10_2 was increased to 10 µg. then plasma Ca^{45} levels were comparable to those obtained from chicks which had received 2.5 µg. vitamin D_3. No further increase in calcium absorption was observed when larger doses of AT-10_2 were administered. If the deposition of Ca^{45} in bone was taken as a measure of activity, the the results in Table 8 show that AT-10_2 was more active on a weight basis than vitamin D_3.

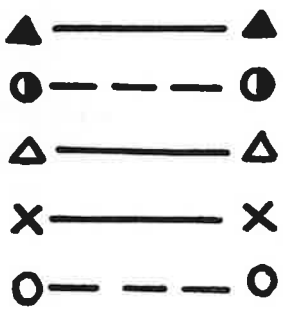
In earlier experiments it was noted that vitamin D_2 was relatively ineffective in raising plasma calcium levels in the chick. An explanation for this observation might be postulated on structural grounds. If the structure of the side-chain were of prime importance to the process of calcium transport, then AT-10_3 should be more active in this respect than AT-10_2. The previous experiments were carried out with a sample of AT-10_2 from Lights. To compare the effect of AT-10_2 and AT-10_3 on calcium absorption, crystalline samples of these compounds were obtained from Philips-Roxane and this material has been used in the following <u>in vivo</u> and <u>in vitro</u> experiments.

AT-10_3, when given by mouth 16 hours prior to studying calcium absorption from <u>in vivo</u> duodenal loops, was found to possess the same activity as AT-10_2 (Table 9). Both AT-10_2 and AT-10_3 were ineffective in increasing calcium uptake when given at a level of 2.5 µg. intra-

FIGURE 17

THE EFFECT OF DIHYDROTACHYSTEROL ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops. The number of birds is given in parenthesis.



rachitic (8)

2.5 µg. AT-10_2 (8)

10 µg. AT-10_2 (4)

100 µg. AT-10_2 (4)

2.5 µg. vitamin D_3 (8)

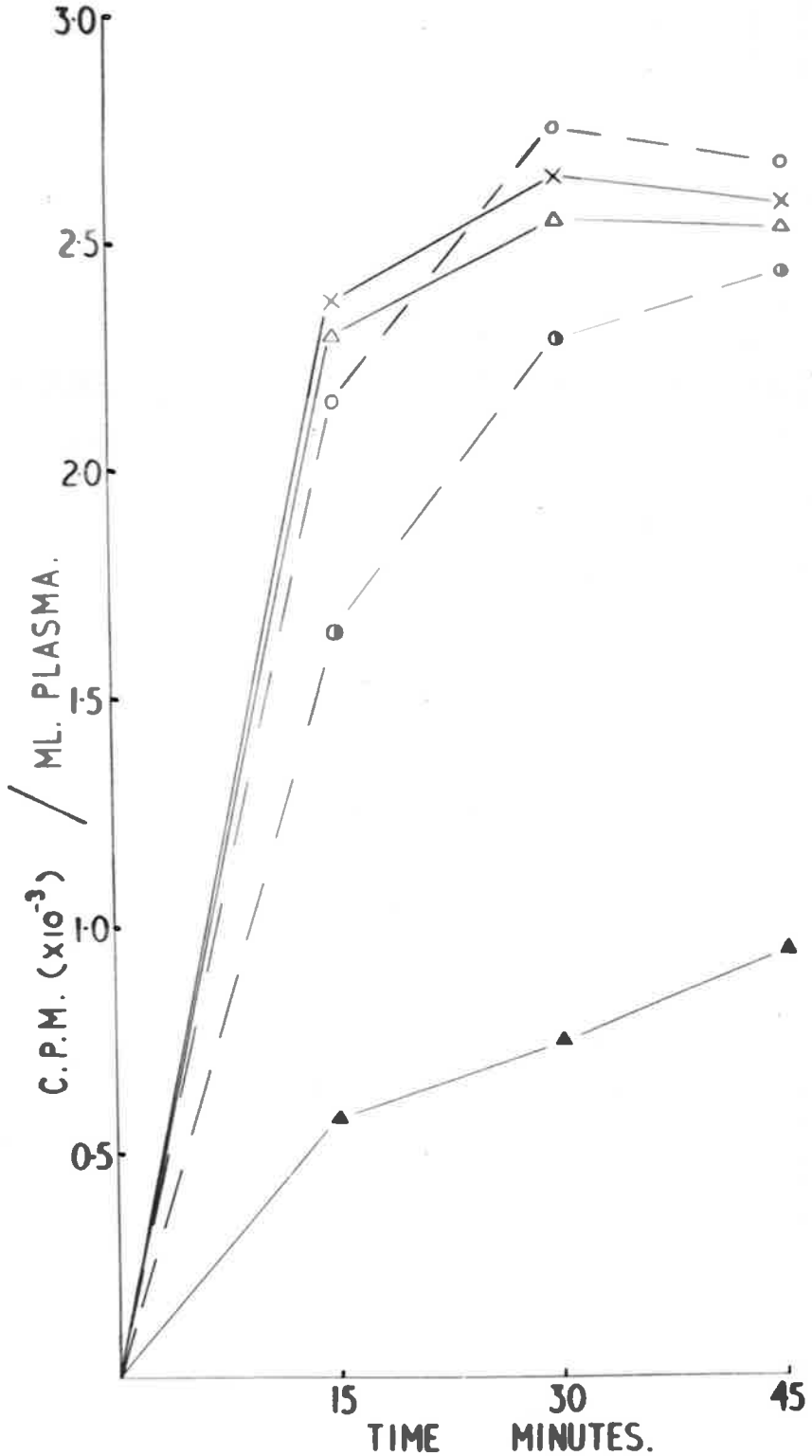

3·0
2·5
2·0
1·5
1·0
0·5
C.P.M. (x10⁻³) / ML. PLASMA.
15
30
45
TIME MINUTES.

TABLE 8

THE EFFECT OF AT-10_2 AND VITAMIN D_3 ON Ca^{45} DEPOSITION IN BONE

The substances were given by mouth 16 hours before preparing *in vivo* duodenal loops. Tibia were removed after 2 hours. The figures represent the values of 4 birds.

Treatment	Bone Ca^{45} c.p.m. X 10^{-4}/ashed tibia
-	2.45 ± 0.31
2.5 µg. D_3	6.50 ± 0.90
2.5 µg. AT-10_2	10.99 ± 1.70
10.0 µg. AT-10_2	12.83 ± 3.50

TABLE 9

A COMPARISON OF THE EFFECT OF VITAMIN D_3, AT-10_2 AND AT-10_3 ON CALCIUM ABSORPTION WITH THE ROUTE OF ADMINISTRATION

The compounds were given either orally in oil 16 hours before the experiment or intracardially in propylene glycol 1 hour prior to studying calcium absorption. Plasma Ca^{45} levels were measured after preparing *in vivo* duodenal loops.

Treatment	No. of birds	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins) 15	30	45
-	8	0.72	0.80	0.85
D_3 (orally)	8	2.50	3.00	2.83
AT-10_2 (orally)	4	2.20	2.35	2.40
AT-10_3 (orally)	4	2.33	2.42	2.45
AT-10_2 (intracardially)	4	0.76	0.83	0.77
AT-10_3 (intracardially)	4	0.63	0.86	0.96

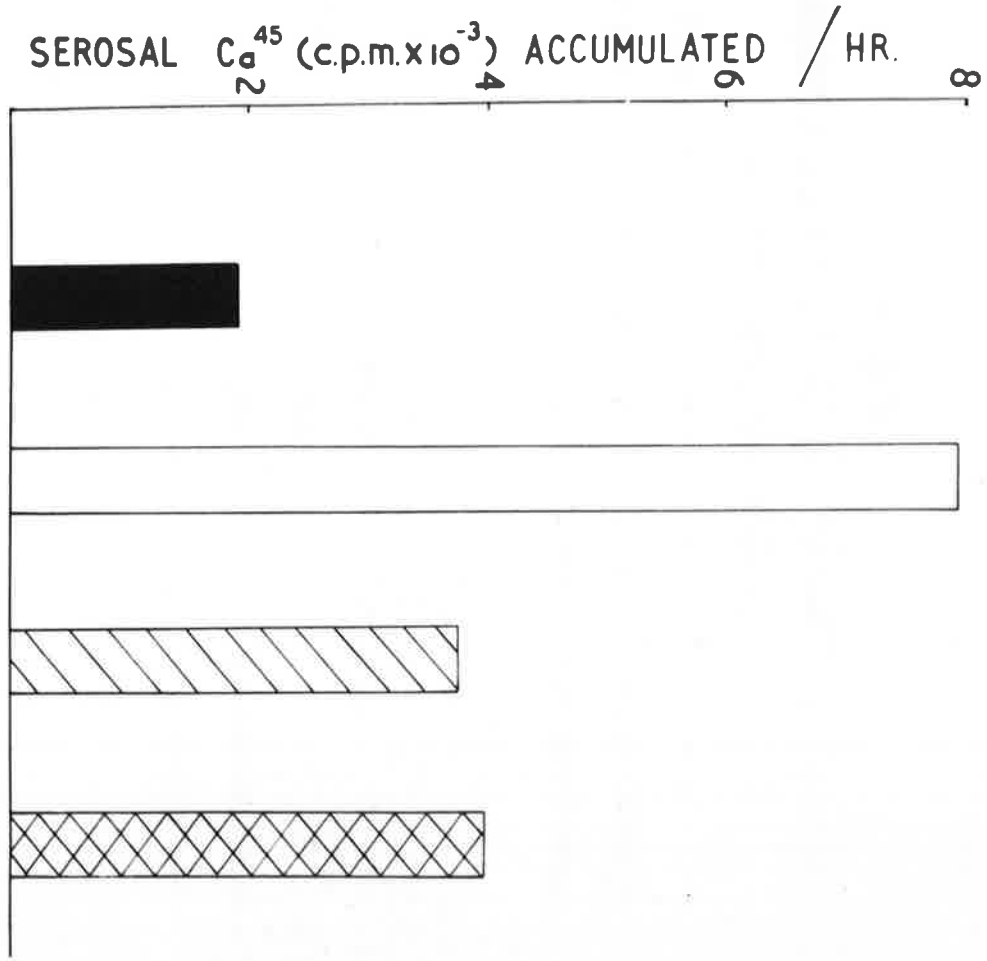
cardially 1 hour before inserting the $Ca^{45}Cl_2$ into the duodenal loop. *In vitro* studies (Method E) with everted distal segments of intestine obtained 16 hours after treatment of the chicks with 2.5 µg. $AT\text{-}10_2$ or 2.5 µg. $AT\text{-}10_3$ indicated that these substances were equally active in increasing calcium accumulation in the serosal fluid (Figure 18). However, the amount of Ca^{45} accumulated in the serosal fluid of gut sacs from these chicks showed only a twofold increase over the rachitic group, whereas a fourfold increase was obtained after treatment with vitamin D_3.

FIGURE 18

A COMPARISON OF THE EFFECTS OF AT-10_2, AT-10_3 AND VITAMIN D_3 ON CALCIUM TRANSPORT IN VITRO

Ca^{45} was measured in the serosal fluid of everted distal sacs of small intestine after 1 hour. Histograms represent the mean of the uptake from 6 sacs.

Blacked	rachitic
Blank	vitamin D_3
Diagonal hatched	AT-10_2
Cross hatched	AT-10_3


SEROSAL Ca^{45} (c.p.m.x 10^{-3}) ACCUMULATED / HR.
2
4
6
8

DISCUSSION

Several techniques have been described for studying calcium absorption from the small intestine. When given in small amounts to rachitic chicks 16 hours earlier, vitamin D_3 stimulated a marked increase in the amount of calcium absorbed from the small intestine both *in vivo* and *in vitro*. The ability to measure *in vivo* such changes in calcium transport enables a much more physiological approach to the manner in which this ion is transported, as both the blood supply and the absorptive mechanism remain intact.

It is obvious however, that the *in vivo* methods described are time consuming and this seriously limits the number of birds which may be examined in any one day. Rachitic and vitamin D_3-treated (100 I.U. - 16 hours) chicks were included in all experiments to establish accurately the D_3/rachitic ratio for the particular batch of chicks under study. Although this ratio itself was relatively constant, the level of radioactivity in the plasma varied according to the age and rachitic condition of the birds. Coates and Heldsworth (1961) have also pointed out the importance of feeding conditions during the experimental period.

When intestinal segments were perfused *in vivo* using a gas-flow technique (Method B), the results were not consistently reproducible. On occasions, small tissue particles accumulated at the glass pore of the vessel and caused irregular movement of fluid through the gut. There was also a marked tendency for frothing with this type of system. Both these hazards were however eliminated when circulation was controlled by a peristaltic pump. A constant flow was ensured and the absence of constrictions allowed the free passage of any solid material which might

be present in the perfusing fluid.

The importance of an *in vitro* technique for studies on calcium transport was manifest from an early stage. Beyond demonstrating the dependence on vitamin D, previous studies *in vivo* gave little information concerning the transport mechanism and the mode of action of vitamin D in this process. Attempts were therefore made to provide a system whereby isolated segments of intestine could be readily examined. Although it was possible to demonstrate an effect of vitamin D on calcium transport using a modified form of the apparatus described by Salomon and Johnson (1959) (Method D), differences previously noted with similar studies *in vivo* were not observed under these conditions. This loss of 'sensitivity' in the test system made preliminary studies with metabolic inhibitors extremely difficult to interpret.

During this phase of the work, Harrison and Harrison (1960) published a report describing a method for studying calcium transport in everted sacs of rat small intestine. When this technique (Method E) was applied to similar investigations in the chick it was found to be entirely satisfactory and reproducible results could be obtained.

Numerous investigators have studied the influence of vitamin D on the *in vivo* absorption of calcium in the rat (Greenberg, 1945; Harrison and Harrison, 1951; Linquist, 1952; Nicolaysen and Eeg-Larsen, 1953; Carlsson, 1954, a). Calcium uptake from the small intestine is increased, deposition of calcium in bones occurs and there is a marked rise in the plasma calcium level. More recently these *in vivo* results have been confirmed and extended by *in vitro* experiments using isolated everted segments of rat small intestine. In many respects, rats do not seem very suitable for work related to vitamin D and calcium metabolism

as rickets can only be produced if these animals are fed on a diet in which the Ca/P ratios have been grossly altered. Further, rickets so produced can be healed simply by restoring the ratios to those found in the natural diets even without vitamin D. Dietary factors are known to exert a major influence on the results of such experiments. Often the data may be difficult to interpret, due to this interplay of dietary effects and vitamin D action.

These factors may account for the variable results reported by Schachter and Rosen (1959). When calcium transport was studied in vitro with everted sacs of small intestine, Ca^{45} was transferred from the outside (mucosal) medium to the inside (serosal) medium. The Ca^{45} concentration ratio inside medium / outside medium was expressed as I/O. In their early experiments, rats were fed a vitamin D-free diet containing a low P, high Ca ratio and these animals developed rickets in 14 days. The final I/O for control animals varied from 5.2 - 3.2 and active transport could only be shown to occur in the proximal one-fifth section of the small intestine. However, a more recent investigation (Kimberg, Schachter and Schenker, 1961) indicates that when rats were fed a low Ca, high P diet, rickets was not observed within the same time period, the I/O was 2.0 and active transport could be demonstrated in all parts of the small intestine. Schachter, Dowdle and Schenker (1960 b) have also emphasised the great variation in results obtained with everted gut sacs prepared from rat small intestine. These authors state that "the gut-sac preparation is limited in its usefulness by the variability in calcium transport among gut sacs from different rats and from different segments of the intestine in the same animal."

Many of these difficulties are eliminated when chicks are used for experimental studies of this nature, as was recently shown by Gershoff and Hegsted (1956). Further, the duodenal loop of the chick is of uniform size and allows reproducible lengths of tissue to be studied. The chicks used for the present investigations became rachitic after 18 days on a diet containing approximately 0.8% Ca and 0.4% P, and this diet was complete with the exception of vitamin D. The experiments described in this chapter indicate the ability of the rachitic chick to respond to a low level of vitamin D_3 (100 I.U.). Coates and Holdsworth (1961) have recently shown that calcium absorption can be restored to levels approaching that in normal birds 16 hours after giving 100 I.U. vitamin D_3, and is in contrast to the large unphysiological amounts of vitamin D_2 normally used in rat experiments.

Calcium absorption was observed to take place in the small intestine and the action of vitamin D could be demonstrated by both *in vivo* and *in vitro* techniques. This is the first direct evidence that vitamin D_3 can influence calcium transport by *in vitro* preparations of chick small intestine in a manner similar to that observed *in vivo*.

The *in vivo* absorption of calcium from a loop of chick small intestine was studied by measuring the disappearance of calcium from the loop or, by the appearance of Ca^{45} in the plasma or by counting the Ca^{45} in bone ash at the conclusion of the experiment. With distal loops from rachitic chicks only 8% of the initial 4 mg. calcium was absorbed but this was increased seven-fold to 56% when 100 I.U. vitamin D_3 was given 16 hours earlier. With duodenal loops the increase recorded was only three-fold (16% to 48%). It was interesting to note, however, that

the vitamin D_3-treated distal loop gave only 17% better absorption than similarly treated duodenal segments. This finding is in agreement with the work of Coates and Holdsworth (1961) who have suggested that calcium absorption takes place along the entire length of the small intestine. Similar increases were found when Ca^{45} was measured in plasma. The absorbed Ca^{45} was also deposited in bone giving a D_3/rachitic ratio of 5/1 when using distal loops.

The transport of calcium obtained with segments of intestine *in vitro* is qualitatively similar to the reported findings *in vivo*. The amount of calcium transported was assessed to be the amount appearing in the serosal fluid of the everted loop since this is more comparable to measuring serum calcium *in vivo*. At the usual dosage of 100 I.U. vitamin D_3 16 hours before the experiment the ratio D_3/rachitic in distal loops was 3.5/1. When 2,000 I.U. vitamin D_3 was given over a period of 48 hours in an experiment designed to measure the maximum amount of calcium that accumulated in the tissue and serosal fluid, the ratio D_3/rachitic was increased to 7.6/1. The greatest transport was obtained with distal loops, smaller amounts being transported by the middle and duodenal segments respectively. Schachter and Rosen (1959) and Schachter, Dowdle and Schenker (1960 a) have reported similar findings for the hamster: everted gut sacs of hamster ileum and duodenum transferred the calcium against concentration gradients but in contrast to rats, calcium transport in the hamster was greater in the ileum than in the duodenum.

The rather constant amount of calcium bound by the mucosal cells from each of the three equal segments of intestine suggests that calcium may be saturating a combination of tissue-binding material (e.g. protein)

and a calcium carrier system. The increase in the binding of the calcium after giving vitamin D_3 may be due to the increase in both these mechanisms but more probably to an increase in the carrier system. A more rapid rate of calcium turnover seems to occur with the distal portion of the intestine, leading to a greater transfer of calcium to the serosal fluid.

Both *in vivo* and *in vitro* the greatest effect of vitamin D_3 has been obtained with distal loops and for many purposes the use of this tissue for studying calcium absorption was the more sensitive technique. If only *in vitro* studies had been made it would have been easy to conclude that in the chick the greatest amount of absorption takes place in the distal part of the small intestine. However, the *in vivo* results show that the duodenal portion from vitamin D_3-treated chicks is only slightly less effective than the distal segment and suggests that under natural conditions calcium absorption takes place effectively along the entire small intestine. Although it has been established in the rat that the proximal fifth of the small intestine is very largely responsible for calcium uptake *in vitro*, Kimberg et al. (1961) have shown that calcium absorption can take place along the entire small intestine.

Attempts to show an immediate effect of vitamin D_3 on calcium absorption by having the vitamin present as an aqueous colloidal suspension either in loops in the live bird or added to the mucosal medium *in vitro* were unsuccessful. Coates and Holdsworth (1961) and Dowdle et al. (1960) with similar experiments have also been unable to obtain any response. The shortest time period for which it was possible to show a positive effect with vitamin D_3 was 4 hours. A recent report by Harrison and

Harrison (1960) indicates that in rats given 1,000 I.U. vitamin D_2 there is a time lag of 4 to 10 hours before an effect can be seen. It is suggested however that this time lag may be shortened when a much larger dose e.g. 50,000 I.U. is given.

The intracardial method of administration ensured large circulating levels of vitamin D_3 in the blood and would thus eliminate any time lag due to slow absorption. That there is a time lag before the vitamin can be shown to influence calcium transport would suggest that either there is a change in the mucosal cells themselves or that vitamin D_3 is converted into a more active form or indeed that both these transformations are necessary.

Other factors were considered that could have an important bearing on calcium uptake. The pH of the duodenal and distal loop was measured and confirms the recent findings of Coates and Holdsworth (1961). The solubility of calcium phosphate is very low under physiological conditions of pH and ionic strength and thus it would appear that unless its solubility is altered by some unknown factor, the calcium must remain insoluble as the phosphate. The presence of a more acid medium in the duodenal loop did not influence the absorption of calcium.

Lehmann and Pollack (1941-42) have suggested from indirect evidence that amino acids can promote calcium absorption by virtue of an increased solubility of calcium in their presence. Wasserman, Comar, Schooley and Lengemann (1957) however, do not believe that such a concept is tenable in view of the distinct species difference in the ability of these substances to promote calcium absorption. They point out that the general environmental conditions in the intestinal tract of both the

rat and the chick appear to be similar (Dukes, 1955).

The addition of L-lysine or L-arginine to loops containing $Ca^{45}Cl_2$ did not increase the plasma Ca^{45} levels in rachitic chicks. These results are in agreement with the findings of Wasserman et al. (1957) and are in direct contrast to results from similar studies with rats (Wasserman, Comar and Nold, 1956). The latter workers have suggested that the response in rats is brought about by the influence of these amino acids on the metabolism of the intestine.

It should be pointed out however that under the conditions of the test in these present studies the true effect of the amino acid may not be readily demonstrable as the calcium is already present in a soluble form. Amino acids present under natural conditions could perhaps influence the solubility and hence the absorption of calcium in the manner suggested by Lehmann and Pollack (1941-42).

The vitamin D_3-effect appeared to be relatively specific to the transport of calcium ions. Sodium transport *in vivo* was not influenced by vitamin D_3 and this finding gives support to results obtained from a similar type of *in vitro* study (Harrison and Harrison, 1960). Similarly, phosphate transport *in vivo* was not greatly altered after vitamin D_3-treatment. Although there was possibly a small effect of vitamin D_3 in raising the plasma phosphorus levels, this was negligible in comparison to the effect of vitamin D_3 on calcium absorption under similar conditions, so that vitamin D_3 does not appear to be primarily involved in the transport of the phosphate ion. Nicolaysen and Eeg-Larsen (1953) also suggest that any effect of vitamin D on phosphorus absorption must be considered secondary to its effect on calcium transport.

Carlsson (1954 a) has observed from *in vivo* studies an improved absorption of phosphorus under the influence of vitamin D, but he believes that this effect is probably indirect and that it depends upon an increased absorption of calcium. In more detailed studies *in vitro*, Harrison and Harrison (1961) have also concluded that vitamin D can influence phosphate absorption, providing calcium ions are present.

The value of AT-10 in promoting calcium absorption and in curing rickets has become a controversial issue. Albright, Bloomberg, Drake and Sulkowitch (1938) and Albright, Sulkowitch and Bloomberg (1939) from clinical observations, could find no evidence to suggest that AT-10 could cure rickets, despite the observed increase in calcium absorption. These workers postulated that the primary effect of AT-10 was not on calcium absorption but rather on phosphorus excretion (in contrast to the vitamin D effect), which they suggest, could explain the difference in antirachitic potency observed between these two compounds.

Shohl, Fan and Farber (1939) have interpreted their results as being in agreement with this hypothesis. These workers found that AT-10 could provide a more effective cure for rickets in rats if the phosphorus intake was high and the calcium was low. Correll and Wise (1942) demonstrated that large doses of AT-10 could heal rickets in rats and chicks and that the effectiveness of AT-10 in chicks was much greater than vitamin D_2. In more recent investigations, Carlsson and Lindquist (1955 b) compared the value of AT-10 and vitamin D in curing rickets in rats and they found that the dose of AT-10 required to cure rickets was much higher than vitamin D_2.

Dowdle et al. (1960) have compared the effectiveness of vitamin

D_2, vitamin D_3 and AT-10 on calcium transport *in vitro*, using everted sacs of rat small intestine. AT-10 increased the amount of calcium transported above that shown by vitamin D_2 and vitamin D_3. The present investigations with chicks also demonstrate the effectiveness of AT-10 in increasing calcium transport both *in vivo* and *in vitro*. In a review on calcium metabolism, Nicolaysen and Eeg-Larsen (1953) have stated that AT-10 has less effect than vitamin D on calcium absorption. The present findings suggest however that on an equal weight basis, AT-10 is only slightly less effective than vitamin D_3 in promoting calcium absorption. However, in relation to calcium deposition in bone AT-10 would appear much more active than vitamin D_3.

Vitamin D_2 was found to have only a small percentage of the activity of vitamin D_3. This confirms a previous report by Bills (1935). The structural difference between these two vitamins lies in the side-chain at C_{17} (Figure 1): AT-10_2 (side-chain like vitamin D_2) and AT-10_3 (side-chain like vitamin D_3) are both equally as active in increasing the absorption of calcium in the chick. These substances are only slightly less active than vitamin D_3 when tested *in vivo*. However, the results suggest that caution is necessary when interpreting the effect of AT-10 on calcium metabolism. Ca^{45} absorbed under the influence of this compound can be deposited in the bone (Table 8) and yet the same batch of AT-10_2 showed an activity of less than 1 I.U. when tested for anti-rachitic potency in chicks (Dr. M.E. Coates, N.I.R.D., Shinfield, personal communication).

It does appear unlikely that the influence of the side-chain is functionally important to the transport of calcium. However, the change

of vitamin D_2 to an active calcium carrier may be slow in the chick due to the difference in the side-chain between vitamin D_2 and vitamin D_3 (Figure 1). The rat however would appear to be capable of converting either side-chain as both vitamin D_2 and vitamin D_3 are equally as effective in this animal.

SUMMARY

1. *In vivo* and *in vitro* systems for observing calcium transport by chick small intestine have been described. All systems demonstrate a marked increase in calcium transport in rachitic birds given 100 I.U. vitamin D_3 16 hours previously.

2. The small intestine has been examined for the site of maximum absorption of calcium. Calcium transport occurred along the entire length of the small intestine, but it was found that the distal loop was a more sensitive system for demonstrating the influence of vitamin D_3 on calcium uptake.

3. 16 hours were required between the oral administration of vitamin D_3 and the giving of $Ca^{45}Cl_2$ in order to stimulate a maximum response. There was no evidence to suggest vitamin D_3 could produce an immediate increase in calcium uptake. The minimum time required appeared to be 4 hours. Large doses of vitamin D_3 given intravenously were without effect in the short period they were studied.

4. Calcium absorption from duodenal loops was unaffected by a more acid pH than that found under natural conditions.

5. L-lysine and L-arginine did not promote a more efficient uptake of calcium from duodenal loops of rachitic chicks.

6. The transport of sodium and phosphate ions in rachitic chicks was not affected by the presence of vitamin D_3.

7. When vitamin D_2 (2.5 µg.) was given at the same dose level as vitamin D_3 (2.5 µg.) there was little stimulation of calcium absorption. Much larger amounts (2.5 mg.) were necessary to produce a plasma calcium level comparable to the vitamin D_3-treated birds.

8. Both AT-10_2 and AT-10_3 were equally as effective in promoting calcium absorption both *in vivo* and *in vitro*. When compared to vitamin D_3 on a weight basis, these compounds were not as active in promoting absorption of Ca^{45} as measured by plasma Ca^{45} levels. However, AT-10 in comparison to vitamin D_3, exerted a greater influence on the deposition of Ca^{45} in bone tibia.

CHAPTER 3

CALCIUM METABOLISM IN RELATION TO VITAMIN D_3 AND ADRENAL FUNCTION

Introduction

Materials and Methods

Results

Discussion

Summary

CHAPTER 3

CALCIUM METABOLISM IN RELATION TO VITAMIN D_3 AND ADRENAL FUNCTION.

INTRODUCTION

The conclusions of the previous chapter, and the work of other investigators (Lindquist, 1952; Harrison and Harrison, 1960; Coates and Holdsworth, 1961; Schachter et al. 1961) suggest that before vitamin D_3 can produce an effect on calcium absorption it must first of all undergo a change to a more active form. The greatest amount of vitamin D activity is normally found in the liver (Kodicek, 1956 a) and thus it would seem a logical place to explore for the modified forms of the vitamin.

However, Cruickshank, Kodicek and Armitage (1954) have demonstrated that, 48 hours after the giving of an oral dose of vitamin D to rats, the adrenal glands contained this substance in a concentration approaching that found in the liver. Raoul and Gounelle (1958) have also reported finding 80% of the initial dose administered to rats in the adrenal gland within 30 minutes. More recent work by Kodicek, Cruickshank and Ashby (1960) using C^{14}-labelled vitamin D, did not support this claim, as only 1% of the vitamin could be located in the adrenals after 20 minutes. These reports suggested that the adrenal gland could play an important role in regulating calcium metabolism. Hypertrophy of the adrenal cortex has been observed when birds were maintained for long periods on calcium deficient diets (Urist, 1959).

The effects of cortisone on calcium metabolism have not yet been thoroughly investigated. Cortisone is known to increase calcium excretion in the urine and faeces (Irving, 1957). In patients suffering

a hypercalcaemia from sarcoidosis or from a hypervitaminotic condition caused by toxic doses of vitamin D, Anderson, Dent, Philpot and Harper (1954) found that cortisone could restore to normal these elevated levels of serum calcium. Fanconi (1956) has also reported on the beneficial effect of cortisone in cases of hypercalcaemia. Thus, cortisone is regarded as a specific antidote for vitamin D poisoning (Dent, 1956) and Anderson et al (1954) have postulated that cortisone acts as an antimetabolite to vitamin D. Rats, however, do not show a similar response to cortisone treatment, as was recently pointed out by Kodicek (1956 b), who in collaboration with Cruickshank could find no evidence to suggest that cortisone was acting as an antimetabolite (Cruickshank and Kodicek, 1958). These latter workers suggested that cortisone may act by altering the metabolism of certain tissues on which the vitamin appears to exert its influence.

Evidence is presented in this chapter which offers an explanation for the action of cortisol and a number of other steroids in regulating calcium balance. The results may best be interpreted by assuming that the adrenal cortex is concerned in the change of vitamin D_3 to its active carrier form. Although no attempt has been made to critically examine the influence of the parathyroids on calcium metabolism it was of interest, in view of the important interrelationship existing between the parathyroid and the adrenal gland, to study the influence of a parathyroid extract on calcium absorption. These findings have also been included in this chapter.

MATERIALS AND METHODS

The influence of adrenal steroids on calcium metabolism was examined using *in vivo* isolated loops (Method A) and *in vitro* everted sacs (Method E) of small intestine. Ca^{45} was measured in plasma as previously described (P 27, (a) i). Unless otherwise stated, chicks receiving vitamin D_3-treatment were given an oral dose of 100 I.U. vitamin D_3 16 hours prior to preparing the segment of intestine. Chicks receiving only vitamin D_3-treatment as described above are referred to as the vitamin D_3 control group.

Short-term administration of steroids

The steroid to be studied was given as an intracardial injection (0.15 ml.) 1 hour before the calcium test.

Long-term administration of steroids

Rachitic chicks were given an intramuscular injection (0.15 ml.) of the steroid daily for 3 days. On the evening of the third day, the usual pretreatment with 100 I.U. vitamin D_3 was given. Calcium uptake from the intestinal segment was studied on the fourth day after commencing steroid treatment.

o,p-DDD

1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (o,p-DDD) was obtained from the Aldrich Chemical Co. Inc., Wisconsin, U.S.A. For administration, gelatin capsules containing 50 mg. crystalline o,p-DDD were given by mouth over the required time period. Control groups received empty capsules under the same conditions.

Steroids

Crystalline cortisol and 11-deoxycorticosterone (DOC) were

obtained from L. Light and Co. Ltd. (Colnbrook, England). The following steroids were kindly provided by Dr. Folkers of Merck, Sharp and Dohme Research Laboratory, New Jersey, U.S.A.: aldosterone-21-acetate, 9α-fluoro-1,4-pregnadiene 11β, 16α, 17α, 21-tetrol-3,20-dione (Δ^1-9α-fluoro-16α-hydroxy-hydrocortisone); 9α-fluoro,4-pregnene-11β,17α, 21-triol-3,20-dione, 21-acetate; (9α-fluorohydrocortisone acetate); 1,4-pregnadiene-11β,17α-21-triol-3,20-dione (Δ^1-cortisol). For intracardial or intramuscular injection, the steroids were dissolved in ethanol/propylene glycol mixture, the final concentration of ethanol being less than 0.5%. Control groups not receiving steroid were treated with the appropriate amount of vehicle.

Eschatin

A standardised extract of the adrenal cortex was a product of Parke, Davis and Co., Detroit, Michigan.

Parathyroid extract

The product used was a standardised solution of parathyroid extract from beef glands prepared for injection by Eli Lilly and Co. Indianapolis, U.S.A.: its activity was not less than 100 U.S.P. parathyroid units per ml.

RESULTS

The influence of an adrenal extract on calcium absorption

Eschatin - a purified natural extract of the adrenal cortex, was tested for its effect on calcium absorption in the chick. An intracardial injection of 0.1 ml. Eschatin was given 1 hour before the $Ca^{45}Cl_2$ was placed in *in vivo* duodenal loops of rachitic and vitamin D_3-treated chicks. The calcium absorption was studied by following the level of circulating plasma calcium at varying time intervals and Figure 19 presents these results. The adrenal extract stimulated a small increase in plasma calcium above the level shown by the vitamin D_3 control group. A relatively larger stimulation of uptake was observed in rachitic chicks.

Cortisol and vitamin D

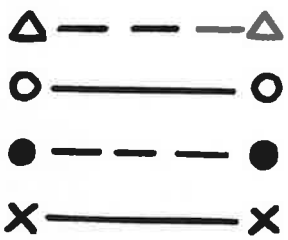
Calcium absorption studies with cortisol indicated that the results were profoundly affected by the conditions of pretreatment with cortisol before the application of the *in vivo* calcium uptake test. It has, therefore, been convenient to group the results according to the time period of pretreatment with cortisol.

Short-term administration. Cortisol (500 µg.) was given intracardially to both rachitic and vitamin D_3-treated chicks 1 hour before inserting the $Ca^{45}Cl_2$ into *in vivo* duodenal loops. A marked increase in the level of circulating calcium occurred with the cortisol group receiving in addition vitamin D_3, as compared with the control group receiving the vitamin alone (Figure 20). The level of plasma calcium from the rachitic group remained unchanged following the cortisol injection: these values have been omitted from the figure to avoid unnecessary confusion with the

FIGURE 19

THE EFFECT OF AN ADRENAL EXTRACT ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing in vivo duodenal loops. 0.1 ml. adrenal extract (Eschatin) was given intracardially. Each point is the mean of 7 birds.



Eschatin treated vitamin D_3-dosed chicks

vitamin D_3-dosed controls

Eschatin-treated rachitic chicks

rachitic controls.

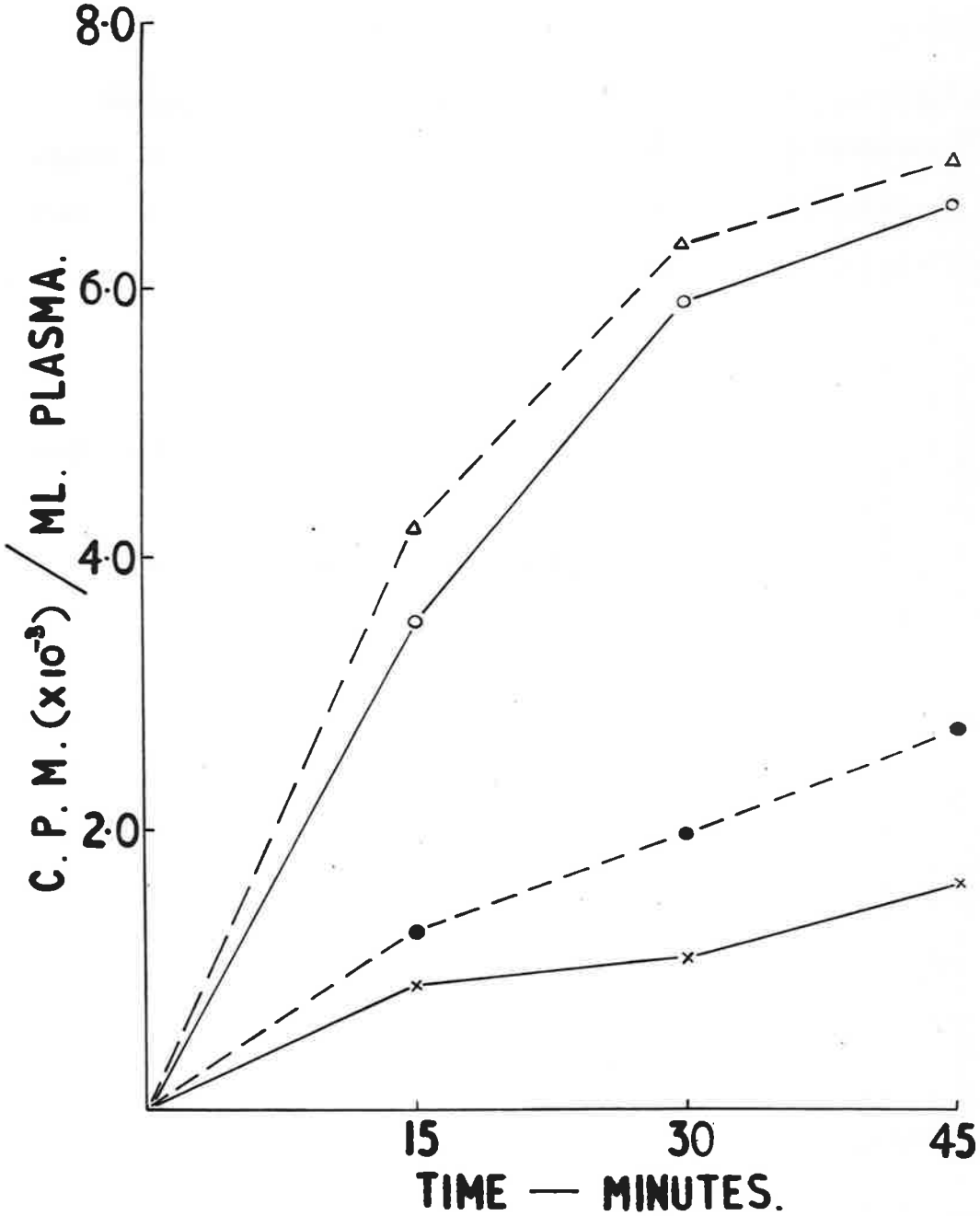
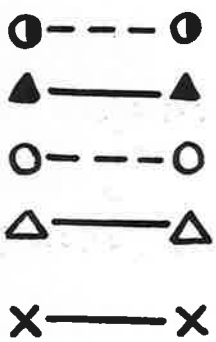

8·0
6·0
4·0
2·0
C. P. M. (x10³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

FIGURE 20

INFLUENCE OF SHORT OR LONG-TERM TREATMENT WITH CORTISOL ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops. With short-term treatment, chicks were given 500 µg. intracardially. Long-term treatment was by intramuscular injection of 2 mg./day for 3 days. Number of birds given in parenthesis. Not included on figure are the effects of cortisol on rachitic chicks for short or long-term, since these results were not significantly different from the rachitic controls.



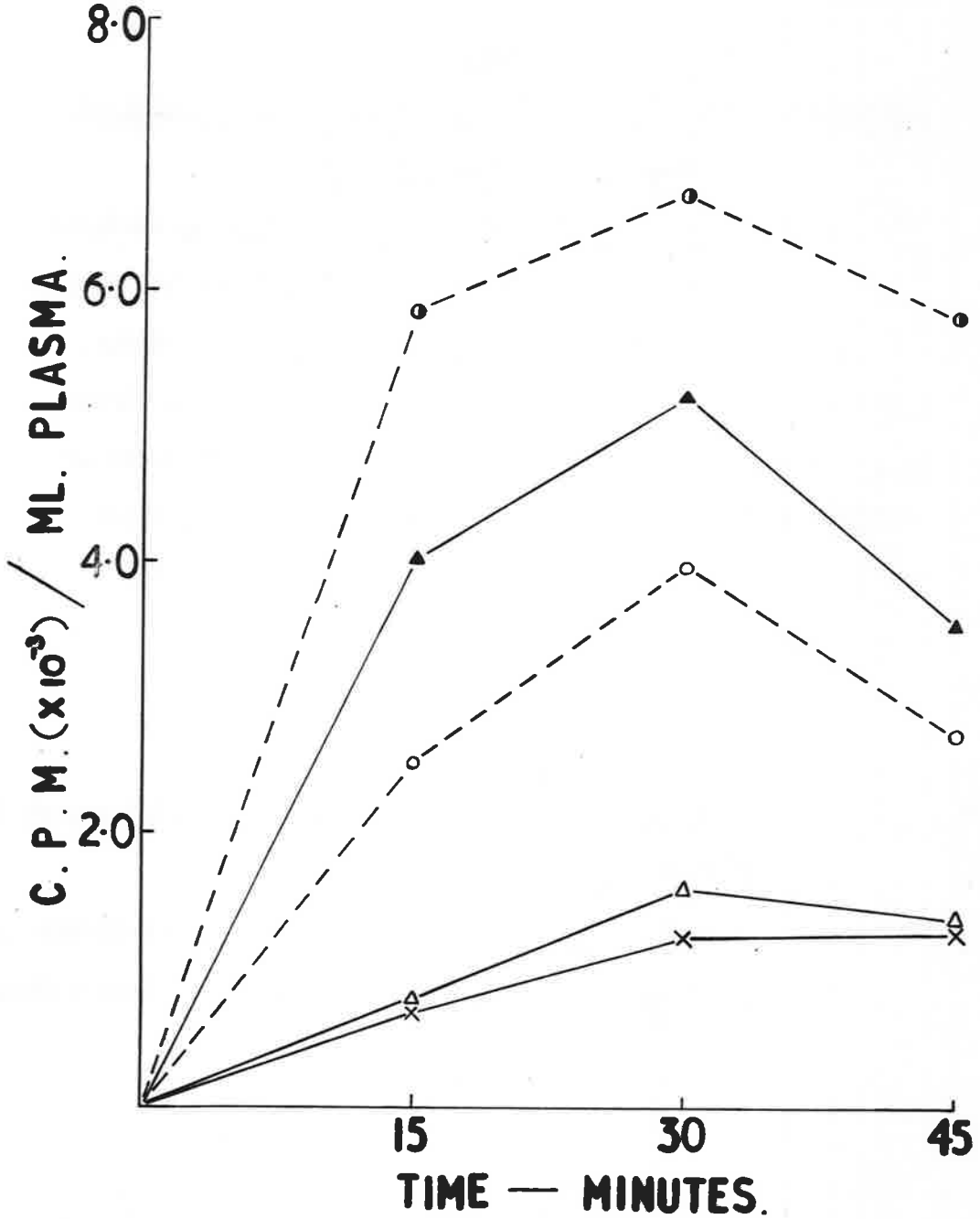
cortisol short-term with vitamin D_3-treated chicks (13)

vitamin D_3-treated controls (23)

cortisol long-term with vitamin D_3-treated chicks (10)

cortisol and vitamin D_3 given together 1 hour before the test (4)

rachitic controls (23)


8·0
6·0
4·0
2·0
C. P. M. (x 10³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

legends. A small, but possibly significant increase in plasma Ca^{45} was recorded when both cortisol and vitamin D_3 were given intracardially to rachitic chicks 1 hour before inserting the $Ca^{45}Cl_2$ solution into the duodenal loop.

Further data in support of these findings are summarised in Table 10. Vitamin D_3 stimulated a threefold increase in calcium absorption, 52% of the initial calcium being absorbed from the segments after 2 hours while in the untreated rachitic group the corresponding value was 17%. However, in the presence of cortisol, calcium uptake was increased to 62% with the vitamin D_3-treated group. There was no significant effect on the absorption from rachitic chicks when cortisol alone was given intracardially. Ca^{45} measurements from ashed tibia confirmed that deposition of Ca^{45} was greater in vitamin D_3-treated chicks receiving cortisol (32.7×10^4 c.p.m.) than in the vitamin D_3 control group (27.8×10^4 c.p.m.). Again, a small increase in both the absorption of Ca^{45} from the loop and also the deposition in the bone can be shown for chicks given vitamin D_3 and cortisol at the same time.

The influence of short-term cortisol treatment on vitamin D_3-treated birds was also studied with *in vitro* everted distal sacs of small intestine. The results which are summarised in Table 11 indicate that cortisol had no significant effect on calcium transport *in vitro* when given in short-term experiments.

Long-term administration. Rachitic chicks received 2 mg. cortisol daily for 3 days in the manner described above and calcium absorption was then studied with *in vivo* isolated duodenal loops. A marked decrease in circulating calcium was observed in the plasma of chicks treated with cortisol as compared with the usual plasma calcium

TABLE 10

THE INFLUENCE OF SHORT- AND LONG-TERM CORTISOL TREATMENT ON CALCIUM ABSORPTION AND DEPOSITION IN BONE

In short-term experiments 500 µg. cortisol was given intracardially 1 hour before the calcium test. Long-term treatment consisted of an intramuscular daily dose of 2 mg. cortisol over a period of 3 days. The vitamin D_3-treated chicks, with the exception of that group receiving both cortisol and vitamin D_3 1 hour before the $Ca^{45}Cl_2$, were given 100 I.U. vitamin D_3 16 hours before the test. Control groups received only the vehicle. Uptake and deposition of Ca^{45} was measured 2 hours after commencing the test. The results of each group are expressed as the mean of 4 birds.

Route of cortisol administration	Time period of cortisol dosage	D_3 (I.U.)	% calcium absorption	Bone deposition c.p.m. X 10^{-4} ashed tibia
-	-	-	17	10.5
-	-	100	52	27.8
Intracardial	1 hr.	-	16.5	9.7
Intracardial	1 hr.	100	62	32.7
Intramuscular	3 days	-	16.0	11.1
Intramuscular	3 days	100	45	23.7
Intracardial	1 hr. together with D_3	100	22	14.0

TABLE 11

THE EFFECT OF SHORT-TERM CORTISOL TREATMENT ON CALCIUM TRANSPORT IN VITRO

Cortisol (500 µg.) was injected intracardially into vitamin D_3-treated birds 1 hour before measuring calcium transport. Everted sacs of distal small intestine were prepared from rachitic and vitamin D_3-treated chicks.

Treatment	No. of birds	Ca^{45} Serosal c.p.m. X 10^{-3} accumulated/hour
-	4	3.551
D_3	6	8.740
D_3 + cortisol	6	8.424

level obtained with chicks similarly treated with vitamin D_3 but receiving no cortisol (Figure 20). Cortisol given under these conditions had no effect on calcium absorption in the rachitic control group.

These findings are emphasised in Table 10 where the percentage absorption of calcium is recorded. Calcium absorption, evaluated as 52% for the vitamin D_3 control group, has been reduced to 45% by long-term cortisol treatment. Similarly, deposition of Ca^{45} in bone has decreased from 27.8 X 10^4 c.p.m. with vitamin D_3 alone to 23.7 X 10^4 c.p.m. from chicks receiving both cortisol and vitamin D_3.

Effect of mineralocorticoids

The possibility that steroids possessing mineralocorticoid activity can influence the metabolism of cations other than sodium and potassium has never been fully established. It was of interest, then, to study the influence of 11-deoxycorticosterone, aldosterone-21-acetate and several synthetic steroids in promoting calcium absorption from the small intestine of the chick.

11-deoxycorticosterone

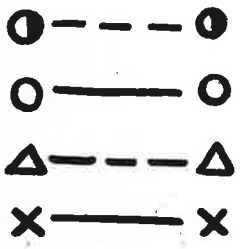
The effects of this steroid are also dependent upon the conditions used in respect to its administration.

Short-term administration. When 500 µg. DOC was given intracardially 1 hour before inserting $Ca^{45}Cl_2$ into *in vivo* distal loops of vitamin D_3-treated chicks there was a considerable increase in the plasma Ca^{45} level above that shown by the vitamin D_3 control group (Figure 21). When DOC was given in a similar manner to the untreated rachitic chicks, no increase in circulating Ca^{45} was observed. These findings are similar to those obtained from studies with short-term cortisol administration.

FIGURE 21

THE INFLUENCE OF SHORT-TERM TREATMENT WITH 11-DEOXYCORTICOSTERONE ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* distal loops. 500 µg. DOC was given intracardially. Each point is the mean of 8 birds.

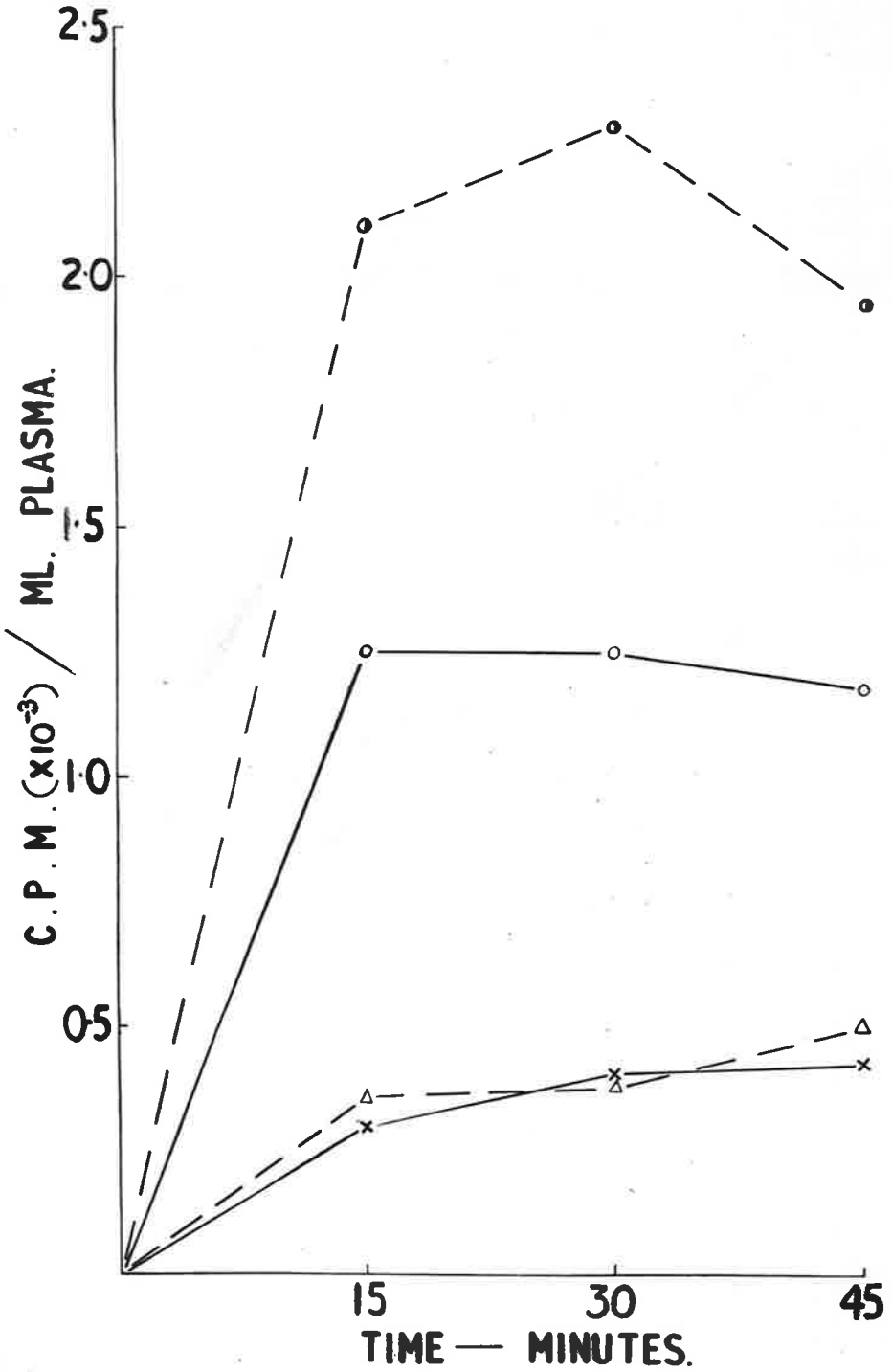


DOC treatment of vitamin D_3-treated chicks

vitamin D_3-treated controls

DOC treatment of rachitic chicks

rachitic controls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2·0
1·5
1·0
0·5
C. P. M. (x10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

Long-term administration. An intramuscular dose of 2 mg. DOC daily for 3 days followed by the usual vitamin D_3 pretreatment and the *in vivo* distal loop technique decreased the plasma Ca^{45} concentration from that given by the vitamin D_3 control group, as indicated in Figure 22. The results suggest, then, that DOC has no influence as a mineralocorticoid in raising plasma calcium levels. The relationship of this steroid to calcium transport would suggest that it is involved in a much more widespread phenomenon, possibly implicating the adrenal glands with the vitamin D_3 effect.

Aldosterone

This steroid has been recognised as one of the most potent substances involved in the regulation of mineral metabolism. It was demonstrated from earlier experiments that the transport of sodium across the small intestine was not under the influence of vitamin D_3. As aldosterone is known to exert such a profound effect on sodium balance in the body it was of interest to observe the effect of this steroid on sodium transport when given intracardially 1 hour before perfusing *in vivo* duodenal segments of small intestine (Method C). Table 12 demonstrates that aldosterone does not influence the intestinal absorption of sodium in either rachitic or vitamin D_3-treated chicks.

That mineralocorticoids are themselves incapable of increasing calcium absorption is evident from the results of an *in vivo* experiment using isolated distal loops from both rachitic and vitamin D_3-treated groups (Figure 23). Aldosterone (250 µg.) given as an intracardial injection 1 hour prior to placing $Ca^{45}Cl_2$ into the loop, showed no significant effect in increasing plasma calcium levels of either the

FIGURE 22

THE INFLUENCE OF LONG-TERM TREATMENT WITH 11-DEOXYCORTICOSTERONE ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* distal loops. 2 mg. DOC was given by intramuscular injection daily for 3 days. Each point represents the mean of 5 birds.

O——— O vitamin D_3-treated controls

X— — — X DOC treatment of vitamin D_3-treated chicks

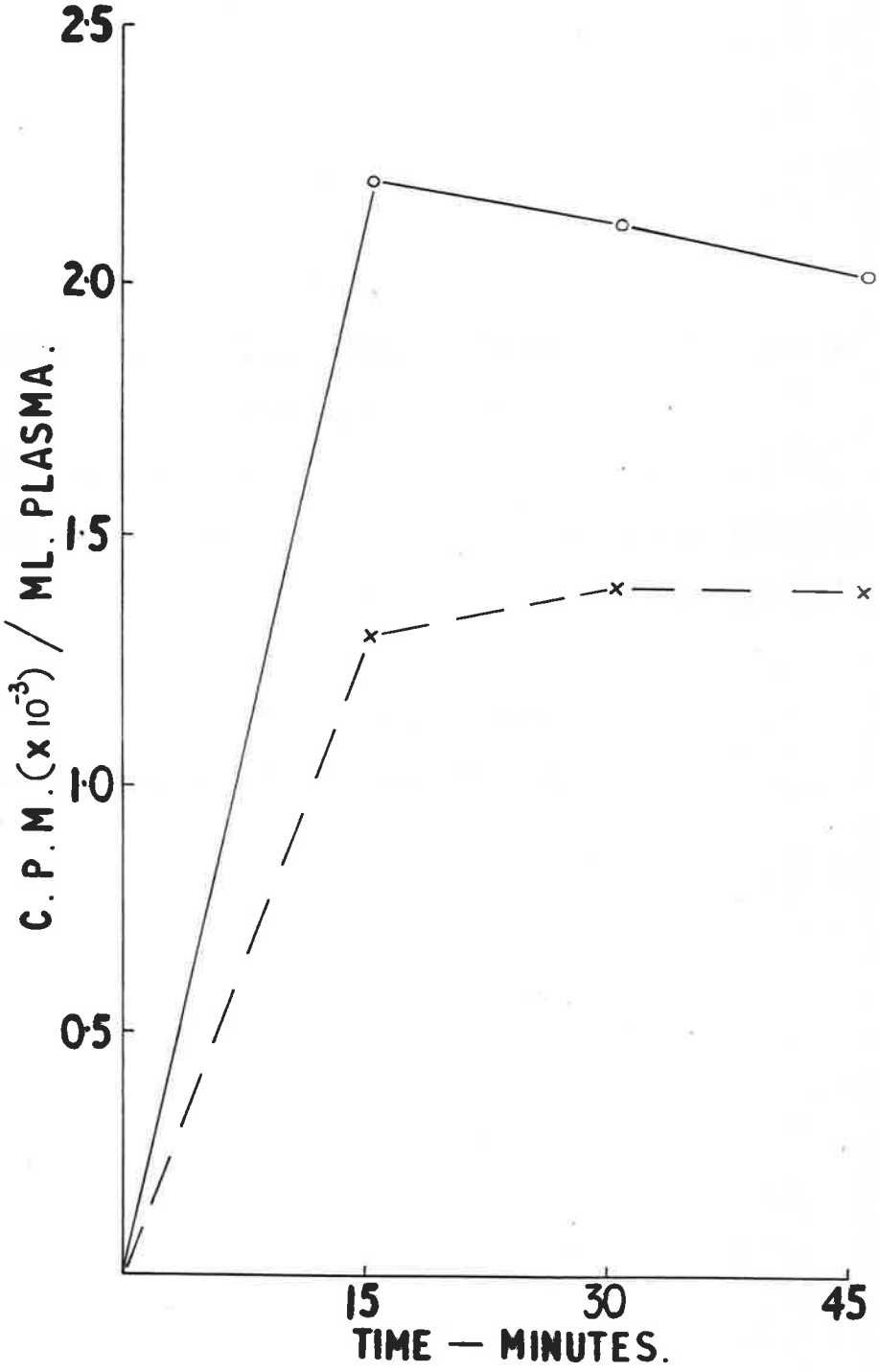

2·5
2·0
1·5
1·0
0·5
C. P. M. (x 10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

TABLE 12

THE INFLUENCE OF ALDOSTERONE ON SODIUM TRANSPORT IN RACHITIC AND VITAMIN D_3-TREATED CHICKS

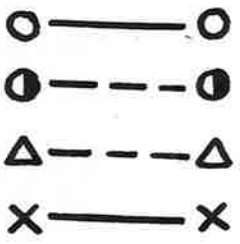
Aldosterone (250 µg.) was given intracardially 1 hour before the experiment. Na^{22} was measured in blood after perfusing duodenal loops from rachitic and vitamin D_3-treated chicks *in vivo* (Method C). Each value is the mean of 8 birds.

Treatment	Na^{22} c.p.m. X 10^{-3}/ml. blood TIME (mins)		
	10	20	45
-	1.93	3.89	10.07
Aldosterone	1.89	4.16	9.97
D_3	1.85	4.04	9.07
D_3 + aldosterone	1.75	4.15	9.35

FIGURE 23

THE EFFECT OF ALDOSTERONE ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* distal loops. 250 µg. aldosterone acetate was given intracardially. Each point is the mean of 8 birds.

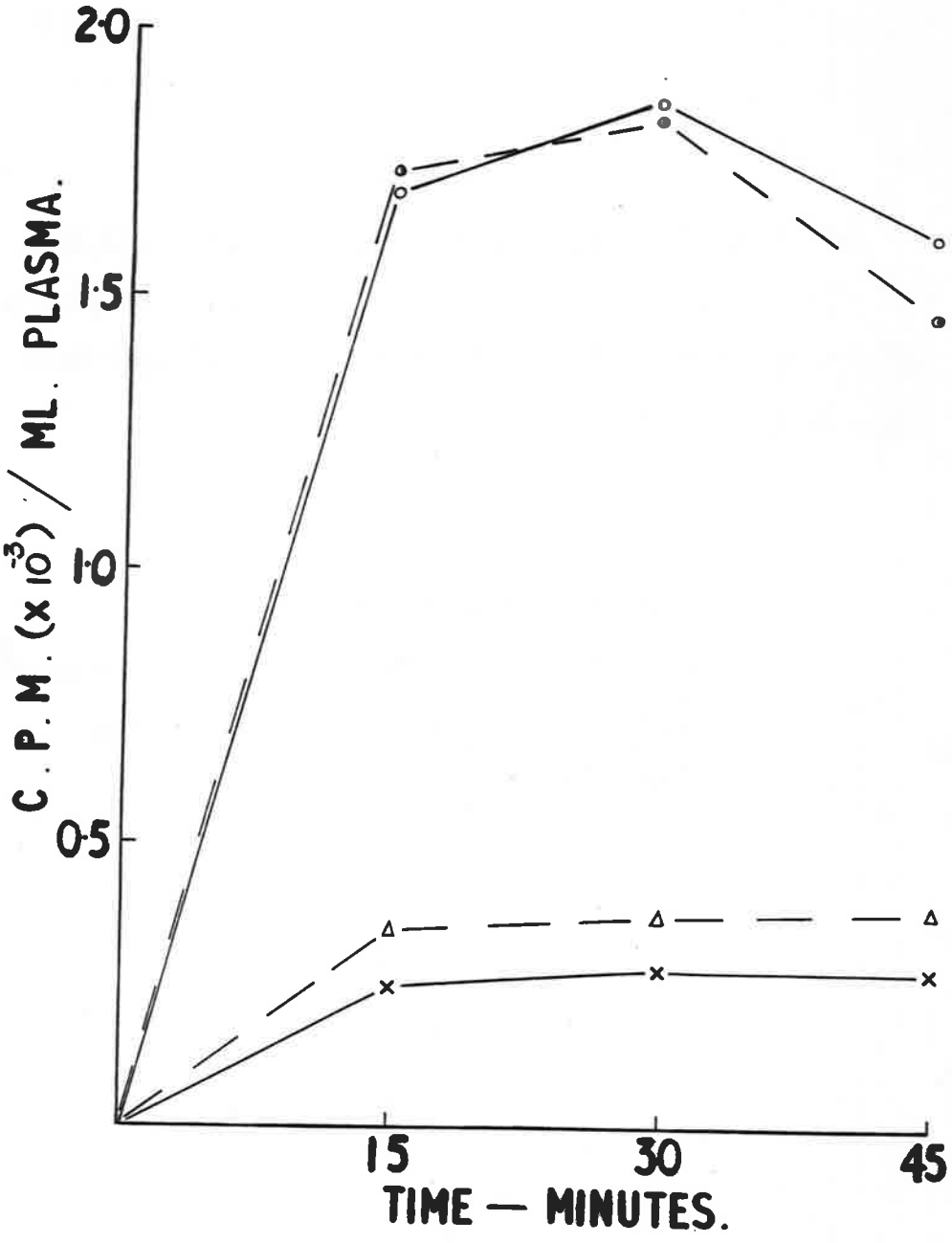


○——○ vitamin D_3-treated controls

◑- - -◑ aldosterone treatment of vitamin D_3-treated chicks

△- - -△ aldosterone treatment of rachitic chicks

×——× rachitic controls


2·0
1·5
1·0
0·5
C. P. M. (x 10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

vitamin D_3 control group or the untreated rachitic group. A similar experiment, but using only 50 µg. aldosterone, also showed no effect. Further evidence to substantiate these findings is presented in Table 13. Ca^{45} deposition in bone tibia was not influenced by the presence of aldosterone. Similarly, residual calcium present in the gut after 2 hours was unchanged from the control levels following aldosterone treatment.

Synthetic steroids

Of recent years many new synthetic steroids have been prepared and examined for their usefulness with regard to gluco- or mineralocorticoid activity. Three of these steroids have been studied for their effect on calcium absorption from the small intestine of the chick. 0.5 mg. of the respective steroid was given by intracardial injection 1 hour before inserting $Ca^{45}Cl_2$ into *in vivo* isolated distal loops and recording plasma Ca^{45} levels at varying time intervals. Figure 24 summarises the effect of Δ^1-cortisol, 9α-fluorohydrocortisone acetate and Δ^1-9α-fluoro-16α-hydroxyhydrocortisone in altering the plasma calcium level of vitamin D_3-treated chicks. Δ^1-cortisol was the only steroid which stimulated an increase in plasma calcium above that shown by the vitamin D_3 control group.

The influence of parathyroid hormone on calcium absorption from the small intestine

The effect of the parathyroid gland in raising the concentration of plasma calcium has been adequately described in the literature (McLean and Urist, 1955; Neuman and Neuman, 1958; Fourman, 1960). Following the action of parathyroid hormone calcium can be made available as the

TABLE 13

THE INFLUENCE OF ALDOSTERONE ON THE ABSORPTION AND DEPOSITION IN BONE OF CALCIUM IN RACHITIC AND VITAMIN D_3-TREATED CHICKS

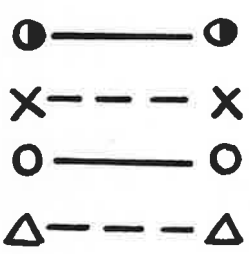
Treated birds were given 100 I.U. vitamin D_3 16 hours prior to the calcium test. Aldosterone (250 µg.) was given intracardially 1 hour before the test. Uptake and deposition of calcium was measured after 2 hours from *in vivo* distal loops containing 4 mg. calcium. Each value is the mean of 4 birds.

Treatment	% calcium absorption	Bone Ca^{45} c.p.m. X 10^{-4} / ashed tibia
-	7	5.31
Aldosterone	7	5.38
D_3	56	24.90
D_3 + aldosterone	54	25.70

FIGURE 24

THE INFLUENCE OF SYNTHETIC STEROIDS ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing in vivo distal loops of vitamin D_3-treated chicks. Each point is the mean of 4 birds. Chicks were given 500 µg. of the steroid by intracardial injection.

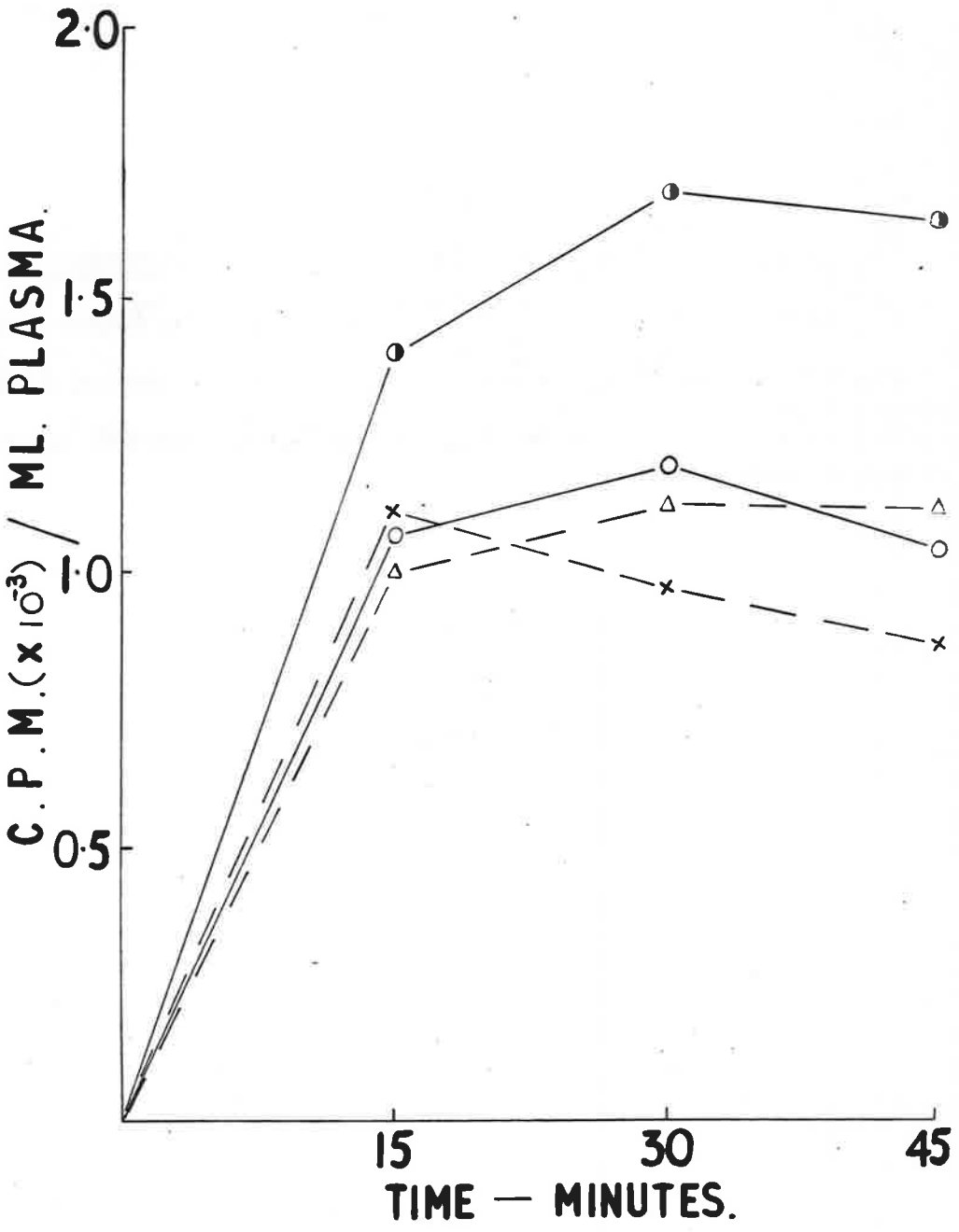


Δ^1-cortisol

9α-fluorocortisol acetate

vitamin D_3 controls

Δ^1-9α-fluoro-16-hydroxycortisol


2·0
1·5
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C.P.M.(x 10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

result of a bone resorption phenomenon. There is no real evidence to suggest however that the increased calcium levels observed are the direct result of an increased calcium absorption from the gastrointestinal tract. In view of the present studies which have clearly demonstrated the importance of adrenal function for vitamin D_3 action, and also from a consideration of the interrelationship existing between the parathyroid and adrenal gland, calcium absorption from the small intestine was measured following the giving of a parathyroid extract.

Rachitic and vitamin D_3-treated chicks received parathyroid extract (0.2 ml.) by intracardial injection 1 hour before inserting $Ca^{45}Cl_2$ into in vivo isolated duodenal loops. Ca^{45} present in the plasma was measured and Figure 25 presents these findings. When plasma Ca^{45} levels of birds treated with parathyroid extract were compared with their respective control groups there was no significant difference in the amount of radioactivity present. An analysis of the Ca^{45} present in ashed bone tibia demonstrated that the extract had not influenced the deposition of calcium in the bone (Table 14). Further data, also presented in this Table, confirmed that there was no increase in the amount of calcium absorbed from the gut following treatment with the extract.

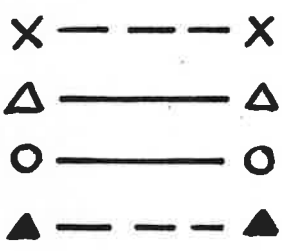
Inhibition of the vitamin D_3 effect

In the light of the experimental data gained from the studies with the various adrenal steroids it was considered necessary to examine more closely the possibility that an active form of vitamin D_3 was being produced by the adrenal gland. To study this aspect o,p-DDD, a known specific inhibitor of adrenal cortical function, was used as a means to determine whether vitamin D_3 could still influence calcium absorption to

FIGURE 25

THE INFLUENCE OF A PARATHYROID EXTRACT ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops. Parathyroid extract (0.2 ml.) was given intracardially. Each point is the mean of 4 birds.



rachitic

rachitic given parathyroid extract

vitamin D_3-treated

vitamin D_3-treated given parathyroid extract

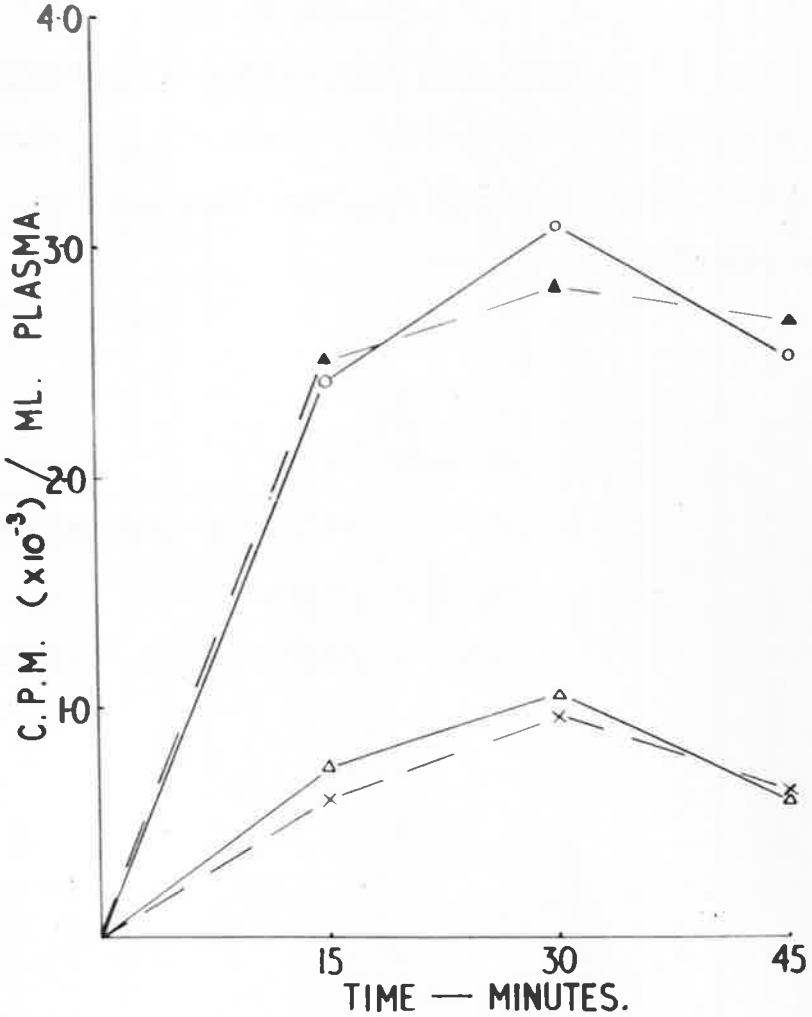

4·0
30
20
10
C.P.M. (x10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

TABLE 14

THE INFLUENCE OF PARATHYROID EXTRACT ON CALCIUM ABSORPTION

Parathyroid extract was injected intracardially 1 hour before the addition of $Ca^{45}Cl_2$ to the loop. Absorption and deposition of calcium was measured 2 hours after preparing *in vivo* duodenal loops. Values represent the mean ± standard error of the mean for 4 chicks.

Treatment	% calcium absorption	Bone Ca^{45} c.p.m. X 10^{-4}/ ashed tibia
-	15.5	9.7 ± 1.9
Parathyroid extract	17.0	9.5 ± 0.7
D_3	51.0	33.9 ± 2.3
D_3 + parathyroid extract	51.0	31.2 ± 4.1

the same extent even if the adrenal function were impaired. Capsules containing o,p-DDD were fed daily to rachitic chicks for a period of 3 days in the manner outlined under Methods. Each chick then received 100 I.U. vitamin D_3 16 hours before performing the calcium absorption test with *in vivo* isolated distal loops. The data from one such experiment are presented in Figure 26 and have been confirmed on two other occasions. After 30 minutes the plasma Ca^{45} level given by the vitamin D_3 control group was five times greater than the value obtained for the rachitic group. However, those chicks which received o,p-DDD treatment in addition to vitamin D_3 showed a complete inability to raise the calcium level above that shown by the untreated rachitic control group. Table 15 emphasises still further the magnitude of this inhibition. Of the initial calcium present, 56% was absorbed by the vitamin D_3 control group and only 7% of the calcium was taken up by chicks receiving the o,p-DDD treatment. This compares favourably with 6% absorption from the loops of untreated rachitic chicks.

Similar studies with o,p-DDD were also carried out *in vitro* using everted sacs. Although not producing such a dramatic inhibition of calcium transport, nevertheless o,p-DDD reduced the normal level of transport shown by the positive control group by as much as 60% (Table 16).

Histological examination of the adrenal gland

That the inhibition of calcium transport following o,p-DDD treatment is associated with adrenal function is strongly suggested from a histological examination of adrenal slices. The inhibitor was fed for 3 days and after giving a single dose of 100 I.U. vitamin D_3 overnight the adrenal glands were carefully dissected free and immediately

FIGURE 26

THE INHIBITION OF THE VITAMIN D_3 EFFECT BY o,p-DDD

Ca^{45} was measured in plasma after preparing *in vivo* distal loops in rachitic and vitamin D_3-treated chicks. 50 mg. o,p-DDD was given by mouth on each of 3 days. Vitamin D_3-treated chicks received 100 I.U. by stomach tube on the third day 16 hours before the calcium test. The number of birds is given in parenthesis.

O - - - O vitamin D_3-treated controls (8)

Δ ——— Δ o,p-DDD treatment of vitamin D_3-treated chicks (10)

O ——— O o,p-DDD treatment of rachitic chicks (4)

X - - - X rachitic controls (8)

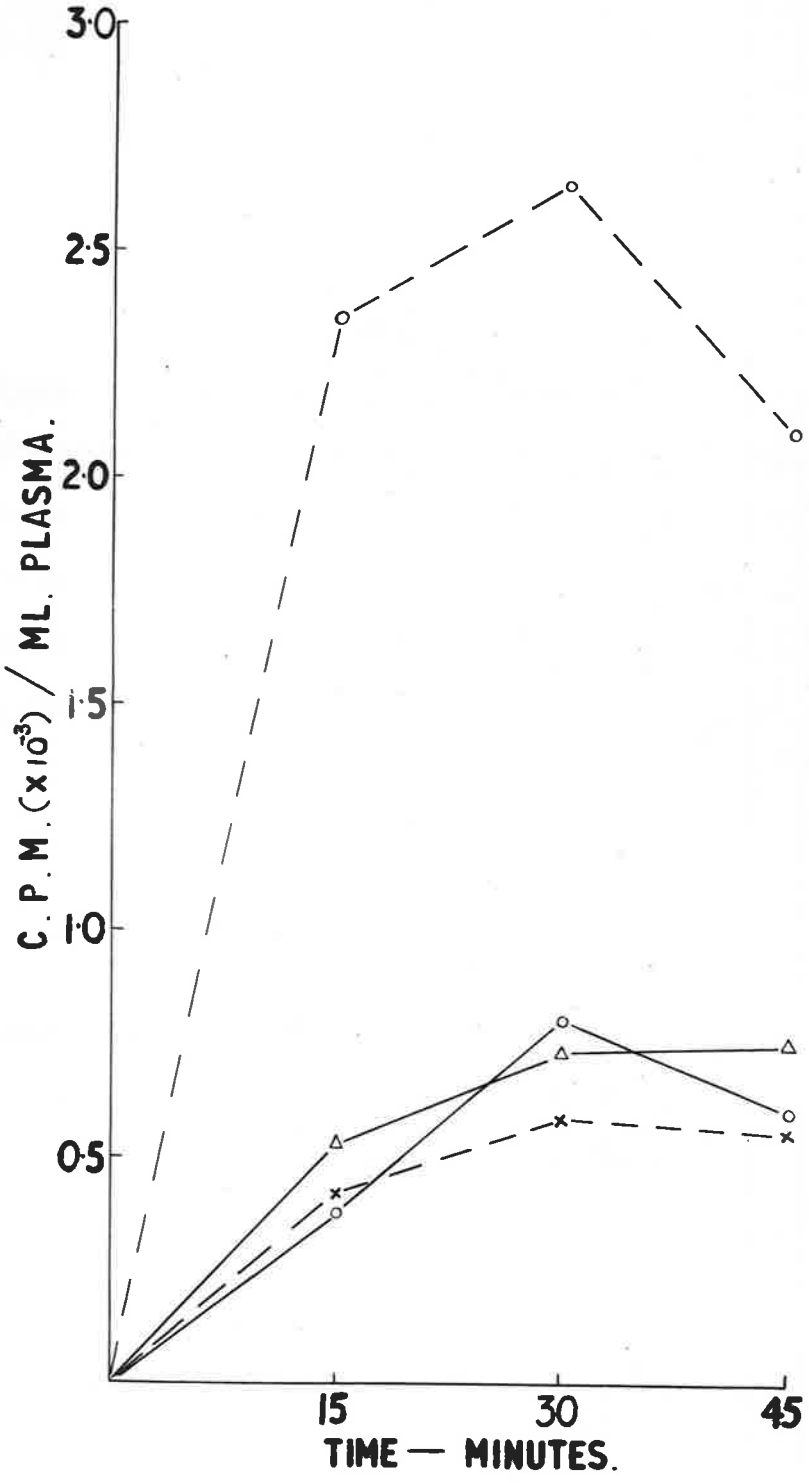

3·0
2·5
2·0
1·5
1·0
0·5
C. P. M. (x 10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

TABLE 15

THE INFLUENCE OF o,p-DDD ON THE EFFECT OF VITAMIN D_3 ON CALCIUM ABSORPTION

Vitamin D_3 was given orally either 100 I.U. 16 hours before the Ca^{45} test or 1,000 I.U. over a period of 7 days. 50 mg. o,p-DDD was fed daily for either 2 or 3 consecutive days prior to the giving of $Ca^{45}Cl_2$ on the fourth day. Calcium absorption from *in vivo* distal loops was measured after 2 hours.

Treatment	Period of D_3 treatment	Period of o,p-DDD treatment	No. of chicks	% calcium absorption
-	-	-	4	6
D_3 (100 I.U.)	16 hr.	-	5	56
D_3 (100 I.U.)	16 hr.	4 days	5	7
D_3 (1,000 I.U.)	7 days	-	4	50
D_3 (1,000 I.U.)	7 days	3 days	4	50

TABLE 16

o,p-DDD INHIBITION OF THE VITAMIN D_3 EFFECT ON CALCIUM TRANSPORT IN VITRO

Everted distal gut sacs from rachitic and vitamin D_3-treated chicks were prepared (Method E). o,p-DDD was fed for 3 days prior to measuring calcium transport on the fourth day. Chicks receiving vitamin D_3 were given 100 I.U. by mouth 16 hours before preparing the gut sacs. Each value represents the mean ± standard error of the mean for 10 birds.

Treatment	Ca^{45} Serosal c.p.m. X 10^{-3} accumulated/hour
-	3.654 ± 0.8
D_3	8.200 ± 1.0
D_3 + o,p-DDD	5.220 ± 0.7

immersed in Bouin's fixative medium. Paraffin embedded sections were then prepared by Mr. F. Fraser (C.S.I.R.O., Adelaide) and stained with haemotoxylin-eosin.

Figure 27 compares sections of adrenal tissue from o,p-DDD treated and untreated vitamin D_3-dosed birds. The definite pattern of cell structure in the peripheral areas observed in the control preparations was no longer apparent after o,p-DDD treatment. The adrenal cortical cells appeared to have been disrupted and to have contracted away from the capsule. A marked band of chromaffin tissue was evident beneath this capsule suggesting atrophy in this area. With haemotoxylin-eosin staining, the chromaffin tissue appeared strongly basophilic whilst the strands of interrenal tissue were much paler in appearance.

Weight of adrenal glands.

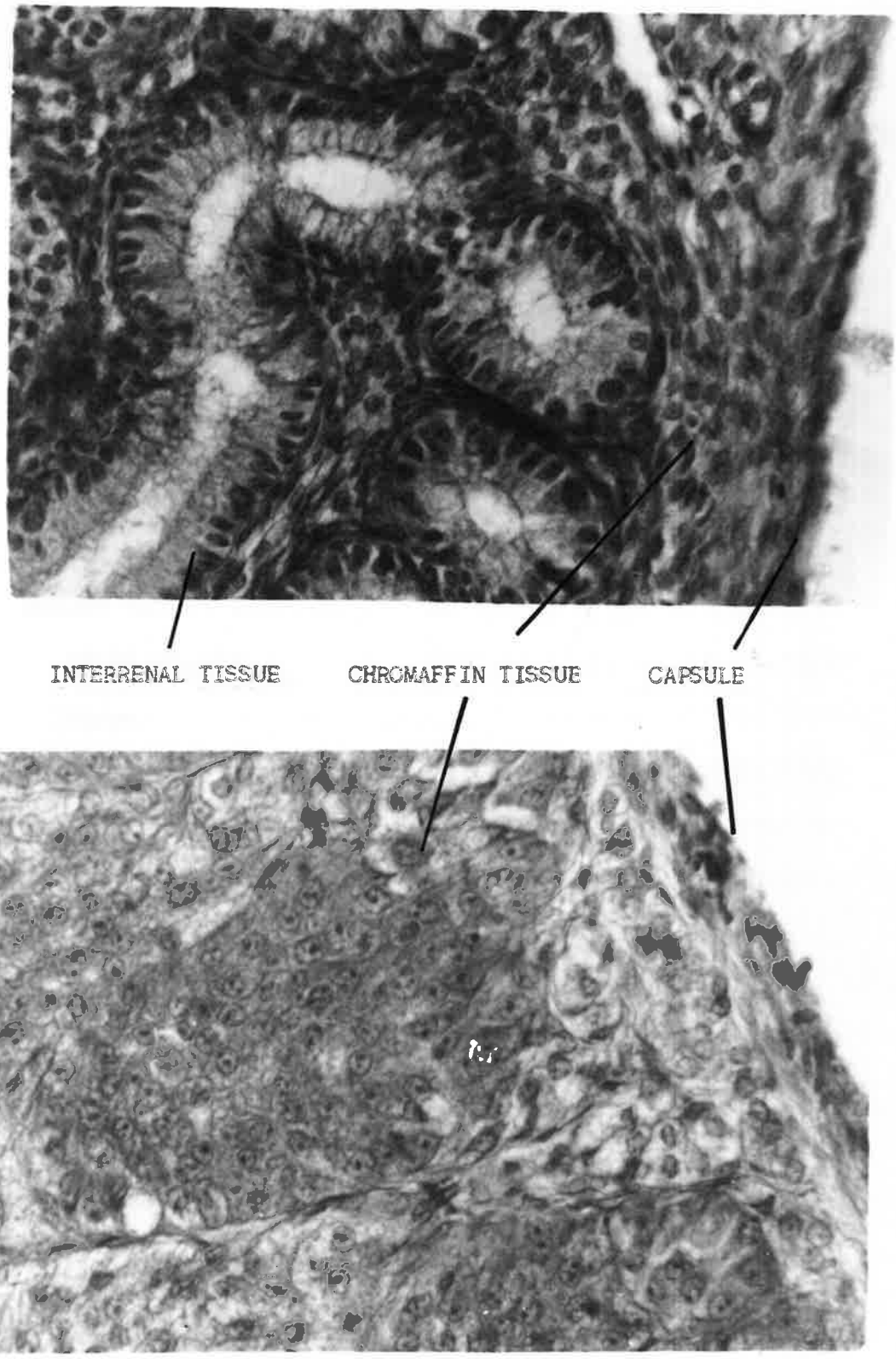
Newcomer (1959) demonstrated an increase in weight of the adrenal gland after chicks had received 2 mg. o,p-DDD over a period of 16 days. When 200 mg. o,p-DDD was given over a period of 4 days to rachitic chicks (i.e. 50 mg. daily) the majority of the birds died on the fifth day. However, when this treatment was given for only 3 days and the birds were allowed to live for a further 7 days death seldom resulted.

The weight of adrenals was determined in a group of 5 chicks given o,p-DDD for 3 days followed by 16 hours pretreatment with 100 I.U. vitamin D_3. These values were compared with a control group receiving vitamin D_3 only. The mean value obtained (expressed as mg. % body weight) for the group receiving o,p-DDD was 25.6 ± 7.5 whilst the figure for the control group was 21.3 ± 7.6. These values would suggest that treatment with o,p-DDD had increased the weight of the adrenal glands.

FIGURE 27

HISTOLOGICAL APPEARANCE OF THE ADRENAL GLAND AFTER TREATMENT WITH o,p-DDD

The photograph compares the typical appearance of an outer section of the adrenal gland of a chick given o,p-DDD treatment with a similar section from an untreated bird. Chicks were given 50 mg. o,p-DDD daily for 3 days followed by 16 hours pretreatment with 100 I.U. vitamin D_3. The control birds were given only vitamin D_3. The cells were stained with haematoxylin-eosin. The photograph was taken through a Leitz ortholux microscope, magnification X 320.


INTERRENAL TISSUE
CHROMAFFIN TISSUE
CAPSULE

However, the results obtained were not statistically significant ($P > 0.05$).

Although o,p-DDD is believed to exert a specific action on the adrenal cortical tissue the possibility remained that the inhibition of calcium transport was due to a toxic effect on the gut mucosa. Further experiments were therefore conducted to eliminate this possibility by the feeding of 1,000 I.U. vitamin D_3 over a period of 7 days before applying the calcium test, thereby ensuring an adequate supply of active carrier, providing that such a compound was relatively non-labile. Capsules of o,p-DDD (50 mg.) were fed on 2 consecutive days prior to examining the in vivo absorption of calcium from distal loops. Plasma Ca^{45} levels are shown in Table 17 and indicate that the vitamin D_3 effect could still be observed following the feeding of o,p-DDD. Measurement of the amount of calcium absorbed from the loop confirmed that treatment with o,p-DDD under these conditions was not inhibitory to the vitamin D_3 effect (Table 15).

TABLE 17

THE EFFECT OF o,p-DDD TREATMENT OF VITAMIN D_3-DOSED CHICKS ON CALCIUM ABSORPTION

Vitamin D_3-treated chicks received 1,000 I.U. over a period of 7 days, the final dose being given 16 hours before the test. 50 mg. o,p-DDD was fed daily for 2 days prior to the giving of $Ca^{45}Cl_2$ on the third day. Plasma Ca^{45} levels are recorded at varying time intervals after giving the calcium.

Treatment	No. of birds	Period on D_3	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins)		
			15	30	45
-	8	-	0.38	0.54	0.52
D_3	8	7 days	1.71	2.22	1.93
D_3 + o,p-DDD	8	7 days	1.55	2.19	1.98

DISCUSSION

The effect of vitamin D_3 on the absorption of calcium in rachitic chicks has been clearly demonstrated from the experiments described in Chapter 2. That there is a time lag before vitamin D_3 can exert an effect would suggest that this compound is being transformed to a more active form. The localisation of vitamin D in the adrenal gland of the rat has been reported by Kodicek (1960) but the importance of this finding is at present obscure. If, however, the transformation of vitamin D were to occur in this organ, then evidence of the localisation of vitamin D in a particular region of the adrenal gland would gain new significance. The possibility of active material being present in adrenal extracts was therefore considered.

When a commercial preparation of adrenal cortical extract was given intracardially to rachitic and vitamin D_3-treated chicks there were small but significant increases in calcium uptake. The small response detected may have been due either to the use of the less sensitive technique of duodenal rather than distal loops, or perhaps to the small biological potency of the extract. No further work was done with this extract since it could have contained a number of steroids, known or unknown. Instead, a number of known adrenal steroids were studied in relation to their influence on calcium metabolism *in vivo*. Cortisol has been identified as a major component of chick adrenal secretion (Professor Chester-Jones, personal communication). Recently, de Roos (1960) has demonstrated the *in vitro* production of corticosterone, cortisol and aldosterone by cock adrenals. Cortisol, given by intracardial injection 1 hour before the experiment, had no effect on the uptake of calcium from the intestine or rachitic

chicks, but if these chicks had received vitamin D_3 16 hours previously, there was a marked stimulation of the calcium uptake to an amount 20% greater than that given by the vitamin alone, as calculated from calcium absorption values, plasma Ca^{45} levels, or deposition of Ca^{45} in the bone.

Long-term administration of adrenal steroids has been shown to produce atrophy of the adrenal glands in rats (Ingle, Higgins and Kendall, 1938; Martini, Pecile and Guiliani, 1960; D'Arcy and Howard, 1958). In view of the results obtained from the short-term experiments further studies were undertaken to investigate the influence of the intramuscular injection of the compound for 3 days. Although this treatment cannot in the strictest sense be called a long-term one, the results indicate that under such conditions cortisol exerts a profound influence on calcium absorption. The increase in calcium absorption *in vivo* normally observed after vitamin D_3-treatment was noticeably decreased following 3 days of cortisol treatment.

Harrison and Harrison (1960), from *in vitro* studies have also demonstrated that cortisol when fed daily to rats over a period of 3 days leads to a reduced level in the active transport of calcium. These latter workers suggest that cortisol treatment antagonises the vitamin D effect on the diffusion of calcium. Both cortisol and vitamin D are believed to influence calcium transport by their action on the permeability of the intestinal cell surfaces to calcium. Since the experiments on long-term cortisol treatment were completed, Williams, Bowser, Henderson and Uzgiries (1961) using *in vitro* gut sacs from rats have shown an inhibition of the vitamin D effect when cortisone was given over a period of 6 days. Long-term cortisone treatment in rachitic rats has also been shown to produce

profound changes in bone structure (Storey, 1960).

A number of workers have demonstrated that cortisone can restore to normal the elevated serum calcium level resulting from hypervitaminosis or sarcoidosis and this effect has led Anderson et al. (1954) to suggest that cortisone antagonises vitamin D and thus inhibits calcium absorption from the gut. More recent studies on the effect of cortisone treatment in rats fed excess vitamin D, however, have discouraged the idea of a substrate competition mechanism (Wilson, Care and Anderson, 1957; Cruickshank and Kodicek, 1958; Thomas and Morgan, 1958). The present evidence from the short-term studies with cortisol also suggests that a structural antagonism is unlikely. A possible clue to this apparently anomalous behaviour of cortisol is provided in a further communication from Dent (1956) where he pointed out that treatment of patients with 150 mg. cortisone for 10 days led to a depression of adrenal cortical activity. This point will be discussed further after consideration of the effects of the other steroids.

DOC gave a similar response to cortisol. Intracardial injection 1 hour before the calcium absorption test showed a marked enhancing of the vitamin D_3 activity, whereas intramuscular injection for 3 days led to a decreased effect of vitamin D_3 on calcium absorption. DOC by itself had no effect (short-term experiment) on the uptake of calcium by rachitic chicks. It is apparent, therefore, that the effect depends on the presence of vitamin D_3. If the stimulation of calcium uptake were the result of mineralocorticoid activity, then short-term injection of a more potent mineralocorticoid should stimulate an even greater increase in calcium absorption. Aldosterone is approximately 25 times more active

as a mineralocorticoid than DOC, as measured by the sodium retention test (Desaulles, Tripod and Schuler, 1953) yet, aldosterone at levels of 50 µg. or 250 µg. had no effect on calcium uptake in either the presence or absence of vitamin D_3 when tested by the short-term procedure.

Several synthetic steroids were tested in an attempt to establish some general pattern of activity. Δ^1-cortisol gave very similar results to cortisol in short-term experiments, but neither 9α-fluorohydrocortisone acetate nor Δ^1-9α-fluoro-16α-hydroxy hydrocortisone had any effect on calcium uptake. The substitution of the 9α-fluoro group in the cortisol molecule causes a marked increase in both gluco- and mineralocorticoid activity, and the further introduction of the 16α-hydroxy group had no effect on the glucocorticoid activity but abolished the mineralocorticoid effect (Fried and Borman, 1958). It is clear from the results however, that the property of enhancing the effect of vitamin D_3 activity is not connected in any way with the possession of gluco- or mineralocorticoid properties. Many of these observations may be explained if those steroids enhancing or decreasing the vitamin D_3 effect are doing so through a specific effect on the adrenal cortex.

In the rat, prolonged administration of cortisone and Δ^1-cortisol causes extensive atrophy in the zona fasciculata of the adrenal cortex with depletion of the sudanophilic lipids (D'Arcy, 1961). The deposition of C^{14}-labelled vitamin D_2 in rat adrenals was shown by radio-autography to be located in the zona fasciculata (Kodicek, 1960). Thus the disfunction of the cortex brought about by cortisol could lead to interference with the site of formation of active material from vitamin D_3. The similar effects produced by DOC are more difficult to explain since,

in the rat, this compound appeared to reduce the sudanophilic lipids in the zona glomerulosa (D'Arcy, 1961). However, in the chick the zones are not clearly differentiated and the specific effects of cortisol and DOC on the chick adrenal cortex have yet to be determined. From the results, then, it would appear that long-term administration of cortisol and DOC led to a diminished production of active carrier from vitamin D_3 and, hence, to the observed decrease in calcium transport from the gut.

To account for the short-term effects of cortisol and DOC, it is postulated that these steroids stimulated an increased secretion from the adrenal cortex of active material formed from the 100 I.U. vitamin D_3 given 16 hours previously. This "flushing out" effect may account for the observation that vitamin D_3 and cortisol, given together 1 hour prior to the test, had a small effect on calcium uptake. As reported in the previous Chapter, there was no increase in calcium absorption in the rachitic birds when vitamin D_3 was given by itself 1 hour before the test. It is more difficult to explain the absence of any short-term cortisol effect on calcium transport <u>in vitro</u>. If there is a continual "flushing out" of active material, then the preservation of the blood supply may be an important factor in transmitting this effect.

One obvious way to prove the hypothesis that the adrenals are essential for vitamin D_3 activity would be to demonstrate that vitamin D_3 has no influence on adrenalectomised rachitic chicks. Adrenalectomy by the usual surgical procedures or by cauterisation was, however, found to be impracticable in 4 week old chicks due to the inaccessibility of the glands which are embedded close to the aorta and posterior vena cava. In this respect, rats would appear to be much more suitable subjects as

the adrenals are easily removed. There have been few investigations directed toward a study of the influence of adrenalectomy on calcium metabolism. Conway and Hingerty (1946) observed a slight hypocalcaemia in adrenalectomised rats. Kimberg et al. (1961) found that adrenalectomy enhanced the transport of calcium *in vitro* when rats were fed a low calcium diet. If, however, the rats were maintained on a high calcium diet, transport was not affected.

Gaunt, Chart and Renzi (1961) have discussed the principle of specific chemical interference with certain glands as in the well known case of alloxan causing atrophy of the cells of the pancreas. The chemical o,p-DDD was reported to cause atrophy of the adrenal cortex (Nichols and Green, 1954; Brown, 1960). Newcomer (1959) tested this compound in chicks and found that it led to a decrease in 17-hydroxy steroid output with a subsequent increase in the weight of the adrenal gland which he attributes to an increased accumulation of Δ^4-3 keto-17-hydroxy steroids.

When o,p-DDD was fed to rachitic chicks for 3 days the enhancing effect of vitamin D_3 on calcium uptake could not be observed from *in vivo* segments of intestine. Everted segments from these treated chicks also showed a decreased ability to transport calcium. In the small group of chicks examined, there was no significant increase ($P>0.05$) in the weight of the adrenal gland following o,p-DDD treatment.

It is difficult to assess the effect of o,p-DDD in relation to the weight of the adrenal gland. Although Newcomer (1959) has demonstrated a mean increase in the weight of the adrenals taken from birds treated with o,p-DDD, the conclusions from these data are based on a small group of chicks in which the size and weight of the adrenals could have varied

considerably. The results would obviously gain more significance if larger groups of birds were used and the data obtained could then be examined statistically. It is interesting to note, however, that the present determination of the weight of the adrenal gland/body weight of the chick is comparable to Newcomers's estimation.

The adrenal gland was examined histologically to determine the effect of o,p-DDD on the cell structure. Unlike the mammalian gland, there is no definite zonation in the fowl adrenal. The interrenal tissue (equivalent to the mammalian adrenal cortex) appears as double rows or cords of cells which loop as they approach the capsule although they seldom reach the capsule itself, being separated from the latter by tongues of chromaffin tissue. In places, this tissue occupies the surface of the gland beneath the capsule (Professor Chester Jones, personal communication). Chromaffin tissue, a medullary tissue, shows no definite cell arrangement.

The histological picture outlined above was observed in adrenal sections from the control group. In contrast, however, there was a marked change of cell structure in areas associated with the interrenal tissue after treatment with o,p-DDD. The importance of the zona fasciculata in accumulating vitamin D in the rat has already been mentioned. These findings would suggest that the transformation of vitamin D_3 may occur in this particular region of the mammalian gland. It would therefore appear worthwhile to continue these studies in rats where it may be possible to locate more specifically the site of action of o,p-DDD.

Thus these results support the previous findings and suggest that disfunction of the adrenal cortex brought about by o,p-DDD, cortisol

or DOC interferes with the production of active material from vitamin D_3. Attempts have been made to isolate active carrier material from chicks and details of these experiments will be described in a later chapter. It does, however, seem worthwhile to point out that the adrenal gland appears to be an admirable site for a transformation of the vitamin D_3 structure in view of the capacity of this organ for steroid synthesis.

To show that o,p-DDD was not having a toxic effect on the gut itself, this compound was fed to chicks that had been receiving vitamin D_3 for several days prior to commencing the treatment. There was no indication of an inhibition of the vitamin D_3 effect following the feeding of o,p-DDD. These experiments could be interpreted to mean that active material made in the adrenal cortex is used for the transport of calcium by the intestine and that o,p-DDD does not interfere with the function of preformed carrier. The observation by Thomas and Morgan (1958) that cortisone treatment did not relieve the symptoms of hypervitaminosis in rats when vitamin D was fed concurrently, could also be explained by this hypothesis.

The delay before an effect of vitamin D_3 can be shown on calcium uptake (reported in Chapter 2) may well be due to the necessity to build up a concentration of the active carrier material in the mucosal cells, and the evidence from these present investigations suggests that this transformation takes place in the adrenal cortex.

The participation of parathyroid hormone in calcium uptake appears to be a controversial issue. In recent reviews Irving (1957) and Neuman and Neuman (1958) have suggested that the intestinal absorption of calcium

is not under the direct control of the parathyroid gland. Rasmussen (1959), using everted duodenal sacs from parathyroidectomised rats, demonstrated a decreased active transport of calcium in these preparations as compared with the control group. Talmage and Elliott (1958) reported similar results from *in vivo* studies with parathyroidectomised rats.

More recent studies by Gran (1960), however, indicated that removal of the parathyroid glands did not affect calcium absorption. Further, the absence of parathyroid hormone did nor prevent the vitamin D_3 stimulation of calcium absorption. These findings by Gran have been collaborated by Wasserman and Comar (1961). These latter workers demonstrated that parathyroidectomy was without effect on the intestinal absorption of calcium *in vivo* or on calcium transfer across everted intestinal sacs *in vitro*.

When a parathyroid extract was given to chicks by intracardial injection 1 hour before the calcium absorption test there was no increase in calcium absorption from the intestine. Calcium absorption, then, does not appear to be under the influence of parathyroid function. Of more importance, the results demonstrate that the effects on calcium absorption previously noted in studies related to the adrenal gland are not mediated through the parathyroid gland.

SUMMARY

1. The effects of adrenal steroids on calcium transport have been studied. Cortisol, Δ^1-cortisol and DOC in short-term experiments enhanced the effect of vitamin D_3 on calcium transport *in vivo*.

2. Administration of cortisol or DOC for 3 days brought about a depression of the vitamin D_3 effect.

3. Aldosterone, 9α-fluorohydrocortisone acetate and Δ^1-9α-fluoro-16α-hydroxyhydrocortisone had no effect on calcium transport when studied by short-term administration.

4. Aldosterone did not increase sodium absorption from the small intestine.

5. o,p-DDD, when fed 3 days prior to giving vitamin D_3, inhibited the vitamin D_3 effect on calcium absorption. When the adrenal gland was examined histologically following this treatment, marked changes in the interrenal tissue were observed. There was no significant increase in the weight of the adrenal gland.

6. When o,p-DDD was fed to chicks previously dosed for several days with vitamin D_3(1,000 I.U.), calcium transport was not appreciably affected.

7. The effects observed could be correlated with interference with the adrenal function by o,p-DDD, cortisol or DOC treatment of rachitic chicks, which diminished the vitamin D_3-enhancement of calcium transport.

8. The theory is proposed that vitamin D_3 is transformed into a more active form in the adrenal cortex.

9. Parathyroid extract did not influence the absorption of calcium from the intestine.

CHAPTER 4

THE MECHANISM OF CALCIUM TRANSPORT

Introduction

Materials and Methods

Results

Discussion

Summary

CHAPTER 4

THE MECHANISM OF CALCIUM TRANSPORT

INTRODUCTION

Before the commencement of this thesis information concerning the mechanism of the transport of calcium was practically non-existent in the literature. Neuman and Neuman (1958) have recently attempted to describe a mechanism from the meagre facts available but they have also emphasised the difficulty of this task with so little knowledge of the metabolism of the intestinal mucosa. Although the effects of vitamin D have been adequately described, the mode and site of action of this substance are still unknown (Nicolaysen and Eeg-Larsen, 1953, 1956). However, since *in vitro* techniques have been developed for studying calcium transport great strides have been made in elucidating the steps involved in this process.

During these present investigations Schachter and Rosen (1959) published a report describing the movement of calcium across everted segments of rabbit, rat and guinea pig intestine. These workers postulated the existence of an active transport mechanism which was dependent upon oxidative phosphorylation. Further, this process was partially dependent on vitamin D. Despite these important observations, Schachter and Rosen point out the limitations of these studies in providing information on the importance of an active transport mechanism during the absorption of calcium *in vivo*. Since the work of Schachter and Rosen (1959), several publications have appeared describing calcium transport by mammalian small intestine (Harrison and Harrison, 1960; Schachter et al., 1960 a, b; Dowdle et al., 1960; Kimberg et al., 1961; Schachter, Kimberg and

Schenker, 1961). However, some of these reports present conflicting evidence on the mechanism of active calcium transfer so that it has been extremely difficult to gain a true assessment of the situation both as to the energy source involved and as to the role of vitamin D_3 in this process.

The phenomenon of calcium transport under the influence of vitamin D_3 has therefore been re-investigated in the chick. An attempt was made to locate more specifically the source of the energy supply. The experimental findings are considered in relation to the data available from similar studies with mammalian tissue. The application of an *in vitro* transport mechanism to the absorption of calcium *in vivo* is also discussed.

MATERIALS AND METHODS

In vitro preparations

Everted and non-everted gut sacs. Everted sacs of the distal portion of small intestine from both rachitic and vitamin D_3-treated chicks (100 I.U. - 16 hours) were prepared and tested in the manner already described (Method E). Where anaerobic conditions were employed, the flask contents were gassed with a mixture 95% N_2 - 5% CO_2 (oxygen content was less than 0.002%). When non-everted sacs were tested they were subjected to the same procedures and conditions as described for the everted sacs.

Calcium transport at 0° C. Everted gut sacs were prepared and placed in ice-cold incubation medium contained in flasks surrounded by cracked ice. These flasks were then shaken for 1 hour under aerobic conditions.

Starvation. The effect of starvation on calcium transport was studied in chicks deprived of food for a period of 24 hours prior to preparing everted gut sacs.

Omission of glucose. As described in Chapter 1, everted gut sacs were normally incubated in a medium containing 0.02 M glucose (Method E). In an *in vitro* experiment designed to observe the effect of a glucose deficiency on calcium transport glucose was omitted from both the mucosal and serosal incubation media.

In vivo preparations.

Inhibitor studies. Distal segments of small intestine from both rachitic and vitamin D_3-treated (100 I.U. - 16 hours) chicks were perfused *in vivo* in the manner previously described (Method C). The

loops were perfused for 45 minutes with 10 ml. Krebs-Ringer solution containing 2 mg. Ca^{40}/ml. and 2 µc. Ca^{45}/ml. together with the inhibitor to be studied.

Chelation of calcium. The disodium salt of EDTA was used to chelate calcium. The absorption of calcium was studied from *in vivo* duodenal loops of rachitic and vitamin D_3-treated (100 I.U. - 16 hours) birds. A solution of 0.1 mMoles Ca^{45}-EDTA was introduced into the loop. Control birds received 0.1 mMoles Ca^{45} in o.9% saline.

Inhibitor solutions.

When necessary, inhibitor solutions were adjusted to pH 7.0 with sodium hydroxide for *in vitro* experiments, and pH 6.0 for *in vivo* studies. A solution of sodium cyanide was prepared by dissolving the required amount of this compound in ice-cold distilled water and then neutralising with hydrochloric acid at 0° C. A solution of sodium arsenite was prepared by dissolving the required amount of arsenious oxide in a small volume of sodium hydroxide and, after suitable dilution with water, hydrochloric acid was added to adjust the pH to 7.4 before making to volume.

Estimations.

Ca^{45} in serosal fluid. Radioactivity was estimated as previously described (P27, (b) i).

Ca^{45} in blood. Ca^{45} in plasma obtained during *in vivo* studies in the presence of EDTA was measured by end-window counting as described earlier (P27, (a) i). During inhibitor studies *in vivo* with perfused isolated loops, Ca^{45} was measured in acid extracts of plasma by scintillation counting (P27, (a) ii).

Endogenous respiration of tissue slices

The consumption of oxygen of respiring slices of chick small intestine was measured in a Warburg apparatus. Groups of rachitic and vitamin D_3-treated (100 I.U. - 16 hours) chicks (4 chicks/group) were used in the experiment. After rinsing the distal portion of the small intestine free of food contents with physiological saline, this section was removed from the body and immersed in an ice-cold solution containing 0.13 M NaCl and 0.02 M KCl. The gut wall was slit longitudinally along the anti-mesenteric surface and then several times horizontally so that a number of sections approximately 1 cm^2 were obtained. The procedure was repeated in order to obtain sufficient slices to study each group in triplicate.

The slices from the rachitic chicks were pooled separately, as were those from the vitamin D_3-treated chicks, so that the final population of slices in each group was randomised by thorough mixing. After briefly blotting on filter paper, four slices were added to each Warburg vessel containing 2.8 ml. Krebs-Ringer-bicarbonate medium with glucose and calcium added in final concentrations of 0.02 M and 2.5×10^{-3} M respectively. The centre well contained a filter paper strip soaked in 0.2 ml. 5 N potassium hydroxide solution. A thermobarometer recorded atmospheric and temperature changes.

After a 5 minute equilibration period, the flasks were incubated with shaking (90 oscillations per minute) at 37° C for 1 hour. After incubation the slices were lifted out of the flasks, excess fluid removed by briefly blotting with filter paper, and the tissue then placed in a desiccator until dry. The dry weight of tissue was determined gravimetric-

ally. $Q(O_2)$ is defined as µl. O_2 taken up per mg. dry weight tissue per hour.

Lactic acid

Formation of lactic acid. Everted distal sacs from groups of three rachitic and vitamin D_3-treated (100 I.U. - 16 hours) chicks were prepared as described earlier (Method E). Fluid bathing the mucosal surface consisted of 5.0 ml. Krebs-Ringer-bicarbonate buffer together with glucose and unlabelled calcium at concentrations of 0.02 M and 2.125×10^{-3} M respectively. The everted sacs were filled with 1.0 ml. buffer medium also containing glucose (0.02 M) and calcium (2.25×10^{-3} M). The preparations were incubated with shaking at 37° C for 1 hour and were gassed with a mixture of 95% N_2 - 5% CO_2 to maintain anaerobic conditions so preventing any further breakdown of lactic acid formed during this period.

At the end of the incubation the sacs were removed and the serosal and mucosal fluids were pooled and kept at 0°. The tissue itself was homogenised for 2 minutes in ice-cold distilled water using a Potter-Elvejhem homogeniser fitted with a teflon plunger. This homogenate was added to the pooled fluids before diluting with distilled water to a known volume. Samples from each group were then taken to determine the amount of lactic acid present.

Estimation of lactic acid. Lactic acid was determined by the method of Barker and Summerson (1941). The protein was precipitated from the sample by treatment with perchloric acid (final concentration 4%) and removed by low speed centrifugation (2,000 X g. for 10 minutes). A portion of the supernatant (5.0 ml.) was neutralised with 4 N sodium hydroxide before the addition of 1.0 ml. 20% copper sulphate solution. The total

volume was made to 10 ml. with distilled water, 100 mg. calcium hydroxide was added and the mixture vigorously shaken before allowing to stand for 30 minutes. The copper calcium complex was removed by centrifugation and a sample of the supernatant retained for colour development with an alkaline solution of p-hydroxydiphenyl. The intensity of colour produced was determined on a Unicam spectrophotometer (model SP 350) at 560 mμ.

Reagent blanks and tubes containing standard amounts of lactic acid were carried through the entire procedure to estimate contamination and efficiency of the method. A standard curve was also prepared with known amounts of lactic acid not subjected to the above treatment. Recoveries of no less than 98% were given by this technique.

Succinic dehydrogenase activity

Preparation of mitochondria. Mitochondria were isolated from loops of distal small intestine of rachitic and vitamin D_3-treated (100 I.U. - 16 hours) birds. Each group consisted of 3 chicks. Following the usual surgical procedure the tissue was dissected free and immediately immersed in ice-cold phosphate buffer medium. After everting each loop the tissue was cut into small pieces and placed in 20 ml. ice-cold phosphate buffer pH 7.7 preparatory to homogenising in a Potter-Elvejhem homogeniser. The homogenates in each group were pooled.

The mitochondria were prepared by differential centrifugation of the homogenate in a manner similar to that described by Hogeboom, Schneider and Pallade, 1948. To remove cellular debris the homogenate was centrifuged in the cold at 800 X g. for 15 minutes. The supernatant was decanted and recentrifuged at 5,000 X g. for 20 minutes at 0° C to separate the mitochondria. The supernatant fluid was then carefully

removed by aspiration and the mitochondrial pellet resuspended in an equal volume of phosphate buffer. The suspension was centrifuged again at 0° C for 10 minutes at 10,000 X g. After removal of the supernatant, the mitochondria were finally resuspended in a small volume of ice-cold phosphate buffer.

Determinations of enzyme from each group were carried out in triplicate in a manner similar to that described by Nachlas, Margulies and Seligman (1960). The reaction mixture consisted of the following reagents: sodium succinate (0.2 M), 0.5 ml; phosphate buffer (0.1 M) pH 7.7, 0.75 ml; gelatin (0.1%), 0.25 ml; 2,3,4-triphenyltetrazolium chloride (TTC, 6 mg./ml.) 0.5 ml; enzyme solution, 0.5 ml. and distilled water, 0.25 ml. Control tubes contained either sodium fumarate (0.1 M) 0.5 ml., in place of succinate or alternatively the reaction was carried out in the absence of any substrate. Details of the complete incubation are summarised in Table 18.

The reaction mixture was preincubated at 37° C for 5 minutes before adding the enzyme, and the complete mixture incubated at 37° C for 90 minutes. Further control data were also obtained from tubes incubated in an ice-bath at 0° C for the same period of time.

The incubation period was terminated by the addition of 40% trichloracetic acid (0.2 ml.). Tetrahydrofuran (3.0 ml.) was then added, and the mixture shaken mechanically in centrifuge tubes fitted with polythene stoppers until all the colour had been removed from the precipitated protein (approximately 10 minutes). After removal of the protein by centrifugation, the colour present in the supernatant was estimated in a Unicam Spectrophotometer (Model SP 350) at 540 mμ against tetrahydrofuran.

TABLE 18

THE DETERMINATION OF SUCCINIC DEHYDROGENASE ACTIVITY

Mitochondria were prepared from the distal small intestine of rachitic and vitamin D_3-treated chicks. The enzyme preparation from the rachitic chicks is designated as R and that from the vitamin D_3-treated chicks is N. The addition of reagents to the incubation tubes is indicated by a positive sign. Incubation of the mixtures was carried out at 37° C or 0° C for 90 minutes.

	Vol. added	37°C														0°C					
Tube No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Sodium Succinate 0.2 M	0.5	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
Sodium Fumarate 0.2 M	0.5	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+
Gelatin 0.1%	0.25	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphate Buffer 0.1 M	0.75	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T.T.C. 6 mg./ml.	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Enzyme	0.5	R	R	R	N	N	N	R	R	R	N	N	N	R	R	N	N	R	N	R	R	N	N

Succinic dehydrogenase activity was recorded as the optical density change per mg. protein.

Protein was estimated by the biuret method (Gornall, Bardawill and David, 1949) using normal dried serum albumin (human) supplied by the Commonwealth Serum Laboratories as a standard.

RESULTS

Non-everted gut sacs

To show that the accumulation of calcium was a uni-directional process, calcium transport was studied using non-everted distal sacs of small intestine. Under these conditions, calcium transport took place from the serosal surface to the mucosal surface, and the accumulation of Ca^{45} was measured in the fluid bathing the mucosal cells. The results from this experiment are presented in Figure 28.

Less calcium was transported with non-everted rachitic sacs than the everted rachitic controls. This decreased transport may be a consequence of the lower transport of water observed which was a net gain of 0.6 g. water per hour per everted sac, but a net loss from the original 2 g. of 0.3 g. water per hour per non-everted sacs. There was a tendency for the non-everted sacs to shrink considerably in size during the incubation period whereas no change in length was observed with the everted sacs.

More interesting was the absence of any influence of vitamin D_3 on the transport of calcium using the non-everted preparations. That the vitamin D_3 phenomenon is an active process is shown by the increased accumulation of calcium in the serosal fluid from the vitamin D_3-everted sacs, as compared to that from the rachitic-everted sacs (Figure 28).

Transport at 0° C

Evidence for the enzymic nature of calcium transport may be deduced from Table 19 from which it is evident that there was very little transport of calcium at 0° C. If calcium transport was mainly by diffusion, then lowering the temperature would not be expected to cause such a marked inhibition of transport, although there would possibly be

FIGURE 28

THE UNIDIRECTIONAL TRANSPORT OF CALCIUM BY IN VITRO DISTAL LOOPS

Ca^{45} was measured in the serosal fluid after 1 hour. Histograms represent the mean of the uptake from 6 sacs.

Diagonal hatched	rachitic everted distal intestine
Blank	vitamin D_3-treated everted distal intestine
Cross hatched	rachitic non-everted distal intestine
Blacked	vitamin D_3-treated non-everted distal intestine

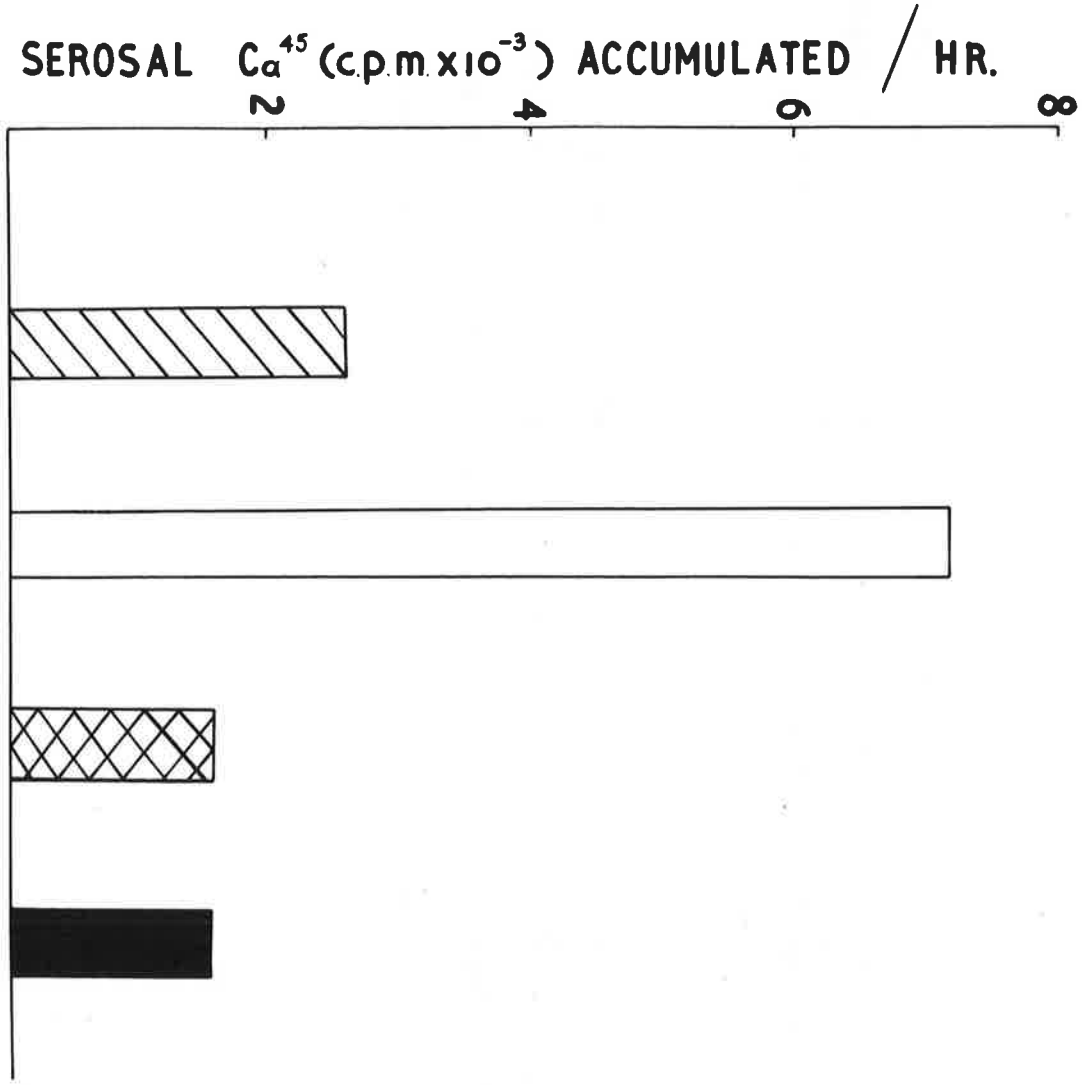

SEROSAL Ca^{45} (c.p.m. x 10^{-3}) ACCUMULATED / HR.
2
4
6
8

TABLE 19

THE INFLUENCE OF VITAMIN D_3 ON CALCIUM TRANSPORT AT 0° C

Transport was measured at 37° and 0° by the accumulation of Ca^{45} into serosal fluid from everted distal small intestine. The vitamin D_3-treated chicks received 100 I.U. vitamin D_3 16 hours prior to preparing the gut sacs. Each value is the mean of 6 birds.

Treatment	Temperature	Ca^{45} Serosal c.p.m. X 10^{-3} accumulated/hour
-	37°	3.160
D_3	37°	8.852
D_3	0°	0.320

some small drop from the normal rate of diffusion.

Starvation and the effect of glucose

During preliminary experiments on calcium absorption in chicks, Coates and Holdsworth (personal communication) found that a period of starvation decreased the ability of vitamin D_3-treated chicks to transport calcium. When everted distal sacs from chicks starved for 24 hours were tested for calcium transport *in vitro*, the vitamin D_3-treated chicks showed a slightly diminished ability to absorb calcium. Sacs from starved rachitic birds gave no change from the rachitic control level. Similarly, gut sacs from chicks not starved but incubated without glucose in the standard medium, showed that the endogenous substrate can support 62% of the normal rate of absorption. These results, which are shown in Table 20, were obtained with vitamin D_3-treated chicks. Incubation of rachitic sacs under either of these conditions produced little or no change from the rachitic control level.

Metabolic inhibitors

Various inhibitors were used both *in vivo* and *in vitro* in an attempt to identify the source of energy used in calcium transfer. Table 21 presents the results from a series of *in vitro* studies. These results have been expressed as a percentage inhibition of the active transport process, by which is inferred the difference between vitamin D_3-treated and rachitic transport.

This seems justified as the data obtained at 0° C and also from studies with the non-everted gut sacs preparations suggested that calcium transport in the rachitic chicks was probably the result of passive diffusion and exchange processes. Further, preliminary studies on the

TABLE 20

THE EFFECT OF STARVATION OR THE ABSENCE OF GLUCOSE ON CALCIUM TRANSPORT IN VITRO

Transport was measured by the accumulation of Ca^{45} in the serosal fluid from everted distal sacs. Sacs from vitamin D_3-treated chicks (100 I.U.-16 hours) incubated in standard medium were assigned an arbitory value of 100%.

Treatment	No. of birds	% D_3 transport
-	6	28
Starved 24 hours	4	27
No glucose	4	25
D_3 starved 24 hours	4	65
D_3 no glucose	4	62

effect of iodoacetate under anaerobic conditions, on calcium transport in the rachitic chick, indicated that there was no further decrease in the amount of calcium transported. In contrast, the vitamin D_3 enhanced transport was greatly inhibited under these conditions (Table 21).

2,4-Dinitrophenol gave only 50% inhibition at 2×10^{-4} M. That energy was not derived solely from oxidative phosphorylation was further suggested from the fact that even in the presence of 1×10^{-2} M. cyanide there was still a considerable amount of calcium transport taking place (60%). The greatest inhibition was obtained with an inhibitor of glycolysis, namely 2×10^{-3} M. iodoacetate under anaerobic conditions. Other well known glycolytic inhibitors, sodium fluoride (2×10^{-3} M.), phlorizin (2×10^{-4} M.), and sodium arsenite (2×10^{-3} M.) gave approximately 50% reduction in the amount of calcium transported.

It seems most probable then, that both glycolytic and oxidative phosphorylative processes are required to maintain a metabolically active membrane. If energy were derived equally from substrate level and oxidative phosphorylations, it might be possible by a combination of inhibitors, to prevent any calcium from being transported. When phlorizin and 2,4-dinitrophenol were both added to the mucosal fluid bathing the everted sacs, a small percentage of calcium still accumulated in the serosal fluid (Table 21).

The results obtained with mercuric chloride cannot be interpreted because this substance caused the mucosal fluid to become turbid with sloughed cells. Turbidity of the mucosal fluid was also observed but to a lesser extent, when everted sacs were incubated under anaerobic conditions. Ouabain, a cardiac glycoside and a potent inhibitor

TABLE 21

THE EFFECT OF INHIBITORS ON THE ACTIVE TRANSPORT OF CALCIUM IN VITRO

Active transport was taken to be the difference between the calcium transported into the serosal fluid by everted distal sacs from rachitic chicks as compared to similar sacs from chicks treated with 100 I.U. vitamin D_3 16 hours previously. Inhibition is expressed as a percentage of this active transport. For details of the incubation conditions, refer to the Methods section in this Chapter. The inhibitors were added to the mucosal fluid and are recorded as final concentrations.

Inhibitor	Inhibitor conc. M	No. of birds	% inhibition of transport
N_2/CO_2 in gas space	-	7	37
Sodium cyanide	5×10^{-4}	4	8
Sodium cyanide	1×10^{-2}	4	39.6
2,4-dinitrophenol	2×10^{-4}	8	50
Sodium iodoacetate/N_2	2×10^{-3}	8	80
Sodium fluoride	2×10^{-3}	8	54
Sodium arsenite	2×10^{-3}	4	55
Phlorizin	2×10^{-4}	8	48
Phlorizin + 2,4-dinitrophenol	2×10^{-4}) 2×10^{-4})	6	67
Mercuric chloride	2×10^{-3}	4	95*
Ouabain	6.8×10^{-5}	8	0

* cells sloughed off.

of sodium transport, was also tested for its effect on calcium transport. Evidence presented in Table 21 shows that calcium accumulation under the influence of vitamin D_3 was not influenced by the presence of ouabain. On the contrary, transport was slightly stimulated by this substance.

An attempt was made to correlate the *in vitro* findings with similar experiments performed on the live birds. It is obvious however, that the range of inhibitors which may be tested satisfactorily *in vivo* is severely limited, e.g. it was not possible to determine the influence of cyanide. The results from these studies are presented in Table 22. Contrary to the *in vitro* findings, there was little change in calcium absorption as measured by plasma Ca^{45} levels (from vitamin D_3-treated birds) in the presence of iodoacetate, iodoacetamide, and 2,4-dinitrophenol. The lack of effect with the addition of 1×10^{-4} M 2,4-dinitrophenol to the perfusion fluid might be explained if this substance were being rapidly absorbed, or diluted to such an extent by body fluids as to be incapable of exerting an inhibitory effect. To test this possibility, a sample of perfusate was withdrawn on the completion of the experiment and the amount of 2,4-dinitrophenol present was measured spectrophotometrically. There was little or no change from the concentration of 2,4-dinitrophenol originally present.

When phlorizin was added to the perfusing fluid there was a 50% inhibition of transport, confirming the previous *in vitro* findings. It is apparent however, that these results must be interpreted with caution. During the first 15 minutes, there was a marked decrease in the amount of radioactivity appearing in the blood stream. At longer time intervals there was a gradual increase in the amount of calcium absorbed.

TABLE 22

THE EFFECT OF METABOLIC INHIBITORS ON THE ABSORPTION OF CALCIUM IN VIVO

Plasma Ca^{45} levels were measured during *in vivo* perfusion of distal loops from rachitic and vitamin D_3-treated chicks (Method C). Inhibitors were added directly to the perfusion fluid and are recorded as final concentrations.

Treatment	Inhibitor	Conc. M	No. of birds	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins)		
				15	30	45
D_3	-	-	20	1.475	2.308	2.666
D_3	2,4-dinitrophenol	2×10^{-4}	8	1.408	2.045	2.684
D_3	iodoacetate	1×10^{-3}	4	1.562	2.174	2.639
D_3	iodoacetamide	1×10^{-3}	4	1.274	2.140	2.643
D_3	phlorizin	1×10^{-4}	4	0.864	1.542	2.216
-	-	-	10	0.247	0.277	0.324

Chelation of calcium

Chelation of calcium present in isolated *in vivo* duodenal loops by means of EDTA caused a marked decrease in calcium absorption from both rachitic and vitamin D_3-treated groups (Figure 29). It thus seems likely that the calcium must be in the ionic form before it is absorbed from the gut.

Measurement of glycolysis

To determine whether vitamin D_3 itself had any effect on glycolysis, everted distal gut sacs were incubated under anaerobic conditions and after 1 hour the amount of lactic acid formed was determined. The results of two such experiments are recorded in Table 23. Similar amounts of lactic acid were produced by the rachitic and vitamin D_3-treated gut sacs.

Endogenous respiration

Oxygen consumption by slices of distal small intestine from rachitic and vitamin D_3-treated chicks was measured. The basal $Q(O_2)$ value for slices from the rachitic group was 1.57 and the corresponding value for the vitamin D_3 control group was 1.50. Each value represents the mean of 3 determinations.

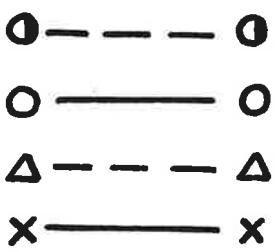
Succinic dehydrogenase activity

Mitochondria prepared from small intestine of rachitic and vitamin D_3-treated chicks was examined for the presence of succinic dehydrogenase activity. A tetrazolium salt was used as an electron acceptor in the succinoxidase system. After 90 minutes incubation, values of 3.53×10^{-2} (O.D./mg. protein) and 3.30×10^{-2} (O.D./mg. protein) were obtained for succinic dehydrogenase activity of the rachitic and vitamin

FIGURE 29

THE EFFECT OF EDTA ON CALCIUM ABSORPTION IN VIVO

Ca^{45} was measured in plasma after preparing *in vivo* duodenal loops from rachitic and vitamin D_3-treated chicks. Loops were given 0.1 mMoles Ca^{45} EDTA and control loops 0.1 mMoles $Ca^{45}Cl_2$ in saline. Each point is the mean of 7 birds.



vitamin D_3-treated and EDTA

vitamin D_3-treated controls

rachitic and EDTA

rachitic controls

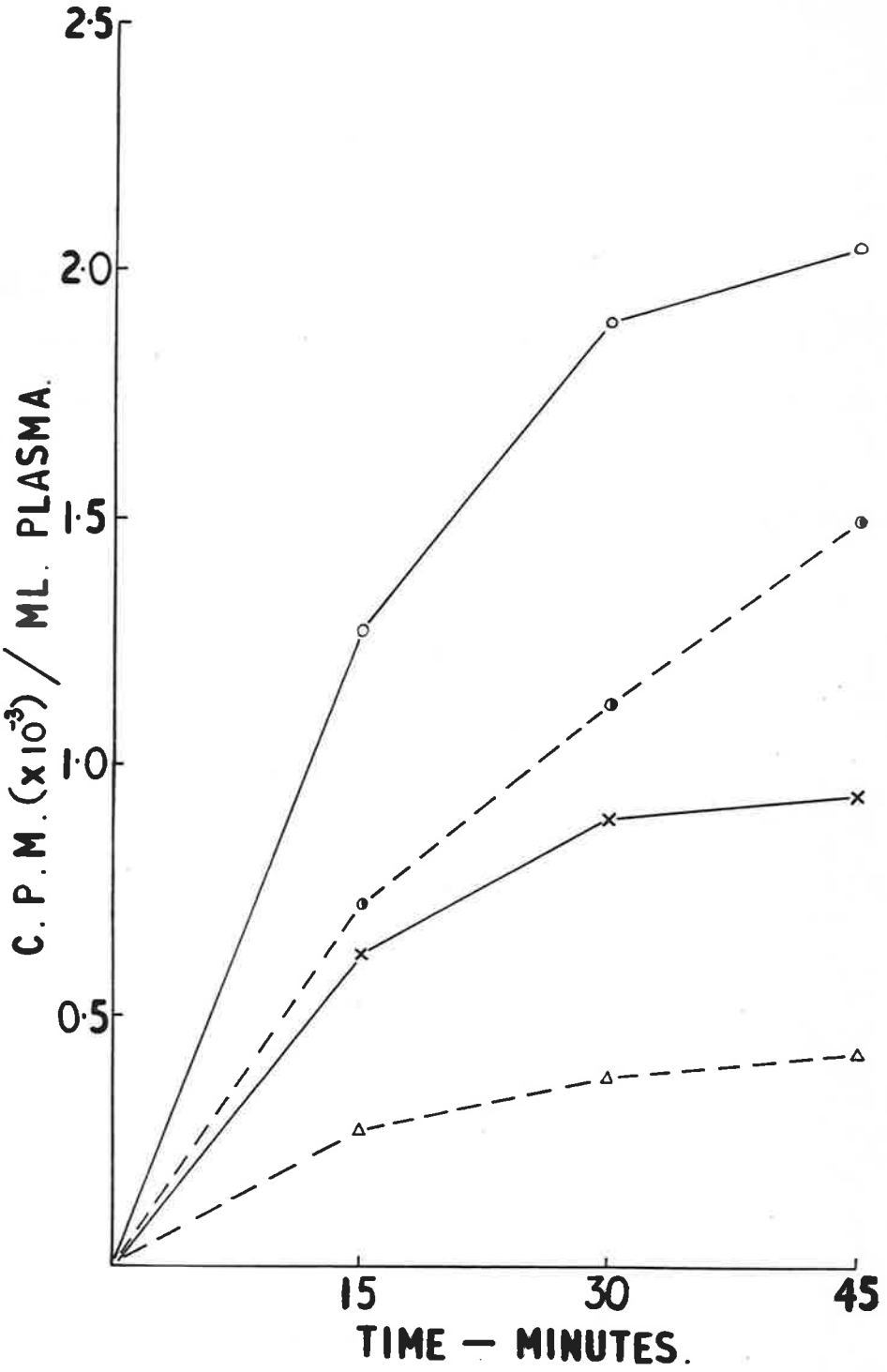

2·5
2·0
1·5
1·0
0·5
C. P. M. (x 10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

TABLE 23

THE INFLUENCE OF VITAMIN D_3 ON THE ACCUMULATION OF LACTIC ACID IN THE SMALL INTESTINE

Everted sacs of distal small intestine from rachitic and vitamin D_3-treated chicks were incubated *in vitro* for 1 hour, under anaerobic conditions, in 5.0 ml. Krebs-Ringer-bicarbonate glucose solution containing 85 µg./ml. calcium. Each value represents the mean ± standard error of the mean for 3 determinations.

Experiment	Treatment	µg. lactic acid/hour/12.5 cm. distal loop
1	-	18.3 ± 2.1
	D_3	18.1 ± 2.1
2	-	14.0 ± 1.3
	D_3	14.8 ± 2.4

B_3-treated preparations respectively. These results show that there has been no increase in the activity of this enzyme following the giving of vitamin B_3. No activity was detected at 0° C or in the presence of fumarate.

DISCUSSION

It is generally accepted that substances may move across biological membranes by two main processes, namely (a) passive diffusion or (b) active transport, or a combination of both. Taggart (1958) has broadly defined both these terms. Simple diffusion signifies that the movement of molecules occurs along a concentration gradient and conforms to Fick's laws of diffusion. Active transport is a term normally applied to those systems in which a substance is transported across a biological membrane, apparently against a concentration gradient (often referred to as uphill transport), at the expense of energy derived from both aerobic and anaerobic cell metabolism. It must be realised, however, that these definitions are not rigid. Active transport may also occur down a concentration gradient. Steric factors are of importance in this type of transport which is often referred to as facilitated transport.

The relative contribution of each of the above processes to the transport of calcium has influenced interpretations on the mechanism of calcium transport and the mode of action of vitamin D. Harrison and Harrison (1960) suggest that when vitamin D is incorporated into the cell surface of the small intestine the permeability properties of the membrane are so altered that an increased rate of diffusion of calcium is possible. These workers believe that vitamin D is mainly concerned with influencing the diffusion of calcium (a process independent of oxidative metabolism) although it may in part also affect the energy dependent active transport system. When the rate of calcium transfer was measured at 10° C it was found to be considerably reduced, although the vitamin D effect still persisted at this temperature. In a further experiment,

Harrison and Harrison (1960) studied the net flux of calcium across the intestinal mucosa using *in vitro* preparations. The outside fluid contained 3 mM calcium and the fluid bathing the serosal surface was 1 mM. Vitamin D was found to increase the rate of calcium transfer across the proximal intestinal wall when the concentration was the driving force. There was, however, only a small increase in the distal portion. This finding is difficult to explain if the process of calcium transport is purely a diffusion phenomenon, as Kimberg et al. (1961) have also demonstrated calcium transport under the influence of vitamin D in the distal portion of rat small intestine. However, Harrison and Harrison could find no evidence to support the alternative concept of an active transport mechanism. Cyanide (5×10^{-4} M) and anaerobic conditions (N_2/CO_2) only partially inhibited the transfer of calcium indicating that the transfer mechanism was not wholly dependent on oxidative phosphorylation.

Other workers have, however, postulated an active mechanism for calcium transport. Schachter and Rosen (1959) found that everted gut sacs from the rabbit transported little calcium at 5° C. Further, the presence of sodium cyanide, or 2,4-dinitrophenol in the medium completely abolished calcium transport. This evidence strongly supported the hypothesis that calcium transport was an active process. Much of the evidence put forward in support of a diffusion or active transfer process is derived from such studies with metabolic inhibitors. In many respects the literature reporting the effects of inhibitors on *in vitro* calcium transport is rather confusing.

In their early paper Schachter and Rosen (1959) claimed that nitrogen inhibited the active accumulation of calcium in rabbit everted

gut sacs whereas more recently Kimberg et al. (1961) found that although nitrogen inhibited transport in the duodenal segments it stimulated calcium uptake in the lower segments of rat small intestine. Studies by Schachter et al. (1960 b), who measured the accumulation of calcium by rat duodenal slices, indicated only partial inhibition of the transport process by 10^{-3} M cyanide. It is obvious that the portion of intestine used to study calcium absorption in the rat is a factor which influences the effect obtained with the metabolic inhibitor and, therefore, any scheme which is postulated to account for calcium transport must also envisage an absorption process for calcium in all parts of the small intestine. Investigations by Wasserman (1960) emphasise this point. Although sodium cyanide, iodoacetic acid and 2,4-dinitrophenol decreased calcium movement from the mucosa to the serosa in duodenal segments of rat small intestine, these substances did not influence calcium absorption in distal segments. Similarly, incubation of everted distal segments under nitrogen did not influence calcium transport in this region.

Schachter and Rosen (1959) suggested on the basis of their experimental observations that the transfer of calcium may be accomplished by a series of carefully controlled steps in the following manner:

(a) uptake of Ca^{45} by the intestinal mucosa from the lumen

(b) the accumulation of Ca^{45} in the interstitial fluid of the gut wall

(c) the subsequent transfer of calcium through the mucosal and serosal layers to the serosal fluid.

A requirement for high energy phosphate is postulated for steps (a) and (b), whereas (c) is thought to be controlled by diffusion. These workers

also found that the bulk of the calcium transferred to the inside medium was in the ionised state and suggest that such evidence supports the participation of a cation transport mechanism analogous to the sodium pump.

From more detailed *in vitro* studies Schachter et al (1960 a) also postulated that at least two steps are involved in the transfer of calcium. The first is the rapid absorption of calcium from the intestine or mucosal fluid which, they suggest, may result from the binding of calcium ions to anionic receptor sites on or in the mucosal cells. The second step involves the transfer of this bound calcium to the fluid bathing the serosal surface. Schachter et al. (1960 a) also claim that their results support the hypothesis of a cation transfer mechanism being involved. An alternative hypothesis will be considered further after discussion of the present findings from transport of calcium across chick small intestine. Because the characteristics of the *in vitro* transport mechanism do not appear entirely similar to the process as studied in the live animal the effects observed with these two systems will be considered separately.

The results presented in this chapter suggest that *in vitro* calcium is transported by an active process. Experiments with non-everted gut sacs indicated that the passage of calcium from serosal to mucosal fluid was small. The amount of calcium accumulated could be regarded as a measure of the passive diffusion and exchange taking place. Further, the process of calcium transport from the serosal to the mucosal side of the membrane was not influenced by prior treatment with vitamin D_3, in contrast to the marked effect observed when calcium transport was

measured from the mucosal to the serosal surface. The accumulation of calcium then under the influence of vitamin D_3 was a unidirectional process. Everted sacs from rachitic chicks transported slightly more calcium than did non-everted sacs from similar birds, and this may have been due to the difficulty of ensuring that the chick was completely deficient in vitamin D_3.

Further data supporting the concept of an active transport mechanism were revealed when calcium transfer was studied at a reduced temperature. There was little accumulation of calcium in the serosal fluid at 0° C when enzyme activity could be regarded as negligible. This evidence would tend to argue against a purely passive diffusion phenomenon as although the rate of diffusion drops with a reduction in temperature one would not expect to find an almost complete inhibition of calcium transport.

In all the <u>in vitro</u> experiments reported, calcium was transferred against a slight concentration gradient, thereby reducing simple diffusion along a concentration gradient to a minimum. The unidirectional increase in transport above the rachitic value brought about by vitamin D_3 has been ascribed to the active transport of calcium. As the diffusion process was not under the control of vitamin D_3 the data from the present studies do not support the views expressed by Harrison and Harrison (1960). It was established in Chapter 2 that the vitamin D effect was also relatively specific for calcium ions. Therefore, the effect cannot be regarded as a non-specific alteration of the permeability of the intestinal wall.

In an attempt to establish the energy source used in the active

transfer process, transport of calcium was studied in the presence of a number of metabolic inhibitors. There was only an 8% inhibition of active calcium transport in distal segments in the presence of 5×10^{-4} M cyanide. Even when the concentration of this substance was greatly increased to 10^{-2} M accumulation of calcium still continued under the influence of vitamin D_3 to such an extent that 60% of the normal calcium transport was measured. This demonstration suggests that the transport process is not dependent on aerobic conditions. Under anaerobic conditions it was also possible to demonstrate the capability of the system in transporting calcium.

The accumulation of calcium in the presence of high concentrations of cyanide does not entirely rule out the possibility of high energy phosphate being formed during the passage of electrons down the electron transport chain. The enzyme succinic dehydrogenase is not inhibited by cyanide and thus the succinoxidase system could play an important role in supplying the necessary energy for transport. When distal small intestine was examined for succinic dehydrogenase activity, however, there was no evidence to suggest that vitamin D_3 was influencing the activity of this enzyme.

That oxidative phosphorylation is not the sole contributor of energy for the transfer process is also suggested by data obtained from studies with 2,4-dinitrophenol. The effect of this inhibitor on oxidative metabolism is well known. Loomis and Lipmann (1948) and Cross, Taggart, Covo and Green (1949) have shown that 2,4-dinitrophenol reversibly uncouples phosphorylation from oxidation. 2,4-Dinitrophenol does not inhibit respiration except at high concentrations. When 2,4-dinitro-

phenol was added to the mucosal medium during *in vitro* experiments, calcium transport was inhibited by only 50%. This finding does not correlate with the *in vitro* results obtained by Schachter et al. (1960 b) who claim a greater inhibition of calcium transfer with this reagent.

After assessing the data obtained in the presence of nitrogen, cyanide and 2,4-dinitrophenol it seems unlikely, then, that calcium transport *in vitro* in the chick small intestine is dependent upon oxidative phosphorylation. Another possible source of energy does exist, however, since the energy requirement could be satisfied by substrate level phosphorylation during anaerobic glycolysis.

Since iodoacetate 2 X 10^{-3} M inhibited transport of calcium under anaerobic conditions by 80%, it would appear that the major energy supply *in vitro* is from glycolysis. Iodoacetate inhibits the formation of 1,3-diphosphoglyceric acid, an intermediate required for substrate level phosphorylation in the following manner:

1,3-diphosphoglyceric acid + ADP $\rightleftharpoons$ 3-phosphoglyceric acid + ATP

(phosphorylglycerate kinase)

Other well known inhibitors of glycolysis, fluoride, phlorizin and arsenite, reduced the level of calcium transport under the influence of vitamin D_3 by as much as 50%.

For many years, it was assumed that all active transport mechanisms gained their energy requirement from oxidative phosphorylation. More recently, it has been shown from *in vitro* studies that a number of substances rely equally upon glycolysis and aerobic oxidation to promote their active transport. Breuer and Whittam (1957) and del Monte (1961) have also suggested that glycolysis supplies the energy for the transport

of glucose, water, sodium and potassium. That vitamin D_3 does not affect transport by altering the amount of glycoysis is shown by the production *in vitro* of equal amounts of lactic acid from rachitic and vitamin D_3-treated distal small intestine.

Vitamin D_3 was also without effect on the $Q(O_2)$ measured in respiring slices of intestinal tissue. This demonstration substantiates the findings from the *in vitro* inhibitor experiments, in that aerobic conditions do not seem necessary for active transfer. Zar and Irving (1959) in a recent report were also unable to show any effect of vitamin D on the respiration of oral tissue from rachitic rats.

The addition of ouabain, a cardiac glycoside, did not interfere with calcium transfer *in vitro*. Daniel and Robinson (1960) state that ouabain interferes with sodium and potassium exchange in rabbit uterine preparation. Csaky, Hartzog and Fernald (1961) have shown that this drug completely inhibits the active transport of 3-methylglucose and they suggest that the inhibitory action of ouabain is primarily on the 'sodium pump'. If this is correct, then the finding of an inhibitory effect of ouabain on calcium transport *in vitro* would indicate that the uptake is independent of the 'sodium pump'.

In vivo studies in the presence of metabolic inhibitors are much more difficult to interpret. 2,4-Dinitrophenol and iodoacetate were without effect on the transport of calcium. From these data, energy does not appear to come from either glycolysis or oxidative phosphorylation. Only phlorizin reduced the level of transport to any extent. In Chapter 2, the different characteristics displayed by *in vivo* and *in vitro* experiments concerning the site of calcium absorption were discussed. Similarly, with

inhibitor studies it may well be that the chick is capable of overcoming, *in vivo*, the effects of the reagent either by detoxication or, as already pointed out, if energy is gained from more than one source, by utilising more efficiently alternate metabolic pathways to allow the active transfer of calcium to occur at a normal rate.

Although the observations made in the presence of inhibitors are of great value toward interpreting the nature of the process such studies do not provide direct proof of a specific reaction being involved. Many of the inhibitors are not specific for a single enzyme reaction. For example, the ability of phlorizin to inhibit the phosphatases that hydrolyse hexose phosphates is well known. However, Shapiro (1947) and Lotspeich and Keller (1956) have also shown that phlorizin inhibits the oxidative reactions of the citric acid cycle and the generation of ATP which normally accompanies these reactions. More detailed work would be required to confirm the primary action of these inhibitors. In a recent review, Lotspeich (1960-61) has discussed the action of phlorizin in inhibiting glucose transport. The effect of this inhibitor on oxidative metabolism is thought to be due to an alteration in the permeability of the mitochondrial membrane. High energy phosphate is normally required to maintain the permeability of this membrane. Glucose is believed to be transported through a biological membrane by combination with a membrane carrier and phlorizin may inhibit at this site. An active carrier has already been postulated for calcium transport so that an analogous situation may well exist which could partly explain the inhibition observed with phlorizin. Rosenberg and Wilbrandt (1957) visualise phlorizin as a steroid analogue, binding

a membrane carrier through metal chelation or hydrogen bonding.

DeLuca and Engstrom (1961) have recently published observations on calcium uptake in kidney mitochondria. The presence of ATP, magnesium ions and an oxidisable substrate are necessary for this process which does not appear to be under the influence of vitamin D. Although ATP is required, inhibitor studies suggested that the process was not directly dependent on oxidative phosphorylation nor the operation of the entire electron chain.

These findings are interesting in the light of the evidence available from these present investigations. If the levels of ATP are altered under rachitic conditions then the presence of vitamin D may be an important factor in controlling the cellular level of ATP. The enzyme adenosine triphosphatase may also play an important role in view of the more recent observations on the mechanism of sodium transport (Whittam and Wheeler, 1961).

It would appear from these present studies that active transport under anaerobic conditions, in the absence of glucose, is dependent upon the oxidation of endogenous substrates. When the level of these substrates is reduced, as in starvation, the calcium transport in the presence of added glucose is also reduced.

The present investigations with chicks strongly supports the conclusion that a metabolically active membrane is required for calcium transport. It is not possible with the data available to describe in detail the mechanism of this process. As pointed out by Taggart (1958), the demonstration that a transport mechanism can be inhibited by unfavourable metabolic conditions such as anaerobiosis or selective enzyme

inhibition, provides only suggestive evidence of the active nature of the transport. The maintenance of cellular structure is also dependent upon continuous metabolic activity and subtle changes in the membrane structure, for example, could modify diffusion processes as well as active transport.

It would appear likely that calcium transport in vivo can occur either by active transport or diffusion. However, conditions in the small intestine, under normal dietary status, would seem unfavourable to the presence of high concentrations of calcium in solution and calcium uptake by diffusion must therefore be a very slow process. A mechanism that rapidly moves the small amount of calcium ion across the membrane would allow further dissociation of the sparingly soluble calcium salts to take place and thus lead to a more rapid uptake of calcium. Hence, this may be why the vitamin D active transport mechanism is necessary for the transport of this small ion.

Robertson (1960) has postulated a general scheme to explain the active transport of ions. He believes that the driving force behind the movement of ions is the separation of a positive and negative charge. The electron so separated is thus free to move through an array of electron carriers present in a lipoidal membrane. A structure such as mitochondria has been shown to be hypothetically suitable for the separation of the charges.

Much of the evidence implicating oxidative phosphorylation in an active transport mechanism stems from inhibitor studies with 2,4-dinitrophenol. As pointed out by Robertson, however, such evidence does not necessarily prove that ATP formed in oxidative phosphorylation

is an intermediate in the process of transport. An alternative explanation could be that the separation of charges and the formation of ATP may be separate steps which, individually, may or may not be inhibited by 2,4-dinitrophenol. This possibility may, therefore, account for active calcium transport still taking place across chick small intestine in the presence of 2,4-dinitrophenol.

To enable transport of ions to take place, Robertson visualises a carrier capable of reversible oxidation and reduction in a lipoidal membrane. The properties of this compound are important, for not only is it necessary for the carrier to complex with the ion but it must also be capable of releasing this ion on the side of the membrane through which its efflux is slow relative to its influx by active transport. Combination of the ion with a carrier would thus allow movement of the ion across a lipoidal membrane which was previously impermeable to the free ion. Specificity of transport may also be explained in this hypothesis as a carrier in a non-aqueous membrane would have a different affinity for a different ion.

Mitchell (1961) in an extension of Robertson's hypothesis, has proposed a coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. This worker also visualises a compound present inside the cell capable of accepting or donating electrons which, in combination with a reversible adenosine triphosphatase system, can transport ions in either direction.

On the basis of these ideas, a tentative scheme may be postulated for active calcium transport across chick small intestine. The accumulated evidence from studies in this thesis suggests that, provided

there is present an oxidisable substrate capable of producing energy for the transport and an active carrier compound, then calcium transfer across the membrane barrier can proceed normally and may be demonstrated *in vivo* or *in vitro*. Although calcium transport *in vitro* relies upon energy from glycolysis, neither glycolysis nor oxidative phosphorylation are specifically required for transport under physiological conditions. As suggested by DeLuca and Engstrom (1961), it may well be that only a portion of the electron chain is required. From investigations reported in Chapter 2, it was concluded that the delay between giving vitamin D_3 treatment and the observed improvement in calcium absorption could be explained if vitamin D_3 were being changed to a more active form. Further studies outlined in Chapter 3, suggested that the adrenal gland was the site of this transformation.

Evidence presented in this chapter clearly shows that calcium is transported as the result of an active process. To fulfil the requirements for such a mechanism the calcium carrier must be incorporated into the cell membrane. Further, in accordance with the hypothesis proposed by Robertson (1960), this carrier must also be capable of undergoing reversible oxidation and reduction and thus permit the calcium to be complexed.

It is postulated then that the following steps are involved in the general scheme of calcium absorption:

(a) vitamin D_3 is changed in the adrenal gland to a form more active in the transport of calcium

(b) active material is rapidly secreted from the adrenal gland and incorporated into the mucosal membrane of the small intestine.

(c) oxidation and reduction reactions within the membrane make energy available for the transfer of calcium and also allow the calcium ion to complex with the carrier.

(d) calcium is transferred to the serosal surface of the membrane where specific reactions permit release of the calcium and the return of the carrier to the site of calcium uptake.

The formation and possible chemical nature of an active calcium carrier is discussed further in the following chapter.

SUMMARY

1. The transport of calcium across the small intestine was found to be unidirectional. There was little transport of calcium from the serosal to the mucosal surface and vitamin D_3-treatment did not affect this movement.

2. Calcium was not transported at 0° C even under the influence of vitamin D. It was considered unlikely that calcium transport, under the influence of vitamin D_3, was the result of a diffusion process. An active transport mechanism was postulated.

3. Under starvation conditions or in the absence of added substrate, the amount of calcium accumulated in the serosal fluid was partially reduced. From this evidence, it was concluded that endogenous substrate can support at least 60% of normal calcium transport.

4. Ouabain did not inhibit movement of calcium across everted distal sacs of vitamin D_3-treated chicks. This observation suggested that calcium transport was independent of the 'sodium pump'.

5. Several metabolic inhibitors were studied for their effect on calcium transport *in vitro*. Anaerobic conditions, 2,4-dinitrophenol or cyanide (10^{-2} M) did not completely eliminate active movement of calcium. Iodoacetate under anaerobic conditions gave the greatest inhibition (80%). From these studies, it was postulated that the major portion of the *in vitro* energy for the active transfer of calcium was being derived from reactions linked to the glycolytic cycle. Oxidative phosphorylation could, however, contribute to a small extent.

6. Inhibition of calcium absorption was also studied *in vivo*. 2,4-dinitrophenol, iodoacetate and iodoacetamide were without effect.

Phlorizin inhibited to a small extent. Energy for the *in vivo* transport of calcium thus appeared independent of either glycolysis or oxidative phosphorylation.

7. Lactic acid present in the small intestine was estimated as a measure of glycolytic activity. Vitamin D_3 did not increase glycolysis.

8. There was no increase in succinic dehydrogenase activity or in the $Q(O_2)$ value of respiring slices of small intestine following vitamin D_3-treatment.

9. Chelation of calcium with EDTA decreased the amount of calcium absorbed from duodenal loops *in vivo*. It was thought that calcium was normally in the ionic form for absorption.

10. The experimental findings were discussed in relation to the present proposed theories of active transport of ions. A tentative scheme has been postulated to account for calcium transport across the small intestine under the influence of vitamin D.

CHAPTER 5

ATTEMPTED PREPARATION, IDENTIFICATION AND ISOLATION OF ACTIVE CARRIER MATERIAL FOR CALCIUM TRANSPORT

Introduction

Part I: Attempted preparation of ketone$_{250}$ and the calcium enolate of ketone$_{250}$.

Part II: An examination of various tissues and fluids for the presence of active carrier material.

Discussion

Summary

CHAPTER 5

ATTEMPTED PREPARATION, IDENTIFICATION AND ISOLATION OF ACTIVE CARRIER MATERIAL FOR CALCIUM TRANSPORT

INTRODUCTION

A scheme was postulated at the end of Chapter 4 to indicate the mode of action of vitamin D_3 and the mechanism of calcium transport. The calcium ion was thought to combine with a carrier compound activated for the movement of calcium across the mucosal membrane. The theory was postulated that the active calcium carrier could be derived from a parent compound such as vitamin D_3 by transformation of this substance in the adrenal gland.

The chemical nature and properties of this proposed carrier, however, still remain to be defined. Raoul and his co-workers (Raoul, LeBoulch, Baron, Chopin and Guerillot-Vinet, 1954; Baron, LeBoulch and Raoul, 1955; Raoul, LeBoulch, Baron, Bazier and Guerillot-Vinet, 1956; Raoul, 1958) have prepared derivatives of vitamin D possessing potent antirachitic properties. They have demonstrated that one of these compounds, known as ketone$_{250}$, can chelate calcium. The calcium complex so formed was found to possess an antirachitic potency in the chick equivalent to that of vitamin D_3. This compound would appear admirably suited for the role of a carrier compound as it is capable of undergoing keto-enol tautomerism, thus permitting calcium to be readily complexed and released.

Further investigations on the preparation and properties of ketone$_{250}$ and its calcium complex are reported in Part 1 of this Chapter. Following the synthetic preparation from cholesterol of a calcium complex,

a number of tissues and fluids were examined for the presence of material active in increasing the transport of calcium. Material was only considered 'active' if it stimulated an immediate improvement of calcium absorption in the rachitic chick. Attempts to isolate this material are described in Part 1 of this Chapter.

PART I

THE ATTEMPTED PREPARATION OF KETONE$_{250}$ AND THE CALCIUM ENOLATE OF KETONE$_{250}$

MATERIALS AND METHODS

Preparation of ketone$_{250}$* from cholesterol

The procedure followed, with one exception, was essentially as described by Raoul et al. (1954). As floridine XXS was not readily available, this catalyst was replaced by Fuller's earth which was activated by heating at 300° C for 2 hours. Cholesterol (10 g.) was dissolved in 400 ml. of carbon tetrachloride and after the addition of 80 g. Fuller's earth the mixture was refluxed continuously for 5 to 6 hours until the mixture turned black. The colour changes previously described by Bills (1926) were observed during this procedure.

After separating the Fuller's earth by vacuum filtration the filter cake was washed several times with carbon tetrachloride and then allowed to stand for 15 minutes with 50 ml. of dry peroxide-free ether, stirring occasionally during this period. The orange-yellow ether extract was filtered and the extraction and filtration cycle was repeated several times until there was little colour appearing in the extract. Finally, the Fuller's earth was rinsed once with 50 ml. of acetone and this washing was added to the pooled extracts.

The organic solvent was removed in vacuo in a flash-evaporator, keeping the temperature below 25° C. The residue was dissolved in carbon tetrachloride and chromatographed on a column (180 mm. X 25 mm.) of neutral Woelm alumina deactivated to Brockmann grade IV by the addition

* The chemical nomenclature used during this preparation is that of Raoul et al. (1954). This does not infer however, that the products obtained are necessarily identical to those obtained by the latter workers.

of water. Continuous washing with carbon tetrachloride removed a highly coloured fraction whilst a brown band remained adsorbed to the alumina. When the eluate became colourless, the solvent was changed to pure ether, and the new eluate appeared as an orange-red liquid.

The ether eluate was again taken to dryness and redissolved in a mixture of petroleum ether (b.p. 40°-60°) and ether (9:1, v/v). The compounds present in this mixture were separated by chromatography on a column (160 mm. X 15 mm.) of neutral Woelm alumina deactivated to Brockmann grade II by the addition of water. On washing the column with the petroleum ether - ether mixture, an orange coloured substance was removed (8'). When this solvent was replaced with ether the eluate again appeared orange-red in colour (Pc).

After taking the eluate nearly to dryness, the product (Pc) was treated with 5 ml. of 5% methanolic potassium hydroxide. The mixture was boiled for exactly 1 minute on a water bath and distilled water then added to give a total volume of 100 ml. A yellow precipitate was formed. The alkaline hydrolysate and the precipitate were washed several times with ether in a separating funnel and then acidified with sulphuric acid until just slightly acid to litmus. The aqueous layer was extracted with 50 ml. of ether and the ether extract was washed once with distilled water and then taken to dryness in vacuo.

The product was dissolved in petroleum ether and again chromatographed on a column (150 mm. X 10 mm.) of Woelm alumina deactivated to Brockmann grade II by the addition of water. The fraction adsorbed on the column was washed with petroleum ether and finally eluted with a mixture of petroleum ether and ether (1:9, v/v). When this eluate was

taken to dryness the product obtained was an orange-brown waxy material (ketone$_{250}$). The average yield of material was 25 mg.

Preparation of the calcium complex of ketone$_{250}$ (compound 8_3)

The ether extract containing compound Pc was taken to dryness in a flash evaporator and the residue (150 mg.) was boiled for 1 minute with 5 ml. of 5% methanolic potassium hydroxide. This mixture was diluted to 100 ml. with a solution containing 20 mg. calcium and 20 µc. Ca^{45} and then allowed to extract into 100 ml. of peroxide-free ether over a period of 24 hours. The ether layer was removed and evaporated to dryness in vacuo. The residue (90 mg.) was redissolved in a known volume of ether and the Ca^{45} present was estimated by scintillation counting.

Biological assay of active material

Rachitic chicks were dosed orally with compound 8_3 dissolved in arachis oil 16 hours before preparing *in vivo* isolated duodenal loops (Method A). Each chick received 2 mg. of this compound. Radioactivity in the plasma was measured by an end-window geiger counter (P 27 (a) i).

Spectrophotometric assay

The IR spectra were recorded on a double-beam recording spectrophotometer. This work was carried out by Mr. A.L. Moritz of the Department of Organic Chemistry, University of Adelaide. Absorption in the UV range was determined with a Shimadzu recording spectrophotometer.

RESULTS

The yield of ketone$_{250}$ was poor, being less than 0.5%. Although this compound was prepared six times, the maximum amount of material obtained from any single preparation using 10 g. of cholesterol was 50 mg. The substitution of the Fuller's earth for floridine XXS may possibly account for these results. When the IR spectrum of the ketone$_{250}$ was examined, an absorption band of high intensity was seen in the region 5.7 - 5.8 μ. which suggested the presence of a carbonyl grouping (Figure 30). Deformations due to OH and $-CH_2-$ frequencies were prominant in the regions 2.9μ. and 3.4μ. respectively. The IR spectrum was strikingly similar to that reported by Raoul (1958).

An attempt was also made to identify the product from its absorption in the UV range. The product was dissolved in absolute ethanol and its absorption determined from 230 mμ. to 320 mμ. There was no indication of strong absorption at 250 mμ which Raoul (1958) previously observed with ketone$_{250}$. Further, the product appeared extremely labile under these conditions. The initial UV absorption spectrum of this compound could not be reproduced once the solution had been briefly exposed to the beam of UV light in the spectrophotometer (Figure 31).

The semicarbazone and 2,4-dinitrophenol hydrazone could not be prepared, contrary to the claims made by Raoul and his co-workers who have put forward apparently convincing chemical evidence in support of the proposed compound. As the substance isolated was certainly not pure, an elementary analysis of the product was not obtained.

During the preparation of compound 8_3, the addition of Ca^{45} instead of Ca^{40} to the reaction mixture following alkaline hydrolysis

FIGURE 30. Infra-red spectrum of ketone$_{250}$. The material was analysed as a nujol mull using a NaCl prism at 26° C.

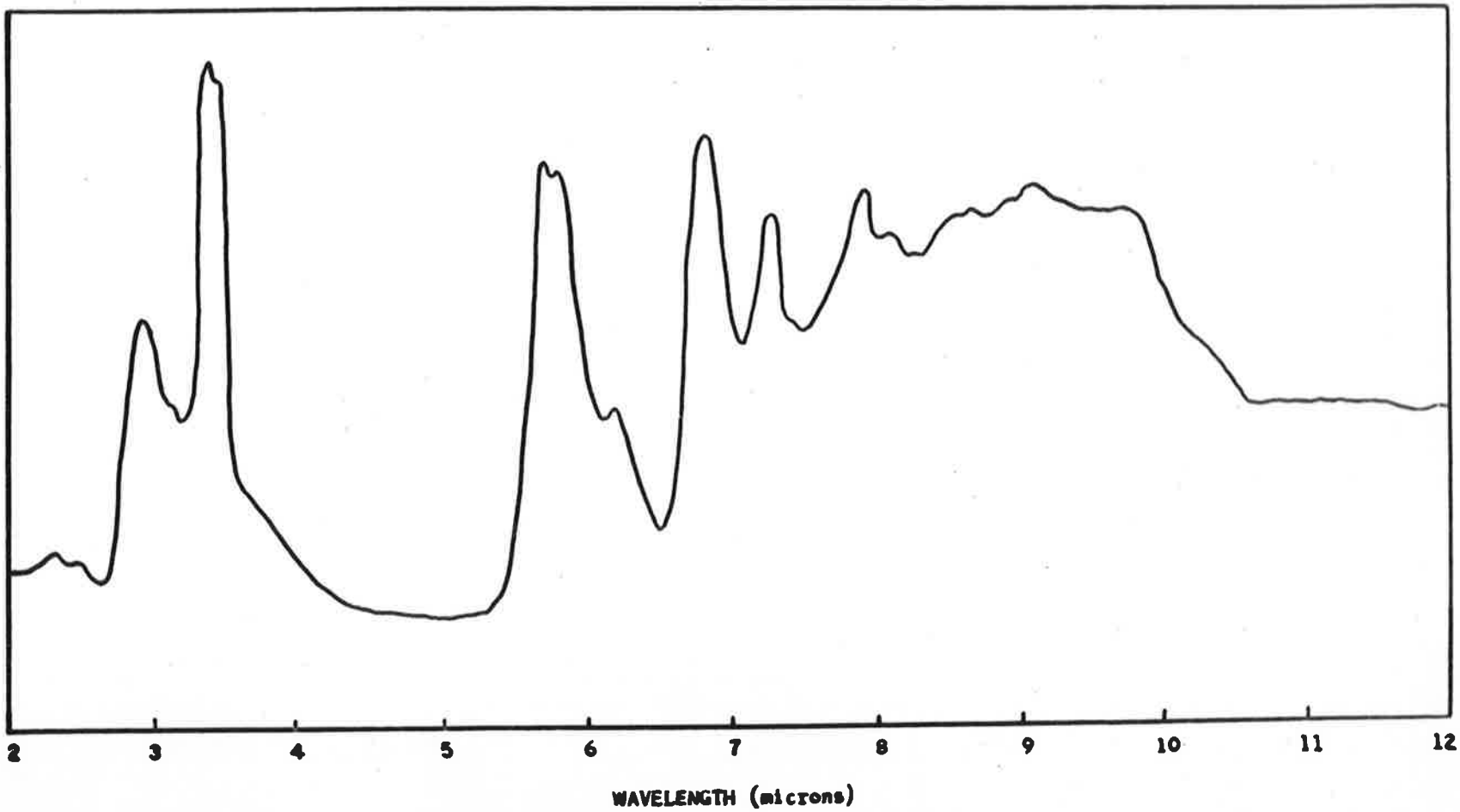
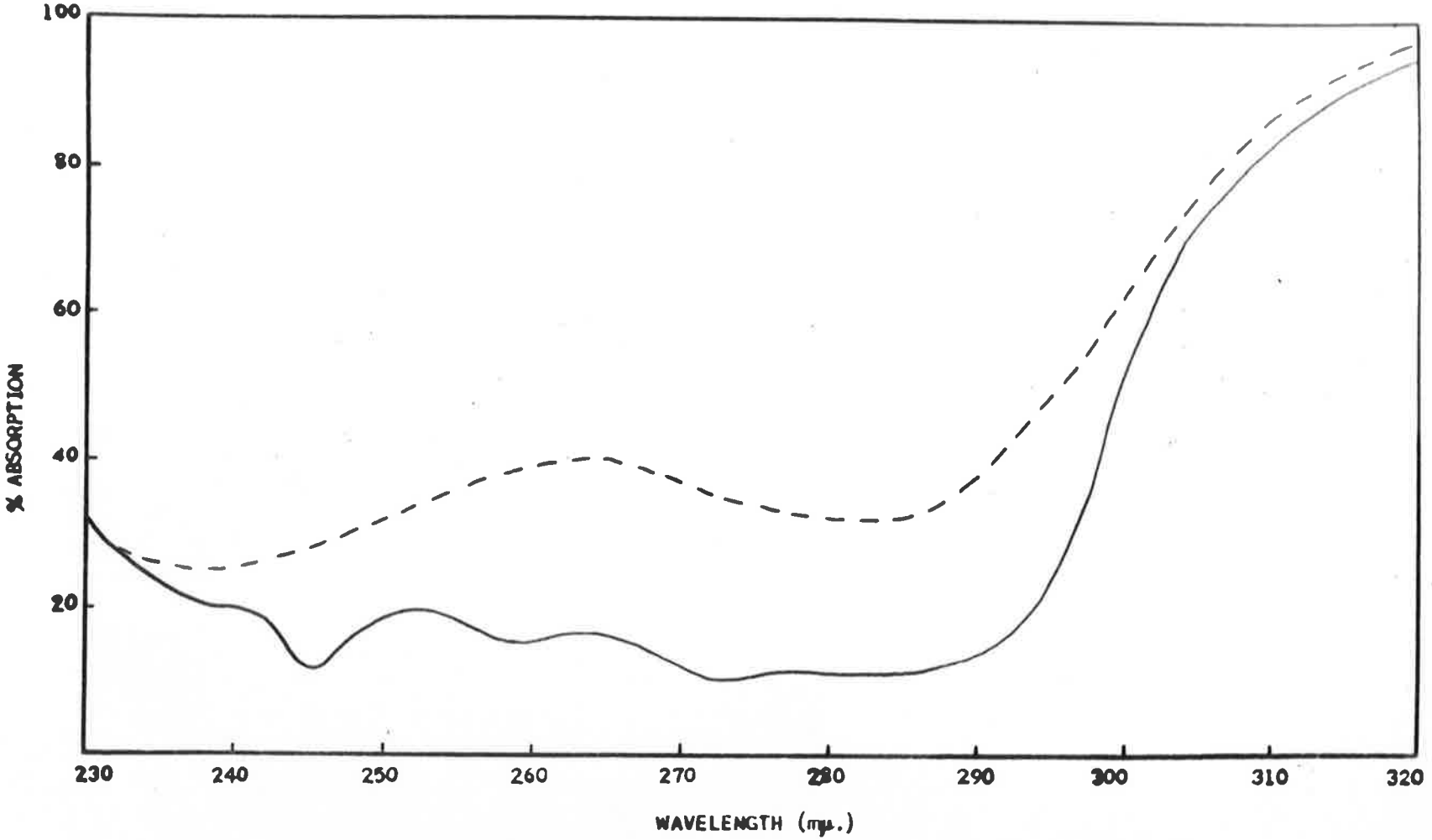


FIGURE 31. Ultra-violet spectrum of ketone$_{250}$ (in ethanol). The complete line is the initial spectrum, and the broken line indicates a rescan of the same material after one exposure of the solution in the spectrophotometer to UV light.



provided a means of demonstrating the formation of a calcium complex. When an ether extract was made of the original hydrolysate which contained 1 μc. per mg. calcium, the amount of radioactivity estimated in this extract was 45,000 c.p.m./ml. After applying the necessary background, quenching and efficiency corrections, the total counts per minute at 100% efficiency present in the extract were 556,200. As 1 μc. is equivalent to 2.2×10^{-6} disintegrations per minute at 100% efficiency, it is evident from the data available that the extract contained 0.25 μc. Ca^{45} or 250 μg. calcium in a total weight of 90 mg. material. Under similar conditions, except for the absence of any cholesterol derivative, no radioactivity was detected in the ether extract.

The UV spectrum of this compound (8_3) was also determined. In this case it was first necessary to initially extract the alkaline hydrolysate with ether to remove any unhydrolysed Pc. Such a procedure is practicable as Pc is rapidly taken up by ether whereas Raoul and his colleagues (1954) have shown that the calcium complex is only slowly extracted into ether. There was no evidence of strong absorption at 265 mμ in ether, as previously descibed for this compound. A peak was observed at 232 mμ (Figure 32).

The IR spectrum of this compound is reproduced in Figure 33. When compared with the IR spectrum observed with $ketone_{250}$ (Figure 30), the spectrum shows that the carbonyl grouping, previously noted in the region 5.7 - 5.8 μ. has shifted to 5.8 - 6.0 μ. and is also reduced in intensity (comparatively only). When tested for its ability to increase calcium absorption in chicks, there was no evidence to suggest that compound 8_3 was active in this regard. Table 24 shows that there was

FIGURE 32. Ultra-violet spectrum of the calcium complex of ketone$_{250}$ (in ether).

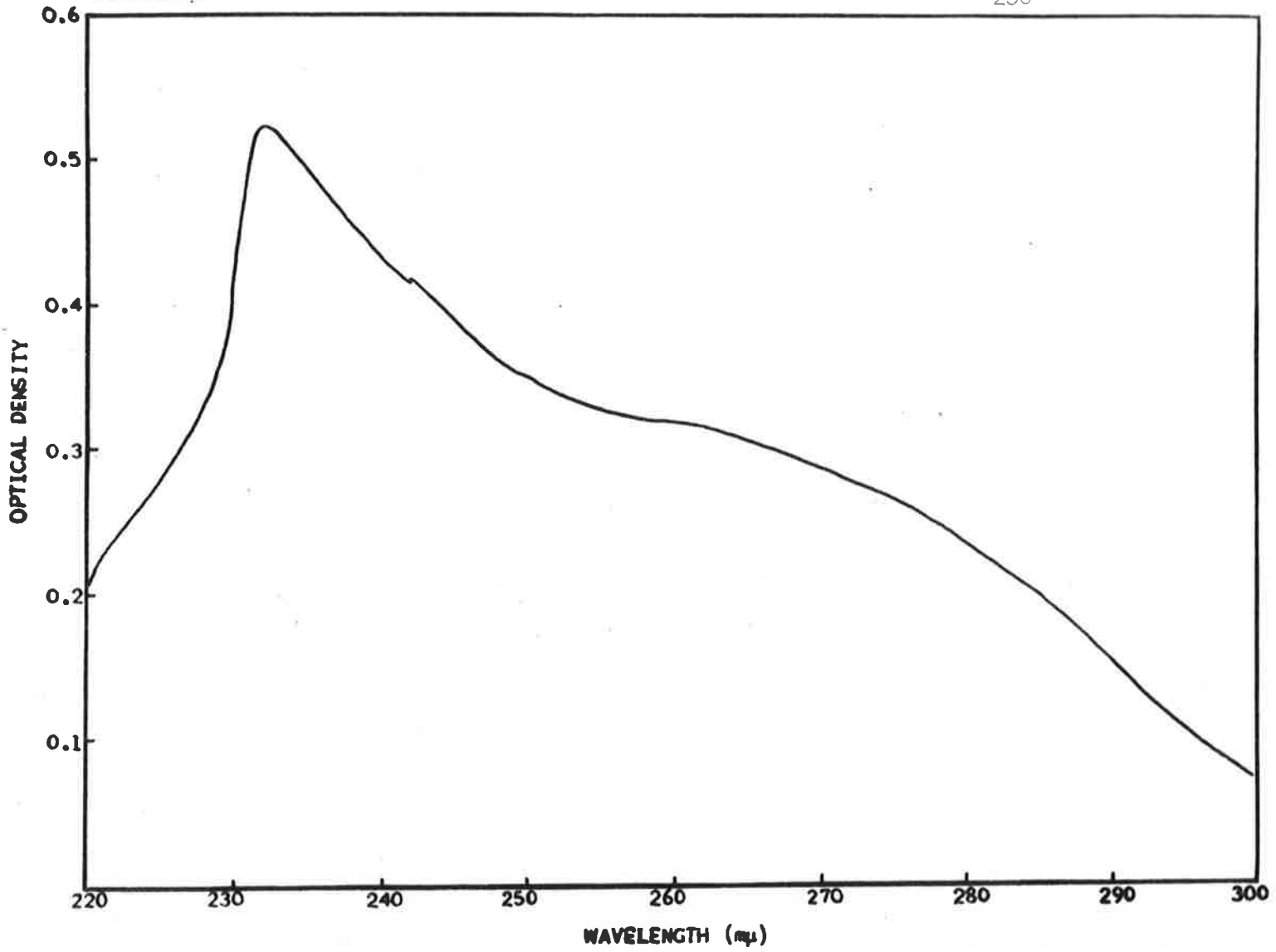


FIGURE 33. Infra-red spectrum of the calcium complex of ketone$_{250}$. The material was analysed as a nujol mull using a NaCl prism at 26° C.

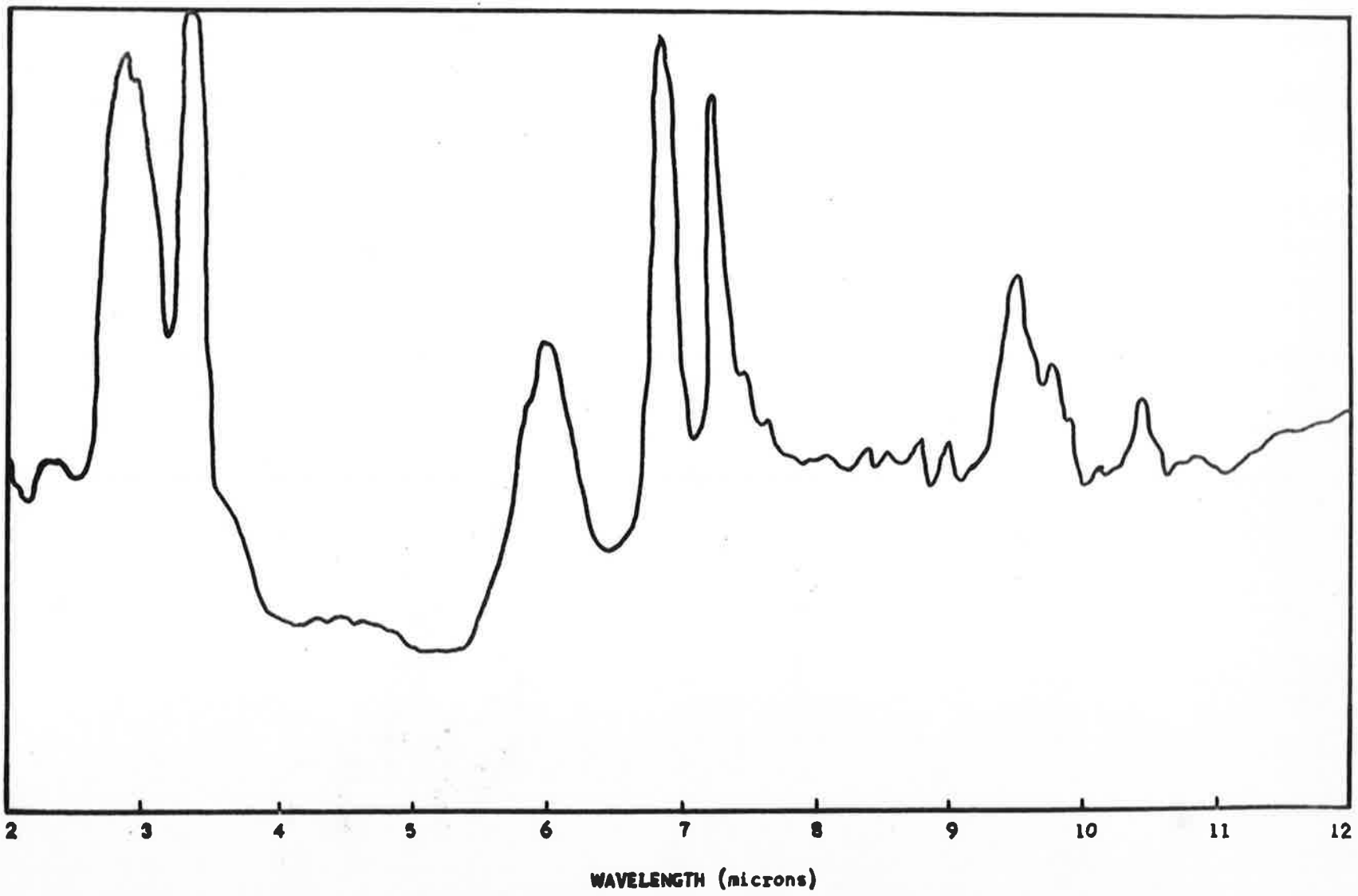


TABLE 24

THE EFFECT OF A CALCIUM COMPLEX (8_3) ON CALCIUM ABSORPTION FROM DUODENAL LOOPS OF RACHITIC CHICKS

The calcium complex was dissolved in arachis oil and rachitic chicks received 2 mg. by stomach tube 16 hours prior to introducing $Ca^{45}Cl_2$ into the loop. Plasma Ca^{45} levels were measured after preparing isolated duodenal loops *in vivo*. Each value is the mean of 4 birds.

Treatment	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins)		
	10	20	30
-	0.700	0.725	0.850
8_3	0.650	0.700	0.825
D_3	1.177	2.307	2.407

no increase in the level of plasma Ca^{45} above the rachitic control value in the group receiving oral treatment with the calcium complex.

PART II

AN EXAMINATION OF VARIOUS TISSUES AND FLUIDS FOR THE PRESENCE OF ACTIVE CARRIER MATERIAL

Kodicek and his colleagues have recently investigated the pattern of C^{14}-labelled vitamin D metabolism in the rat (Kodicek, 1956 a; Kodicek, 1960). The data obtained following the giving of an oral dose of 1 mg. C^{14}-labelled vitamin D_2 to rachitic rats indicated that, of the total radioactivity recovered, 70.9% was in the form of breakdown products and only 30.3% was associated with vitamin D_2. A similar pattern of distribution could also be demonstrated with much smaller doses of vitamin D (10 µg.).

More interesting, however, was the relationship of time with the distribution of the vitamin in the tissues following its administration intravenously. Table 25 reproduces some results obtained by Kodicek, Cruickshank and Ashby (1960). Very little of the vitamin appeared in the adrenal gland, contrary to a recent report by Raoul and Gounelle (1958). Within 20 minutes the major portion of the dose accumulated in the liver and even after 5 hours the liver still retained one half of the dose. A considerable amount of radioactive material believed to be vitamin D associated with serum proteins (Innes Chalk and Kodicek, 1960), continued to remain in circulation.

Bile may also play an important part in the metabolism of vitamin D as it may be involved in the absorption and re-excretion of the vitamin in mammals (Greaves and Schmidt, 1933; 1934 a, b; Taylor, Weld and Sykes, 1932; 1935; Heymann, 1937 b). Kodicek (1956 a) has also suggested that the vitamin D breakdown products found in the faeces

TABLE 22

THE METABOLISM OF C^{14}-LABELLED VITAMIN D_2 INJECTED INTRACARDIALLY INTO RATS

(Reproduced from the data of Kodicek, Cruickshank and Ashby, 1960)

Two rats were injected with 200 µg. C^{14}-labelled vitamin D_2 and the tissues analysed at varying time intervals. The figures in parenthesis represent the percentage of the dose recovered as vitamin D.

	% total C^{14}-recovered		
	TIME		
	20 min.	40 min.	5 hours
Adrenals	1.3 (1)	0.2	0.5 (0.2)
Liver	54.3 (48.9)	60.1 (55.4)	50.0 (47)
Blood	10.7	11.3	9.1
Kidneys	0.3	3.9	2.3
Intestinal tissue	1.5	0.4	1.3

originate from the liver and that these products are then excreted through the bile. These interesting observations suggested that an examination of the various tissues and fluids after vitamin D_3 administration to chicks might assist in the isolation and identification of active carrier material.

BILE

The influence of bile on calcium absorption in rachitic chicks

Materials and methods

Bile was obtained from the gall-bladders of freshly killed rachitic and vitamin D_3-treated chicks. The chicks which had been treated with vitamin D_3 received an oral dose of 100,000 I.U. vitamin D_3 over a period of 4 days. Bile from these birds has been designated 'normal' bile, whilst bile from the rachitic chicks will be referred to as 'rachitic' bile. Isolated duodenal loops were prepared from both rachitic and vitamin D_3-treated (100 I.U. - 16 hours) chicks (Method A). The influence of bile on calcium absorption was observed in rachitic chicks only, the vitamin D_3-treated group serving as controls to estimate the extent of calcium absorption due to the presence of bile.

An aqueous solution (0.5 ml.) containing 4.0 mg Ca^{40}, 8.0 µc. Ca^{45} and 0.1 ml. bile was inserted into the ligated duodenal segment. In place of the bile solution, rachitic and vitamin D_3-treated control groups received the $Ca^{45}Cl_2$ test solution containing 0.1 ml. saline (0.9%). Calcium absorption was measured by following plasma Ca^{45} levels at varying time intervals. Prepared plasma samples were then counted using a thin end-window geiger tube (P 27 (a) i).

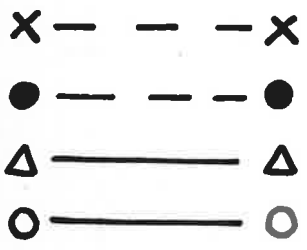
Results

Figure 34 presents the results of this experiment. Bile stimulated an

FIGURE 34

THE EFFECT OF BILE ON CALCIUM ABSORPTION IN VIVO

Bile was obtained from rachitic chicks (rachitic bile) and from chicks dosed over a period of 4 days with 100,000 I.U. vitamin D_3 (normal bile). Ca^{45} was measured in plasma after preparing duodenal loops *in vivo*. 0.1 ml. of the respective bile solution together with 0.4 ml. $Ca^{45}Cl_2$ solution was inserted into the prepared loop. Controls consisted of rachitic and vitamin D_3-treated chicks who received 0.1 ml. saline in place of the bile solution. Each point represents the mean of 4 birds.

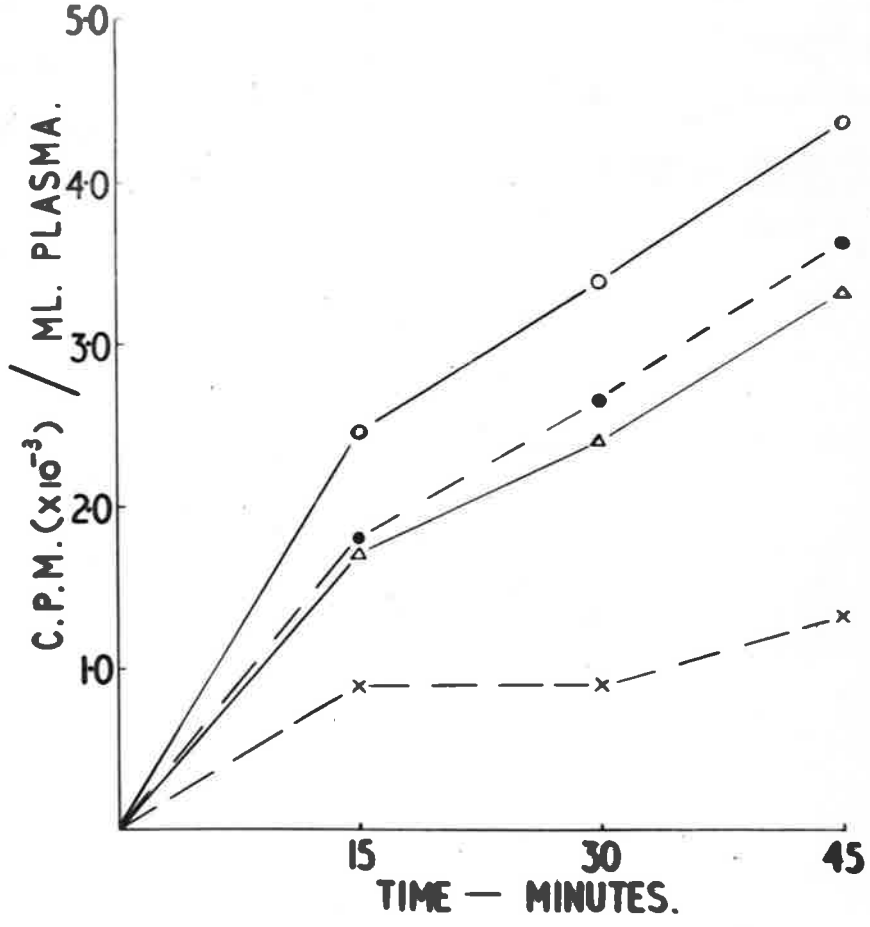


rachitic controls

rachitic chicks with rachitic bile

rachitic chicks with normal bile

vitamin D_3-treated controls


5·0
4·0
3·0
2·0
1·0
C.P.M. (X10⁻³) / ML. PLASMA.
15
30
45
TIME — MINUTES.

increase in circulating plasma calcium above the level of the rachitic control group. The increase, however, was not as marked as the effect observed after vitamin D_3-treatment. 'Rachitic' bile was equally as effective as 'normal' bile in promoting calcium absorption.

The isolation and bioassay of active material in bile

Materials and methods

Eight-week old cockerels which had been raised from birth on a diet adequately supplemented with vitamin D_3, were obtained from a local hatchery. These birds were heavily dosed with vitamin D_3 (100,000 I.U.) over a period of 3 days before removing bile from the gall-bladders. The bile was immediately poured into 50 ml. of a mixture of boiling ethanol and ether (1:1, v/v). The precipitate was removed, washed once with the same solvent and the combined extracts were then taken to dryness in vacuo. Finally, the residue was redissolved in 3.0 ml. of propylene glycol with gentle warming.

The material obtained was tested for its biological potency in promoting calcium absorption in rachitic chicks. One hour after administering the extract by intracardial injection to rachitic chicks, *in vivo* duodenal loops were prepared (Method A), and calcium absorption was studied by following the level of plasma Ca^{45}. Samples of plasma were counted as previously described (P 27 (a) i).

Results

No increase in circulating Ca^{45} was observed in the rachitic chicks receiving the bile extract as compared to their rachitic controls (Table 26).

TABLE 26

THE INFLUENCE OF PLASMA, EXTRACTS OF BILE AND THE ADRENAL GLAND ON CALCIUM ABSORPTION IN RACHITIC CHICKS

Ca^{45} was measured in plasma after preparing *in vivo* isolated duodenal loops of rachitic and vitamin D_3-treated chicks (Method A). 0.5 ml. plasma plus 0.25 ml. $Ca^{45}Cl_2$ test solution was inserted directly into the ligated loops or rachitic birds. Controls were given 0.5 ml. saline (0.9%) in place of the plasma. Extracts of bile and the adrenal gland (0.15 ml.) were injected intracardially into rachitic chicks 1 hour before inserting Ca^{45} in the ligated segment. Controls received 0.15 ml. propylene glycol under the same conditions. Each value is the mean of 4 birds.

Treatment	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins)		
	15	30	45
- (both controls)	0.54	0.82	1.49
D_3	3.54	4.50	4.20
Bile extract	0.73	0.88	1.45
Plasma	0.60	0.82	1.40
Adrenal extract	0.68	0.80	1.42

PLASMA

The influence of plasma on calcium absorption in rachitic chicks

Materials and methods

A group of rachitic chicks approximately 35 days old were dosed orally with 7,000 I.U. vitamin D_3 over a period of 1 week. The birds were killed by removing blood from the heart, using a heparinised syringe. The blood was immediately centrifuged and the plasma pooled and stored at 4° C until required. *In vivo* duodenal loops were prepared in rachitic chicks (Method A) and each bird received directly into the ligated segment 0.5 ml. plasma plus 0.25 ml. of a solution containing 4.0 mg. Ca^{40} and 8.0 µc. Ca^{45}. Rachitic controls were given 0.25 ml. of the $Ca^{45}Cl_2$ solution and 0.5 ml. saline (0.9%). At given time intervals, samples of blood were removed and the radioactivity in the plasma was measured (P 27 (a) 1).

Results

It is evident from the results summarised in Table 26 that plasma obtained from the vitamin D_3-treated chicks did not improve calcium absorption when tested in rachitic chicks.

LIVER

The influence of a liver extract on calcium absorption in rachitic chicks

Materials and methods

Preparation and isolation of active material from liver. Three twelve-week old cockerels were obtained from a local poultry farm. These birds had received a diet adequately supplemented with vitamin D_3 since hatching. The birds were killed, the livers excised and immediately chilled on an ice-cold petri dish. After cutting into small pieces, a

liver mince was prepared by forcing the tissue through a stainless steel grid (30 mesh) held in a 20 ml. polythene syringe. The mince was divided into three parts, and 7 g. were added to each flask containing the following incubation mixtures:-

Flask No.	Incubation medium	D_3 (75 µg.)	Temp.
1	4.8 ml.	+	37°
2	4.8 ml.	-	37°
3	4.8 ml.	+	37°

The incubation medium consisted of a Krebs-Ringer-bicarbonate solution which was prepared as described by Umbreit, Burris and Stauffer (1949) with the exception that no calcium was added. To this medium was added a solution of vitamin D_3 (0.2 ml. ≡ 75 µg. D_3) dissolved in propylene glycol.

The flasks were incubated in a metabolic shaker incubator for 1 hour under aerobic conditions. At the end of this period, the contents of each flask were homogenised in a Lourdes topdrive homogeniser at 18,000 r.p.m. for 30 seconds with cold ether. The homogenate was then centrifuged at 10,000 X g. for 15 minutes at 0° C, and the aqueous and ether layers removed. After washing the aqueous layer twice with small portions of cold ether the combined ether extracts were taken to dryness in vacuo and the residue redissolved in 1.5 ml. propylene glycol. Residues obtained from incubation flasks 1, 2 and 3 will be designated as extracts A, B and C respectively in the following experiment.

Biological activity of the liver extracts. The biological

potency of extracts A, B and C prepared as above was determined by studying calcium absorption from in vivo duodenal loops of rachitic chicks (Method A). Groups of chicks received 0.15 ml. of the appropriate extract by intracardial injection 1 hour before the calcium test. Rachitic and vitamin D_3-treated control groups were given 0.15 ml. propylene glycol in a similar manner. The absorption of calcium was followed by measuring the amount of radioactivity present in the plasma after inserting the test dose of $Ca^{45}Cl_2$ into the loop. Ca^{45} in plasma was measured as described earlier (P 27 (a) i).

Results

The experimental findings are summarised in Table 27. Following overnight treatment with vitamin D_3, there was a marked increase in circulating calcium in comparison to the rachitic controls. Extract A, which might have been expected to give a positive response if active carrier material were present, did not stimulate an increased uptake of calcium. Likewise, plasma levels of Ca^{45} from rachitic chicks receiving extracts B and C showed no significant increase above the original rachitic value.

ADRENAL GLAND

The influence of an adrenal gland extract on calcium absorption in rachitic chicks

Materials and methods

Preparation of an adrenal extract. Three twelve-week old white leghorn cockerels obtained from a local hatchery were heavily dosed with vitamin D_3 (100,000 I.U.) over a period of 5 days. The birds were then killed, both adrenal glands excised and immediately immersed in 3.0 ml. ice-cold propylene glycol. Using a Potter-Elvejhem

TABLE 27

THE INFLUENCE OF LIVER EXTRACTS ON IN VIVO CALCIUM ABSORPTION FROM DUODENAL LOOPS

Liver extracts were injected intracardially into rachitic chicks 1 hour before the calcium test. Details concerning the liver extracts have been described under the Methods section. Plasma Ca^{45} levels were measured after preparing *in vivo* duodenal loops from rachitic and vitamin D_3-treated chicks. Each value is the mean of 4 birds.

Treatment	Liver extract	Ca^{45} c.p.m. X 10^{-3}/ml. plasma TIME (mins) 15	30	45
D_3	-	2.580	3.550	2.870
-	-	0.920	1.410	1.390
-	A	0.970	1.600	1.580
-	B	0.680	1.050	1.370
-	C	0.950	1.470	1.240

teflon homogeniser the tissue was homogenised for 3 minutes at 0° C. The homogenate was transferred to chilled centrifuge tubes and the debris removed by centrifugation at 80,430 X g. for 15 minutes at 0° C, using a Spinco refrigerated centrifuge (model L, rotor No. 40-2). The supernatant was used in the biological assay described below.

The activity of an adrenal extract in rachitic chicks. Of the supernatant prepared as above, 0.2 ml. was injected intracardially into rachitic chicks 1 hour prior to the calcium test. In vivo duodenal loops were prepared and the $Ca^{45}Cl_2$ test solution placed in the ligated segments (Method A). At given time intervals, blood was withdrawn and plasma samples prepared in the usual manner (P 27 (a) i). Both a rachitic and a vitamin D_3 group were included in the experiment, each chick receiving 0.2 ml. propylene glycol in place of the adrenal extract in the manner described above.

Results

When a crude adrenal extract was injected intracardially into rachitic chicks there was no indication that such a preparation could improve calcium absorption within 1 hour of its administration. The data presented in Table 26 shows that there has been an increase above the rachitic level in circulating Ca^{45} following the giving of the extract. Under the influence of vitamin D_3 however, the amount of radioactivity circulating in the plasma has increased approximately five-fold.

INTESTINE

Attempted extraction of a calcium complex from the intestine

Materials and methods

Extraction with ether. A group of rachitic chicks was given

an oral dose of 10,000 I.U. vitamin D_3 on two consecutive nights prior to the experiment. In vivo duodenal loops from both rachitic chicks and the vitamin D_3-treated group were then prepared and perfused with $Ca^{45}Cl_2$ as described earlier (Method B). After 15 minutes, the perfusion apparatus and loops were drained of all fluid. The tissue was cut into small pieces and quickly immersed into a flask containing ice-cold ether. After overnight extraction at -16° C the ether extract was washed twice with distilled water and then taken to dryness in vacuo. The residue was redissolved in 1.0 ml. chloroform and 0.2 ml. aliquots were spread on 1 cm^2. aluminium planchets and allowed to dry under an infra-red drying lamp. Radioactivity in the samples was determined using an end-window geiger counter. Samples of plasma obtained at the completion of the perfusion were also counted in the same manner to ascertain the effect of the vitamin treatment.

Extraction from a preparation of freeze-dried intestine

(a) Preparation. Everted gut sacs prepared from rachitic and vitamin D_3-treated chicks (100 I.U. - 16 hours) were incubated in the presence of $Ca^{45}Cl_2$ as described earlier (Method E). After 1 hour, the loops were removed, rinsed once with physiological saline and the tissue was then placed in a freeze-drying apparatus until dry. The tissue was removed, finely ground in a pestle and mortar, and then stored at 4° C, in a desiccator containing sulphuric acid, in the dark until required.

(b) Perchloric acid extract. 10 mg. dried tissue from vitamin D_3-treated chicks was digested with 1.0 ml. 0.5 N perchloric acid at 70° C for 2 hours. The residue was removed by centrifugation at 2,000 X g. for 10 minutes. Radioactivity in the supernatant was measured by scintill-

ation counting.

(c) Papain extract.

(i) Preparation. 200 mg. tissue from vitamin D_3-treated chicks, suspended in 9.0 ml. water, was digested with 1.0 ml. papain (25 mg./ml. in 0.03 M cysteine) at 70° C for 2 hours and the supernatant liquid was retained for further study. Protein in a portion of the supernatant was precipitated by the addition of an equal volume of 0.5 N perchloric acid, and the radioactivity in the acid extract was determined by scintillation counting.

(ii) Ultrafiltration of the papain digest. A small portion of the supernatant prepared as above was passed through 8/32 visking tubing in vacuo. The radioactivity in the ultrafiltrate was measured by scintillation counting. The sample was added directly to the scintillator phosphor contained in the counting vessel.

(iii) An ether extract of the papain digest ultrafiltrate. An equal volume of ether was added to a small portion of the ultrafiltrate. The mixture was allowed to stand for 24 hours, and then separated. Ca^{45}, extracted into the etherial layer, was measured by scintillation counting.

Results

As indicated in Table 28, Ca^{45} was detected in small amounts in the residue obtained from both groups. Slightly less radioactive material was extracted from the intestine of the vitamin D_3-treated chicks as compared to the rachitic group, suggesting that the amounts of extractable material were not influenced by vitamin D_3 treatment. That vitamin D_3 has been effective in promoting calcium absorption during the 15 minute perfusion is demonstrated from the increased plasma

TABLE 28

ORGANIC EXTRACTION OF Ca^{45} FROM THE INTESTINE AFTER SHORT-TERM IN VIVO PERFUSION OF DUODENAL LOOPS

Duodenal loops from rachitic and vitamin D_3-treated (100 I.U. - 16 hours) chicks were perfused *in vivo* with $Ca^{45}Cl_2$ (Method B) for 15 minutes. Ca^{45} was then estimated in plasma as a measure of the influence of the vitamin D_3-treatment on calcium absorption. The intestine was extracted for several hours with ether and the extract evaporated to dryness. Ca^{45} was measured in the ether residue. Each value is the mean of 3 birds.

Treatment	Ca^{45} c.p.m. X 10^{-3}/ml.	
	Plasma	Intestinal extract
-	1.12	2.00
D_3	2.98	1.50

counts in the treated group (Table 28).

Table 29 summarises data obtained during experiments with a preparation of freeze-dried intestine from vitamin D_3-treated chicks. On the assumption that perchloric acid treatment liberates all of the tissue bound calcium, digestion with papain released 50% of the calcium. It would appear also that the majority of the calcium liberated during the incubation was either ionic calcium or was calcium bound to a low molecular weight material. This conclusion is based on the rapid escape time (approximately 50% in 30 minutes) of the calcium from the dialysis sac. Attempts to extract the soluble calcium into ether were unsuccessful.

The influence of serotonin on calcium transport

In recent investigations, Woolley (1958) and Woolley and Campbell (1960) have demonstrated that *in vitro*, in the absence of any cells or cell particles, calcium may become fat-soluble and move through a lipid phase. This form of calcium transport required the presence of serotonin and a crude lipid extract which Woolley has shown may be extracted from hog duodenal tissue. From these important observations, Woolley has postulated that serotonin can combine with a specific lipid in the cell membrane (serotonin-receptor) and that calcium can then be complexed by this serotonin-receptor compound. The resulting complex which is thus rendered fat-soluble, is free to diffuse across the membrane. As a result of specific enzymic reactions, the calcium could then be released at the new site.

This remarkable demonstration of a fat-soluble calcium complex and the proposed mechanism for calcium transport in a lipid phase, has many features in common with the hypothesis outlined in Chapter 4,

TABLE 29

EXTRACTION OF Ca^{45} FROM PREPARATION OF FREEZE-DRIED INTESTINE

Everted gut sacs from vitamin D_3-treated chicks were incubated in the presence of $Ca^{45}Cl_2$ (Method E). After 1 hour, the sacs were removed and the tissue was freeze-dried. Ca^{45} was extracted from the preparation with 0.5 N perchloric acid and the radioactivity in the extract was determined. The tissue was also digested with papain (25 mg./ml.) and Ca^{45} was measured (a) in a perchloric acid extract of the supernatant from the papain digest (b) after ultrafiltration of the digest (c) in an ether extract of the ultrafiltrate.

Treatment	Ca^{45} c.p.m./ mg. tissue	% efficiency of the treatment
Perchloric acid extract	92.82	100
Papain digest supernatant	46.0	49.6
Ultrafiltrate of papain digest	34.6	37.3
Ether extract of the ultrafiltrate	-	-

involving an active calcium carrier. It was therefore important to examine more closely, the effect of serotonin on calcium transport and in particular, the participation of vitamin D_3 in such a serotonin-receptor-calcium transport mechanism.

Materials and methods

Preparation of serotonin-receptor. Crude serotonin-receptor material was prepared essentially as described by Woolley (1958). Immediately after death, duodenal and distal segments of small intestine were excised from groups of rachitic and vitamin D_3-treated birds. Each group consisted of 4 chicks. The segments were split longitudinally and then cut into pieces approximately 1 cm^2. and allowed to fall into a mixture of ether and absolute ethanol (2:1, v/v) kept at 0° C. For each g. tissue 15 ml. of solvent was used.

During the extraction of the serotonin-receptor material, the mixture was stored at -16° C for 24 hours and was occasionally shaken during this period. The residue was then separated by filtration in the cold, washed once with 50 ml. of solvent, and the combined solvent extracts were taken to dryness in a flash-evaporator with the temperature below 30° C. The material obtained was immediately dissolved in a small volume of a mixture of equal parts of n-butanol-benzene.

Assay. Formation of the serotonin-receptor-calcium complex was carried out in a 10 ml. graduated centrifuge tube containing 5.0 ml. of benzene-butanol extract, 4.0 ml. of Ringers-calcium solution and 0.1 ml. of $Ca^{45}Cl_2$ (0.2 µc. and 20 µg. Ca^{45}). The mixture was shaken vigorously for 5 minutes and then centrifuged at 2,000 X g. for 10 minutes to separate the organic layer from traces of the aqueous phase. Samples

of the organic phase were retained to measure radioactivity present.

Controls. Two sets of control tubes were included in the experiment. Controls without the addition of rachitic or vitamin D_3-treated extract to the calcium solution measured the amount of calcium transferred to the benzene-butanol solvent in the presence or absence of serotonin. Further control tubes which lacked serotonin but contained the tissue extract, tested the ability of the extract to combine with calcium and form a fat-soluble complex.

Ringers-calcium solution contained NaCl (900 mg.), KCl (42 mg.), $NaHCO_3$ (50 mg.), glucose (50 mg.), serotonin (5-hydroxy tryptamine creatinine sulphate (2.5 mg.), Ca^{40} (45 mg.) and water to make 100 ml.

Estimation of Ca^{45}. Radioactivity present in the organic phase was measured by scintillation counting. A sample of the extract was added directly to the scintillator phosphor contained in the counting vessel. As the benzene-butanol layer was highly coloured, quenching measurements were carried out by the addition of a standard solution of $Ca^{45}Cl_2$ to the counting fluid. The necessary quenching corrections have been taken into consideration in the final evaluation.

Incubation of everted intestinal sacs. Everted sacs of distal small intestine were prepared from rachitic and vitamin D_3-treated chicks (Method E). Incubation medium in the test flasks contained serotonin (25 µg./ml.). Accumulation of Ca^{45} in the serosal fluid was measured after 1 hour and compared with data obtained from sacs incubated under similar conditions but in the absence of any added serotonin. Radioactivity was estimated by scintillation counting (P 27 (b) i).

Results

Table 30 demonstrates that calcium may be made fat-soluble in the presence of an extract from chick small intestine. The formation of this complex does not appear to be under the influence of vitamin D_3 as there was no significant variation in the level of radioactivity using extracts from rachitic or vitamin D_3-treated intestine. Further the presence of serotonin in the incubation mixture did not improve the transfer of Ca^{45} from the aqueous to the organic phase.

There was no transfer of Ca^{45} in the absence of tissue extract whether serotonin was present or absent from the medium. A relatively greater amount of calcium was transferred with extracts of duodenal tissue as compared to distal tissue. This would suggest higher concentrations of the receptor substance in this region of the small intestine.

Similar observations were made when calcium transport was studied with <u>in vitro</u> everted sacs (Table 31). Under the influence of vitamin D_3, there was an increased accumulation of Ca^{45} in the serosal fluid. The addition of serotonin to the mucosal fluid did not stimulate further transport in either rachitic or vitamin D_3-treated preparations. On the contrary, the results suggest that there has been a slight inhibition of calcium movement in the presence of serotonin.

TABLE 30

THE FORMATION OF A FAT-SOLUBLE Ca^{45} COMPLEX IN THE PRESENCE OF SEROTONIN AND AN EXTRACT OF SMALL INTESTINE

Accumulation of Ca^{45} in a mixture of n-butanol and benzene was measured after incubation of a Ringers $Ca^{45}Cl_2$ solution with serotonin and a cell-free extract of small intestine from either rachitic or vitamin D_3-treated chicks. The cell-free extract was contained in the mixture of n-butanol and benzene. The addition of serotonin to the Ringers solution is indicated by a positive sign.

Treatment	Origin of tissue	Serotonin	Ca^{45} c.p.m. X 10^{-2}/ g. tissue
-	distal	+	1.99
D_3	distal	+	2.22
-	distal	-	2.30
D_3	distal	-	2.18
-	duodenal	+	5.16
D_3	duodenal	+	4.95
-	no extract	+	-
-	no extract	-	-

TABLE 31

THE INFLUENCE OF SEROTONIN ON CALCIUM TRANSPORT IN VITRO

Accumulation of Ca^{45} was measured in the serosal fluid of everted distal gut sacs from rachitic and vitamin D_3-treated chicks. Serotonin (25 µg./ml.) was added to the mucosal fluid. Each value represents the mean of 8 birds ± standard error of the mean.

Treatment	Serotonin	Ca^{45} Serosal c.p.m. X 10^{-3} accumulated/hour
-	-	1.872 ± 0.23
-	+	1.429 ± 0.56
D_3	-	6.120 ± 0.50
D_3	+	4.926 ± 0.70

DISCUSSION

The importance of a carrier compound capable of complexing calcium has been stressed from previous considerations on the mode of action of vitamin D_3 and the mechanism of calcium transport. Using as starting material cholesterol, vitamin D_2, vitamin D_3 or dihydrotachysterol, Raoul and his co-workers (Raoul et al., 1954; Baron et al., 1955; Raoul et al., 1956; Raoul, 1958) have prepared a number of derivatives, the most important of which appears to be a 9,10-seco-steroid with hydroxy groups in positions C_7 and C_{19} of the original molecule. This compound may be stabilised in the presence of calcium to form a lipid-soluble calcium complex. Such a product, when tested for its antirachitic potency in the rachitic chick, gave a response equivalent to that of vitamin D_3.

Considering the important properties of this compound, an attempt was made to isolate a quantity of this active material for further investigations of its actions in the rachitic chick. When cholesterol was treated with Fuller's earth the colour changes during the reaction and the subsequent separation of the components of the reaction mixture appeared to follow the pattern described by Raoul et al. (1954). There was, however, no confirmation that the final product obtained, in the absence of calcium, was a compound of the proposed structure of ketone$_{250}$. The IR spectrum suggested the presence of a carbonyl grouping, but the UV absorption at 250 mμ which Raoul (1958) claims to be characteristic for this compound was absent. If the two compounds are identical, then it is hard to imagine how one can successfully determine its absorption in the UV region with such a labile

compound. There was no chemical evidence that the compound prepared possessed a ketonic functional grouping suggesting that the carbonyl detected by IR must be heavily hindered.

Studies with the calcium complex proved a little more fruitful. The formation of a fat-soluble calcium complex was demonstrated by means of Ca^{45}. This in itself is a noteworthy feat as calcium ions alone did not penetrate into the organic phase. Again it was difficult to compare the compound with the biologically active calcium complex previously reported by Raoul (1958). The IR spectrum of this compound showed that the carbonyl absorption was reduced in intesity and that there had been a slight shift in the position of the maximum absorption. If enolisation were required before the calcium complex was formed, one would expect to find significant changes in the spectrum, with no evidence of a carbonyl grouping. When given to rachitic chicks, this compound gave no indication of being biologically active in promoting calcium absorption from the small intestine.

Recent investigations by Irmscher, Wirts and Daehne (1959), who have also been unsuccessful in preparing $ketone_{250}$, cast some doubt as to the authenticity of the products obtained by Raoul and his colleagues. The former workers isolated a number of dehydration products after floridine treatment of cholesterol but 9,10-seco-steroids were not found. These products appeared as resins with UV absorption at 250 mμ. and gave IR spectrum similar to $ketone_{250}$. Vitamin D_3 was also isomerised into iso-tachysterol which was further converted by atmospheric oxidation to give a compound absorbing at 250 mμ. and with a similar IR spectrum to that of $ketone_{250}$. However, the product so formed did not have the

properties of a 3,10 diol nor that of an α,β-unsaturated ketone.

Kodicek and Ashby (1960) from an examination of biologically active fractions from rat tissues, were not able to detect the presence of any ketone$_{250}$. Despite this lack of collaborating evidence the possibility of a change in the vitamin D molecule to a more active form of the type proposed by Raoul presents an attractive hypothesis. Such a transformation would give opportunities of oxidation and reduction or of keto-enol tautomerism that would permit the carrier to release its calcium, for example, in the manner required in the theory of cation transport recently postulated by Robertson (1960).

Heymann (1937 a) and more recently Kodicek (1960) have studied the distribution of vitamin D in various organs and fluids of the body. Observations from these experiments suggested a basis for further investigations on the nature of the material postulated as being required for the active transport of calcium.

In 1904, Pavlov, and later Wisner and Whipple in 1922, implicated bile salts in the absorption of calcium after noting that dogs with biliary fistulas developed extensive osteoporosis. It was assumed that bile salts, which were thought to promote vitamin D and calcium absorption, could account for this condition. Greaves and Schmidt (1932), and Taylor, Weld and Sykes (1935), claimed that bile enhanced calcium uptake only indirectly as the result of a greater absorption of vitamin D. Heymann (1937 b) found that vitamin D was not present in the serum of dogs when the bile duct was ligated. This author thus concluded that vitamin D was not absorbed unless bile was present in chyme. Verzar and McDougall (1936) offered a possible

explanation for the effect of bile on calcium absorption when they observed the hydrotropic action of bile on calcium soaps. They have suggested that calcium, bile fatty acid and bile acids form a soluble, diffusible complex in the intestine which, when broken down in the liver, leads to the liberation of calcium in the blood stream.

More recently, Lengemann and Dobbins (1958) have re-investigated the role of bile on calcium absorption. When bile flow was stimulated by injection of sodium taurocholate, there was an increased calcium absorption from the intestine. Ligation of the bile duct removed the influence of sodium taurocholate. The effect of the bile could not be correlated with a change in the intestinal pH. Vaughan and Filer (1960) were unable to confirm this reported effect of bile on calcium absorption. When bile was inserted directly into ligated duodenal and ileal segments of rat small intestine, there was no increase in calcium absorption.

If a physiologically more active form of vitamin D were being re-excreted into the intestine then the appearance of such a compound in bile would offer an explanation for the observed increase in calcium absorption. The importance of earlier observations by Russell and Chichester (1931) and Russell, Taylor and Chichester (1934) are, however, difficult to evaluate with this concept. These workers observed that the gall-bladders in rachitic chicks were much larger and that the amount of bile was much greater than in normal chicks. Whether an increase in bile flow is the result of a compensatory mechanism for the lack of carrier material and hence the lack of calcium for skeletal requirements, is perhaps a thought worthy of further consideration.

Although the present investigations confirm the effect of bile on the uptake of calcium, vitamin D did not influence this process.

Similar findings have been reported by Coates and Holdsworth (1961). When bile salts were precipitated from the bile extract and the material extracted was then given intracardially 1 hour before measuring calcium absorption from the intestine, there was no increase in the absorption of Ca^{45} as measured by the plasma Ca^{45} levels. The positive response produced with bile would thus appear to be associated with the bile salts themselves, which may be exerting a hydrotropic action on the gut, allowing greater transport of the calcium ion. It is obvious, however, that an adequate explanation for the influence of bile on calcium absorption is still lacking. Further investigations on the role of bile in calcium metabolism could prove very interesting.

The importance of liver function in calcium metabolism was emphasised from early clinical observations. Gerstenberger in 1933 postulated that the liver could play a decisive role in the functioning of vitamin D. Severe skeletal changes have been observed when liver function is impaired, which has led Atkinson, Nordin and Sherlock (1956) to suggest that the destruction of the liver cells may be interfering with the storage and metabolism of vitamin D. Heymann (1937 a) found that vitamin D was still present in the liver 8–12 weeks after feeding vitamin D to rabbits. From studies with C^{14}-labelled vitamin D, Kodicek (1960) has also reported that a considerable proportion of an oral dose is retained in the liver.

During investigations of the action of vitamin D and the role of the liver, Heymann (1937 c) severely limited the function of the liver by obstructive biliary cirrhosis and also from feeding carbon tetrachloride. When vitamin D was fed to rachitic rats receiving this treatment, there

was a decreased antirachitic potency observed with this substance, which Heymann concluded was the result of severe hepatic damage.

Whipple and his associates have demonstrated that osteoporosis in dogs, developed as a result of biliary fistulas, can be prevented by feeding liver to such animals (Whipple, 1922; Wisner and Whipple, 1922; Hawkins and Whipple, 1935). From this evidence he postulated the existence of an unknown substance elaborated by the liver and absorbed through the intestinal tract which prevented decalcification of bone.

The present investigations have attempted to show experimentally the presence of 'an unknown substance' or a more potent active carrier compound in liver. A liver mince was incubated in the presence of vitamin D_3 in an attempt to synthesise some of this material. Heymann (1937 a) has previously demonstrated that saponified extracts from animals treated with vitamin D are active when assayed for their antirachitic potency in rachitic rats.

Extracts of chick liver incubated in the manner described were not saponified because in all probability, if the active carrier were of the kind proposed by Raoul (1958), then prolonged contact in an alkaline medium could readily destroy such material. Further, an extraction of the liver mince with ether over a period of several hours should be sufficient to remove any calcium complex. The effect of vitamin D_3 in the test system can be ruled out as previous experiments have failed to show an effect with this substance within 4 hours. Again it must be emphasised that the success of the experiment was dependent upon an immediate effect by the liver extracts. That the extracts failed to give a positive response in rachitic chicks would suggest that the

liver was not the site either for the synthesis of active material or its ultimate storage.

Serum was also tested for the presence of material which could promote a rapid absorption of calcium. Heymann (1937 a) found that after feeding vitamin D the blood level remained high for several months after its administration which suggested that there was a considerable storage of the vitamin. Observations by Thomas, Morgan, Connor, Haddock, Bills and Howard (1959) and more recently by Innes Chalk and Kodicek (1960) indicate that vitamin D is attached to serum proteins during its transport.

When serum from vitamin D_3-dosed chicks was inserted directly into duodenal loops of rachitic chicks there was no significant increase in calcium absorption observed. Similar studies with a prepared adrenal extract also failed to increase the uptake of calcium from the intestine.

Although only short-term experiments were carried out in an attempt to stimulate an immediate improvement in calcium absorption, the negative results obtained do not necessarily rule out the existence of active carrier material. In Chapter 3, it was postulated that the delay observed for vitamin D_3 activity was due to the building up of sufficient concentration of carrier compound at the site of calcium absorption. However, such a delay may be attributed to (a) the rate of synthesis of carrier material from vitamin D_3 (b) the incorporation of carrier material into the intestinal cells or (c) as a result of both these processes. If concept (b) were the prime factor causing the delay, then an immediate effect may not be evident when carrier material is presented to the rachitic chick in short-term experiments.

If calcium were being actively transported across the intestinal membrane by a specific carrier substance then it seemed logical to examine this tissue for the presence of such material. Short-term perfusion experiments were carried out to label the carrier with Ca^{45}, and the tissue was then extracted with ether. Although it was obvious from the plasma Ca^{45} levels that vitamin D_3-treatment of rachitic chicks was stimulating a greater absorption of Ca^{45}, there was no increase in radioactivity with tissue extracts from the vitamin D_3-treated birds, as might have been expected, if active carrier material were present. The radioactivity detected in the extract was probably the result of a non-specific binding of Ca^{45} with fatty acid material.

Accumulation of Ca^{45} in mucosal cells has already been demonstrated from previous in vitro investigations on calcium transport (Chapter 2). This finding was confirmed during these present studies, when twice as much Ca^{45} was accumulated by the mucosal cells of the vitamin D_3-treated preparation as compared to the amount present in tissue from the untreated chicks. Enzymic digestion of mucosal cells obtained from the vitamin D_3-preparation indicated that 50% of the bound Ca^{45} could be released by digestion of the peptide linkage. Ultrafiltration of the digestion mixture suggested that a large portion of the released calcium was either in the form of ionic calcium or attached to a small molecular weight particle. This conclusion would seem feasible if the calcium complex were of the type proposed by Raoul (1958).

The existence of a compound extractable from the intestinal mucosal cells and capable of forming a fat-soluble calcium complex has been demonstrated by Woolley (1958). Chelation of calcium was found to re-

quire the presence of serotonin. Garattini, Grossi, Paoletti, Paoletti and Poggi (1961) claimed that serotonin increased calcium absorption both *in vivo* and *in vitro*. Their experiments were carried out on normal animals.

If the formation of a serotonin-receptor-calcium complex were necessary for the normal intestinal absorption of calcium, then under rachitic conditions the lack of either component might offer an explanation for the impaired efficiency of calcium uptake. When extracts of the intestine were made with an organic solvent, calcium was rendered fat-soluble. Vitamin D did not influence the amount of crude receptor compound extracted as similar amounts of fat-soluble calcium complex were formed with tissue from either rachitic or vitamin D_3-treated chicks. Further the absence of serotonin did not prevent the transfer of Ca^{45} to the organic phase. Woolley and Campbell (1960) have also observed a similar phenomenon and have interpreted these findings as a non-specific binding of calcium with material present in the crude extracts. They suggest that 'an impurity such as phosphatidic acid could combine with Ca^{45} in the aqueous phase, thus allowing solubilisation of calcium phosphatidate in the organic solvent".

Similar studies with everted sacs *in vitro* also demonstrated the ineffectiveness of serotonin to stimulate calcium transport. Whether the positive response previously seen in rats is peculiar to this species as, for example, has been shown with amino acids, remains a debatable point. It must therefore be concluded that the formation of a serotonin-receptor-calcium complex does not explain the mechanism of calcium transport under the influence of vitamin D_3.

SUMMARY

1. When cholesterol was treated with Fuller's earth, a steroid derivative was prepared with an IR absorption spectrum strikingly similar to the compound previously described by Raoul (1958) as ketone$_{250}$. However, the UV spectrum indicated the absence of a peak of maximum absorption at λ = 250 mμ. Chemical evidence was also lacking to identify this derivative as ketone$_{250}$.

2. A fat-soluble calcium complex was prepared from a precursor of ketone$_{250}$. The calcium chelate was not biologically active when tested in the rachitic chick. The UV spectrum of this compound showed a peak at λ = 232 mμ.

3. Bile was active in promoting calcium absorption in the rachitic chick. The effect obtained appeared independent of the influence of vitamin D_3 as bile from rachitic chicks was equally as effective as bile from vitamin D_3-dosed chicks. It is postulated that the effect is caused by the hydrotropic action of the bile salts.

4. Extracts of liver mince were tested for their biological potency in stimulating uptake of calcium from the intestine. The results indicated that active carrier material was not present in these extracts.

5. When freeze-dried intestinal tissue obtained from vitamin D_3-dosed chicks was incubated with papain, 50% of the bound calcium was released by digestion of the peptide linkages. Ultrafiltration studies suggested that the majority of the calcium released was ionic calcium or calcium attached to a low molecular weight particle.

7. Material was extracted from intestinal mucosal cells which, in the presence of calcium, formed a fat-soluble calcium complex. The

activity of the extract was tested by measuring the transfer of Ca^{45} from an aqueous to an organic phase. Serotonin did not influence the transfer process. Extracts of rachitic tissue were equally as effective as extracts from vitamin D_3-treated chicks. It is suggested that the phenomenon observed is the result of a non-specific binding of calcium with material present in crude intestinal extracts.

8. The presence of serotonin in the medium during <u>in vitro</u> studies on the movement of calcium across everted intestinal sacs, did not stimulate calcium transport. The effect of vitamin D_3 appeared independent of the formation of a serotonin-receptor-calcium complex.

CONCLUSIONS

CONCLUSIONS

The work presented in this thesis has been an attempt to elucidate the mode of action of vitamin D_3 in the metabolism of calcium in the chick. Previous investigations in this field have thrown little light on this phenomenon as workers were hampered by the lack of suitable test systems. A number of *in vivo* and *in vitro* methods, which proved invaluable for further investigations on calcium transport, were described in Chapter 1. For the first time it has been possible to directly compare results from *in vitro* studies with those obtained under more physiological conditions.

Chicks were selected for the experimental studies because of the conditions under which these birds developed rickets. This disease is the result of a deficiency of vitamin D and is manifest clinically by an inadequate calcification of the skeleton together with a number of biological abnormalities. Rickets was produced in chicks by rearing the birds in the absence of sunlight on a diet containing normal ratios of calcium and phosphorus but lacking vitamin D. The diet used was capable of supporting the optimal growth of the bird. The rickets produced in chicks then was the result of a single deficiency. This is in sharp contrast to rickets in rats which is normally associated with a number of variables.

Calcium was found to be poorly absorbed from the small intestine in the vitamin D-deficient chicks. This impaired efficiency offered an explanation for the decreased calcium content of the tibia observed in the rachitic bird. However, as reported in Chapter 2, when these birds were given an oral dose of 100 I.U. vitamin D_3 (2.5 µg), calcium absorption was restored to normal within 16 hours and it was therefore concluded that

vitamin D promoted greater absorption of calcium from the intestine. This positive response of the rachitic chick to vitamin D_3-treatment provided a basis for the study of the influence of a number of compounds on calcium transport.

When the small intestine was divided into three equal segments and everted and the movement of calcium observed in vitro, the greatest transport of calcium under the influence of vitamin D occurred with the distal segment. This finding was noted to be in contrast to the results obtained from similar studies with rats (Harrison and Harrison, 1960; Schachter et al., 1960 a) and suggested a distinct species difference. In vivo, calcium was absorbed almost equally as well in the upper or duodenal portion of the small intestine, although the distal segment presented a more 'sensitive' site for observing the vitamin D_3 effect. Under physiological conditions, then, it was concluded that calcium was effectively absorbed along the entire length of the small intestine.

A rapid rise in plasma Ca^{45} was demonstrated when 2.5 µg. dihydrotachysterol series 2 ($AT-10_2$) or dihydrotachysterol series 3 ($AT-10_3$) was given to rachitic chicks. Despite the increased deposition of calcium in the bone, $AT-10_2$ possessed less than 1 I.U. per mg. of antirachitic potency. Albright and Reifenstein (1948) have proposed that the influence of AT-10 is primarily on phosphate metabolism rather than on calcium absorption. If this hypothesis is correct, then it must be concluded that although calcium is present at the bone site the ratio of calcium: phosphorus is detrimental to the conditions required for the continued formation of calcified bone.

The influence of vitamin D_2, vitamin D_3, $AT-10_2$ and $AT-10_3$ was also considered in relation to the structure of their side-chain. When

compared with vitamin D_3, on an equal weight basis, vitamin D_2 had little influence on calcium transport. AT-10_2 was found to be equally as active as AT-10_3, but neither of these compounds increased calcium absorption to the same extent as an equal quantity of vitamin D_3. Vitamin D_2 and AT-10_2 have identical side-chains attached to the parent molecule, as do the pair AT-10_3 and vitamin D_3. It was concluded from these experimental observations that the constitution of the side-chain was not of prime importance to the transport of calcium.

Although it was firmly established that treatment with vitamin D_3 could restore calcium absorption to normal a definite time interval was required before the effects of this compound became apparent. Even when unphysiological amounts of the vitamin were placed near the site of calcium absorption or were circulating in the blood stream there was no increase in the uptake of calcium. At least 4 hours were needed before an effect with 100 I.U. vitamin D_3 could be noted.

From these observations, two possibilities were considered. Either, vitamin D_3 was being incorporated into the intestinal cells and the permeability of these cells was then altered in some special manner or vitamin D_3 was being transformed to another type of compound. Both these changes would require a definite time interval. If a new compound were being formed then the isolation and subsequent feeding of such a compound to rachitic chicks should promote an immediate improvement in calcium absorption and thus eliminate any time lag previously observed with vitamin D_3. These postulates were examined in later chapters.

Vitamin D has been located in the adrenal glands of rats (Kodicek, 1960). In view of the capacity of this organ for steroid synthesis, the

adrenal gland was considered a likely site for the transformation of vitamin D and investigations of this hypothesis were presented in Chapter 3.

When the function of the adrenal gland was severely impaired by the chemical destruction of the cells comprising the interrenal tissue the influence of vitamin D on calcium metabolism was abolished. The interrenal tissue in chick adrenals is analagous to the adrenal cortex in the mammalian gland. This is the first known report of the adrenal being implicated as a mediator of the vitamin D_3 effect.

Further confirmatory evidence of the major role of the adrenal was obtained from studies with a number of known adrenal steroids. Cortisol and 11-deoxycorticosterone, when fed over a period of several days, also substantially reduced the influence of vitamin D_3 on calcium absorption. The effects of these steroids could not be explained by their possessing cortico- or mineralocorticoid properties but were believed to be due to a specific effect on the adrenal cells.

When cortisol or 11-deoxycorticosterone was given for only a short interval before studying the absorption of calcium in vitamin D_3-treated chicks, there was a surprising increase in the amount of calcium absorbed from the gut. This observation could also be explained if these steroids caused a rapid secretion of active carrier material from the adrenal. It was stressed that the effects of cortisol or 11-deoxycorticosterone were only apparent in the vitamin D_3-treated chicks. Thus, disruption of adrenal function could explain the peculiarities observed after treatment with adrenal steroids, together with many other puzzling observations previously reported in the literature.

Because of the structure of chick adrenal glands it was not

possible to locate any specific zone in which vitamin D_3 was transformed to a more active form. Kodicek (1960) has located vitamin D in the zona fasciculata of rat adrenals and, in view of this demonstration, it is tempting to suggest that this region of the mammalian cortex is concerned in the change of vitamin D. Further investigations with mammalian tissue are obviously required to confirm this speculation. The evidence from these adrenal studies supported the conclusion that the delay before an effect of vitamin D_3 can be shown on calcium uptake may well be due to the necessity to synthesise active material in the adrenal cells and thus build up a sufficient concentration of this material in the mucosal cells of the small intestine.

The mechanism of transport of the calcium ion across the small intestine was investigated. The process did not appear to be the result of a diffusion phenomenon. It was previously noted that the influence of vitamin D was relatively specific to the movement of calcium. This evidence, together with the experimental findings discussed in Chapter 4, discouraged the theory that a time-lag in vitamin D_3 action was caused by a delay in changing the permeability properties of the transporting membrane. Instead a transport mechanism was invoked in which calcium was moved against a concentration gradient in one direction only, and that this transport required an energy source. Such evidence was interpreted to support an active transport mechanism.

From studies with well-known metabolic inhibitors, it was suggested that the energy source for the transport of calcium <u>in vitro</u> was different from that used under <u>in vivo</u> conditions. The reactions linked to the glycolytic cycle appeared to supply the major portion of the <u>in vitro</u> energy

although oxidative phosphorylation was believed to contribute to some small extent. Energy for the *in vivo* transport of calcium did not appear dependent upon glycolysis or oxidative phosphorylation. It was therefore postulated that alternate pathways might function more efficiently to maintain an energy supply *in vivo* and that perhaps only a small portion of the electron transport chain was specifically required in this process.

Vitamin D_3 did not influence calcium transport by altering the level of respiration or glycolysis in the small intestine. The accumulated evidence suggested that providing there was present an oxidisable substrate capable of producing energy, and an active carrier compound, then calcium transport across the mucosal barrier could proceed normally.

Although many investigators have postulated the existence of carriers to explain the transport of substances and ions across an impermeable membrane, the actual demonstration of these compounds has been extremely difficult and in most cases their existence can only be regarded as hypothetical. Robertson (1960) has recently postulated a mechanism for anion transport which requires the presence of an active carrier. The carrier, he suggests, should be capable of undergoing reversible oxidation and reduction within the lipoidal membrane. The application of this type of system to calcium transport was discussed in Chapter 4 and a tentative scheme was put forward to explain the mechanism of calcium transport under the influence of vitamin D_3. This scheme envisaged the presence in the intestine of active calcium carrier material, preformed from vitamin D_3 by specific reactions in the adrenal gland. Oxidation and reduction reactions within the lipoidal membrane permitted calcium to complex with the carrier and also supplied the energy necessary for calcium transfer

from the mucosal surface to the serosal side of the membrane where calcium could be released and made available for general metabolism.

Harrison and Harrison (1961) from studies on the influence of vitamin D on phosphorus and calcium transport, have suggested by analogy to sugar transport that the transporting mechanism may be located in the brush border membrane of the small intestine. McDougal, Little and Crane (1960) have deduced from the histochemical localisation of galactose in various layers of hamster intestine that the mechanism for sugar transport is located in the epithelial membrane. Similar investigations on the localisation of calcium carrier complex in specific cell layers of the chick small intestine could also prove equally rewarding.

Raoul (1958) has drawn attention to the existence of a new antirachitic compound, ketone$_{250}$, which may be prepared synthetically from a variety of substances including vitamin D. The properties of ketone$_{250}$ stimulated considerable interest as Raoul had found that this compound was capable of forming a fat-soluble complex with calcium which possessed potent antirachitic properties when tested in the chick. It is believed that calcium stabilises the enol form of the molecule as the proposed structure of the compound would readily permit keto-enol tautomerism. If calcium ions were being complexed by such a carrier compound present in the intestinal cells, then hypothetically tautomerism of this kind would also readily allow the complex to release its calcium after transfer across the membrane.

The attempted preparation and isolation of active carrier material was described in Chapter 5. Although a fat-soluble calcium complex could be prepared by treatment of cholesterol with an acid type

silicious earth, it was not possible to conclude from this demonstration that the compound prepared was identical to the product obtained by Raoul et al. (1954). There was no evidence from studies reported in this thesis to support the conclusion that this compound was biologically active in promoting calcium transport in the rachitic chick.

Several tissues and fluids were examined for the presence of active carrier material. Bile was found to stimulate calcium absorption. However, bile taken from rachitic chicks was equally as effective as that obtained from chicks receiving vitamin D_3. It was concluded that active carrier material was not causing the stimulation but rather, the effect could be explained by the hydrotropic action of the bile salts. There was no immediate improvement of calcium absorption when serum, or extracts of liver from vitamin D_3-treated chicks were tested in the rachitic chick. There was a similar lack of response after administering an extract prepared from adrenal glands of vitamin D_3-treated chicks.

Conclusions from studies reported in Chapter 3 indicated that the adrenal was the site for the transformation of vitamin D_3. In the light of the present findings it seemed doubtful that the adrenal was also a store for active material. A homeostatic mechanism may be involved in which vitamin D, stored in the liver, is released in response to increased demands for the carrier from the site of active calcium absorption. As the active carrier is prepared in the adrenal, this material might be rapidly secreted to the small intestine and briefly stored or used immediately. This type of mechanism would minimise the risk of destroying the activity of the carrier material e.g. by prolonged storage. In view of the extremely small quantities (0.1 µg.) of

vitamin D_3 which can influence calcium absorption, it is obvious that the isolation of active material must be extremely difficult.

An attempt was made to label the proposed calcium-carrier complex with radioactive calcium and thus permit ready identification of the compound during extraction from the small intestine. Accumulation of calcium within the mucosal cells was observed and vitamin D_3 was found to influence the amount of calcium accumulated. Extraction of this tissue with ether did not remove calcium carrier material.

Woolley (1958) has postulated that calcium may be moved through a lipoidal membrane by combination with a serotonin-receptor compound. A substance was extracted from the intestine which in the absence of any cells or cell particles, was capable of chelating calcium. The complex so formed permitted transport of calcium from an aqueous to an organic phase. Transport of calcium in this system could be observed in the absence of serotonin. Vitamin D_3 did not influence the amount of chelating substance present in the intestine. The proposal put forward by Woolley and Campbell (1960) that there may be a non-specific binding of calcium with fatty acid (e.g. phosphatidic acid) present in the intestine, could explain the movement of calcium observed under these conditions. When calcium transport was studied with <u>in vitro</u> everted gut sacs, from rachitic and vitamin D_3-treated chicks, serotonin did not improve the accumulation of calcium in the serosal fluid. It has therefore been concluded, that the formation of a serotonin-receptor-calcium complex, does not explain the mechanism of calcium absorption under the influence of vitamin D_3.

BIBLIOGRAPHY

ALBRIGHT, F., BLOOMBERG, E., DRAKE, T. and SULKOWITCH, H.W.
J. Clin. Invest. (1939) 17, 317.

ALBRIGHT, F. and REIFENSTEIN, E.C.
The Parathyroid Glands and Metabolic Bone Disease. (1948)
Williams and Wilkins Company, Baltimore.

ALBRIGHT, F. and SULKOWITCH, H.W.
J. Clin. Invest. (1938) 17, 305.

ALBRIGHT, F., SULKOWITCH, H.W. and BLOOMBERG, E.
J. Clin. Invest. (1939) 18, 165.

ANDERSON, J., DENT, C.E., PHILPOT, G.R. and HARPER, C.
Lancet. (1954) 267, 2 720.

ASKEW, F.A., BRUCE, H.M., CALLOW, R.K., PHILPOT, J. St.L. and WEBSTER, T.A.
Nature. (1931) 128, 758.

ATKINSON, M., NORDIN, B.E.C. and SHERLOCK, S.
Quart. J. Med. (1956) 25, 299.

BARKER, S.B. and SUMMERSON, W.H.
J. Biol. Chem. (1941) 138, 2, 535.

BARON, C., LeBOULCH, N. and RAOUL, Y.
Bull. Soc. Chim. (1955) 22, 2 948.

BARTTER, F.C.
Ann. Rev. Physiol. (1954) 16, 429.

BAUER, G.C.H., CARLSSON, A. and LINDQUIST, B.
Kgl. Fysiograf. Sallsk. Lund. Forh. (1955) 25, 1.

BETHKE, R.M., KENNARD, D.C., KICK, C.H. and ZINZALIAN, G.
Poultry Science. (1929) 8, 257.

BILLS, C.E.
J. Biol. Chem. (1926) 67, 756.

BILLS, C.E.
Physiol. Rev. (1935) 15, 1.

BILLS, C.E.
The Vitamins. Vol. II (1954) p. 132.
Academic Press, New York. Ed. Sebrell and Harris.

BOURNE, G.H.
The Biochemistry and Physiology of the Bone. (1956).
Academic Press, New York.

BRAY, G.A.
Anal. Biochem. (1960) 1, 279.

BREUER, H.J. and WHITTAM, R.
J. Physiol. (1957) 135, 213.

BROCKMANN, H. and BUSSE, A.
Naturwissenschaften (1938) 26, 122.

BROCKMANN, H. and BUSSE, A.
Z. Physiol. Chem. Hoppe-Seyler's. (1938) 256, 252.

BROWN, J.H.V.
Nature. (1960) 187, 985.

CARLSSON, A.
Acta Physiol. Scand. (1952) 26, 212.

CARLSSON, A.
Acta Physiol. Scand. (1954 a) 31, 301.

CARLSSON, A.
Acta Physiol. Scand. (1954 b) 31, 308.

CARLSSON, A. and HOLLUNGER, G.
Acta Physiol. Scand. (1954) 31, 317.

CARLSSON, A. and LINDQUIST, B.
Acta Physiol. Scand. (1955 a) 35, 53.

CARLSSON, A. and LINDQUIST, B.
Acta Paed. (1955 b) 44, 548.

COATES, M.E. and HOLDSWORTH, E.S.
Brit. J. Nutr. (1961) 15, 131.

COLEMAN, R.D., BECKS, H., KOHL, F. van N. and COPP, D.H.
Arch. Path. (1950) 50, 209.

CONWAY, E.J. and HINGERTY, D.
Biochem. J. (1946) 40, 561.

COPP, D.H.
Amer. J. Med. (1957) 22, 275.

CORRELL, J.T. and WISE, E.C.
J. Nutr. (1942) 23, 217.

CROSS, R.J., TAGGART, J.N., COVO, G.A. and GREEN, D.E.
J. Biol. Chem. (1949) 177, 655.

CRUICKSHANK, E.M. and KODICEK, E.
J. Endocrin. (1958) 17, 35.

CRUICKSHANK, E.M., KODICEK, E. and ARMITAGE, P.
Biochem. J. (1954) 58, 172.

CSAKY, T.Z., HARTZOG III, H.G. and FERNALD, G.W.
Amer. J. Physiol. (1961) 200 (3) 459.

DANIEL, E.E. and ROBINSON, K.
J. Physiol. (1960) 154, 445.

D'ARCY, P.F.
Mem. Soc. Endocrin. (1961) 10 Prog. in Endocrin. II, 46.

D'ARCY, P.F. and HOWARD, E.M.
J. Endocrin. (1958) 16, VI.

D'ARCY, P.F. and HOWARD, E.M.
J. Endocrin. (1958) 17, V.

DAY, H.G. and McCOLLUM, E.V.
J. Biol. Chem. (1939) 130, 269.

De LUCA, H.F. and ENGSTROM, G.W.
Proc. Nat. Acad. Science N.Y. (1961) 47, 11.

De LUCA, H.F., GRAN, F.C. and STEENBOCK, H.
J. Biol. Chem. (1957) 224, 201.

De LUCA, H.F. and STEENBOCK, H.
Science. (1957) 126, 258.

Del MONTE, U.
Biochim. et Biophys. Acta. (1961) 49, 431.

DENT, C.E.
Brit. Med. J. (1956) 230, 1 Part 1.

DeROOS, R.
Endocrin. (1960) 67, 719.

DESAULLES, P., TRIPOD, J. and SCHULER, W.
Schweiz. Med. Wochschr. (1953) 83, 1008.

DICKENS, F.
Biochem. J. (1941) 35, 1011.

DOWDLE, E.B., SCHACHTER, D. and SCHENKER, H.
Amer. J. Physiol. (1960) 198, 2, 269.

DUKES, H.H.
The Physiology of Domestic Animals. (1955)
Comstock Publishing Associates, Ithaca. New York.

FANCONI, G.
Ciba Found. Symposium on Bone Structure and Metabolism. (1956)
p. 187. Churchill & Co. Ltd., London.

FOURMAN, P.
Calcium Metabolism and the Bone. (1960)
Blackwell Scientific Publications, Oxford.

FREEMAN, S. and CHANG, T.S.
Amer. J. Physiol. (1950) 160, 341.

FRIED, J. and BORMAN, A.
Vitamins and Hormones. (1958) XVI, P. 303.
Acad. Press, New York.

GARATTINI, S., GROSSI, E., PAOLETTI, P., PAOLETTI, R. and POGGI, M.
Nature. (1961) 191, 185.

GAUNT, R., CHART, J.J. and RENZI, A.A.
Science. (1961) 133, 3453, 613.

GERSHOFF, S.N. and HEGSTED, D.M.
Amer. J. Physiol. (1956) 187, 203.

GERSTENBERGER, H.J.
Monatschr. f. Kinderh. (1933) 56, 217.

GORNALL, A.G., BARDAWILL, C.J. and DAVID, M.M.
J. Biol. Chem. (1949) 177, 751.

GRAN, F.C.
Acta Physiol. Scand. (1960) 49, 211.

GREAVES, J.D. and SCHMIDT, C.L.A.
Proc. Soc. Exp. Biol. & Med. (1932) 29, 373.

GREAVES, J.D. and SCHMIDT, C.L.A.
J. Biol. Chem. (1933) 102, 101.

GREAVES, J.D. and SCHMIDT, C.L.A.
Univ. Calif. Publ. Physiol. (1934 a) 8, 43.

GREAVES, J.D. and SCHMIDT, C.L.A.
Univ. Calif. Publ. Physiol. (1934 b) 8, 49.

GREENBERG, D.M.
J. Biol. Chem. (1945) 157, 99.

HAMILTON, B. and DEWAR, M.M.
Amer. J. Dis. Child. (1937) 54, 548.

HARRIS, L.J. and INNES, J.R.M.
Biochem. J. (1931) 25, 367.

HARRISON, H.E. and HARRISON, H.C.
J. Biol. Chem. (1951) 188, 83.

HARRISON, H.E. and HARRISON, H.C.
J. Paed. (1952 a) 41, 756.

HARRISON, H.E. and HARRISON, H.C.
Yale J. Biol. & Med. (1952 b) 24, 273.

HARRISON, H.C., HARRISON, H.E. and PARK, E.A.
Amer. J. Physiol. (1958) 192, 432.

HARRISON, H.E. and HARRISON, H.C.
Amer. J. Physiol. (1960) 199, (2) 265.

HARRISON, H.E. and HARRISON, H.C.
Amer. J. Physiol. (1961) 201, (6) 1007.

HART, E.B., SCOTT, H.T., KLINE, O.L. and HALPIN, J.F.
Poultry Science. (1930) 9, 296.

HAWKINS, W.B. and WHIPPLE, G.H.
J. Exp. Med. (1935) 62, 599.

HENRY, K.M. and KON, S.K.
Brit. J. Nutr. (1953) 7, 147.

HERRMANN, R.G.
Proc. Soc. Exp. Biol. & Med. (1958) 99, 3 777.

HESS, A.F.
Rickets including Osteomalacia and Tetany. (1929)
Lea & Febiger, Philadelphia.

HEYMANN, W.
J. Biol. Chem. (1937 a) 118, 371.

HEYMANN, W.
J. Biol. Chem. (1937 b) 122, 249.

HEYMANN, W.
Proc. Soc. Exp. Biol. & Med. (1937 c) 36, 812.

HOGEBOOM, G.H., SCHNEIDER, W.C. and PALLADE, G.E.
J. Biol. Chem. (1948) 172, 619.

HOLDSWORTH, E.S. and COATES, M.E.
Clin. Chim. Acta. (1961) 6, 38.

HOLTZ, F.
Arch. Klin. Chir. (abstract) (1933) 177, 32.

HOWARD, J.E. and CONNOR, T.B.
Trans. Ass. Amer. Physicians. (1954) 67, 199.

HOWARD, J.E.
J. Clin. Endocr. (1957) 17, 1105.

INGLE, D.J., HIGGINS, G.M. and KENDALL, E.C.
Anat. Rec. (1938) 71, 363.

INNES CHALK, K.J. and KODICEK, E.
Biochem. J. (1960) 74, 22P.

IRMSCHER, K., WIRTS, H.D. and DAEHNE, W.V.
Z. Physiol. Chem. Hoppe-Seyler's. (1960) 317, 68, 49.

IRVING, J.T.
Calcium Metabolism. (1957) Methuen & Co. Ltd., New York, London.

KEANE, K.W., COLLINS, R.A. and GILLIS, M.B.
Poultry Science. (1956) 35, 6 1216.

KENNY, A.D. and MURSON, P.L.
Endocrin. (1959) 64, 513.

KIMBERG, D.V., SCHACHTER, D. and SCHENKER, H.
Amer. J. Physiol. (1961) 200 (6) 1256.

KODICEK, E.
Ciba Found. Symposium on Bone Structure and Metabolism. (1956 a) p. 161. Churchill & Co. Ltd., London.

KODICEK, E.
Ciba Found. Symposium on Bone Structure and Metabolism. (1956 b) p. 203. Churchill & Co. Ltd., London.

KODICEK, E.
Biochem. J. (1956) 64, 25P.

KODICEK, E.
Fourth Internation. Congress of Biochem.
Vitamin Metabolism. (1960) XI, p. 198. Pergamon Press, London.

KODICEK, E. and ASHBY, D.R.
Biochem. J. (1960) 75, 3 17P.

KODICEK, E., CRUICKSHANK, E.M. and ASHBY, D.R.
Biochem. J. (1960) 76, 1 15P.

KORENSCHEVSKY, V.
Spec. Rep. Ser. Med. Res. Council (London) (1922) 71.

LEHMANN, H. and POLLACK, L.
J. Physiol. (1941-42) 100, 17P.

LENGEMANN, F.W. and DOBBINS, J.W.
J. Nutr. (1958) 66, 1 45.

LINDQUIST, B.
Acta Paediat. (1952) 41, Suppl. 86 1.

LOOMIS, W.F. and LIPMANN, F.
J. Biol. Chem. (1948) 173, 807.

LOTSPEICH, W.D. and KELLER, D.M.
J. Biol. Chem. (1956) 222, 843.

LOTSPEICH, W.D.
The Harvey Lectures. (1960-61) 56, 63.
Academic Press, New York.

MacCALLUM, W.G. and VOEGTLIN, C.
J. Exp. Med. (1909) 11, 118.

McCOLLUM, E.V., SIMMONDS, N., SHIPLEY, P.G. and PARK, E.A.
J. Biol. Chem. (1921) 47, 507.

McCOLLUM, E.V., SIMMONDS, N., BECKER, J.E. and SHIPLEY, P.G.
J. Biol. Chem. (1922) 53, 293.

McCOLLUM, E.V., SIMMONDS, N., BECKER, J.E. and SHIPLEY, P.G.
J. Biol. Chem. (1925) 65, 97.

McDOUGAL, D.B. Jr., LITTLE, K.D. and CRANE, R.K.
Biochim. et Biophys. Acta. (1960) 45, 483.

McLEAN, F.C. and URIST, M.R.
Bone, An Introduction to the Physiology of Skeletal Tissue.
(1955). The University of Chicago Press, Chicago.

MALM, O.J., NICOLAYSEN, R. and SKJELKVALE, L.
Ciba Found. Colloquia Ageing I. (1955) 109
Churchill & Co. Ltd., London.

MARTINI, L., PECILE, A. and GUILIANI, G.
Mem. Soc. Endocrin. (1960) 9 Prog. in Endocrin I, 34.

MELLANBY, E.
Spec. Rep. Ser. Med. Res. Council (London) (1921) 61.

MELLANBY, E.
J. Physiol. (London) (1949) 109, 488.

MIGICOVSKY, B.B.
Can. J. Biochem. & Physiol. (1957) 35, 1267.

MIGICOVSKY, B.B. and EMSLIE, A.R.G.
Arch. Biochem. (1947) 13, 175.

MIGICOVSKY, B.B. and EMSLIE, A.R.G.
Arch. Biochem. (1949) 20, 325.

MIGICOVSKY, B.B. and EMSLIE, A.R.G.
Arch. Biochem. (1950) 28, 324.

MIGICOVSKY, B.B. and JAMIESON, J.W.S.
Can. J. Biochem. & Physiol. (1955) 33, 202.

MIGICOVSKY, B.B. and NIELSEN, A.M.
Arch. Biochem. Biophys. (1951) 34, 105.

MITCHELL, P.
Nature. (1961) 191, 144.

MITCHELL, H.H., HAMILTON, T.S., STEGGERDA, F.R. and BEAN, H.W.
J. Biol. Chem. (1945) 158, 625.

NEUMAN, W.F. and NEUMAN, M.W.
The Chemical Dynamics of Bone Mineral. (1958)
University of Chicago Press, Chicago.

NEWCOMER, W.S.
Amer. J. Physiol. (1959) 196 (2) 276.

NICHOLS, J. and GREEN, H.D.
Amer. J. Physiol. (1954) 176, 374.

NICOLAYSEN, R.
Biochem. J. (1937 a) 31, 107.

NICOLAYSEN, R.
Biochem. J. (1937 b) 31, 122.

NICOLAYSEN, R.
Acta Physiol. Scand. (1943) 5, 200.

NICOLAYSEN, R.
Acta Physiol. Scand. (1951) 22, 260.

NICOLAYSEN, R. and EEG-LARSEN, N.
Vitamins and Hormones. (1953) 11, 29.
Academic Press, New York.

NICOLAYSEN, R. and EEG-LARSEN, N.
Ciba Found. Symposium on Bone Structure and Metabolism. (1956) p. 175. Churchill & Co. Ltd., London.

NICOLAYSEN, R., EEG-LARSEN, N. and MALM, O.J.
Physiol. Reviews. (1953) 33, 424.

NICOLAYSEN, R. and NORDBØ, R.
Acta Physiol. Scand. (1943) 5, 212.

NOSSAL, P.M.
Aust. J. Exp. Biol. & Med. Sci. (1953) 31, 583.

NOWOTARSKI, J.S. and BIRD, H.R.
Poultry Science. (1943) 22, 72.

PATRICK, H. and SCHWEITZER, G.K.
Poultry Science. (1955) 34, 832.

PAVLOV, I.P.
Verhandl. Ges. Russ. Aertz (1904) 72, 314.

RAOUL, Y.
Internationale Zeitschrift Fur Vitamin Forschung (1958) 28, 306.

RAOUL, Y., Le BOULCH, N., BARON, C., CHOPIN, J. and GUERILLOT-VINET, A.
Bull. Soc. Chim. Biol. (1954) 36, 1265.

RAOUL, Y., Le BOULCH, N., BARON, C., BAZIER, R. and GUERILLOT-VINET, A.
C.R. Acad. Sci. (1956) 242, 3 3004.

RAOUL, Y. and GOUNELLE, J.C.
C.R. Acad. Sci. (1958) Paris 247, 161.

RASMUSSEN, H.
Endocrin. (1959) 65, 517.

REED, C.I., STRUCK, H.C. and STECK, I.E.
Vitamin D. (1939) The University of Chicago Press, Chicago.

REITZ, L.L., SMITH, W.H. and PLUMEE, M.P.
Anal. Chem. (1960) 32, 1728.

ROBERTSON, R.N.
Biol. Rev. (1960) 35, 231.

ROSENBERG, TH. and WILBRANDT, W.
Helv. Physiol. & Pharm. (1957) 15, 168.

ROTTENSTEN, K.V.
Biochem. J. (1938) 32, 1285.

RUSSELL, W.C. and CHICHESTER, D.F.
Science. (1931) 74, 437.

RUSSELL, W.C., TAYLOR, M.W. and CHICHESTER, D.F.
J. Nutr. (1934) 8, 689.

SALOMON, L.L. and JOHNSON, J.E.
Arch. Biochem. (1959) 82, 179.

SAVILLE, P.D., NASSIM, J.R., STEVENSON, F.H. and MULLIGAN, L.
Clin. Science. (1955) 14, 489.

SCHACHTER, D. and ROSEN, S.M.
Amer. J. Physiol. (1959) 196 (2) 357.

SCHACHTER, D., DOWDLE, E.B. and SCHENKER, H.
Amer. J. Physiol. (1960 a) 198, 263.

SCHACHTER, D., DOWDLE, E.B. and SCHENKER, H.
Amer. J. Physiol. (1960 b) 198, 275.

SCHACHTER, D., KIMBERG, D.V. and SCHENKER, H.
Amer. J. Physiol. (1961) 200 (6) 1263.

SCHENCK, F.
Naturwissenschaften. (1937) 25, 159.

SCHNEIDER, H. and STEENBOCK, H.
J. Biol. Chem. (1939) 128, 159.

SCHREIER, K. and SCHNEPF, E.
Z. ges. Exp. Med. (1956) 127, 508.

SHAPIRO, B.
Biochem. J. (1947) 41, 151.

SHOHL, A.T. and BUTLER, A.M.
New Eng. J. Med. (1939) 220, 515.
SHOHL, A.T., FAN, C.H. and FARBER, S.
Proc. Soc. Exp. Biol. & Med. (1939) 42, 529.

SHOHL, A.T. and FARBER, S.
J. Nutr. (1941) 21, 147.

SMITH, M.C., GLYNN, H.E., WILKINSON, P.A. and PEEVERS, R.W.
Nature. (1943) 152, 385.

STEENBOCK, H. and BELLIN, S.A.
J. Biol. Chem. (1953) 205, 985.

STOREY, E.
Aust. Ann. Med. (1960) 9, 318.

TAGGART, J.V.
Amer. J. Med. (1958) 24, 774.

TALMAGE, R.V.
Ann. N.Y. Acad. Science. (1956) 64, 326.

TALMAGE, R.V. and ELLIOTT, J.R.
Fed. Proc. (1958) 17, 160.

TAYLOR, N.B., WELD, C.B. and SYKES, J.F.
Trans. Roy. Soc. Can. (1932) V 26, 29.

TAYLOR, N.B., WELD, C.B. and SYKES, J.F.
Brit. J. Exp. Path. (1935) 16, 302.

TELFER, S.V.
Quart. J. Med. (1926-27) 20, 1.

THOMAS, W.C. Jr. and MORGAN, H.G.
Endocrin. (1958) 63, 57.

THOMAS, W.C., MORGAN, H.G., CONNOR, T.B., HADDOCK, L., BILLS, C.E. and HOWARD, J.E.
J. Clin. Invest. (1959) 38, 1078.

UMBREIT, W.W., BURRIS, R.H. and STAUFFER, J.F.
Manometric Techniques and Tissue Metabolism. (1939) p. 119.
Burgess Publishing Co., Minneapolis.

UNDERWOOD, E., FISCH, S. and HODGE, H.C.
Amer. J. Physiol. (1951) 166, 387,

URIST, M.R.
Recent Progress in Hormone Research. (1959) 15, p. 455.
Academic Press, New York.

VAUGHAN, O.W. and FILER, L.J. Jr.
J. Nutr. (1960) 71, 10.

VERZAR, F. and McDOUGALL, E.J.
Absorption from the Intestine. (1938)
Longmans, Green & Co., London.

WAASJO, E. and EEG-LARSEN, N.
Acta Physiol. Scand. (1951) 25 Suppl. 89 p. 84.

WASSERMAN, R.H.
Proc. Soc. Exp. Biol. Med. (1960) 104, 92.

WASSERMAN, R.H. and COMAR, C.L.
Endocrin. (1961) 69, 6 1074.

WASSERMAN, R.H., COMAR, C.L. and NOLD, M.M.
J. Nutr. (1956) 59, 371.

WASSERMAN, R.H. COMAR, C.L., SCHOOLEY, J.C. and LENGEMANN, F.W.
J. Nutr. (1957) 62, 367.

WHIPPLE, G.H.
Physiol. Rev. (1922) 2, 440.

WHITTAM, R. and WHEELER, K.P.
Biochim. et Biophys. Acta. (1961) 51, 622.

WILLIAMS, G.A., BOWSER, E.N., HENDERSON, W.J. and UZGIRIES, V.
Proc. Soc. Exp. Biol. Med. (1961) 106, (3) 664.

WILSON, G., CARE, A.D. and ANDERSON, C.K.
Clin. Science. (1957) 16, 181.

WINDAUS, A.
Nature. (1931 a) 128, 39.

WINDAUS, A.
Proc. Roy. Soc. (1931 b) B 108, 568.

WISNER, F.P. and WHIPPLE, G.H.
Amer. J. Physiol. (1922) 60, 119.

WOOLLEY, D.W.
Proc. Nat. Acad. Sci. (1958) 44, 1202.

WOOLLEY, D.W. and CAMPBELL, N.K.
Biochim. et Biophys. Acta. (1960) 40, 543.

YENDT, E.R. and HOWARD, J.E.
Bull. Johns Hopk. Hosp. (1955) 96, 101.

ZAR, L. and IRVING, J.T.
Nature. (1959) 184, 1410.

PUBLICATIONS

"The influence of vitamin D on calcium absorption in the chick",

J.D. Sallis and E.S. Holdsworth.

Amer. J. Physiol. (1962) in press.

"Calcium metabolism in relation to vitamin D_3 and adrenal function in the chick",

J.D. Sallis and E.S. Holdsworth.

Amer. J. Physiol. (1962) in press.

COMMUNICATIONS

"Calcium absorption in chicks",

J.D. Sallis and E.S. Holdsworth.

Aust. J. Science. (1960) 23, 14.