THE CALCINOGENIC PRINCIPLES OF

Trisetum flavescens

BY

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ABSTRACT

The Calcinogenic Principles of *Trisetum flavescens*.

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Studies have been conducted on the calcinogenic plant *Trisetum flavescens* in order to determine the physiological actions and chemical nature of the toxic substance or substances.

An organic soluble substance was extracted and purified and shown to promote intestinal phosphate transport and increase plasma phosphate levels. This factor was shown to influence the movement of phosphate from the serosa to the bloodstream but not direct uptake from the intestinal lumen. This substance was found to be unlike vitamin D_3 or its hydroxy - metabolites in that it did not promote intestinal calcium transport, or bone resorption when studied *in vitro*. This substance did decrease plasma alkaline phosphatase levels in vitamin D_3-deficient chicks however.

An aqueous soluble extract was partially purified on a Sephadex column and found to promote an increase in plasma phosphate in both vitamin D_3-replete and vitamin D_3-deficient chicks. The extract was also able to influence intestinal phosphate transport in a similar manner to the organic solvent soluble phosphataemic factor. As both the aqueous soluble and organic solvent soluble factors were active in vitamin D_3-deficient chicks they were presumed to act upon the paracellular route for intestinal phosphate uptake. The aqueous soluble factor differed from the organic solvent soluble one in that its administration produced a dramatic decline in the plasma calcium levels of vitamin D_3-deficient chicks. The aqueous soluble factor was without effect upon bone cultured *in vitro*.

A second aqueous soluble extract was shown to increase intestinal transport of calcium in chicks with a metabolic deficiency of vitamin D_3 caused by the inclusion of strontium in the diet. This substance appears to be able to mimic the actions of $1,25(OH)_2$chole-calciferol.

The potent aqueous soluble phosphataemic factor is not related chemically to vitamin D_3 but has the characteristics of an aromatic indole or amine.

It is concluded that the actions of these three substances in raising plasma [Ca x P_i] results in calcinosis.

CHAPTER 1

LITERATURE REVIEW

CHAPTER 1

INTRODUCTION

Calcinosis is a disease caused by the deposition of calcium and magnesium salts in the soft tissues of vertebrates as the result of the breakdown of the calcium regulatory system. Several plants have been implicated as causative agents having been found to promote an unregulated rise in the plasma levels of either calcium and/or phosphate, resulting in salt deposition in soft tissues. The extent of the debility experienced by an affected animal increases with time and seriously affects the economic value to the farmer, in terms of both milk yield and slaughter value.

Research into plant induced calcinosis began seriously in 1967 when the cause of enzootic calcinosis of cattle in the Argentinian province of Buenos Aires was ascribed to a toxic plant (Worker and Carrillo, 1967). This plant, Solanum malacoxylon, was later discovered to contain an active form of vitamin D_3 which caused serious malfunctions of the calcium regulatory system of ruminants. Subsequently, rapid advances were made involving the determination of the mechanism of action of the plant toxin and its identity as $1,25(OH)_2$cholecalciferol. This progress was greatly facilitated because at the same time research into the vitamin D_3 endocrine system was also making rapid strides.

In 1971 $1,25(OH)_2$cholecalciferol was discovered, and its role in calcium regulation in normal animals ascertained (Lawson, Fraser, Kodicek, Morris and Williams, 1971. Following the understanding of the relationship between vitamin D_3 metabolism and actions and Solanum malacoxylon induced calcinosis, other plants were proposed as being responsible

for the disease in other parts of the world where Solanum malacoxylon was not present. Cestrum diurnum (also a member of the Solanaceae), and Solanum torvum have both been found to cause similar signs to Solanum malacoxylon.

In 1973 it was discovered that calcinosis of cattle grazing in the foothills of the Austrian and Bavarian alps was caused by a member of the Graminae family (Dirksen, Plank, Hanichen and Spiess, 1973). This plant, Trisetum flavescens, was found to promote a high plasma phosphate level, with normal or slightly elevated calcium unlike the aforementioned Solanaceae which promoted elevated plasma calcium levels with little effect on phosphate. Thus, it appeared the calcinosis induced by Trisetum flavescens differed from that caused by other plants.

The early research on the calcinogenic activity of this plant was carried out using organic soluble extracts, but no substance was found which could be toxic to the extent noted in affected animals. The present research project was designed to elucidate whether or not organic soluble and/or water soluble calcinogens exist in the plant, and the nature of their physiological actions and chemical structure.

LITERATURE REVIEW

1:1 The Significance of Plant Induced Calcinosis.

Calcinosis in grazing animals is a problem in several parts of the world. The plants responsible for the disease have been found to belong to different families and to originate from both tropical and temperate regions. The existence of calcinosis was first established at the turn of the century (Lignieres, 1898). Lignieres reported on cattle suffering from calcinosis in Argentina. Later, Collier (1926), indicated that the disease was caused by the plant Solanum malacoxylon (syn.Solanum glaucophyllum). Unfortunately the work was overlooked for many years, and it was not until the 1960s that Solanum malacoxylon was "rediscovered" as the causative agent.

Prior to the 1960s Arnold (Arnold and Finchman,1950, Arnold, 1954) described the calcinosis problem found in Jamaica, where the disease is called 'Manchester Wasting Disease'. (The Manchester Uplands, Jamaica, is an area where the disease is commonly found). He did not realise that a plant was responsible but did associate the disease with certain types of grazing and reported the typical macroscopic signs of calcinosis in detail (Arnold, 1954). Affected animals had a stiff gait and found it difficult to lie down and stand up; they became anorexic, emaciated and listless and their milk yield eventually ceased. Young and in-calf heifers seemed to exhibit the most severe problems although no definite age or sex correlation was apparent. Internally, widespread calcifications were noted at the sites of all elastic and collagenous fibres. Of particular note was the deposition

of calcium salts in the alveolar walls of advanced cases. The extent of calcification in the lungs could be so severe that the lungs did not collapse when the thoracic wall was opened. In addition calcifications were visible in the aorta and other major and minor arteries and in the kidneys where crystals containing calcium phosphate and calcium carbonate were found.

In Argentina, the disease (known as "Enteque Seco") was responsible for the loss of about five to ten per cent of an affected herd each year. The incidence of the disease was known to vary with the season and to be associated with certain soil types (Worker and Carillo, 1967). Animals would recover if moved from the area in which the disease was contracted, to one with different ('better') pasture (Tilley, 1967; Worker and Carillo, 1967).

In the early 1960s the low productivity of Argentine cattle was noticed by the United Nations Technical Assistance Board, and a five year project was initiated by the United Nations and the Argentine government to investigate the problem. "Enteque Seco" was quickly singled out for the study by the research group set up. The group identified a plant that was responsible for causing the calcifications and high levels of plasma calcium and phosphate; this was Solanum malacoxylon. This plant is a member of the Solanaceae and was a shrub found in abundance in the grazing areas harbouring the disease (Tilley, 1967). The high toxicity of the plant was of particular note, and it was found that as little as fifty grams per week of dried leaves of Solanum malacoxylon could cause all the signs of "Enteque Seco" in cattle. It was thought that cattle became ill by accidentally grazing the fallen leaves of the plant; they did not graze on the live plant preferentially.

Eradication of the plant from the pasture was the obvious means of dealing with the problem, but this proved impractical, mainly because the plant has an extensive root system which sends up stems at a distance from the parent plant, and so the problem remained unsolved. However, research work into the mode of action of the plant toxin proceeded in various locations, leading rapidly to a clear understanding of the underlying pathophysiology.

Another plant was found to be responsible for the calcinosis found in horses in Florida; this is Cestrum diurnum ("Day-Blooming Jessamine"). The condition was first reported in 1970 by Krook and Wasserman (1975). It was found that horses were severely affected three to four months after showing the first signs . The lesions were the same as those found in the Argentine cattle suffering from Solanum malacoxylon intoxication. Cestrum diurnum was suspected as the cause as it was also a member of the Solanaceae and was found in the areas grazed by affected animals. Cestrum diurnum is a sub-tropical to tropical erect shrub growing up to five metres in height; it was introduced in Florida and California from the West Indies. The affected animals were found to have locomotory problems similar to those found in cattle with "Enteque Seco": they had high plasma calcium and phosphate levels and internally there was calcification of areas such as the aorta, left atrium, ligaments and cartilage.

Signs of calcinosis in cattle have also been reported from Papua, New Guinea by Copland (1975). The affected animals were grazed in pine plantations. These tended to be young, introduced cattle. Again they showed typical calcinosis signs , such as stiff gait, pronounced wasting and increased levels of plasma calcium and phosphate. Several

plants, suspected of being causative agents, were tested (Morris, Simonite, Pullen and Simpson, 1979) but only Solanum torvum (var. Swartz) was found to be calcinotic, causing elevations in serum calcium and phosphate in rats.

The presence of enzootic calcinosis in Austria was first described by Libiseller and Gunhold (1969). In 1970, Dirksen's group described a new form of 'spontaneous' calcinosis in cattle reared on grassland farms in Germany, (Dirksen, Plank, Dammrich and Hanichen, 1970). He described the pathological and anatomical features of the disease and found that they were very similar to those found by Arnold in Jamaica. However, no preponderance of Solanaceous species were found in the pasture.

Research workers presumed that a plant was responsible for the disease because of the earlier discovery of Solanum malacoxylon and also because the other diseases that might be responsible for the symptoms (eg. osteomalacia, manganese deficiency, hyperparathyroidism) could be discounted for one reason or another. The problems, however, resemble hypervitaminosis D_3 and so the comparison could be made with "Enteque Seco" (Dirksen, 1975). Dirksen's group therefore looked to the fodder composition for the answer.

Trisetum flavescens was soon identified as the calcinogenic plant by Dirksen, Plank, Hanichen and Spiess(1972, 1973) who fed rabbits with pure Trisetum flavescens and compared the development of arterial calcifications with groups fed on only Dactylis glomerata and mixed Graminae. Further experiments in sheep (Dirksen, 1975) confirmed Trisetum flavescens as the calcinogenic plant. It was found that in

a severely affected animal, calcifications of the abdominal aorta could be felt in the live animal by palpation via the rectum. In Germany, up to half a herd could be affected, as opposed to the 5-10% of animals affected in Argentina, although the onset of the disease appeared to be slower than in animals grazing the Solanaceous species. There was a time lag of twelve to thirty-six months before cattle grazing on the German pastures became seriously affected. Sheep fed only Trisetum flavescens developed macroscopic calcifications within fifty three days (Simon, Daniel, Hanichen and Dirksen, 1978). Signs were often first noticed at the end of the summer, with the animals initially showing a humped back, (a symptom typical of the disease), difficulties with locomotion, particularly when rising, as well as reduced milk yield and general lethargy.

Signs of macroscopic calcifications in major arteries could be seen in experimental sheep grazed on 100% or 25% Trisetum flavescens pasture, but not Trisetum flavescens - free pasture. The first signs of the development of calcinosis was calcification of the major arteries (Dirksen, Plank, Simon, Hanichen, Daniel and Spiess, 1974).

In affected cattle, serum calcium levels were generally in the upper physiological range, but serum phosphate was very much elevated (up to 12 mg/100 mls). Elevations in urinary phosphate were also noted. The increase in serum phosphate was particularly evident towards the end of the summer, suggesting seasonality in the calcinosis inducing agent. Moving animals to a new environment caused a reduction in serum phosphate levels and they recovered within a few months. Necropsy findings showed calcinosis to be similar to that of animals suffering Solanum malacoxylon or Cestrum diurnum intoxication.

Typically, calcifications occurred in the left atrium (the first site of calcium salt deposition), the arteries and lungs. There was also hardening of the digital flexor tendons where calcium foci were visible. In the arteries it appeared that destruction of elastic and collagenous fibres preceded calcification of these areas; the intima and elastic lamina increased in size, the arterial elastic fibres became swollen and fragmented and occasionally they were finely calcified. Dirksen concluded that metaplasia of the connective tissue was caused by fibroblasts which, when fibres become damaged, regenerate excessive connective tissue (Dirksen,Plank,Hanichen,Daniel and Spiess,1974)

Microscopic examination of the bone tissue showed some major abnormalities. These could be termed as systematised osteo - myelosclerosis, which meant that new layers of bone had been laid down which were more highly mineralised than normal bone. This generalised excess of bone formation was explained as an increase in bone tissue formation rather than to a cessation of bone resorption.

It would appear that the management of pastures in German alpine regions prone to calcinosis is detrimental, in that it encouraged the prevalence of Trisetum flavescens at the most potent stage of its growth (Lichtenegger, Kutsclera, Kohler and Libiseller, 1970). Management involved the mowing of the pasture once or twice a year which prevented maturation of the plant,resulting in the persistence of young leafy plants which are the most calcinogenic. Manuring of the pastures resulted in a reduction of the number of plant species in the pasture, which increased the population of Trisetum flavescens since it competes well under these conditions.

This information suggests that a certain measure of control over the disease could be obtained by altering the pasture management regime to one which diversified the number of species and allowed maturation of the plants. Other studies (Swieboda and Dabrowska, 1976) showed that Trisetum flavescens would not grow in soil containing less than 0.15% nitrogen and that other grasses competed well with it under these conditions (eg Arrhenatherum elatius and Agropyron repens). The method of encouraging differences in soil and plant composition in pastures where Trisetum flavescens induced calcinosis could occur, may be the most sensible way of preventing the disease in these regions.

Various groups of workers agreed that the leaves of the plant held the calcinogenic activity (Libiseller, Kohler, Glawischnig, Sarafidis and Schmid, 1978; Rambeck, Wetzel and Stark, 1979; Wolf and Dirksen, 1976; Kohler, Libiseller, Schmid and Swoboda, 1978; Rambeck, Kreutzberg, Brums-Droste and Zucker, 1981) rather than the flowers, roots or stems. Young leaves were more potent than old leaves. Feeding the grass as silage did not destroy its calcinogenic activity (Heinritzi, Kragenings and Hanichen, 1977; Kohler, Libiseller, Schmid and Swoboda, 1978), neither did 'artificial green drying', (Dirksen, Kragenings, Hanichen and Elmer-Engelhard, 1981). It was also established that all the various commercial cultivars of Trisetum flavescens that are available are calcinogenic, and to roughly the same extent (Rambeck, Kreutzberg, Bruns-Droste and Zucker, 1981). In addition, it was found that Trisetum elatior, a close relative of Trisetum flavescens was inactive. Other experiments showed that the growing of Trisetum flavescens

under different levels of fertiliser had little influence on the subsequent development of calcinosis in test animals (Simon, Daniel, Hanichen and Dirksen, 1978). All these experimental approaches were designed to detect a simple way of either destroying the calcinogen or discouraging the plant, but none were successful.

Trisetum flavescens is a member of the Graminae (grasses) and therefore became the only known plant, not belonging to the Solanaceae, to be calcinogenic.

The following table shows the known distribution of the disease and identifies the plants responsible.

TABLE 1

THE DISTRIBUTION OF PASTORAL CALCINOSIS.		
COUNTRY	LOCAL NAME GIVEN TO CALCINOSIS SYMPTOMS	CALCINOGENIC PLANT
Argentina	"Enteque Seco"	Solanum malacoxylon
Brazil	"Espichamento"	Solanum malacoxylon
Hawaii	"Naalehu Disease"	S. Malacoxylon/S. Torvum or Cestrum diurnum
Jamaica	"Manchester Wasting Disease"	Solanum malacoxylon
Florida	Calcinosis	Cestrum diurnum
Papua	"Enzootic Calcinosis"	Solanum torvum
Austria/Germany	"Weidekrankheit"	Trisetum flavescens
Australia	"Humpy Back"	Solanum sodomaeum (suspected)
South Africa (Affects sheep)	Calcinosis	unknown possibly S. Malacoxylon
Israel (Affects goats)	Calcinosis	unknown

Clearly, the scale of the problem appears to warrant further efforts by farmers and research workers in identifying and eradicating

calcinogenic plants from pastures. The macroscopic studies made by Arnold (1954), which were subsequently extended by other workers, indicated that a serious problem involving the calcium homeostatic mechanism was responsible for the calcinosis of ruminants. Interference with this complex and highly regulated cycle has been confirmed as the cause of plant induced calcinosis.

1:2 Endocrine Control of Calcium Metabolism.

The Vitamin D_3 Metabolites.

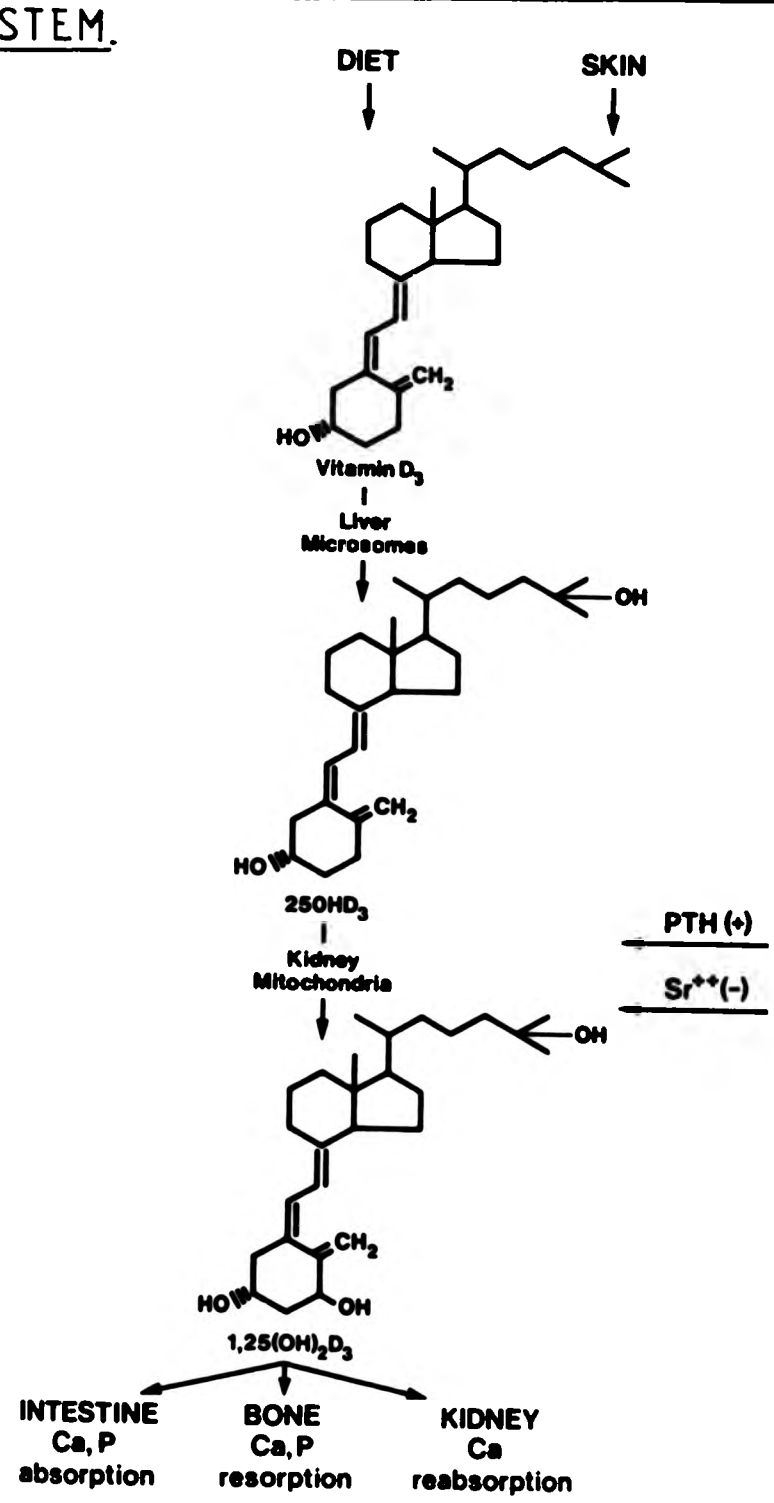
Vitamin D_3 was first characterised in 1932 (Askew, Bourdillon and Bruce, 1932) following the classic experiments of Sir Edward Mellanby (Mellanby, 1919) which showed that a specific diet could induce rickets in puppies. Askew characterised the 'vitamin' as a seco-steroid, that is a steroid that has undergone fission by the severing of a carbon-carbon bond, resulting in the breaking of one of the six member carbon rings thus forming an open-chain structure. In the case of vitamin D_3 the steroid 'B' ring is broken. (See figure 1).

Vitamin D_3 is the only vitamin to be generated by photobiogenesis in the skin, the precursor being 7-dehydrocholesterol. Experiments have shown that light in the 253 nm to 400 nm range transforms 7-dehydro-cholesterol to previtamin D_3. This involves the severing of a C-C bond and a subsequent isomerisation step (a thermally regulated reaction) to produce vitamin D_3. (Holick, Richtand, McNeill, Holick, Frommer, Henley and Potts, 1979). The function of vitamin D_3 (see figure 2) is to maintain serum calcium and phosphate at levels required for bone mineralisation and the prevention of tetany (muscular spasms that occur if serum calcium becomes too low). Vitamin D_3 cannot do this directly, but functions as a precursor for a number of metabolites that help to achieve the homeostasis of calcium and phosphate in the body.

The vertebrate system for maintaining constant blood levels of the mineral ions Ca^{2+} and PO_4^{3-} is very elaborate. These levels are

FIGURE 1

PATHWAY OF BIOSYNTHESIS OF THREE MAJOR METABOLITES OF THE CALCIUM HOMEOSTATIC SYSTEM.



maintained within narrow limits by a metabolic system that functions in a manner akin to the other major endocrine systems of vertebrates. This means that a complete array of glands, hormones and feedback inhibition mechanisms are in evidence. The calcium homeostatic system requires the presence in the diet of calcium, phosphate and vitamin D_3 (where the animals' skin does not have regular exposure to sunlight) in order to function correctly. Otherwise all the necessary metabolites are synthesised at various sites around the body. The ability of ultra-violet light to convert 7-dehydrocholesterol to pre-vitamin D_3 is one aspect of the metabolism of this so-called vitamin that has led to its more accurate description as a pro-hormone.

The levels of calcium and phosphate in the plasma are maintained at a supersaturated level. The serum calcium is either in free ionic form, or is combined with proteins. Fifty per cent of serum calcium is diffusible; this portion is either Ca^{2+} or is combined with anions such as phosphate or bicarbonate. The other fifty per cent is non-diffusible (5% is combined with an ultrafiltrable anion). Hence it is only partly diffusible through a semi-permeable membrane. When serum calcium is measured using Atomic Absorption Spectroscopy, as in this project, the total level of calcium is measured; i.e. diffusible and non-diffusible. The relationship between diffusible and non-diffusible calcium can be expressed in the following way, where P = protein concentration and Ca d++ = diffusible calcium (presuming all diffusible calcium is ionised).

$$P/CaP = 0.403 + 5.80/Ca\ d{+}{+}$$

which gives a pKa Ca-Prot of 2.44. In contrast, all serum phosphate

(as PO_4^{3-} , HPO_4^{2-} or $H_2PO_4^-$) is ultrafiltrable when calcium and phosphate levels are both normal.However, when levels of either one increase sufficiently, a complex of colloidal calcium phosphate is formed (Greenberg, Larsen and Tufts, 1934: McLean and Hinrichs, 1938). The latter found that the intravenous injection of phosphate caused an increase in the levels of 'colloidal calcium phosphate complex' until the product $[Ca \times PO_4]$ of the plasma approached 3 mM/litre, at which point precipitation of calcium phosphate salt begins to occur. It then became clear that calcium and phosphate in serum obey the solubility product law.

The solubility product relation applies to a sparingly soluble salt in the solid state and its ions in solution. The law can be represented in the following way - where [MA] = a saturated solution of binary electrolyte in contact with an excess of undissolved solid at constant temperature.

$$\frac{[M+][A-]}{[MA\ solid]} = \text{constant}$$

therefore $[M+][A-]$ = constant = the solubility product of [MA] at that temperature. This quantitative relation occurs between serum calcium and phosphate and it was found that in normal plasma the Total Calcium x Total Phosphate = 40 mg% (this is equivalent to 40 mg per hundred mls of plasma) or more. But in rachitic animals it is 35 mg% or less (Howland and Kramer, 1922). This is expressed more accurately as : $[Ca^{2+}]3 \times [HPO_4^{2-}]2$.

Comparisons made between the $[Ca \times PO_4]$ products of pure solution and blood plasma showed that plasma is super-saturated with respect to these ions. The calcium x phosphate product of an aqueous solution

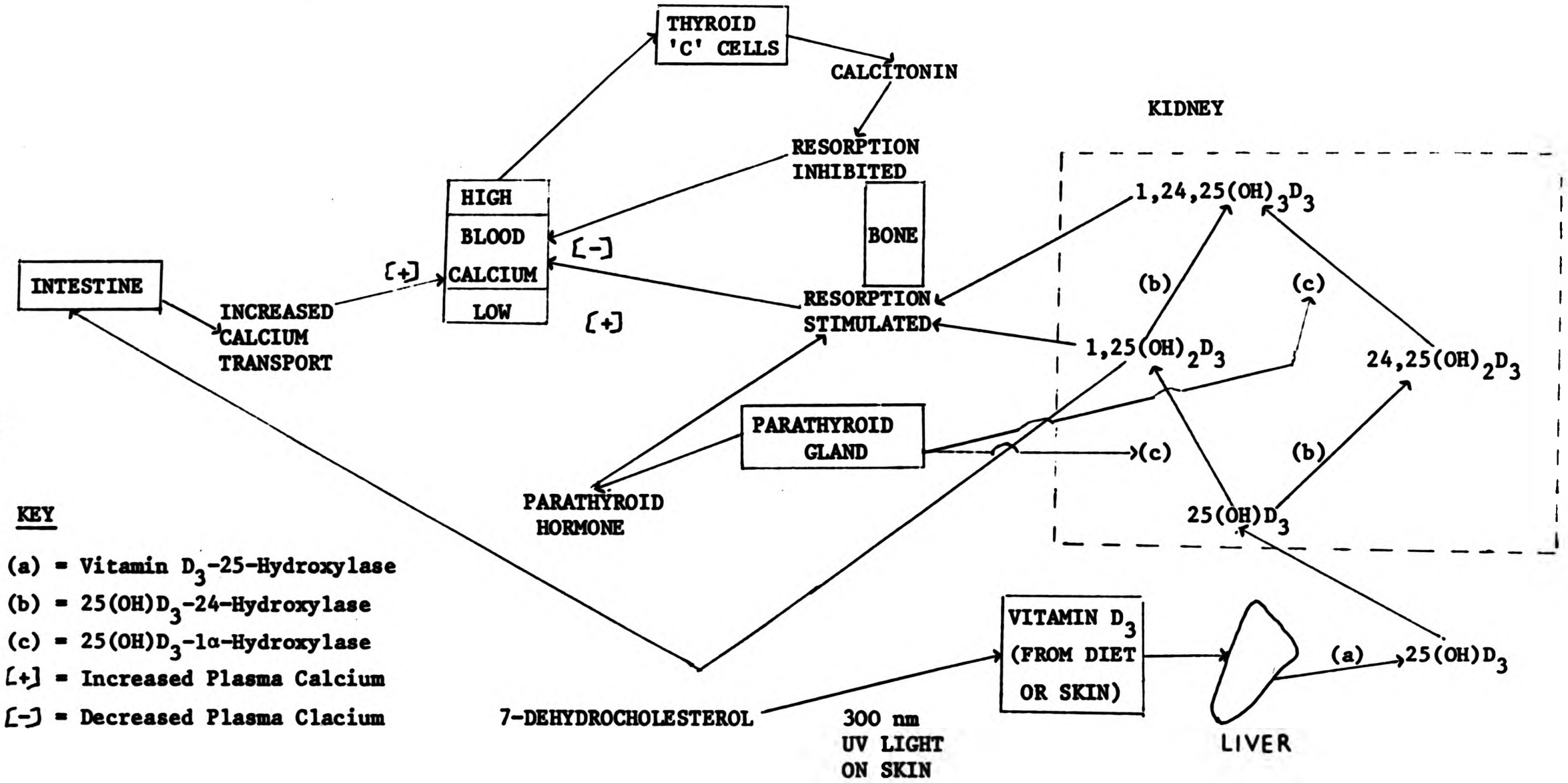
of these ions in the laboratory = 10^{-26} whereas that of plasma = 10^{-23} The reason for this super-saturation was shown to be the further equilibration of carbonate ions with calcium and phosphate leading to the more accurate representation of the equilibrium as follows:

$$[Ca^{2+}]7 \times [PO_4^{3-}]4 \times [CO_3^{2-}]$$

It can be seen from this that calcium and phosphate concentrations in the blood are interdependent, and if the concentration of either one increases so as to exceed the solubility product, there will be precipitation of solid salt. This is the ultimate cause of calcinosis and when precipitates have formed extensively throughout the body, then the condition is exceedingly difficult to reverse. Precipitation of calcium phosphate is often accompanied by other salts. Animals with calcinosis are found to have precipitates of other salts in their tissues. These include magnesium carbonate and calcium carbonate. Magnesium can be substituted for calcium in the formation of salts. The presence of carbonates is not surprising, since, as shown above, the carbonate ion is involved in a three way equilibrium with calcium and phosphate.

The regulatory mechanisms that form the basis of the control of calcium metabolism are shown in figure 2. It can be seen that the processes are regulated by negative feedback based on the level of calcium in the blood. A low level of calcium initiates the production and secretion of parathyroid hormone, which acts on bone to increase resorption, and on the kidney (discussed further in the section on parathyroid hormone) to stimulate production of the crucial enzyme 25(OH)cholecalciferol $-|\ \alpha$ - hydroxylase. This important enzyme

FIGURE 2 THE CALCIUM HOMEOSTATIC CYCLE



KEY

(a) = Vitamin D_3-25-Hydroxylase

(b) = $25(OH)D_3$-24-Hydroxylase

(c) = $25(OH)D_3$-1α-Hydroxylase

[+] = Increased Plasma Calcium

[-] = Decreased Plasma Clacium

converts 25(OH) cholecalciferol to $1,25(OH)_2$ cholecalciferol in the kidney mitochondria, the only site of calcitriol production. Calcitriol (or $1,25(OH)_2$ cholecalciferol) is the metabolically active form of vitamin D_3. It was discovered in 1971 by Lawson, Fraser, Kodicek, Morris and Williams (1971), using mass and ultra violet spectroscopy to confirm the chemical structure. Holick, Schnoes and DeLuca (1971) demonstrated that a previously unknown metabolite of vitamin D_3 was produced when tritium labelled vitamin D_3 was given to chicks, and intestinal extracts from these birds subjected to detailed chromatography. Subsequent mass spectroscopy showed evidence of a vitamin D_3 metabolite previously unknown. The enzyme 25(OH) cholecalciferol-1α-hydroxylase is responsible for the insertion of an hydroxyl group at carbon 1. It is a mixed function monoxygenase, which requires molecular oxygen, magnesium ions and internally generated NADPH. 25(OH)cholecalciferol -1α-hydroxylase contains cytochrome P450. 25(OH)cholecalciferol -1α-hydroxylase has been found to be present in the kidney of every class of vertebrate.

The detailed transformation of the precursor, vitamin D_3 into the active form $1,25(OH)_2$ cholecalciferol is shown in figure 1. The presence of the hydroxyl group at carbon 1 is the major determinant of biological activity, although the hydroxyl group at carbon 25 does assist with the binding of the molecule to its receptor sites in the intestine. Steroid competition assays implied that the hydroxyl group at carbon 25 binds with the receptor to allow the correct positioning of the hydroxyl groups at positions 1 and 3 on the receptor (Norman and Henry, 1979). Molecules such as 25(OH)cholecalciferol and $24,25(OH)_2$ cholecalciferol have very low activity in

comparison. The production of 25(OH)cholecalciferol-1α-hydroxylase is controlled by parathyroid hormone and the secretion of the latter is initiated by low blood calcium. Following the discovery that calcitriol (1,25$(OH)_2$cholecalciferol) is synthesised from vitamin D_3 and is released into the body under the control of parathyroid hormone, calcitriol could legitimately be classified as a hormone; thus vitamin D_3 became a pro-hormone. Calcitriol (1,25$(OH)_2$ cholecalciferol) obeys the prerequisites of a hormone, in that it is synthesised and released in response to the calcium and phosphate status of animals, and its interaction with its target organs resembles the situation encountered with classic steroid hormones. Further it is liberated directly into the blood stream by its gland (the kidney) from where it is carried to its target organs: the intestine, bone and possibly kidney, and is regulated by negative feedback.

The calcium regulatory cycle is geared to respond to either an increase or a decrease in serum calcium. 25(OH)cholecalciferol is the major circulating form of vitamin D_3. It has virtually no biological activity, but acts as a pool of substrate ready for conversion to the active form. However, 25(OH)cholecalciferol is largely converted to 24,25$(OH)_2$cholecalciferol, a circulating metabolite, the concentration of which is inversely related to that of 1,25$(OH)_2$ cholecalciferol. The synthesis of 25(OH)cholecalciferol is increased in normo-calcaemic or hyperphosphataemic conditions (Kumar 1984). This metabolite has a limited ability to activate the intestinal steroid receptor complex but has been shown to inhibit parathyroid secretion and increase bone mineralisation (Miravet et al,1976). The results of Miravet et al (1976) and Stern, DeLuca and Ikekawa (1975)

contradict the work of Dziak (1978) who has shown that both 25(OH)cholecalciferol and 24,25$(OH)_2$cholecalciferol decrease calcium uptake in isolated bone cells.

Conversion of 24,25$(OH)_2$ cholecalciferol to 1, 24, 25 $(OH)_3$ cholecalciferol occurs when the serum calcium level drops and secretion of parathyroid hormone causes the specific repression of the 24(OH)-hydroxylase and induces the 1α-hydroxylase (Tanaka, Lorenc and DeLuca, 1975) in order to increase levels of 1,25$(OH)_2$cholecalciferol.

24, 25$(OH)_2$ cholecalciferol is produced in the kidney mitochondria by 25(OH) cholecalciferol-24-hydroxylase, and also in the intestine (Castillo, Tanaka, DeLuca and Ikekawa, 1978). However, several possible roles for 24, 25$(OH)_2$ cholecalciferol have been suggested. One such suggestion is that it may function as a step in the excretion pathway of vitamin D_3 (Garabedian, Lieberherr, N'Guyen, Corvol, Bailly Du Bois and Balsan S, 1978). According to another group of researchers (Bates, Care, Peacock,Mawer and Taylor, 1975) the low levels of 24, 25$(OH)_2$ cholecalciferol in the plasma of normocalcemic humans inhibits parathyroid secretion by the parathyroid gland, thereby implicating this metabolite in calcium homeostasis. Evidence has also been presented to suggest that 24, 25$(OH)_2$ cholecalciferol has a specific action on bone mineralisation (Ornoy, Goodwin, Noff and Edelstein, 1978). Recent reviews on vitamin D_3 metabolites (eg Kumar,, 1984) however, refer to the continuing lack of understanding of the true role of this metabolite.

The most convenient method of studying the effects of various vitamin D_3 metabolites, the substances related to vitamin D_3 metabolism and calcinogens from plant sources on bone, was found to be an <u>in vitro</u>

bone culture technique first described by Reynolds and Dingle (1970). This involves dissecting the calvariae from five to seven day old mice and maintaining them in an appropriate tissue culture medium. Reynolds found that calcitriol was one hundred times more potent than 25(OH)cholecalciferol at promoting bone resorption and that vitamin D_3 was ineffective. Stained sections of the treated calvariae showed that 1 ng/ml of calcitriol caused almost complete resorption of the immature bone already laid down. However, this would not happen in vivo without the presence of parathyroid hormone. This does not affect the validity of the technique but allows isolated study of a chosen metabolite.

THE INTESTINAL STEROID-RECEPTOR COMPLEX

By 1979 the appearance of calcitriol in intestinal mucosal cells was shown conclusively using frozen section autoradiography (Stumpf, Sar, Reid, Tanaka and DeLuca, 1979). There is clear evidence in the autoradiographs of the subcellular localisation of tritiated calcitriol in the intestinal mucosa of rats. Similar work showed that calcitriol could be detected autoradiographically in the chick duodenum four hours after the administration of labelled calcitriol. (Zile, Bunge, Barsness, Yamada, Schnoes and DeLuca, 1978). The radioactivity was concentrated around the nuclei of absorptive cells in the villi. Calcitriol was also detected in the liver after four hours exposure. The intestinal steroid-receptor complex for calcitriol is a protein of sedimentation value 3.75 situated within target intestinal mucosal cells. When calcitriol enters the cell, it attaches to a binding site on the receptor. There is then a process of activation before the steroid-receptor complex

migrates to the nuclear chromatin fraction where it joins to an acceptor site on the DNA itself. The DNA then codes for a messenger RNA specific for calcium binding protein. The affinity of the complex for DNA is characteristic of the steroid hormones. Saturation analysis demonstrates saturable binding of calcitriol to its receptor. The binding of the receptor complex to chromatin is temperature dependent (Procsal, Okamura and Norman, 1975) and saturation of the chromatin binding sites has also been demonstrated. The stereochemistry of vitamin D_3 is very important in determining its interaction with its receptors. The main features are the conformation of the 'A' ring which carries the vital hydroxyl group; this is in the 'chair' conformation and oscillates continually between the axial and equatorial planes. (In calcitriol the equatorial to axial ratio is 55:45). This means that all vitamin D_3 group receptors must be capable of accepting this conformational mobility.

Experiments conducted by Norman and Henry (1978) have shown that for calcitriol to be recognised by the intestinal receptor system it must be a 9, 10 seco-steroid. They also showed that the hydroxyl groups at positions 1 and 25 must be present if the molecule is to be active. In tests on animals 1(OH)cholecalciferol or 25(OH) cholecalciferol were shown to possess 1/1000 the activity of calcitriol. The intestinal receptor complex was found to be very intolerant of changes in the length of the side chain. On interaction with the receptor protein, it was thought that the 'A' ring was 'frozen out', that is stabilised to prevent oscillation within the steroid/receptor complex.

An *in vitro* chick intestinal receptor system demonstrated the relative ability of various calcitriol analogues to compete for the binding sites on chromatin (Procsal, Henry, Hendricksen and Norman, 1976). These workers incubated increasing concentrations of cholecalciferol analogues with a reconstituted cytosol-chromatin receptor system in the presence of tritiated $1,25(OH)_2$ cholecalciferol. Hence, the amount of radioactivity bound to the chromatin could be assessed for each analogue. This technique allowed the structural requirements for binding of calcitriol to chromatin to be elucidated. The 3βOH group is less important for binding than the 1αOH or 25OH groups. The 5, 6 trans form of vitamin D_3 has very little ability to attach to the binding sites. 1αOH vitamin D_3 is 900 times less effective at competing for the binding site than calcitriol indicating the great importance of the 25(OH)group.

VITAMIN D_3 AND CALCIUM TRANSPORT IN THE GUT

The process of calcium transport across the gut is necessarily achieved by a process of active transport because there is a positive electrical potential difference between the serosal and mucosal surfaces. The calcium transport mechanism that exists appears to have two rate limiting steps: one which allows entry of calcium ions into the mucosal cells by a process of diffusion, and a second which allows exit from them. This latter process involves an active cation pump.

The early evidence for the existence of a specific calcium binding protein came from experiments which showed that there were higher

levels of radiocalcium in the supernatant of centrifuged homogenates of intestinal mucosa from vitamin D_3-replete animals than in that of rachitic animals (Wasserman and Taylor, 1963). These results corresponded with others that showed the presence of a prominent protein band of high electrophoretic mobility in a polyacrylamide gel electrophoresis experiment, which separated proteins in the supernatant fluid of vitamin D_3-replete chicks allowing comparison with vitamin D_3-deficient chicks. It was clear that this protein band was absent in the vitamin D_3-deficient chicks (Taylor and Wasserman, 1967). Hence it was established that vitamin D_3 was involved in the production of this protein.

The protein could be highly purified by a three-step process developed by Wasserman and co-workers (Wasserman, Corradino and Taylor, 1968) involving: i) precipitation with $(NH_4)_2SO_4$, ii) gel filtration column chromatography and finally, iii) preparative discontinuous gel electrophoresis. The amino acid composition and molecular weight of calcium binding protein varies between species, but in the chick the protein was found to have a molecular weight of about 28000 (generally only 10000 to 13000 in mammals). The chick calcium binding protein contains four high affinity binding sites for calcium plus a larger number of low affinity sites. Calcium binding protein will also bind other alkaline earth metal ions such as strontium, barium and magnesium (in that order of preference). Calcium binding protein is not produced prior to an increase in intestinal calcium transport as might be expected, (Spencer et al, 1976). Vitamin D_3-deficient animals, dosed with vitamin D_3,

show an increase in calcium transport several hours before calcium binding protein is detected.

Other workers were also able to show that the rate of calcium binding protein formation and calcium absorption following vitamin D_3 administration, are not directly comparable (Harmeyer and DeLuca, 1969). The reason for this effect is not known.

In the hen high levels of calcium binding protein occur in the intestine and the uterus and these proteins are identical (Fullmer, Brindak, Bar and Wasserman, 1976). The protein appears in the uterus when ovulation and egg laying commence and there is a need for a large amount of calcium to form eggshells. Calcium binding protein is also found in the kidney. In the chick,which has calcium binding protein comprising 2.8% of total body weight,kidney calcium binding protein is identical to intestinal calcium binding protein and is also formed in response to calcitriol, Intestinal calcium binding protein is located at the microvillar border of duodenal enterocyte and goblet cells. Hence it is possible that the goblet cell is the site of synthesis of calcium binding protein (Taylor and Wasserman, 1970).

The synthesis of calcium binding protein occurs de novo (there is no precursor protein molecule) in response to calcitriol by the transcription of a specific messenger RNA. The necessity for the vitamin is illustrated by an experiment in which intestinal polysomes from rachitic chicks were found to be unable to direct the <u>in vitro</u>

synthesis of calcium binding protein whereas those from vitamin D_3-replete chicks could (Emtage, Lawson and Kodicek, 1973) do so.

Following the injection with calcitriol of vitamin D_3-deficient birds it was found that nascent polypeptide chains could be found on intestinal polysomes as early as two hours after dosing, rising to a maximum level of production thirteen hours after dosing (that is, two hours is sufficient time for the transcription of the messenger RNA (MW 700,000)). Corresponding studies showed that two hours was enough time for injected, labelled calcitriol to be detected in the intestinal nuclei of rachitic chicks. These studies lend further weight to the probability that calcitriol regulates a subset of expressed genes which initiate production of calcium binding protein.

Other vitamin D_3 metabolites thought to stimulate calcium transport were 24, $25(OH)_2$ cholecalciferol and 25, $26(OH)_2$ cholecalciferol. This only occurred in animals on a low calcium diet (Miravet, Redel, Carre, Queille and Bordier, 1976). 24, $25(OH)_2$ cholecalciferol is the most abundant vitamin D_3 metabolite to be found in normal or hypercalcemic plasma (Bates et al, 1970). 24, $25(OH)_2$ cholecalciferol and 25, $26(OH)_2$ cholecalciferol were also proposed as being able to stimulate osteoblastic activity in rachitic animals.

PARATHYROID HORMONE AND THE PARATHYROID GLANDS

Parathyroid hormone is a peptide hormone secreted by the parathyoid glands in response to a decrease in circulating calcium ions (Potts et al, 1966). The parathyroid gland itself is able to detect small changes in the levels of serum calcium (ie it is the calcium 'sensing' organ).

Parathyroid hormone acts upon the kidney and the bone and indirectly upon the intestinal calcium absorptive mechanism via its control of calcitriol production. It is responsible for stimulating the production of 25(OH) cholecalciferol 1αhydroxylase and this makes it a vital link in the calcium regulatory mechanism. Parathyroid hormone is also responsible for renal reabsorption of calcium as an indirect effect of its action in stimulating calcitriol production, leading to calcium binding protein production in the kidney. Finally (as described below) parathyroid hormone acts on bone in conjunction with calcitriol, to promote resorption and so increase serum calcium levels.

Parathyroid hormone has been shown to induce the release of calcium from bone explants in culture, two hours after application (Reynolds and Dingle, 1970). At the bone site, the movement of calcium from the bone fluid compartment to the extra-cellular fluid compartment is dependent upon the combined action of both parathyroid hormone and calcitriol. A later response is that parathyroid hormone will stimulate the formation of multinucleate osteoclasts which will release more calcium. This multi-purpose hormone is, therefore, very effective in reversing any tendency towards hypocalcaemia. Because it is responsible for controlling the production of another hormone, ie calcitriol, parathyroid hormone can be classified as a 'tropic' hormone.

Many workers have shown that parathyroid hormone is completely ineffective on the intestine, its action in stimulating intestinal calcium absorption being mediated by calcitriol (Corradino, 1976).

There is a mechanism by which parathyroid hormone and calcitriol act together to cause resorption of the bone - either one on its own is not very effective in this respect, (Garabedian, Tanaka, Holick and DeLuca, 1974). The precise mechanism of this process is not known. The levels of cyclic AMP of isolated bone cells were found to be raised in the presence of calcitriol (or 25(OH) cholecalciferol) plus parathyroid hormone, but not when in the presence of calcitriol (or 25(OH)cholecalciferol) alone (Mahgoub and Sheppard, 1975) thereby implicating (according to the authors) vitamin D_3 analogues as modulating the action of parathyroid hormone on cultured bone cells.

A vitamin D_3 deficient animal has high levels of circulating parathyroid hormone which is released in an attempt to stimulate calcium resorption and 1αhydroxylase in the kidney but remains hypocalcaemic. It cannot maintain the levels of calcium without the accompanying presence of calcitriol. The animal will eventually die from hypocalcaemic tetany. When administered to bones cultured in vitro both parathyroid hormone and calcitriol will resorb bone calcium, but in vivo they must both be present for resorption to occur.

It has been observed that in thyroparathyroidectomised (TPTX) rats pulsed with ^{45}calcium twenty days previously, there were significant increases in circulating radiocalcium levels, when either calcitriol, parathyroid hormone or 1(OH)cholecalciferol were injected (Boris, Hurley and Trmal, 1980). The results were in conflict with those of

Garabedian et al (1974), who demonstrated the lack of response to calcitriol of thyroparathyroidectomised (TPTX) rats fed a low calcium diet. It appears most likely that this is the result of the large, unphysiological doses of calcitriol used by Boris et al (1980) to obtain the response (up to 4000 ng of calcitriol were used to inject each rat in this experiment) and may account for the discrepancy.

Further evidence indicating the synergistic effect of parathyroid hormone and calcitriol was obtained in an experiment using TPTX rats, dosed either with calcitriol alone or in concert with parathyroid hormone (Garabedian, Tanaka, Holick and DeLuca, 1974). The results showed that the rats, fed a low calcium diet, and dosed with calcitriol alone could not maintain their serum calcium levels which fell by 40% in 142 hours. This contrasted with TPTX rats which were dosed with both parathyroid hormone and calcitriol who maintained their normal levels (around 6.0 mg/100 mls) of serum calcium after 56 hours. This experiment also showed that the TPTX rats were unable to synthesise the 25(OH) cholecalciferol - 1α-hydroxylase enzyme as found by Fraser and Kodicek(1973).

Parathyroid hormone, therefore, plays a leading role in the calcium regulatory system, firstly in initiating kidney 25(OH) cholecalciferol-1-α-hydroxylase and then by acting with calcitriol (in an undetermined process) to mobilise calcium from bone. A rise in serum calcium is detected by the parathyroid gland which will cease to produce parathyroid hormone thereby 'switching off' the major processes by which serum calcium is increased. (Boris, Hurley and Trmal, 1980).

An injection of parathyroid hormone in a vitamin D_3-deficient animal caused a temporary increase in plasma phosphate because of the release of calcium phosphate from bone. Later phosphate levels dropped because of the increased kidney excretion of phosphate brought about by parathyroid hormone (Henze, 1962). Further work showed that an injection of calcitonin could lower serum calcium within thirty minutes. Calcitonin is secreted by the 'C' cells of the thyroid in response to high levels of serum calcium. The function of calcitonin is to cause an immediate block of cell mediated calcium release by suppressing the bone resorptive effects of parathyroid hormone and calcitriol and promoting bone formation. An injection of calcitonin will cause an immediate fall in plasma calcium levels (Reynolds, 1972). This suppression can be demonstrated with mouse calvariae, cultured in vitro, that have been pre-labelled with ^{45}calcium when, one and a half hours following treatment with calcitonin, the calvariae show a fifty per cent reduction in resorption as measured by the release of radiocalcium into the medium.

Reynolds,(1972), considered that calcitonin may act as a brake on the action of calcitriol by inducing the formation of new osteoblasts and reducing the effectiveness of existing osteoclasts. It was thought that bone resorption may only occur when calcitonin secretion is cut off entirely.

The mode of action of calcitonin has been clarified a great deal by workers who studied the ability of calcitonin to suppress bone resorption over a period of time and in the presence of parathyroid hormone.

Calcitonin was found to have an unusual characteristic (Raisz, Wener Trumell, Feinblatt and Wyw, 1972) in that it was able to induce a prolonged inhibitory effect on resorption (confirmed by Reynolds, 1972) following the administration of a single dose of calcitonin even in the presence of parathyroid hormone.(This was termed 'induction'by Raisz et al (1972). . However, with time there appears to be a change in the sensitivity of bone cells to calcitonin which occurs after about four to six days of in vitro culture of bone tissue. Resorption begins again in the presence of parathyroid hormone even when calcitonin is administered at levels that were initially effective. This phenomenon was termed 'escape' by Raisz et al (1972).

Other substances found to inhibit bone resorption are:(i) mithramycin and (ii) dichloromethylene diphosphonic acid (Reynolds, 1972). Mithramycin is thought to interfere with the differentiation of new osteoclasts, thereby preventing parathyroid hormone from stimulating the production of new bone resorptive cells. Dichloromethylene diphosphonic acid is thought to act by inhibiting the dissolution of hydroxyapatite, ie it acts as a crystal poison. Whether calcitonin's mode of action is related to the action of either (i) or (ii) is not known, but (ii) would be more likely because the time course of the action of mithramycin is not comparable with that of calcitonin.

PHOSPHATE TRANSPORT

An early idea on the mechanism of transfer of phosphate across the gut was that it was transported with calcium as an accompanying anion and in equimolar proportions with it (Harrison and Harrison, 1961).

That this was not the case, began to become apparent in the early 1960's when Wasserman (1962) showed that the simultaneous presence of calcium and phosphate in the gut mutually inhibited the absorption of both ions. Furthermore, the absorption profiles did not follow an identical pattern not explainable by the formation of insoluble calcium phosphate. The use of a calcium chelator (Sodium EGTA) in the gut had no effect on phosphate transport (Wasserman and Taylor, 1973). Although some stimulation of phosphate absorption by vitamin D_3 was noted in the early stages, the general picture presented by Wasserman was that phosphate absorption was independent of vitamin D_3. Later research was to disprove this.

Harrison and Harrison (1961) and Kowarski and Schacter (1969), found that vitamin D_3 could stimulate the uptake of phosphate in chick or rat duodenum maintained in organ culture. The response however was not detected before twelve hours, uptake continuing for 24 to 48 hours after administration of the vitamin and resulting in a much higher concentration of phosphate in the tissue than in the culture medium. Phosphate was transported at a linear rate. The transport of phosphate was found to be reduced by the administration of uncouplers of oxidative phosphorylation to the gut sacs.

The active transport of phosphate was presumed to be reliant upon protein synthesis (Peterlik and Wasserman, 1976) as compounds such as cycloheximide and actinomycin D reduce it and eliminate the effect of vitamin D_3 in promoting its absorption by the gut

(Peterlik and Wasserman, 1980). Vitamin D_3 must therefore have the ability to control gene expression in the intestine in order to induce phosphate transport, as it does in the better known mechanisms by which it influences calcium transport.

It has been suggested that vitamin D_3 acts directly on the intestine to promote phosphate transport (Kowarski and Schacter, 1969; Peterlik and Wasserman 1978). It is now unequivocally established that calcitriol also has a significant effect upon phosphate transport by the gut (Peterlik and Wasserman, 1980), including the jejunum (Peterlik and Wasserman, 1976) although inhibition of $1,25(OH)_2$cholecalciferol synthesis by strontium did not affect inorganic phosphate uptake by the jejunum. Thus another vitamin D_3 metabolite appears to be implicated. It was clear also, that a step involving protein synthesis was necessary before phosphate uptake occurred in response to calcitriol. Peterlik and Wasserman (1980), also demonstrated that phosphate influx into chick jejunum was influenced by diet, and a diet low in either calcium or phosphate would result in increased phosphate uptake. The mechanism by which calcitriol influences phosphate transport is unclear.

Vitamin D_3 will stimulate phosphate uptake in rachitic animals (Peterlik and Wasserman, 1976; O'Doherty and DeLuca, 1976) and this action is therefore accepted as being independent of its effect on either calcium transport, calcium binding protein or alkaline phosphatase (Peterlik and Wasserman, 1976).

The potency of calcitriol in stimulating phosphate uptake by the kidney is very high as demonstrated by one report (Liang, Barnes, Balakir, Cheng and Sacktor, 1981) where only a few molecules of calcitriol were effective in stimulating the uptake of phosphate in isolated renal cells.

Vitamin D_3 has been found to be less effective at promoting phosphate transport than almost any of its hydroxy-derivatives in the renal environment. Phosphate transport is not affected by strontium inhibition of the renal 25(OH)cholecalciferol 1αhydroxylase because of the ability of metabolites such as $24,25(OH)_2$ cholecalciferol to stimulate phosphate transport. Calcitriol, however, is by far the most potent,known vitamin D_3 metabolite as far as promotion of phosphate transport is concerned.

The transport of phosphate is not solely dependent on the presence of vitamin D_3 or its derivatives, as shown by some experiments on genetically hypophosphataemic mice. These mice have abnormally high rates of renal excretion of phosphate and low rates of intestinal uptake of phosphate. Their intestinal calcium transport is unaffected (O'Doherty and DeLuca, 1976). When dosed with calcitriol, such mice showed an increase in calcium transport but no increase in intestinal phosphate transport. They appeared therefore to have a defect unrelated to vitamin D_3 metabolism and related solely to phosphate transport in kidney and intestine. It has been repeatedly shown that phosphate uptake by the gut is a saturable process (Wasserman and Taylor, 1973; Peterlik and Wasserman, 1976; Peterlik, 1978), the rate limiting step being the rate of diffusion from

the mucosal to the serosal levels of the gut (Peterlik and Wasserman, 1976,1978).

Entry at the lumenal side is an active process, akin to a phosphate 'pump'. In vitro Vmax varies between 0.15 and 0.37 moles Pi/min/g tissue in vitamin D_3-deficient and vitamin D_3-replete gut segments respectively (Peterlik and Wasserman, 1976). Despite this variation in Vmax, the Km of the reaction is not affected by vitamin D_3 metabolites (Peterlik, 1978). This vitamin D_3-influenced phosphate transport is dependent upon the presence of sodium ions and requires metabolic energy; vitamin D_3 metabolites merely increase the velocity. It has been hypothesised that vitamin D_3 metabolites increase the number of carrier proteins for phosphate. Another, completely separate pathway for phosphate transport has been identified which is thought to be para-cellular, and diffusional transport here occurs at a lower rate than the active sodium-dependent process, and is the only route for phosphate entry in vitamin D_3-deficient animals (Fuchs and Peterlik, 1979; Birge and Avioli ,1981). The microtubular cytoskeleton has been implicated in the transfer of phosphate from the mucosa to the serosa since cytochalasin B and other microtubule inhibiting drugs will reduce the mucosal to serosal transfer in vitamin D_3 replete animals (Fuchs and Peterlik, 1979A).

The two pathways of phosphate entry into the gut were investigated by Birge and Avioli (1981) and they found that the first is energised by a sodium gradient in a high affinity process operating at a pH of less than 6.8. The second process is not sodium-dependent but

acts optimally at a pH of 8.0 or above and is stimulated by calcium. It has become clear that alkaline phosphatase is somehow involved in phosphate transport (Birge and Avioli, 1981). The administration of calcitriol to vitamin D_3 deficient chicks causes an increase in phosphate uptake which is paralleled soon afterwards (3 hours) by a strong increase in alkaline phosphatase in the brush border membrane vesicles. It is thought organic and inorganic phosphate transport changes with alkaline phosphatase activity (Moog and Glazier, 1972). Another intestinal constituent affected by vitamin D_3 metabolites is calcium dependent ATPase, although this enzyme has not yet been distinguished from intestinal alkaline phosphatase. Alkaline phosphatase and ATPase are not thought to participate in calcium transport as L-phenylalanine (a potent alkaline phosphatase inhibitor) does not affect either calcium transport or phosphate transport (Birge and Avioli ,1981). The role of alkaline phosphatase in phosphate transport, if any, remains unknown.

1:3 The Relationships Between the Vitamin D_3 Endocrine System and the Known Calcinogenic Plants.

Solanum Malacoxylon.

Following the discovery of this calcinogenic plant, research began on the aetiology of the disease and the nature and mode of action of the toxic principle(s). It was already known (Tilley, 1967) that diseased animals had high serum calcium and phosphate levels. As little as five grams of dried leaf of the plant was shown to cause an increase in blood serum calcium and serum phosphate in cattle (Worker and Carillo, 1967). When first exposed to dietary Solanum malacoxylon there is a transient rise in serum phosphate (Campos, Ladizesky and Mautalen, 1973). Continued administration of the plant results in above-normal levels of serum calcium in two days. Serum calcium remains above-normal and serum phosphate declines to normal levels where it stabilises.

By 1972 it was known that Solanum malacoxylon affected the intestinal absorption of calcium and phosphate (Mautalen 1972). Mautalen showed that rabbits treated with Solanum malacoxylon and fed a diet low in calcium and phosphate could transport 80% more radiocalcium across the gut wall than a control group. Similarly, oral doses of radio-phosphate were absorbed fifty per cent more effectively by animals treated with Solanum malacoxylon.

The effects of Solanum malacoxylon upon skeletal resorption were also revealed by the indirect method of measuring the urinary levels of

radiocalcium in rabbits dosed five weeks previously with ^{45}calcium. While the plant was administered, urinary levels of radiocalcium rose four to five times above basal. Labelled calcium in the bone was presumed to have been released by the active factor in Solanum malacoxylon. As a result of this work, Mautalen(1977)was able to deduce that the active factor of Solanum malacoxylon had similar physiological actions to 1,25$(OH)_2$cholecalciferol. It was also shown that Solanum malacoxylon could promote resorption of cultured chick frontal bones (Puche and Locatto, 1974) and aqueous extracts of Solanum malacoxylon also promoted resorption of explanted mouse calvariae (Simonite, Morris and Collins, 1976).

Further evidence that a substance very similar to the active form of vitamin D_3 was involved in "Enteque Seco" was supplied by Wasserman (1974). Wasserman used the so-called "Strontium chick" technique. In 1922 Shippey et al found that the inclusion of strontium in the diet produced rickets in pigs. Subsequently Omdahl and DeLuca (1971) showed that this was due to strontium inhibition of the 1-hydroxylase, and thus the production of 1,25$(OH)_2$cholecalciferol and dietary calcium absorption were impaired. Hence the metabolic basis for strontium rickets was established.

This suggested that the strontium affected animal would be useful for determining whether or not certain substances possessed 1,25$(OH)_2$ cholecalciferol-like activity. If they possessed such activity they should be able to bypass the "strontium block" and restore normocalcaemia.

These results were strengthened by other studies which showed,for instance, that kidney mitochondria from strontium-fed chicks had a

low rate of synthesis of calcitriol (Omdahl and DeLuca, 1972) despite normal levels of 25(OH)cholecalciferol being present. In addition, there was strong evidence thatanother substance (designated peak Va by the authors) was produced in place of calcitriol if excess calcium was present in the diet. The results show that the declining levels of calcitriol in vitamin D_3-replete chicks fed dietary strontium are mirrored by increasing levels of peak Va. It was also confirmed by these workers that strontium fed chicks supplemented with calcitriol showed a normal rate of calcium absorption.

It can be seen from the figure of the calcium regulatory system (figure 2) that the introduction of calcitriol in sufficient quantity in the diet would cause the breakdown of calcium homeostasis because of the control point being by-passed. The 1α - hydroxylase is normally strictly controlled by feedback inhibition and so introduction of the end product in the diet prevents any control of the levels of calcitriol by the regulatory mechanisms. Thus uncontrolled intestinal uptake of calcium takes place. This is exactly what occurs in animals that consume Solanum malacoxylon. The clinical manifestations of the disease begin when the increasing levels of plasma calcium reach a point where precipitation of calcium salt occurs,the normal calcium x phosphate product being exceeded as a result of the uptake of calcium induced by the plant factor. This uncontrolled deposition of calcium salts interferes with the functions of the affected tissues and eventually microscopic lesions are apparent.

Further work revealed that Solanum malacoxylon extract could initiate synthesis of calcium binding protein in embryonic chick duodenum

(Corradino and Wasserman, 1974), the tissue not normally synthesising this protein until the day of hatching. This finding also showed that the active factor of Solanum malacoxylon was more comparable with calcitriol than with vitamin D_3, as only calcitriol and not vitamin D_3 can induce the formation of calcium binding protein in embryonic chick duodenum. Calcitriol-like properties could also be seen in the Solanum malacoxylon factor at the bone site, where the extract promoted calcium resorption from chick frontal bones cultured in vitro (Puche and Locatto, 1974). Peterlik and Wasserman (1975) also determined that the active factor was functionally similar to calcitriol and did not require transformation by the kidney.

Further evidence of the ability of the plant factor to mimic the action of calcitriol was provided when it was demonstrated that competition existed between the plant factor and labelled calcitriol, for the binding sites in the intestinal chromatin (Procsal, Henry, Hendricksen and Norman, 1976). These binding sites are very specific for calcitriol. The same workers also showed that renal 25(OH) cholecalciferol - 1α-hydroxylase levels were normalised in vitamin D_3-deficient animals by the plant factor to levels which existed in vitamin D_3-replete or calcitriol-replete animals. The rachitic controls had high levels of the enzyme. Like calcitriol Solanum malacoxylon was also found to be actinomycin D-sensitive which meant that the active factor worked via DNA transcription and subsequent protein synthesis.

The puzzling feature about the calcinogenic principle in Solanum malacoxylon was that, although it appeared to mimic the actions of

1, 25(OH)$_2$ cholecalciferol, it was water soluble and therefore probably fundamentally different from authentic 1, 25(OH)$_2$ cholecalciferol. This incongruity was solved partly in 1975 by Peterlik and Wasserman. These workers knew that the active principle of Solanum malacoxylon had an apparent molecular weight in excess of 1000, since the distribution of the Solanum malacoxylon factor on a liquid chromatography column containing Sephadex G-25, indicated this (Humphreys, 1973). This led to further analysis of the active factor by Peterlik ,Bursac,Haussler and Wasserman (1976). who were able to cleave the glycoside from the sterol with a β-glycosidase. This produced an organic soluble substance which showed chemical and biological properties identical to calcitriol. By using a biologically active extract of the plant, Peterlik and Wasserman (1975) showed by thin layer chromatography and various chemical spot analyses, that the biologically active factor of Solanum malacoxylon contained a 5, 6 trans triene steroid structure as well as sugar moieties. This substance was not, therefore, pure calcitriol and it was not until the Solanum malacoxylon factor was hydrolysed with mixed glycosidases derived from the liver of Charonia lampus (a sea worm) that complete hydrolysis was achieved and the resulting organic soluble moiety was shown to have a mass spectrum and an ultra-violet absorption spectrum identical to that of calcitriol (Haussler and Wasserman, 1976). This substance was previously thought to have a function in the mammalian and avian kidney only, and it was rather surprising to find it in a plant.

The native plant calcinogen is extremely likely to be hydrolysed by the endogenous glycosidases within the animal prior to its being able

to react with the receptors in the intestine and bone. Calcitriol itself is only active when all three hydroxyl groups are present, as well as an unaltered side chain. Since the carbohydrate sequences are most likely to be linked to hydroxyl groups, hydrolysis is almost certainly a prerequisite of biological activity (Wasserman, Henion, Haussler and McCain, 1976). This has been demonstrated by an experiment which showed that the active factor of Solanum malacoxylon was only effective when dosed orally. Other means of administration, such as direct subcutaneous or intravenous methods were not effective in producing elevations in plasma calcium or phosphate (Ruksan and Carillo, 1976). There was, however, some activity of the factor on bone in vitro (Puche and Locatto, 1974; Simonite, Morris and Collins, 1976). Further evidence of the activating effect of the gut environment was provided by an experiment where Solanum malacoxylon extract was incubated in rumen liquor, and then injected into sheep subcutaneously or intravenously. This produced elevations in plasma calcium and phosphate (Ruksan and Carrillo, 1976).

The discovery that calcitriol was present in Solanum malacoxylon stirred considerable interest among medical researchers as it was seen as having potential therapeutic value for patients suffering from various diseases where there is a disturbance of the calcium regulatory system. Pure calcitriol is time consumimg to synthesise and so the possibility of obtaining relatively large amounts from a plant was very attractive. The diseases that would respond successfully to calcitriol are as follows:

RENAL OSTEODYSTROPHY

HYPOPARATHYROIDISM

VITAMIN D_3 DEPENDENT RICKETS

FIBROMA

OSTEOMALACIA

OSTEOPOROSIS

OSTEOPETROSIS

All these conditions are characterised by a low serum calcitriol level. Of these, hypoparathyroidism, hypophosphataemic rickets and osteopetrosis are conditions where an infusion of parathyroid hormone leads to an increase in calcitriol levels in blood (Aarskog and Aksnes, 1980).

CESTRUM DIURNUM

Studies on the calcinogenic activity of Cestrum diurnum (Wasserman, Corradino, Krook, Hughes and Haussler, 1976) using chicks as the experimental animal indicated that the dried leaf powder could reverse the effects of dietary strontium in a dose related fashion in exactly the same way as Solanum malacoxylon and restore synthesis of intestinal calcium binding protein and calcium transport in vitamin D_3-deficient birds. The active factor of Cestrum diurnum could also interact with the high affinity saturable cytosol receptor of calcitriol in vitro. Chemically, the active factor was found to be extractable with organic solvents and showed very low water solubility. It was estimated to possess the equivalent of 30,000 to 35,000 Iu of cholecalciferol per kilogram of dried leaf powder. There was thus strong evidence that the active factor was again identical to calcitriol. Hughes and co-workers (1977) showed that it is a

$1,25(OH)_2$cholecalciferol - glycoside, but containing shorter carbohydrate side chains than that in Solanum malacoxylon (Hughes, McCain, Chang, Haussler, Villareale and Wasserman, 1977). This explains the lower water solubility of the Cestrum diurnum factor when compared with that of Solanum malacoxylon.

It was noted in the work done by Krook,Wasserman, McEntee, Brokken and Teigland (1975) on horses in Florida, that at autopsy the thyroid gland had a very low level of calcitonin (no detectable hypocalcemic activity: 0.05 MRC u/g tissue)which suggested that there was a high level of secretion although, surprisingly, no calcitonin was detected in the blood by these workers. Work done on histologic changes in the bone tissue of animals suffering from Cestrum diurnum poisoning showed that bone tissue was abnormal, and osteopetrosis occurred (ie death of cells and disintegration of the bony matrix). With time, the general atrophy of bone cells caused by Cestrum diurnum, resulted in severe osteopenia between petrotic bands, and thinning of the cortex (Kasali, Krook, Pond and Wasserman, 1977). The presence of cementing lines in the bone tissue gave further evidence of arrested osteocytic osteolysis.

Cestrum diurnum caused detectable calcinosis of soft tissues within four weeks of feeding 3% dried leaf to both normal and hyperparathyroid animals (Kasali, Krook, Pond and Wasserman, 1977). Similar experiments showed that the bone abnormalities were partly the result of hypercalcitoninism resulting in the blocking of bone resorption and in the advanced cases this led to a reduction in hypercalcaemia resulting in normal calcium levels (Krook, Wasserman, McEntee, Brokken and Teigland, 1975). The potency of dried leaf Cestrum diurnum bears a close similarity to

that of Solanum malacoxylon.

Furthermore, it has been reported that feeding as little as 1.5% dried Cestrum diurnum leaf powder to chicks with strontium-induced vitamin D_3 deficiency resulted in the synthesis of calcium binding protein, and increased radiocalcium absorption (Wasserman, Corradino and Krook, 1975). Nephrectomised rats, treated with Cestrum diurnum were also able to synthesise calcium binding protein and transport calcium across the gut whereas nephrectomised, untreated rats were not. (Walling, Kimberg, Wasserman and Feinberg,1976).Mass spectroscopy has confirmed that the hydrolysed Cestrum diurnum factor is calcitriol(Hughes et al, 1977).

SOLANUM TORVUM

This plant was suspected of causing calcinosis in Papua, New Guinea. Feeding trials showed that Solanum torvum could induce hypercalcaemia and hyperphosphataemia and mild tissue calcification in laboratory rats (Morris, Simonite and Pullen, 1979).

Further work has shown that calcium release from bone with the in vitro culture technique (See "General Methods", Chapter 2) using calvariae from five to seven day old mice, showed that 250 µg/ml of Solanum torvum promoted resorption of the bone, as shown by increases in medium calcium and phosphate (Morris and Levack unpublished observations.) The presence of calcitriol in the plant, attached to a carbohydrate moiety is probable but has not yet been confirmed. The data already obtained does suggest strongly that there is a strong similarity to the calcinogenic action of Solanum malacoxylon.

TRISETUM FLAVESCENS.

The discovery that calcinosis induced by Trisetum flavescens is characterised by high levels of serum phosphate rather than serum calcium, as in calcinosis caused by the Solaneceous species, suggests that a different mechanism is present. Wolf and Dirksen (1976) and Libiseller, Kohler, Glawischnig, Sarafidis and Schmid (1978) demonstrated that Trisetum flavescens induced high plasma phosphate in rabbits but had little effect on plasma calcium. Plasma phosphate returned to normal within a few days if Trisetum flavescens was withdrawn. The plant also reduced the high levels of plasma alkaline phosphatase, an effect reminiscent of the Solanaceous calcinogenic plants.

Feeding whole Trisetum flavescens to experimental animals (Wasserman, Krook and Dirksen, 1977) was found to be effective in reversing the rachitic state generally, as characterised by the induction of calcium binding protein and a rise in plasma calcium. However, these workers were not able to produce a reversal of strontium inhibition of calcium absorption or to demonstrate any reversal of the effects of dietary strontium on the bone. Typical effects of strontium-induced vitamin D_3 deficiency on bone structure is as follows: i) failure of resorption of the metaphyseal cartilage core ii) retarded osteocytic osteolysis and iii) the presence of osteoid seams in metaphyseal trabeculae and inner haversian lamellae of osteons. Later workers were able to show that feeding dried leaf Trisetum flavescens led to a reversal of the strontium inhibition of the kidney hydroxylase enzyme (Peterlik, Regal and Kohler, 1977). This was,however,in contradiction

to the subsequent work of Zucker, Kreutzberg, Nitsche and Bittner (1979) who found that organic extracts of Trisetum flavescens were not able to overcome strontium inhibition of the renal 25(OH) cholecalciferol - 1α-hydroxylase.

Work on the organic soluble extracts of the plant was carried out by Zucker, Kreutzberg and Rambeck (1977) who used a 'curative rat test' as a determinant of calcinogenic activity. This technique involved rearing rats for three weeks on a low calcium, vitamin D_3-deficient diet. They were then changed to one with a very high calcium level (4%) and low phosphorus. This causes a fall from about 10mg% to just 3 mg% in serum phosphate. If vitamin D_3 (or a plant extract with vitamin D_3-like activity) is given at the time of the dietary change, then a rise in serum phosphate after three days is found to be related to the amount of vitamin D_3 dosed. Zucker et al were able to use this technique to show that both whole lyophilised Trisetum flavescens and purified organic extracts produced a significant increase in the serum phosphate of rats treated in the above manner.

A number of experiments, by various research groups, showed that Trisetum flavescens contained a high level of vitamin D_3-like activity (Waser, Meyer, Hanichen and Dirksen, 1983; Rambeck, Wetzel and Stark, 1979; Regal, 1983A; Regal, 1983B) although there was a great variation in their estimates. Waser et al (1983) compared calcifications in sheep fed 100,000 iu vitamin D_3 and grass free of Trisetum flavescens per day, with sheep fed 1.5 kg Trisetum flavescens per day. Results revealed that sheep fed the Trisetum flavescens developed moderate to severe calcifications within sixty two

days, compared with the one hundred and twenty days required for minor calcifications (small calcified plaques visible in the left atrium) to become apparent in sheep fed 100,000 Iu of vitamin D_3 per day. They concluded that 1 kg of the plant was equivalent to 150,000 Iu vitamin D_3. Experiments by Rambeck Wetzel and Stark (1979) and Rambeck and Zucker (1978) estimated that Trisetum flavescens only possessed the equivalent of 3000 to 4000 Iu vitamin D_3 per kg dried plant material. This was carried out using the Japanese Quail egg-shell test, in which the thickness of eggshell produced by the bird is proportional to the level of vitamin D_3 administered (Zucker and Gropp, 1968) Zucker et al, (1977) obtained comparable results using the curative rat test which indicated a level of 4000 Iu/kg of vitamin D_3-like activity in lyophilised Trisetum flavescens and 3000 Iu/kg in the purified organic extract.

Rambeck, Oesterhelt, Vecchi and Zucker (1979) used GC/MS to ascertain that the active organic soluble extract contained vitamin D_3 but discovered that only 1ug cholecalciferol per 10g original plant material was present (equivalent to 0.1 ppm). This is not a sufficient level of the vitamin to cause hypervitaminosis D_3. This analysis was in agreement with Rambecks' biological testing for vitamin D_3 activity in the plant (Rat bioassay and Quail egg-shell test). Hence the cause of the severe calcifications found in affected cattle could be presumed not to be the result of an excess of vitamin D_3. Calcitriol was also discounted as a possible calcinogen as the result of an HPLC analysis by Zucker, Kreutzberg and Rambeck, (1977) which showed that no calcitriol was present in the plant.

The latter finding was not in accord with the results of Peterlik, Regal and Kohler (1977) who presumed that calcitriol must be present because lyophilised Trisetum flavescens induced calcium binding protein synthesis in the "strontium chick". They also concluded that Trisetum flavescens was twenty five times less potent than Solanum malacoxylon in terms of calcitriol-like activity, as measured by the ability to promote the formation of calcium binding protein. These observations did not take into account the high plasma phosphate levels and little affected plasma calcium levels which are not seen in animals with an excess of calcitriol in the bloodstream.

The ratio of vitamin D_3 activity of Solanum malacoxylon to Trisetum flavescens has been estimated at 100:1 and so it is reasonable to assume that Trisetum flavescens contains more than one calcinogen in order to account for its potency in causing calcinosis. This ratio of 100:1 was based on results obtained by Wasserman, Corradino, Krook, Hughes and Haussler, (1976), who used the induction of calcium binding protein in the duodena of vitamin D_3-deficient chicks as a referenee point. The result showed Solanum malacoxylon to have the equivalent of 400 Iu/g of vitamin D_3-like activity. Rambeck, Kreutzberg, Bruns-Droste and Zucker (1981) estimated the activity of Trisetum flavescens at only 4 Iu/g using both animal bioassays and HPLC/GC analyses. The potency of Trisetum flavescens is such that sheep develop macroscopic calcifications of the aorta in only fifty three days (Simon, Daniel, Hanichen and Dirksen, 1978).

Further analytical studies on the organic soluble factor (Rambeck, Oesterhelt,Vecchi and Zucker, 1979) failed to identify hydroxylated

derivatives of vitamin D_3 in the plant. The abscence of calcitriol in organic extracts was demonstrated conveniently by co-chromatography with pure calcitriol. A hypothesis that the Trisetum flavescens active factor might be a 'bound' form of Vitamin D_3 was put forward (Rambeck et al 1979). There is no evidence to support this, however. Other sterols have been detected in the plant (Rambeck, Kreutzberg, Bruns-Droste and Zucker, 1981).

A study by Dirksen et al has shown that the daily administration of 15g of aluminium hydroxide prevents the development of calcinosis when the animals are on a diet of Trisetum flavescens (Dirksen, Sachs, Held, Nowotzin, Hanichen, Meyer, Rambeck and Zucker, 1983). Animals fed with Solanum malacoxylon were similarly affected by the administration of aluminium hydroxide (Wase, 1972); negatively charged phosphate ions are attracted to the aluminium hydroxide, bind to it, and thereby lower the calcium x phosphate product.

The synthesis of cholecalciferol in Trisetum flavescens appears to be light dependent (Zucker, Stark and Rambeck, 1980). Yellow plexiglass filters were used to prevent ultra-violet light reaching the plants following germination. Wavelengths of light below 490 nm were filtered out. It was then shown that the vitamin D_3-like activity was not synthesised. The workers do state however, that not all the activity is removed in this way. Brief exposure of a diethyl ether extract of these plants to ultra-violet light was enough to restore vitamin D_3-like activity as measured by the curative rat test. Trisetum flavescens grown in the absence of ultra-violet light was normal in appearance, but clearly a photosynthetic reaction was responsible for the production of a good proportion of the organic

soluble vitamin D_3-like activity. In 1981, Rambeck *et al* showed that 7-dehydrocholesterol behaved similarly to ether extracts of *Trisetum flavescens*, when both were exposed to ultra-violet light for three minutes. These observations still do not explain the potent calcinogenic activity of the plant, since only 0.1 ppm cholecalciferol is found in plants grown under normal conditions. This implies that another calcinogen may be present in the plant in addition to this photo-synthesised cholecalciferol. Rambeck, Kreutzberg and Zucker (1982) found that incubation of the residue of ether extraction of *Trisetum flavescens* with rumen fluid produced an increase in the serum phos-phate of rats in the 'curative rat test'.

The apparent differences between the modes of action of *Solanum malacoxylon* and *Trisetum flavescens* were not found to result in marked differences in the soft tissue calcifications produced. Regal (1983A) showed that, as in *Solanum malacoxylon*, the calcifications produced by *Trisetum flavescens* were localised in connective tissues and elastic fibres within these organs. Notably, calcium depositions were found in the smooth muscle cells of the aortic media layer.

Regal (1983A) found that feeding Quail with either a high dose of vitamin D_3 (4000 Iu/day) or lyophilised *Trisetum flavescens* resulted in high levels of proteoglycans and high activity of the Ca^{2+}-dependent ATP ase. He also found that calcium deposits were formed in these animals within connective tissue cells of the lamina propria and in mitrochondria of glandular epithelium. These are presumably the first sites of calcium deposition in animals with either hypervitaminosis D_3 or *Trisetum flavescens*

- induced calcinosis. Regal (1983B) has also shown that either 4000 Iu vitamin D_3 or 10% lyophilised Trisetum flavescens induced the formation of calcium binding protein in the Quail uterus. Further experimentation (Regal 1983C) showed that calcium deposits induced by Trisetum flavescens or vitamin D_3 were formed at sites where proteoglycan deposits were found. This suggested that the proteoglycan deposits were the foci of formation of calcium deposits. In particular, calcifications were found in interstitial connective tissue of heart and kidney tissue, as well as the elastic fibres and basal membranes. Regal found that a large increase in proteoglycans and soluble calcium occurred simultaneously in animals treated with either Trisetum flavescens or high doses of vitamin D_3.

SUMMARY

Different research groups have found several major discrepancies in their results during the course of the variety of experimental approaches employed:

i) When the whole plant was used in feeding trials the vitamin D_3-like activity was estimated at 150,000 Iu./Kg, whereas organic extracts only possessed 4000 Iu/Kg.

ii) Controversy also existed concerning the ability of the plant to overcome strontium inhibition of the renal 25(OH)cholecalciferol - 1α-hydroxylase. The literature revealed that the whole plant was able to overcome this inhibition whereas the organic soluble extracts were not.

The likelihood that calcinogenic activity resided in aqueous soluble, as well as organic soluble, extracts of Trisetum flavescens became

apparent. The following chapters document the experimental approaches and the results obtained following investigations into the nature and modes of action of calcinogenic factors within Trisetum flavescens.

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CHAPTER 2

GENERAL METHODS

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GENERAL METHODS

2:1 Preparation of Extracts of Trisetum flavescens Using Aqueous and Organic Solvents

Preparation of an Aqueous Extract of Trisetum flavescens and its Partial Purification Using Sephadex Chromatography

A 10% w/v suspension of lyophilised Trisetum flavescens was stirred at room temperature in ten times its own volume of distilled water for about twenty four hours. The solid material was then removed from suspension by filtration through muslin cloth. The application of mechanical pressure at this stage ensured that as much filtrate as possible was separated from the solid matter. The resultant brown liquid was clarified by centrifugation at 16,000g for ten minutes. Finally the extract was reduced in volume to produce a highly viscous brown fluid. This was done either by freeze-drying or by rotary evaporation (temperature not exceeding 35°C if possible). The concentrate was diluted slightly to produce a known concentration of roughly 2g original dry plant material per ml. The crude aqueous extract was used for administration to animals and for further purification. Samples were stored at -20°C.

Gel Filtration

Sephadex (Pharmacia Fine Chemicals Ltd.), is an extremely hydrophilic and complex molecule, formed by cross linking dextran with epichlorohydrin. It is produced as dry beads varying in diameter

and the degree of cross linkage. This allows molecules within well defined weight ranges to be fractionated on each grade of Sephadex, the principle being that smaller molecules are retained within the bead pores, allowing molecules to be eluted in order of decreasing molecular weight. The gel is very stable both physically and chemically.

The grade of Sephadex found to be most suitable for the partial purification of the crude aqueous extract of Trisetum flavescens was G-25 (fine grade, which is recommended for preparative work) which will fractionate molecules (theoretically presumed to be spherical) between the molecular weight range 1000 and 5000. Larger molecules are eluted in the void volume.

A 70cm x 2.5cm glass chromatography column was used. This size allowed a reasonable sample size to be used (about the equivalent of 1.5 g original dried leaf) while at the same time the flow rate of about 2.0 mls per minute allowed a complete sample to pass through the column in about six to eight hours, with reliable resolution of the peaks. The column was fitted with a glass sinter at the base. A Sephadex 'bed' of 60 cm in length was used (bed volume 319 cm^3). The flow rate preferred was 1.5 cm^3 to 2.0 cm^3 per minute. The column was always eluted with fresh glass distilled water.

A typical preparative run was carried out by firstly, swelling the dry Sephadex beads in water at room temperature. The column was then packed with the Sephadex slurry in a slight excess of

water, and allowed to settle overnight. The column was then equilibrated with the solvent by pumping water through the system for several hours. The water was pumped to the head of the column using a peristaltic pump (Gilson Minipuls 4). When the bed has equilibrated, the water level above the bed was reduced with care (using a Pasteur pipette) to within 1 cm of the bed surface. (It is important that the bed surface remain completely undisturbed). The concentrated, crude aqueous extract produced by the method described above, was then applied to the head of the column; for this purpose the water level was reduced to 1 cm above the gel bed and the flow stopped. About 1 ml of concentrated extract was then carefully pipetted on to the surface of the bed (usually equivalent to about 1.5 g of the original plant material). After the extract had settled the pump was started and the water level very slowly increased at the head of the column, until the sample was washed into the gel bed. The top of the column was then sealed and the filtration allowed to proceed overnight. The next morning the flow of solvent was stopped and the elution profile compared with previous runs. Peaks containing biological activity were then identified.

During the early stages of the work, it was necessary to test the peaks individually for biological activity, subsequently only active fractions were retained.

The eluent was collected using an automatic fraction collector (LKB) in 15ml aliquots. The flow rate was adjusted to 1.5 cm^3 to 2.0 cm^3 per minute. The eluate, on leaving the column,

FIGURE 3

Elution Profile (280nm) of a Crude Aqueous Extract of Trisetum flavescens.

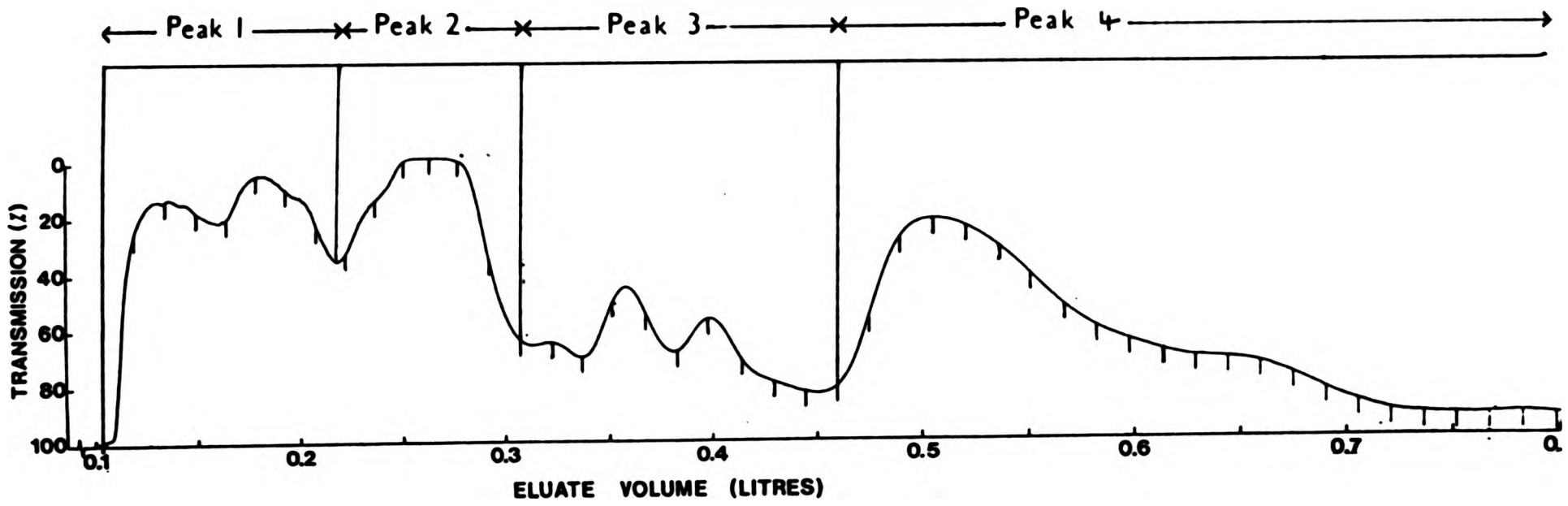


Figure 3 shows the complete absorption profile, monitored at 280nm, of the eluate from the Sephadex column. The figure shows the origin of the materials termed "Peak 3" and "Peak 4" that are used for biological testing in chapter 4. A crude aqueous extract was applied to the column and eluted with pure distilled water. Fractions were collected and were pooled prior to storage at -20°C. Peak 3 and Peak 4 material was stored separately.

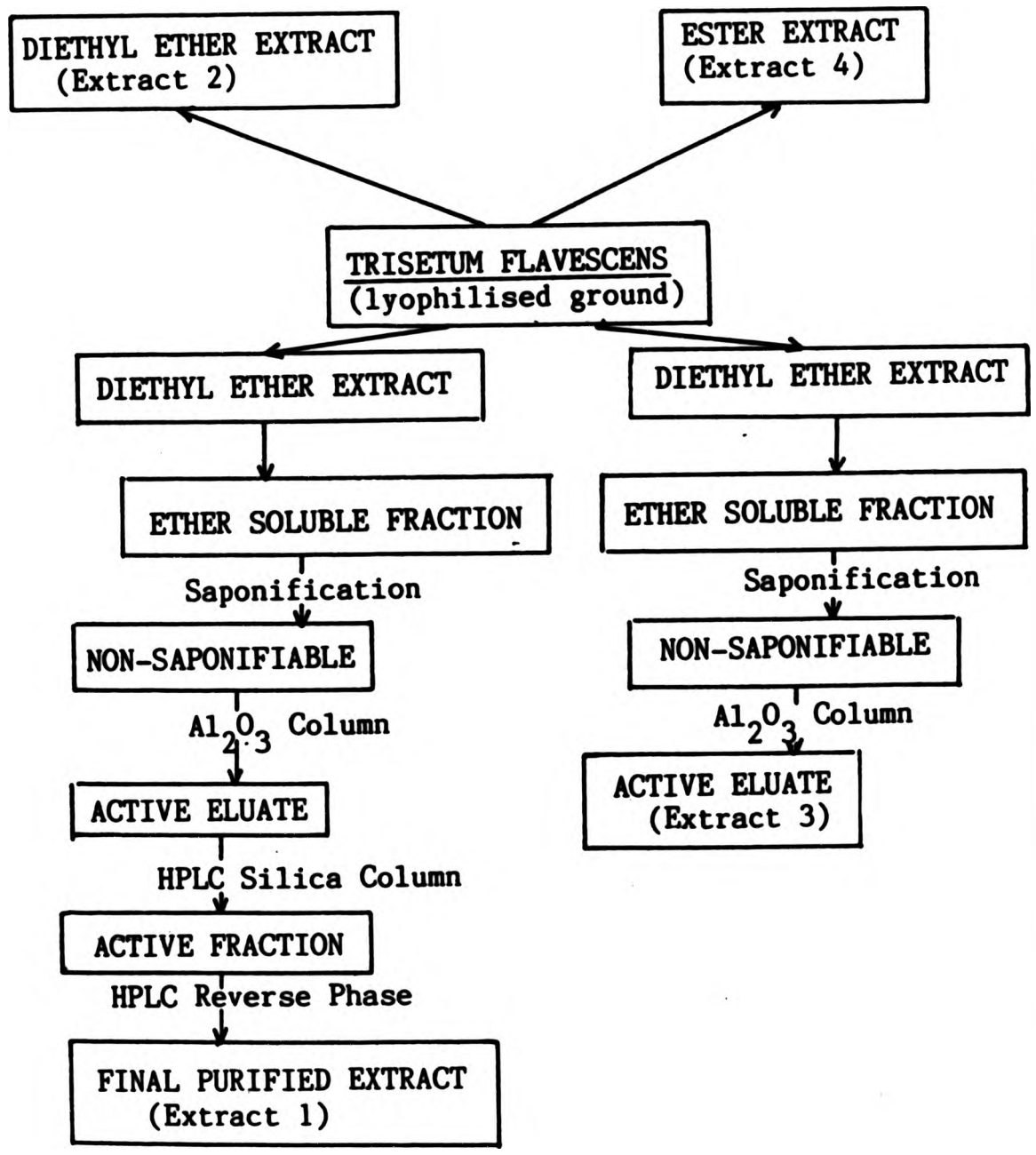
was passed through an ultra-violet detection cell (LKB) set at a constant wavelength of 280 nm. This was then linked up to a chart recorder which drew an elution profile of the run as it proceeded making subsequent identification of the fractions fairly simple. The column had a void volume of 112 cm^3. Once set up the system was fully automatic, and could be left to run overnight as each run took 7 - 8 hours. The fractions found to have biological activity are illustrated in figure 3 which shows the elution profile from a typical run.

Purification of an Organic Solvent Soluble Extract of Trisetum flavescens.

Four extracts were prepared as outlined in figure 4 below.

FIGURE 4

Steps in the Preparation of Organic Solvent Soluble Extracts



Details of the purification procedures were as follows:

Extract 1 was derived from the following purification steps.

100g of freeze-dried Trisetum flavescens was added to 500ml of diethyl ether. After ten hours standing the extract was filtered off and then washed with ether. Evaporation followed using a rotary evaporator. The ether-soluble material was then saponified with

potassium hydroxide in ethanol. The non-saponifiable material was passed down a 20 x 2cm chromatography column packed with Al_2O_3 using increasing amounts of diethyl ether in light petroleum. The fraction containing 9% diethyl ether in light petroleum was found to contain the active fraction . HPLC was carried out on this fraction using a Waters chromatograph. The stationary phase was u-porasil (silica) and the mobile phase was 2.5% isopropanol in hexane. A further separation was done on a reversed phase HPLC system utilising u-bondapak as the stationary phase and acetonitrile as the mobile phase. Monitoring of the effluent from these chromatographic procedures was recorded continuously at 254nm so that individual peaks could be tested for biological activity (Rambeck, Oesterhelt, Vecchi and Zucker, 1979). Extract 1 was collected from the u-bondapak column, following biological testing for the active fraction by Rambeck et al (1979).

Extract 2 was a much cruder preparation than extract 1 and consisted of the ether soluble material prepared directly from the dried grass.

Extract 3 consisted of the active eluate from the Al_2O_3 column step.

Extract 4 consisted of an extraction of the lyophilised plant with ethyl acetate.

The four extracts prepared in the above way were used for the experiments in chapter 3. For <u>in vivo</u> dosing purposes, the extracts, initially in hexane, were dried by passing a stream of nitrogen over them prior to re-dissolving them in propylene glycol. Propylene glycol is used as it is inert in biological systems.

2:2 Techniques for the Analysis of Plasma Minerals and Alkaline Phosphatase

Atomic Absorption Spectroscopy

This technique allows the concentration of metallic elements in solution to be determined with great accuracy. The process consists of spraying the solution into an air/acetylene flame where it is irradiated with radiation from a lamp which generates light at the same wavelength as the spectral emission of the element to be measured. In this way the sample is stimulated to absorb light and so be excited to higher electronic states. The amount of light absorbed is related to the concentration of the element in solution. There is excellent selectivity in terms of absences of spectral interference from other elements because the resonance spectra for elements are of very narrow line width (10 pm).

This technique was routinely used to measure serum levels of calcium, strontium and magnesium for all serum samples and tissue culture media. The sensitivity of the analysis of these elements is 0.003, 0.002 and 0.9 ppm respectively.

Measurement of Plasma Calcium

A stock standard solution of calcium (500μ g/ml) was made up by adding exactly 1.249 g of spectroscopy grade calcium oxide to 50ml of glass distilled water. This was dissolved with a minimum volume of concentrated hydrochloric acid and made up to one litre with

distilled water. A series of standards was set up in the following way using glassware cleaned in 'Decon 90' (a surfactant solution) and well rinsed with glass distilled water.

TABLE 2

Composition of Standard Solutions

Standard (mg%)	Stock Calcium Solution (mls)	Water (mls)	Lanthanum Solution
0	0	2.0	98
2.5	0.1	1.9	98
5.0	0.2	1.8	98
7.5	0.3	1.7	98
10.0	0.4	1.6	98
12.5	0.5	1.5	98
15.0	0.6	1.4	98

Equipment calibration is carried out using a further standard of 4 μ g/ml which gives a typical reading of 0.22 absorbance units. The absorbance levels of the above standards are measured and a graph of concentration versus absorbance drawn up to check for linearity. Both the standards and the samples are diluted fifty times, in order to enable direct comparability and also to be within the detection limits of the Atomic Absorption Spectrophotometer. Lanthanum chloride solution (0.1%) is used in both the standards and the samples to control interferences by other elements in the sample (eg. phosphate and sulphate).

The lanthanum chloride solution is made up by adding 58.64 g of

lanthanum oxide to 50 mls of water, slowly adding 250 ml of concentrated hydrochloric acid and making it up to a litre with distilled water. The 0.1% diluent is prepared by diluting 20 mls of this stock solution to one litre.

For routine serum calcium measurements 0.1 ml of fresh plasma was pipetted (Gilson P200 Autopipette) into duplicate boiling tubes followed by the addition of 4.9 mls 0.1% lanthanum chloride solution. The samples are then mixed thoroughly and the calcium levels measured by aspirating the air/acetylene flame with this solution. The samples were compared with the standard solutions while measurements were being taken, in order to compensate for any fluctuations in the responses of the machine. All measurements were made using the integrated measurement capability of the machine for greater accuracy.

Measurements of Plasma Strontium

This analysis was very similar to that for calcium, the standards being made up from a stock standard solution containing 1000 µg/ml of strontium nitrate. Standards ranging from 0 mg/100 mls to 15 mg/100 mls were set up for calibration of the instrument. Both standards and solutions were made up in a 1% solution of lanthanum chloride in order to control the considerable ionisation of strontium in the air/acetylene flame.

Measurement of Plasma Phosphate

This was most conveniently done using a commercial colorimetric test kit (manufactured by Boehringer Mannheim Gmbh Diagnostica). The colorimetric analysis involved reacting the phosphate with molybdate

and vanadate in nitric acid, to give a coloured complex.

Acid washed glassware was used and the routine analysis was carried out as follows: 0.2 ml of heparinised (Lithium heparin (Sigma)) plasma was pipetted into a 10 ml glass centrifuge tube and 2.0 mls of trichloroacetic acid (1.2M) was added. All samples were analysed in duplicate. After a ten minute interval the tubes were centrifuged at 1156g for 10 minutes and 1.0 ml of the deproteinised supernatant was placed in a clean test tube then 1.0 ml of each of the test solutions was added to each tube. After ten minutes the reaction colour develops fully and the absorbance of the sample can be read against that of the blank in a Cecil spectrophotometer at a constant wavelength of 405 nm. The blank contained 1.0 ml of pure trichloroacetic acid instead of deproteinised supernatant. Results were calculated using the following relation:

$$\text{Concentration of inorganic phosphorus} = 5.5 \times \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}$$

The test kit provided known standard concentrations of inorganic phosphorus.

Measurement of Serum Alkaline Phosphatase

This enzyme was assayed in alkaline solution, using para-nitrophenol phosphate as the substrate. Enzymic cleavage of the phosphate group releases a yellow product (p-nitrophenol) which could be assayed colorimetrically.

Reagents: i) Sodium bicarbonate/Sodium carbonate buffer 30 mM, plus 2 mM magnesium chloride, the final pH of the buffer is adjusted to pH 9.8 by careful addition of sodium hydroxide solution.

ii) Phosphatase substrate 20 mM in water.

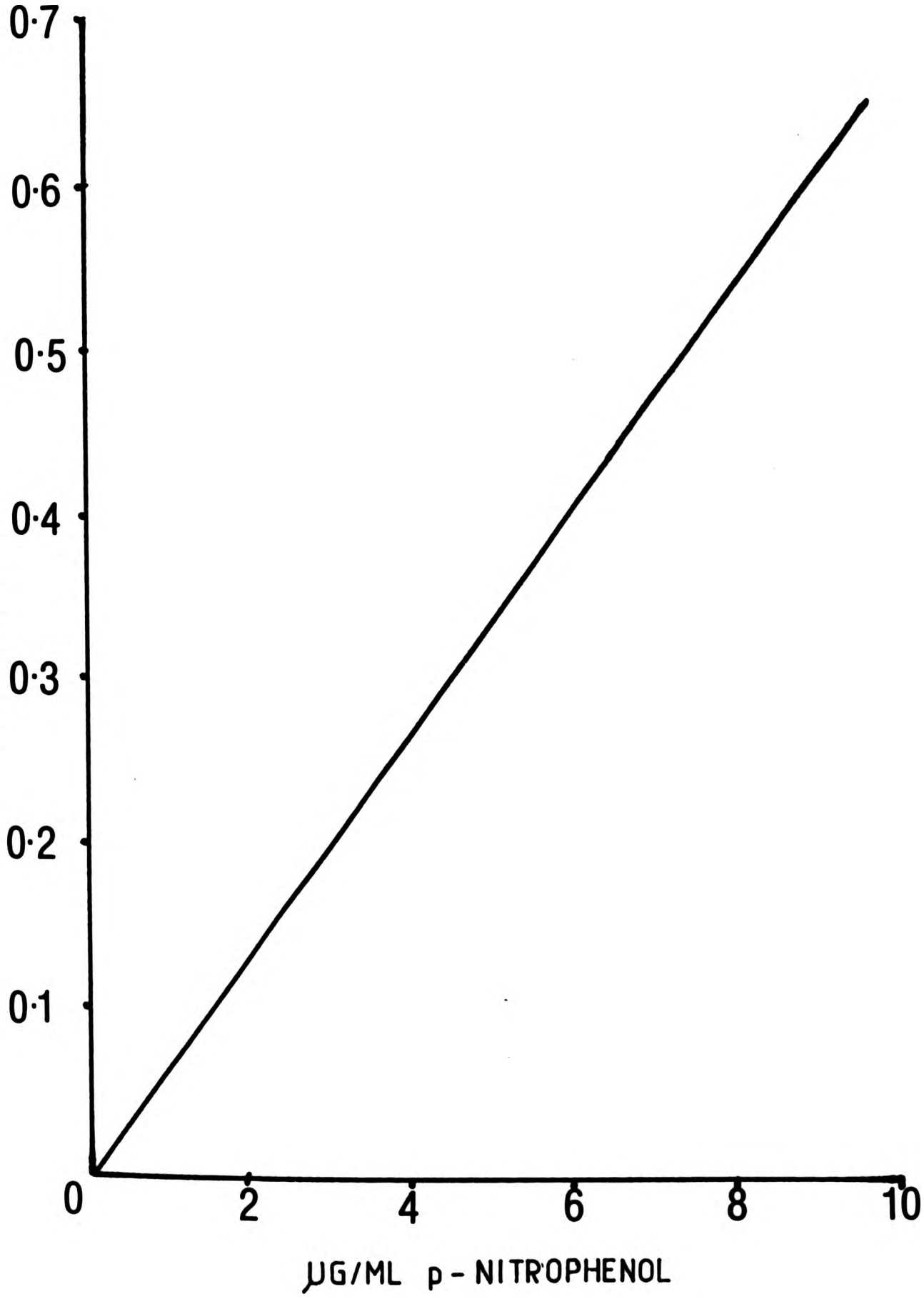
iii) Just prior to the assay, i) and ii) are mixed in equal proportions to give 10 mM substrate.

Assay:- 0.05 ml of plasma are added to test tubes, together with 2.5 ml of the buffered substrate. The tubes are then incubated at 37°C for two minutes. The reaction is then stopped by the addition of 0.5M sodium hydroxide (3 mls) to each tube. The colour intensity is then measured at 405 nm against the blank.

Preparation of the blank: 2.5 ml of substrate is incubated for 2.0 minutes at 37°C followed by the addition of 3.0 mls of sodium hydroxide and finally 0.05 ml of plasma is added. A standard curve was set up using standards containing 0 to 10 μg/ml of p-nitrophenol. The results were then represented as μmoles of PNPP hydrolysed/min/ litre plasma.

FIGURE 5

p - NITROPHENOL STANDARD CURVE



2:3 Liquid Scintillation Counting

This technique was used throughout for the measurement of the concentration of two isotopes, namely ^{45}calcium and ^{32}phosphate. The latter is a high energy beta emitter (Emax 1.71 mev) whereas ^{45} calcium is a low energy beta emitter (Emax 0.254 mev). Because of this quenching of ^{32}phosphate emission is not really a problem and the best counting 'window' for it was already set up on the Beckman LS 7500 counter. The scintillation fluid used for this work was Amersham Triton which is suitable for aqueous samples. The formulation of Amersham Triton was as follows: i) 0.01% (1,4Di{2-5-phenyloxazolyl}-benzene). ii) 0.8% Diphenyloxazole. The solvent is toluene. The above solution is mixed with Triton X 100 in the proportions of 2:1. Amersham Triton can accept up to 1.0 ml of aqueous sample in 10 mls of the scintillant. The following information was gathered with respect to ^{45} calcium, in order to determine the most favourable counting conditions. A number of experiments were performed prior to the use of ^{45}calcium in gut absorption studies, in order to determine the optimum conditions for measuring the radio-calcium levels of solubilised gut material. These were the determination of

i) The counting 'window' of ^{45}calcium.

ii) The 'counting efficiency' of the instrument with regard to ^{45} calcium.

iii) The quenching effects of the tissue solubiliser.

Quenching can be caused by any substance in the vial that interferes with the interactions between the fluors and the solvent. Pure formic

acid was found to be suitable for this purpose. The optimum amount of tissue solubiliser was determined because this must be as small an amount as possible as the acid contributes to 'quench effects' and yet enough must be used for complete solubilisation of the tissue iv) The optimum amount of solubilised tissue to be used in each vial. (This must be enough to give a statistically reliable count and yet not enough to cause significant quenching). Separate determinations of the quenching effects of the solubilised gut tissue and the lumen contents were made.

Results

i) The counting window of ^{45}calcium was determined as described as in the Beckman manual and was discovered to be identical to the counting window of ^{14}carbon.

ii) The counting efficiency of the instrument with regard to ^{45}calcium was determined with greatest accuracy by counting a good number of scintillant-containing vials that have had identical amounts of the isotope added. Thus pipetting errors are minimised and a statistically acceptable result is obtained. In this case 0.0146 μ Ci of ^{45}calcium was added to each of 17 vials which were then hand shaken to mix the isotope with the 'Amersham Triton'. The counts obtained were averaged out and the counting efficiency was obtained by using the following formula:

$$\text{Counting efficiency} = \frac{\text{counts per minute}}{\text{disintegrations per minute}}$$

This resulted in a counting efficiency of 84.3% for the instrument used in the early part of this study and an efficiency of 74.24% for the Beckman (model LS 7500) machine used subsequently.

iii) The quenching effects of the tissue solubiliser and hence the optimum amount of acid to use was determined by adding a known amount of ^{45} calcium to each of 12 vials (0.0148 μ Ci). These vials were then counted before and after the addition of different amounts of formic acid. The results showed that more than 0.3 mls of formic acid per vial caused a detectable reduction in counts per minute. As a result of this it was decided to use 0.2 mls of the solubilised gut tissue per counting vial and 0.5 mls of the diluted solubilised lumen contents (This was equivalent to 0.33 mls of acid).

EXPERIMENTAL RESULTS

TABLE 3 Relationship Between Level of Formic Acid and Quench.

Formic Acid (mls/vial)	% Reduction in counts
0	0
0.1	0
0.2	0
0.3	0.26
0.4	3.63
0.5	19.97

(iv) The optimum amount of solubilised tissue to be used.

For the determination of the optimum amount of gut tissue to be used, a 6.0 cm section of duodenum and another of ileum was taken from a four-week old chick. Each segment was chopped and then

dissolved in 10 mls of 100% formic acid in a boiling tube. Solubilisation was aided by agitation and after 24 hours the tissue had almost completely dissolved. Vials containing 10.0 mls of Amersham Triton were prepared and a radioactive 'spike' of 0.0148 μCi of ^{45}calcium was added to each one. The following amounts of solubilised tissue were added to each of the vials prior to counting:

TABLE 4

Table Showing Optimum Level of Solubilised Tissue For Counting

Solubilised Tissue Sample	Sample Volume (mls)	CPM Tissue	% Reduction In Counts
Control	0	24663	0
Duodenum	0.2	25466	0
Ileum	0.2	24247	0
Duodenum	0.5	26726	0.4
Ileum	0.5	22420	0.4
Duodenum	1.0	21443	17
Ileum	1.0	19492	17

A volume of 0.2 mls per vial was chosen as the most suitable for adding to scintillant, as quenching was minimal. There was no noticeable difference in the level of quench between duodenal or ileal tissue. A similar method was used to determine the optimum amount of solubilised lumen contents to be added to the scintillant. 'Spikes' of 0.0148 μ Ci ^{45}calcium were added to the vials as before.

TABLE 5

The Optimum Level of Lumen Contents per Vial.

Sample Volume (mls)	CPM	% Reduction in counts
0.2	23150	6.2
0.5	24292	1.5
1.0	22020	10.7

A volume of 0.5 mls lumen contents was chosen as the most suitable for addition to the vials.

Technique For Determining Transport of Isotopes Across The Gut Wall

This method was used for measuring the transport of either radiophosphate or radiocalcium across the gut wall of the chick. The overall method was similar for either isotope differing only in the amount of each isotope used, the composition of the salt solutions injected with the isotope and the region of gut used to measure isotope transport. For experiments where ^{45}calcium was used, the 1 ml of isotope solution used contained 10 µCi of radiocalcium plus 1 mg of non-radioactive calcium acetate (Wasserman, 1962). For experiments utilising radiophosphate, the composition of the radioactive solution was as follows: 2mM dipotassium hydrogen phosphate, 150mM sodium chloride and 5µCi of ^{32}phosphorus (as orthophosphate). The pH of the solution was adjusted to pH 7.2 by the careful addition of sodium hydroxide solution. Both radioisotopes were obtained from Amersham.

It was found to be most satisfactory for the transport of calcium to be measured across the duodenum. The duodenum is a major site of calcium transport. However, the transport of ^{32}phosphate cannot be measured across the duodenum because the rate of phosphate absorption is very fast here and would not allow any changes in phosphate absorption to be monitored by this technique (Wasserman, 1962). Therefore the ileum was used (Canas et al. 1977; Wasserman and Taylor, 1973). The portion of ileum used in these experiments was about 8 cm long and began 2 cm proximal to the prominent pair of caecae. Similarly for duodenum, segments eight to ten centimetres long were ligated for testing purposes. Experimental details of the In vivo techniques for measuring the transport of these isotopes are outlined in Chapter 3.

For increased accuracy, quench effects were nullified by the addition of a known amount of isotope, in a 5µl 'spike' to each vial. Vials were then recounted and the degree of reduction in counts of the standard amount of isotope added was then applied directly to the counts per minute of each test sample. This is a popular technique for measuring quench effects and is called the 'internal standard' technique. When corrected for counting efficiency the total disintegrations per minute for each vial could be arrived at.

Example: (using ^{45}calcium)

The following sample vials were counted:-

i) 0.2 mls solubilised gut tissues = 14050 cpm

ii) 0.01095µ Ci ^{45}calcium = 18047 cpm

iii) 0.2 mls gut tissue plus 0.01095µCi ^{45}Ca = 26192 cpm

This means that the standard has been quenched by 32.73% and only yields 12142 cpm as opposed to the initial level of 18047 cpm. The radioactivity in the sample can be presumed to have been quenched in a similar fashion. The true count rate for the sample is thus 20886 cpm or 32.73% above its original value.

In this way values for the total cpm present in each sample of lumen contents or gut tissue could be calculated and when added together, the amount of radioactivity transported out of the gut to the body could be calculated using the following expression:

10 μ Ci - (dpm in luminal fluid + dpm in gut tissue)

= μ Ci transported to body.

μCi transported to body were conveniently represented as a percentage of the initial radioactivity.

2:4 Bone Culture Technique

This tissue culture technique was first introduced by Reynolds and Dingle (1970) who used paired half-calvariae as an integral system, by using one half calvarium as a control to the other which was used as a test bone. It has proved to be a very useful and trouble free method for testing the effects of various agents on bone tissue. The method allows histological examination of the tissue as well as analyses of the bathing medium.

About eight pairs of albino mice were mated in order for litters to be born at convenient times for experimentation. Bones were taken from a minimum number of litters, to aid uniformity of response.

Following decapitation of the mouse, the calvarium was dissected out by removing the skin and cutting around the periphery of the bone.

Extraneous material attached to the bone was carefully removed with fine forceps. Bones were placed on stainless steel grids within an individual multidish 'cell'. The grid size is such that when 2.0 ml of medium was added to the 'cell' it just bathed the underside of the bone.

All necessary equipment for setting up the cultures in sterile conditions was wrapped in aluminium foil where necessary and autoclaved. This included 0.22μ m millipore filters, fine dissecting instruments, glass medicine bottles, steel platforms (1.5 cm x 1.5 cm), gas jars, glass petri dishes and glass pipettes. The plastic multidishes (six 'cells' per tray) were packaged aseptically by the supplier (Linbro).

The culture medium used was medium BGJ_b (Fitton Jackson modification) from Gibco-Biocult. This medium, the composition of which is shown in Appendix 2, was developed for bone culture work, Medium BGJ_b was prepared for use by adding Rabbit Serum (Burroughs Wellcome) to 10% of the final volume, as well as antimicrobial and antibacterial agents: Benzyl Penicillin and Streptomycin Sulphate . The preparation of medium additives and all subsequent operations (except incubation) was carried out in a laminar flow cabinet. The complete medium was then passed through a sterile millipore filter, the entire preparation of the medium being done immediately prior to use. Because the pH of medium BGJ_b changes on exposure to air it was not until all the bones had been dissected out and prepared for pre-incubation that BGJ_b medium was added to the culture dishes. During dissections, prepared bones were placed in a special, pH stable, 'holding' medium. This was medium '199-HEPES' which was maintained at a low temperature

during use by surrounding the dish with ice. (Appendix 2 shows the composition of medium '199-HEPES'). The final composition of medium BGJ_b was as follows:-

TISSUE CULTURE MEDIA

1. 100 mls BGJ_b (Fitton-Jackson modification).
2. 1.0 mls Streptomycin sulphate (10 mg/ml).
3. 1.0 mls Benzyl penicillin (10^4 units/ml).
4. 1.0 mls Fungizone (0.5 mg/ml).
5. 0.7 ml L-Glutamine (0.002 mg/ml).
6. 11.0 mls Rabbit serum.

The final composition of medium '199-HEPES' was as follows:-

1. 82.5 ml sterile water.
2. 10.0 ml Medium 199 (10 x concentrate).
3. 2.0 ml HEPES buffer.
4. 0.342 L-glutamine (0.001 mg/ml).
5. 0.5 ml Sterile 4.4% $NaHCO_3$.
6. 1.0 mls (Streptomycin sulphate (10 mg/ml).
   1.0 mls (benzyl penicillin (10^4 units/ml).
7. 1.0 mls fungizone (0.5 mg/ml).
8. 11.0 mls Rabbit serum.

As with BGJ_b, this medium is passed through a millipore filter. The concentrations of components in the above media represent the final medium concentration.

Following dissection the bones were trimmed to uniform size and rinsed in two changes of ice-cold medium BGJ_b. Each bone was then placed on a grid in a multidish 'cell', containing 2.0 ml of medium, before being quickly placed in a gas jar inside a 37°C incubator. The jars were gassed with 20% oxygen and 5% carbon dioxide to adjust the medium pH to 7.4.

Experimental Incubation

After about 18 hours pre-incubation the gas jar was placed in the laminar flow cabinet, the petri dishes removed and each bone carefully placed on a fresh grid in one of the six 'cells' of a 'Linbro' tissue culture unit. Two millilitres of fresh medium BGJ_b was quickly added, either containing test substances or control medium. The bones were returned to the incubator and were gassed as before. After a prescribed period (usually 48 or 72 hours) the bones were removed from the medium and the medium analysed.

Pre-Labelling Of The Calvariae With ^{45} Calcium

Some experiments involved the _In vivo_ labelling of the skeleton with radiocalcium. Its subsequent release _In vitro_ under the influence of test substances was then monitored.

Pre-labelling was carried out on the mice at one day of age to allow time for the label to be incorporated into the bone tissue prior to experimentation five to seven days later. Each mouse in the litter was injected with 5 μ Ci of ^{45} calcium in 50 μ l of distilled water sub-cutaneously. This dosage level gave counts per minute of about 200,000 per calvarium and over 10,000 counts per 2.0mls

of culture medium, thus giving statistically reliable counts.

When the mice were killed at five to seven days of age, the calvariae were treated as previously described. This method allows any resorption of the bone to be detected accurately throughout the culture period by taking small samples (20µl) of the medium from each culture for ^{45}calcium measurements. These samples were taken from each culture dish using sterile pipette tips, before being added to 0.5 ml 100% formic acid for solubilisation. At the end of the experiment the calvariae themselves were dissolved in 100% formic acid (4 mls per bone) and a 0.1 sample was added to scintillant prior to counting in the Beckman LS7500 machine. The bones took 12 to 24 hours, with frequent agitation, to solubilise completely. Radiocalcium release was expressed as a percentage of that initially available in the bone for release = $\frac{\text{total release} \times 100}{\text{total release + residual}}$

Analyses Of Tissue Culture Medium

It was important to determine that all bones used for experimentation were still viable at the end of the experiment, because if not, then the results would be valueless. The viability of each bone was tested by analysing the amount of glucose utilised during the culture period, thereby ensuring that active respiration had occurred.

Glucose Analysis.

This technique involved reacting glucose oxidase with its substrate and measuring the absorbance of a coloured product in a fixed wavelength spectrophotometer.

Method

Glucose oxidase reagent was made up by adding 5 mg 'o' dianisidine (carcinogenic) to 1 ml acetone. When dissolved this solution was added to 100 ml 0.05 M Tris buffer (pH 7.0) together with 2.0 mg peroxidase (BDH) and 1 ml glucose oxidase (Sigma). Aliquots of tissue culture medium (0.1 ml) from each bone culture were added to 0.9 ml distilled water. This solution was further diluted by adding 20 µl of this to 0.5 ml distilled water. 1 ml of the glucose oxidase reagent was then added to the latter tubes. At this stage the tubes were incubated in a water bath maintained at 37°C for thirty minutes. One millilitre of 7M HCl was then added to all tubes to stop the reaction. Colour developed after ten minutes and the absorbance could be measured in a Cecil fixed wavelength spectrophotometer at 530 nm. Standards were obtained by adding 20 µ l of a standard glucose solution to a reaction tube, instead of diluted tissue culture medium (Glucose absorbance is linear up to a concentration of 1.5 mg/ml, allowing a direct comparison with the standard). Standard glucose solution contained 1 mg/ml in water, giving an absorbance of 0.03. A blank was made by adding 20 µ l of water to a reaction tube.

Sterility Testing

All cultures were tested for contamination by microbes at the end of the culture period. This was done by plating loopfuls of tissue culture medium onto nutrient agar plates, prior to a three day incubation at 37°C. No contamination problems were encountered.

Calcium And Phosphate Analyses

Analyses of non-radioactive calcium and phosphate levels were carried out as previously described.

CHAPTER 3

THE BIOLOGICAL ACTIVITY OF ORGANIC SOLUBLE EXTRACTS OF *TRISETUM FLAVESCENS*

CHAPTER 3

The Biological Activity of Organic Soluble Extracts of Trisetum flavescens

INTRODUCTION

Following the demonstration by Dirksen and colleagues (1973) of the calcinogenic nature of Trisetum flavescens and its ability, when fed to rachitic chicks, to reverse the rachitic state, (Wasserman et al 1977) chemical analyses were performed on organic extracts to determine whether or not the plant contains vitamin D_3. Rambeck and associates (1979A) showed that cholecalciferol (vitamin D_3) was present in purified organic extracts of the plant, but they were unable to demonstrate the presence of hydroxylated metabolites. The biological activity of these extracts was estimated by their ability to raise plasma phosphate levels.

It was thought necessary to confirm these observations using more discriminating biological tests for vitamin D_3-like activity. As an initial step in studying the pathophysiology of Trisetum flavescens induced calcinosis, the ability of diethyl-ether extracts of the leaves, at various stages of purification, to raise plasma phosphate and to promote radio-phosphate transport was determined.

Experiment 1

Demonstration of the Ability of Organic Solvent Soluble Extracts of *Trisetum flavescens* to Promote Radiophosphate Uptake from the Ileum.

Experimental

Plant Extracts

Four organic extracts, prepared in the manner described in the general methods section, were used in this study (see p.63,64). The samples, supplied by Dr. W. Rambeck (University of Munich) in hexane were reduced to dryness under a stream of nitrogen and then taken up in propylene glycol at a known concentration.

The extracts used were as follows:

Extract 1: Final purified, following HPLC.

Extract 2: Crude.

Extract 3: Partially purified following aluminium oxide chromatography.

Extract 4: Ester fraction (not saponified).

Administration of Extracts

Twenty five cock chicks (Rhode Island Red x Light Sussex) were reared to four weeks of age on a vitamin D_3 deficient diet (see appendix 1, page b for diet composition) and five more chicks were reared on the same diet supplemented with vitamin D_3 (2000 Iu/kg). At this time five chicks were assigned to each experimental group in the following manner.

TABLE 6

Dosing Regimen.

GROUP	CHICKS PER GROUP	VITAMIN D_3 STATUS	DOSE
1	5	$-D_3$	EXTRACT 1
2	5	$-D_3$	EXTRACT 2
3	5	$-D_3$	EXTRACT 3
4	5	$-D_3$	EXTRACT 4
5	5	$-D_3$	PROPYLENE GLYCOL
6	5	$+D_3$	PROPYLENE GLYCOL

Each extract contained the equivalent of 1.5g of original plant material dissolved in 1.0ml propylene glycol. Chicks in groups 1 - 4 received 0.5ml of extract sub-cutaneously 48h and 24h prior to estimations of radio-phosphate transport, plasma calcium, phosphate and alkaline phosphatase. Chicks in groups 5 and 6 received 0.5ml vehicle at these times.

Technique for measuring the transport of Radioactive Phosphate or Calcium across the Gut Wall of the Chick.

This procedure was essentially the same for both ^{45}calcium and ^{32}phosphate - the differences are outlined in the General Methods section.

Procedure

Each bird was anaesthetised with 4mg/100g body weight sodium pentabarbitone, which was injected intramuscularly. When unconscious,

feathers were removed from the ventral side of the abdomen. An incision two to three centimetres long was then made diagonally, and slightly to the left of the mid-line. The intestine beneath was then gently pulled through the incision with thick forceps. The desired piece of gut having been located, tied with suture at one end, (care being taken not to damage any of the mesenteric blood vessels) 1ml of ^{32}phosphate solution was then injected into the gut sac while tightening the second suture around the syringe needle during the introduction of the solution. The suture was pulled tight as the needle was withdrawn, so as to prevent any leakage. The exteriorised gut was then carefully replaced within the abdominal cavity, and the wound closed with artery clips. The animal was then kept warm and unconscious, for a further fifteen minutes to allow absorption of the isotope.

At the end of this time, a 4.0ml blood sample was removed from the wing vein and added to a cooled glass centrifuge tube containing 100Iu of solid lithium heparin (sigma). The animal was killed with an overdose of anaesthetic and the gut sac removed from the abdominal cavity and held above a 50ml measuring cylinder. The sac was then slit lengthways with fine scissors and the contents thoroughly washed into the cylinder with a total volume of 40mls of the appropriate washing solution (identical to the radioactive solution injected into the gut segment, but minus the isotope). The rinsed gut tissue was then finely chopped and dropped into a boiling tube containing 15mls of 100% Formic acid. 40mls of the suspended lumen contents (prepared as described above) were then allowed to solubilise for 24 hours (aided by mechanical agitation). The blood sample was then spun at 1156g in an 'MSE Minor' centrifuge for ten minutes and the plasma used for the measurement of isotope, total phosphate, calcium and alkaline phosphatase.

Residual luminal isotope and residual gut wall isotope were determined. Aliquots of solubilised gut tissue and lumen contents were counted in the Beckman LS 7500 liquid scintillation counter as described in the general methods section.

Results

The results of the plasma and radio-phosphate transport measurements are shown in Tables 7 and 8.

TABLE 7

Effects on Blood Parameters in Chicks Treated with Various Organic Extracts of Trisetum flavescens.

Group	Treatment	Vitamin D_3 Status	Plasma Calcium (mg/100mls) mean ± S.E.	Plasma Phosphate (mg/100mls) mean ± S.E.	[Ca x P_i] mean ± S.E.	Plasma Alkaline Phosphatase (Iu) mean ± S.E.
1	Extract 1 (Final Purified)	Deficient	9.12 ± 0.40	5.28 ± 0.67	48.2 ± 8.4	1078 ± 176
2	Extract 2 (Crude Extract)	Deficient	8.14 ± 0.78	6.94 ± 1.26	56.5 ± 15.1	989 ± 133
3	Extract 3 (Partially Purified)	Deficient	8.62 ± 0.51	6.15 ± 0.15	53.0 ± 2.6	997 ± 123
4	Extract 4 (Ester Fraction)	Deficient	8.79 ± 0.48	5.53 ± 0.47	48.6 ± 6.9	1167 ± 172
5	Control	Deficient	8.56 ± 0.94	4.73 ± 0.72	40.5 ± 1.23	1549 ± 221
6	Control	Replete	10.32 ± 0.27	7.60 ± 0.56	78.4 ± 7.1	258 ± 63.6

The chicks were dosed with plant extract or propylene glycol and analyses made of plasma calcium, phosphate and alkaline phosphatase after forty eight hours.

Significance of Differences (t-test)

All pairs of groups were tested and revealed the following notable results.

(i) Vitamin D_3-deficient v vitamin D_3-replete groups

For calcium: ($p < 0.087$ i.e $p > 0.05$)

For phosphate: ($p < 0.015$)

For alkaline phosphatase: ($p < 0.001$)

For $[Ca \times P_i]$: ($p < 0.01$)

(ii) Vitamin D_3-deficient v groups 1-4

For calcium: None of the values differed significantly

For phosphate: Vitamin D_3-deficient v group 3 ($p < 0.067$) otherwise no significant differences.

For alkaline phosphate: No significant differences.

For $[Ca \times P_i]$: vitamin D_3-deficient v group 3 ($p < 0.01$)

(iii) Vitamin D_3-replete v group 2: plasma phosphate did not differ significantly.

(iv) Vitamin D_3-replete v groups 1-4

For $[Ca \times P_i]$, groups 1,3 and 4 ($p < 0.04$) group 2 v Vitamin D_3-replete, not significant.

The results shown in Table 7 indicate that the mean values for each group for plasma calcium are greater than the mean value for the vitamin D_3-deficient controls, with the exception of group 2. However, none of these values is significantly different from the D_3-deficient controls.

The vitamin D_3-replete group has the highest mean plasma calcium level, but this difference is not significantly greater than the vitamin D_3-deficient controls.

Similarly, mean alkaline phosphatase levels for all groups treated with a plant extract, though lower than that for the vitamin D_3-deficient group, were not significantly so. A similar situation, again, is found for plasma phosphate levels; while the mean value for each group is higher than for the vitamin D_3-deficient group, none is so significantly so ($p < 0.05$). The mean value of plasma phosphate for the vitamin D_3-replete group is the highest, but again the difference is not significantly greater than vitamin D_3-deficiant levels. Plasma phosphate levels of the vitamin D_3-replete controls and group 2 are not significantly different. However, all four groups treated with plant extract, show greater mean plasma [Ca x P_i] than vitamin D_3-deficient controls although they are significantly lower ($p < 0.04$) than that for the vitamin D_3-replete group (with the exception of group 2 ($p < 0.05$)).

TABLE 8 To Show the Results for the Measurements of the Transport of ^{32}Phosphate Across the Gut Wall as the Result of Administration of Organic Solvent-Soluble Extracts of T.flavescens.

Group	Treatment (Oral Doses)	% ^{32}P in Gut Tissue Mean ± S.E.	% ^{32}P in Lumen Mean ± S.E.	% ^{32}P Transferred To Body Mean ± S.E.	% ^{32}P /cm Gut Mean ± S.E.	% ^{32}P Transferred /cm Mean ± S.E.	Mean % Total ^{32}P transported from lumen.
1	Extract 1 (Purified)	15.0 ± 4.0	69.6 ± 8.9	16.0 ± 6.0	1.51 ± 0.25	1.34 ± 0.52	31.4
2	Extract 2 (Crude)	11.75 ± 2.2	61.9 ± 5.4	26.3 ± 4.5	1.28 ± 0.3	3.03 ± 0.78	38.1
3	Extract 3 (Partially Purified)	14.7 ± 1.0	70.2 ± 6.7	15.6 ± 5.6	1.62 ± 0.3	1.93 ± 1.0	24.7
4	Extract 4 (Ester)	8.56 ± 2.0	70.7 ± 6.2	20.7 ± 5.3	1.0 ± 0.32	2.59 ± 1.14	29.3
5	Control $-D_3$	10.8 ± 2.0	83.5 ± 3.0	7.84 ± 2.12	1.2 ± 0.2	0.6 ± 0.51	18.64
6	Control $+D_3$	9.6 ± 1.6	72.0 ± 5.1	18.4 ± 4.9	1.14 ± 0.3	2.21 ± 0.86	28.2

Vitamin D$_3$ Deficient

The groups of chicks were dosed with plant extract or propylene glycol at zero time and measurements of their ability to transport ^{32}phosphorus across the gut wall after forty eight hours were made. This involved the measurement of the proportion of a known amount of ^{32}phosphorus leaving the gut lumen within a fifteen minute absorption period. Levels residing in the gut tissue were also assessed.

STATISTICAL ANALYSIS

a) for % ^{32}P in lumen (column 4)

Vitamin D_3 deficient v group 2 ($p < 0.01$)

b) for % ^{32}P transferred to body (column 5)

Vitamin D_3 deficient v group 2 ($p < 0.01$)

Vitamin D_3 deficient v group 4 ($p < 0.05$)

c) % ^{32}P transferred to body/cm gut tissue.

Vitamin D_3 deficient v group 2 ($p < 0.03$)

Otherwise differences between groups are not significant.

Column 8 represents the sum of values in columns 3 and 5.

Levels of ^{32}phosphate in the gut tissue were similar for all groups. However, the mean value for % radio-phosphate remaining in the lumen is highest in the vitamin D_3-deficient group. The value, for this latter group, of 83.5% is 11.5% higher than for the vitamin D_3-replete group and all groups treated with plant extracts have between 12.8% and 21.6% less residual ^{32}phosphate in the lumen than the vitamin D_3-deficient group.

The vitamin D_3-deficient group shows the lowest level of ^{32}phosphate transference to body and is significantly lower than that for group 2 ($p < 0.01$) and group 4 ($p < 0.05$). When the ^{32}phosphate results were adjusted to take the gut lengths into account there was no significant difference between groups with respect to ^{32}phosphate remaining in the gut tissue and the levels of ^{32}phosphate transferred to body per cm gut tissue was again lowest in the vitamin D_3-deficient group. The latter group showed significantly lower ($p < 0.03$) ^{32}phosphate transference to body per cm gut tissue than group 2 animals. The ability of the extracts to stimulate ^{32}phosphate absorption is seen by reference to column 8 (table 8).

FIGURE 6

A Comparison of the Effects of Organic Solvent Soluble Extracts of Trisetum flavescens with Vitamin D_3 in the Chick.

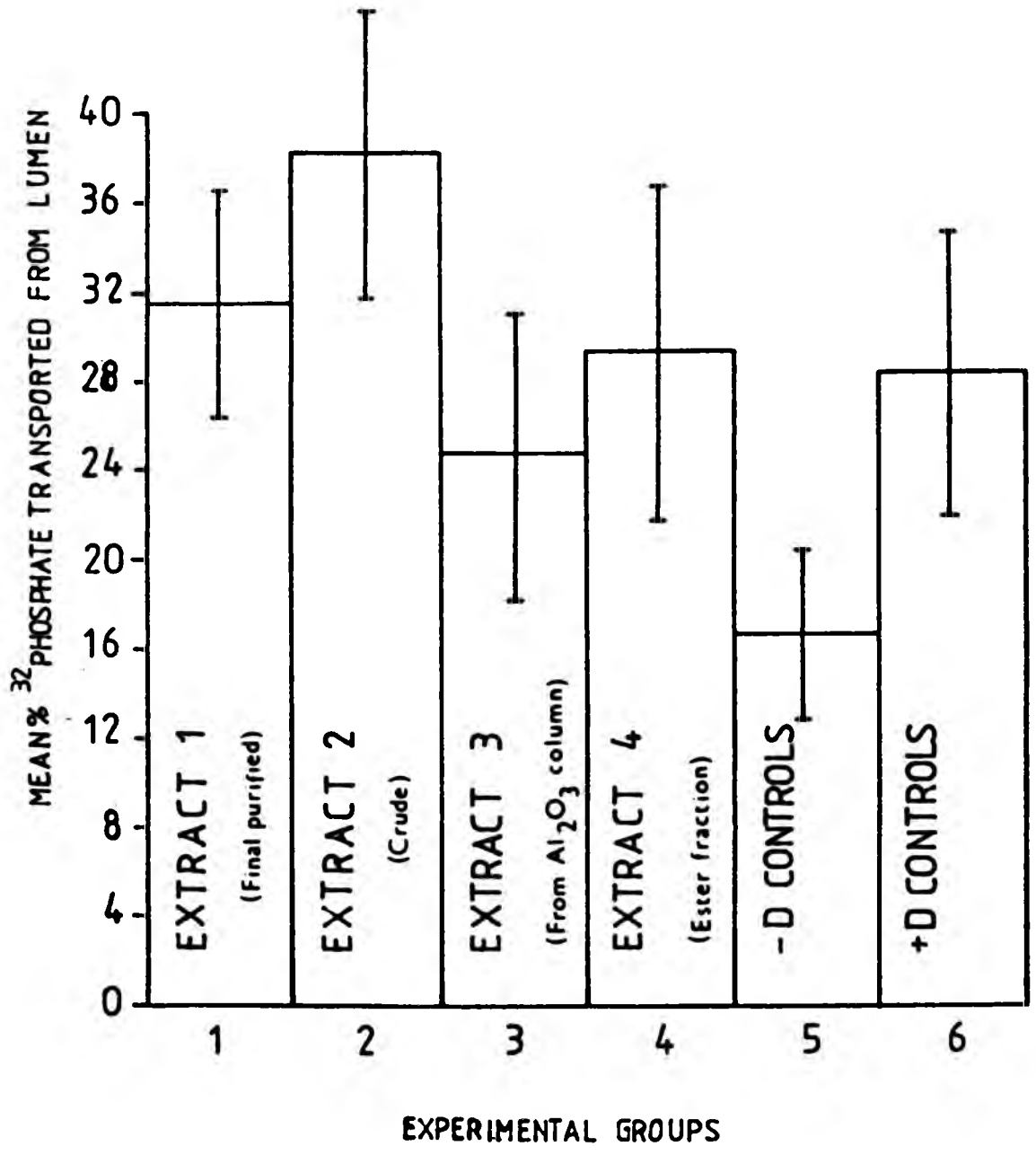


Figure 6 shows the mean % ^{32}phosphate removed from the lumen of vitamin D_3-deficient chicks that were dosed with organic solvent-soluble extracts of Trisetum flavescens. Control groups were either vitamin D_3-deficient or fed a complete diet.

DISCUSSION

These results indicate that the rise in plasma phosphate noted by previous workers (Zucker, Kreutzberg and Rambeck , 1977) was the result of an increase in the intestinal absorption of phosphate. Renal retention cannot be ruled out however.

The extracts appear to promote little change in plasma calcium levels of plant extract-treated chicks.Three treated groups exceed control levels although the differences are not statistically significant. Confirmation of the upward trend in plasma phosphate and the stability of plasma calcium is seen in the values of the calcium x phosphate product for plant extract treated groups, which indicates a reversal of the vitamin D_3-deficient state. The plant extracts also appear able to reduce the abnormally high alkaline phosphatase levels induced by the vitamin D_3-deficient state, which also indicates a partial reversal of vitamin D_3 deficiency. This effect was found by Canas, Ortiz, Asteggiano, Pereira (1977) for Solanum malacoxylon treated chicks. All four extracts are active in promoting intestinal phosphate transport. Indeed, in three of the four groups receiving the plant extract ^{32}phosphate transport is greater than in the vitamin D_3-replete group.

Rambeck, Kreutzberg, Bruns-Droste and Zucker (1981) estimated, in biological assays, that organic extracts of Trisetum flavescens contained only 4 Iu/g of cholecalciferol. The chicks in this experiment were given only 1.5g of original dried leaf which represents, according to Rambeck's figures, only 6 Iu of cholecalciferol per chick. It seems unlikely that 6 Iu of cholecalciferol could produce the extent of reversal of the symptoms of vitamin D_3-deficiency apparent in forty-eight hours. It is therefore likely

that either: a) another biologically active factor, in addition to the cholecalciferol is present or b) Rambeck's estimation of the vitamin D_3 activity is too low.

The uptake of radiophosphate into the gut does not appear to be affected by vitamin D_3 deficiency or the plant extract suggesting that the rate limiting step (and the one influenced by the plant extracts or vitamin D_3) is the passage of phosphate out of the gut and into the bloodstream. It appears that phosphate levels within gut tissue cells are maintained at a constant level regardless of the rate of passage of phosphate through them.

Experiment 2.

An Investigation into the Effects of a Purified Organic Extract of *Trisetum flavescens* on Radio-Calcium Transport Across the Duodenum.

Introduction.

The previous experiment indicated that organic-soluble extracts of *Trisetum flavescens* can stimulate intestinal phosphate transport, an effect reminiscent of vitamin D_3. An increase in the [Ca x P_i] was apparent essentially because of increased plasma phosphate but both plasma calcium and phosphate levels showed some increase.

Because of the work of Zucker, Kreutzberg and Rambeck (1977) which suggested that neither vitamin D_3 nor any of its major hydroxylated derivatives were present in sufficient quantity to be responsible for the biological effects of the plant, it was decided to examine the effects of the purified extract on intestinal calcium transport. The latter is known to be dependent upon the action of hydroxylated vitamin D_3.

Experimental.

The experiment was carried out using the most highly purified organic solvent soluble active fraction of *Trisetum flavescens*. This was extract 1 prepared as detailed on page 63, chapter 2. The extract was dissolved in propylene glycol prior to use.

Ten day-old cock chicks (Rhode Island Red x Light Sussex) were reared to four weeks of age on a vitamin D_3-deficient diet. They were then divided into two groups of five, one group was then dosed with the plant extract and the other was untreated. The dosing regimen is shown in the following table.

TABLE 9 DOSING REGIMEN.

GROUP	VITAMIN D_3 STATUS	NUMBER OF CHICKS	TREATMENT	ADMINISTRATION
1	-D	5	EXTRACT 1 2.7g dried leaf per chick.	Orally, by dosing tube.
2	-D	5		-

Each chick in group 1 received the equivalent of 2.7g of the original dried leaf in two equal doses, forty eight and twenty four hours before testing. Analyses of radio-calcium transport as well as plasma calcium , phosphate and alkaline phosphatase levels were performed.

RESULTS:

TABLE 10 Plasma Calcium and Phosphate Levels in Vitamin D_3-Deficient Chicks Treated with an Active Organic Solvent Soluble Extract of Trisetum flavescens and untreated controls.

GROUP	TREATMENT	PLASMA CALCIUM (mg/100mls) mean ± S.E.	PLASMA PHOSPHATE (mg/100mls) mean ± S.E.	[Ca x Pi] mean ± S.E.
1	Extract 1 (≡ 2.7g plant)	8.50 ± 0.38	6.79 ± 0.63	57.7 ± 3.4
2	Controls (-D)	8.00 ± 0.52	2.36 ± 0.25	18.96 ± 4.7

Two groups of vitamin D_3-deficient chicks were treated with either the purified, biologically active fraction (extract 1) derived from the procedure described on page 63 or were untreated. After a period of forty eight hours, blood samples were taken from each chick for analysis of plasma calcium and phosphate.

TABLE 11

Duodenal ^{45}Calcium Transport in Vitamin D_3-Deficient Chicks Treated with Either Vitamin D_3 or an Organic Solvent Soluble Extract of Trisetum flavescens.

Group	Treatment	Plasma ^{45}Ca per ml. cpm ± S.E.	% ^{45}Ca In Lumen mean ± S.E.	% ^{45}Ca in Gut Tissue mean ± S.E.	% ^{45}Ca Transferred to Body mean ± S.E.
1	Extract 1	519 ± 40.5	60.7 ± 3.67	16.9 ± 1.28	22.3 ± 2.5
2	Controls	392 ± 32.3	53.49 ± 4.20	23.06 ± 2.4	15.17 ± 2.1

Forty eight hours after group 1 was treated with the plant extract, measurements of the chicks abilities to transport ^{45}calcium across the gut wall were made. The control group was untreated. Radio-calcium in the plasma was also monitored.

Statistical analysis.

Students t-tests.

Extract 1 v controls

For plasma calcium: No significant difference.

For plasma phosphate: The groups differ significantly ($p<0.05$).

For [Ca x P_i]: The groups differ significantly ($p<0.05$)

For plasma ^{45}calcium :No significant difference.

For % ^{45}calcium in lumen: No significant difference.

For % ^{45}calcium in gut tissue: No significant difference.

For % ^{45}calcium transferred to body: No significant difference.

Extract 1 produced no significant increase in plasma calcium levels, although the mean plasma calcium level is slightly higher than for the controls. Plasma phosphate, however did show a significant increase above control levels. Measurements of the intestinal calcium transporting ability of both groups revealed that , although the plant extract treated groups have a higher mean level of calcium transport, this is not significantly different to the controls. Similarly, ^{45}calcium levels within the gut tissue and lumen are not significantly different for groups 1 and 2. There is a significantly higher [Ca x P_i] in chicks treated with the plant extract.

DISCUSSION.

The purified extract of Trisetum flavescens possesses phosphataemic activity which was also demonstrated in experiment 1 (chapter 3), and it was able to restore the [Ca x P_i] to normal levels. Plasma calcium levels did not decrease and so this organic solvent soluble extract does not contain the same active factor as the aqeous soluble phosphataemic principle discussed in chapter 4. The latter was found

to cause a dramatic decline in plasma calcium when administered to vitamin D_3-deficient chicks. It may be that the 0.1ppm of vitamin D_3 known to be present in extract 1 is responsible for the apparent stability of the plasma calcium levels seen in this experiment. In conclusion, the organic solvent soluble extract used has no significant effect on ^{45}calcium uptake by the gut . It is able to produce an increase in plasma phosphate and restores the [Ca x P_i] to normal. It is unlikely that the extract contains calcitriol or a more than a very low level of vitamin D_3.

Experiment 3

The Effects of a Purified, Organic Solvent Soluble Extract of *Trisetum flavescens* on Bone Cultured *in vitro*

Introduction

An *in vitro* method for testing the action on bone of various agents was developed by Reynolds and Dingle (1970). This method has since been utilised by workers studying the effects of vitamin D_3 metabolites on bone tissue. (Raisz, Wener, Trummel, Feinblatt and Au 1972). The method has also been found useful by workers studying the calcinogenic plants such as *Solanum malacoxylon* (Simonite Morris and Collins, 1976; Puche and Locatto, 1974).

Since experiments 1 and 2 have revealed that organic extracts of *Trisetum flavescens* are able to increase the [Ca x P_i] of vitamin D_3 deficient chicks, without significantly increasing calcium transport in the gut, it was decided that bone might possibly be the site of action . At this time it was decided to examine the effects of the organic extracts on the release of ^{45}calcium from pre-labelled mouse calvariae cultured *in vitro*.

Experimental

The time course of release of ^{45}calcium from the calvariae of six-day old mice, prelabelled at one-day old, was ascertained over a period of sixty eight hours.

Following the pre-incubation period, test substances were added to groups of culture 'cells' as shown in table 12.

TABLE 12

TREATMENT REGIMEN FOR CULTURED BONES

Culture Number	Additive	Doseage Level	Source of Additive
1 - 6	Control	None	-
7 - 11	$1,25(OH)_2D_3$	2.5ng in Ethanol	Roche Products
12 - 14	$1,25(OH)_2D_3$ Plus PTH	2.5ng in Ethanol/ 3iu PTH	PTH from M.R.C.
15 - 20	Extract 1 (D_3 Ester)	≡ 0.06g Dried Plant	W. Rambeck
21 - 27	Extract 2 (D_3 fraction)	≡ 0.048g Dried Plant	W. Rambeck
28 (No Bone)	Medium only	-	-
29 (No Bone)	Medium plus Extract 1	≡ 0.06g Dried Plant	-
30 (No Bone)	Medium plus Extract 2	≡ 0.048g Dried Plant	

Following the addition of these substances to the cultures, they were returned to the 37°C incubator and maintained in an atmosphere of 5% carbon dioxide in air, and were removed at intervals so that 20µl samples of medium could be removed from the dishes for counting. This was done after gently swirling the tissue culture fluid in order to thoroughly mix the contents. Samples were removed at one, three five, twenty, forty and sixty eight hours after the addition of test substances and assayed for calcium, phosphate, glucose and radio-

calcium. The bones were dissolved in formic acid in order to determine residual radio-calcium.

RESULTS:

TABLE 13 ^{45}Calcium Released from Pre-labelled Calvariae for each Treatment (% Initial Available for Release; Mean ± Standard Error

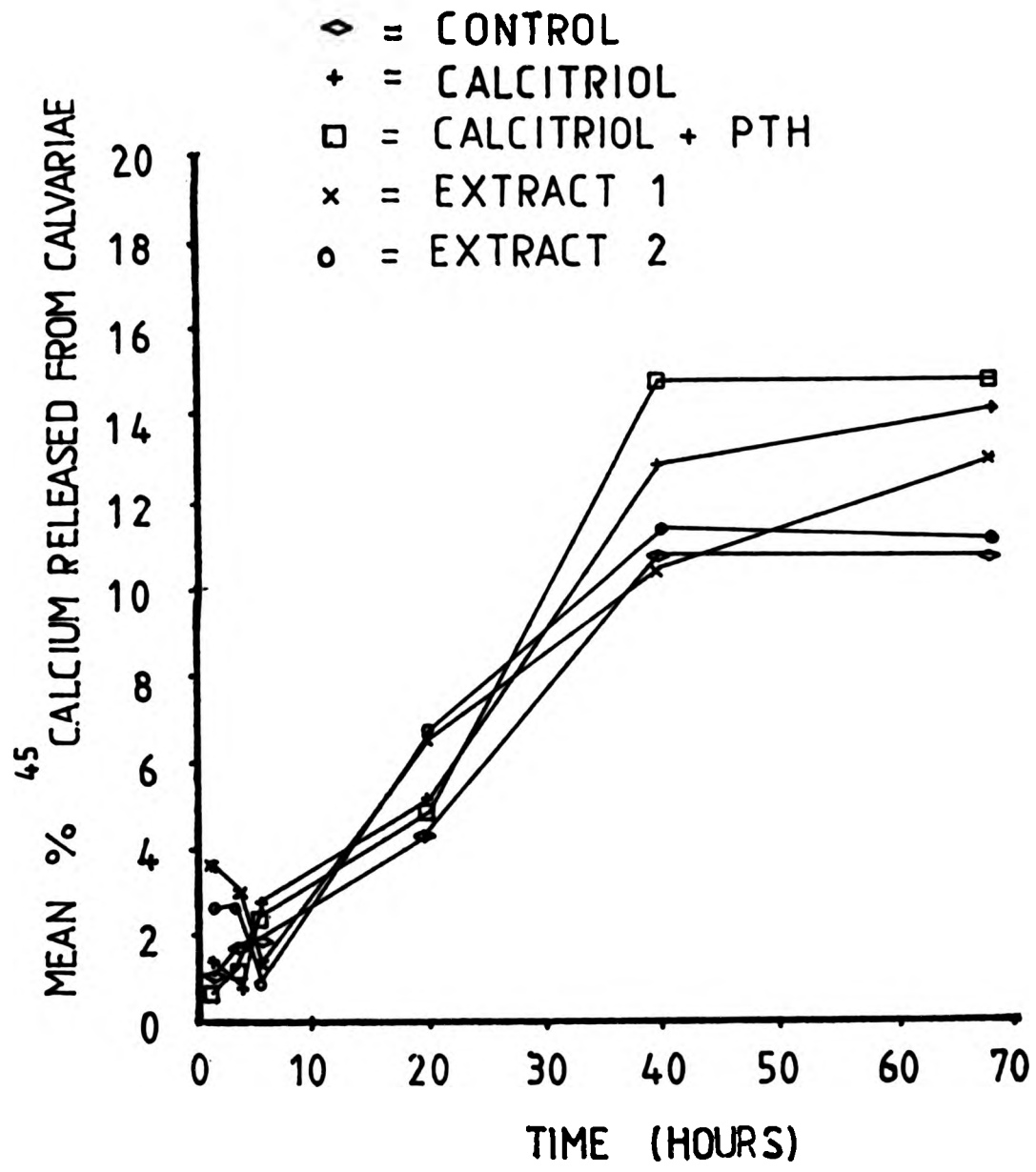
TREATMENT	TIME ELAPSED (HOURS)					
	1	3	5	20	40	68
CONTROL (6)	1.04 ± 0.3	1.96 ± 0.31	1.91 ± 0.76	4.17 ± 1.1	10.75 ± 2.58	11.04 ± 1.64
$1,25(OH)_2D_3$ (5)	1.07 ± 0.22	0.78 ± 0.18	2.36 ± 0.14	5.31 ± 0.22	13.12 ± 0.75	14.07 ± 0.75
$1,25(OH)_2D_3$ + PTH (3)	0.72 ± 0.09	1.37 ± 0.25	2.20 ± 0.34	4.96 ± 1.32	14.75 ± 2.5	15.11 ± 3.1
EXTRACT 1 (6)	3.75 ± 0.71	3.16 ± 0.99	1.41 ± 0.07	6.33 ± 1.04	10.25 ± 1.71	13.44 ± 1.9
EXTRACT 2 (6)	2.56 ± 1.12	2.61 ± 1.23	0.93 ± 0.14	6.40 ± 1.69	11.29 ± 1.29	11.22 ± 0.88

(Numbers in brackets refer to the number of cultured bones per group).

Using an *in vitro* culture technique it was possible to compare the effects of an organic solvent soluble extract of *Trisetum flavescens* with two endogenous metabolites which are known to resorb bone both *in vivo* and *in vitro*.

FIGURE 7

The Effect of Organic Solvent-Soluble Extracts of Trisetum flavescens on ^{45}Calcium Release from Bone in vitro.



Organic solvent-soluble extracts of Trisetum flavescens, calcitriol and parathyroid hormone were tested for their ability to promote the release of ^{45}calcium from prelabelled bone tissue in vitro. Figure 7 shows the increase in isotope levels in the culture medium, with time, for each group. The bone resorbing capabilities of bones treated with either calcitriol, calcitriol plus parathyroid hormone or either of two extracts of Trisetum flavescens are compared.

TABLE 14 Medium Total ^{40}Calcium, Inorganic Phosphate and Glucose Levels of Bones Cultured *in vitro*.

TREATMENT	MEDIUM CALCIUM (mg/100mls)	MEDIUM PHOSPHATE (mg/100mls)	GLUCOSE UTILISED AT 68 HOURS (group mean ± S.E.) in μ moles
CONTROLS	8.91 ± 0.3	3.92 ± 0.17	48.0 ± 3.82
$1,25(OH)_2D_3$	9.43 ± 0.39	3.86 ± 0.29	42.7 ± 2.72
$1,25(OH)_2D_3$ + PTH	9.22 ± 0.52	4.69 ± 0.15	39.8 ± 0.94
EXTRACT 1	8.16 ± 0.6	3.75 ± 0.29	42.5 ± 6.45
EXTRACT 2	8.45 ± 0.24	4.24 ± 0.15	43.6 ± 4.30
MEDIUM ONLY	7.38 ± 0.02	3.22 ± 0.20	-
MEDIUM + EXTRACT 1	7.15	3.21	-
MEDIUM + EXTRACT 2	7.35	3.50	-

Groups of bones were cultured *in vitro* and were treated individually with either a plant extract or an active bone-resorbing metabolite.

STATISTICAL ANALYSIS (Students t-test)

a) for calcium: no significant differences between groups.

b) for phosphate: controls v $1,25(OH)_2$cholecalciferol plus PTH-dosed group ($p < 0.03$) no other groups differed significantly.

c) for % available ^{45}calcium released during the experimental period.

(i) at one hour post-treatment:-

control v extract 1 ($p < 0.005$)

extract 1 v calcitriol ($p < 0.01$)

extract 1 v 1,25 + PTH ($p < 0.023$)

(ii) at three hours post-treatment:-

control v calcitriol ($p < 0.013$)

(iii) at five hours post-treatment:-

extract 1 v extract 2 ($p < 0.04$)

extract 1 v calcitriol ($p < 0.005$)

extract 2 v calcitriol ($p < 0.002$)

extract 2 v 1,25 + PTH ($p < 0.027$)

(iv) at twenty hours - no significant differences between the groups.

(v) at forty hours and sixty hours no two groups differed significantly.

The results for total medium calcium levels show that $1,25(OH)_2$ cholecalciferol and $1,25(OH)_2$ cholecalciferol with parathyroid hormone result in elevated levels of total calcium at sixty-eight hours when compared with controls. The differences are not statistically significant, however. Both extracts 1 and 2 have mean total media calcium levels rather below control levels but not significantly so. Medium phosphate is slightly elevated above control

level in bones receiving extract 2 but again, not significantly so. Only the bones treated with 1,25$(OH)_2$cholecalciferol and parathyroid hormone show a statistically significant elevation of phosphate in the culture medium. All bones used in the experiment were found to be viable as indicated by their utilisation of medium glucose. Results for the measurements of the release of ^{45}calcium to the medium revealed that extract 1 treated bones had released isotope to the culture medium at a level significantly higher than control bones at one hour post addition of test substances ($p<0.005$).

No other group was significantly different to the control group at one hour. At three hours post-treatment only the calcitriol treated group was significantly higher than the control group with respect to isotope levels in the culture medium ($p<0.013$). At five hours no groups differed significantly from the controls although groups treated with calcitriol and/or parathyroid hormone had higher mean levels of medium isotope than all other groups. In contrast plant extract treated groups had the lowest levels of medium isotope at five hours. After twenty hours exposure to the test materials both plant extract treated groups showed higher mean levels of isotope in the medium than any other group although these differences were not statistically significant. After forty and sixty eight hours no significant difference between any pair of groups was found, although calcitriol and calcitriol/parathyroid hormone treated groups had slighty higher mean levels than other groups.

DISCUSSION

The relatively small numbers of bones used in this experiment tend to preclude clear statistical differences but although small differences in the time course of release of ^{45}calcium in groups

treated with the plant extract were apparent, these were not likely to be the result of the action of a resorbing agent. At twenty hours, post treatment, the plant extract treated bones show a small (statistically insignificant) elevation in isotope levels in the medium, although this is not sustained and by forty hours the extract treated bones are no different from control levels while calcitriol and calcitriol/parathyroid hormone treated bones - show an increase over control levels of release at this time. This suggests that the extracts are not causing active resorption of bone. Similarly, at one hour post-treatment both groups treated with plant extract show the highest levels of calcium resorption. The plant extracts are not producing responses similar to either calcitriol or calcitriol in concert with parathyroid hormone and are therefore unlikely to contain calcitriol or a calcitriol analogue. This is further supported by analytical work carried out by German workers on these extracts. Rambeck et al (1979A) could not detect hydroxylated vitamin D_3 derivatives in these plant extracts. No stimulation of medium phosphate levels or calcium levels overall were found in plant extract treated groups indicating that the increases in plasma phosphate noted in the first experiment are not the result of bone resorption of phosphate salts in conjunction with the observed stimulation of intestinal phosphate absorption. In conclusion little effect of the plant extracts on calcium resorption from bone is found there being no significant variations from control levels beyond one hour of treatment. The small amount of vitamin D_3 known to be present in these extracts will not be able to induce bone resorption (Reynolds 1972A).

CHAPTER 4

THE BIOLOGICAL ACTIVITY OF AQUEOUS SOLUBLE EXTRACTS OF *TRISETUM FLAVESCENS*

CHAPTER 4

Examination of the Calcinogenic Activity of Aqueous Soluble Extracts of Trisetum Flavescens Using a Variety of Biological Assay Techniques

INTRODUCTION

The investigations into the mode of action of the organic soluble phosphataemic principle as described in chapter 3, revealed that the factor was unlike vitamin D_3 or calcitriol in its mode of action. German researchers had only looked at organic soluble extracts of Trisetum flavescens and the possibility that a vitamin D_3-type compound might be present in aqueous soluble form (as found within Solanum malacoxylon) did exist. It was necessary therefore to determine whether or not any aqueous soluble calcinogenic activity existed in the plant.

Experiment 1

A Comparison of the Effects of a Crude Aqueous Extract and a Partially Purified Aqueous Extract of Trisetum Flavescens on Vitamin D_3-deficient Chicks.

Introduction

A partially purified extract of *Trisetum flavescens*, the final peak obtained by passing a crude aqueous extract through a Sephadex column ("Peak 4"), was tested by dosing vitamin D_3-deficient chicks and comparing its effects with those of the crude extract. Isolation of fractions that constituted the partially purified extract used in this biological testing, was determined by the ultra-violet absorption spectra of these fractions. "Peak 4" fractions possessed absorption maxima of 269nm which is similar to that of 1,25$(OH)_2$cholecalciferol which has an absorption maximum of 265nm. The blood parameters of total calcium, inorganic phosphate and plasma alkaline phosphatase were assayed at the end of the experiment.

Experimental Procedure

Fifteen day old cock chicks (Rhode Island x Light Sussex) were reared on a vitamin D_3-deficient diet to four weeks of age when signs of vitamin D_3-deficiency were apparent. Five other chicks were reared simultaneously on a vitamin D_3-replete diet to form a control group. The vitamin D_3-deficient birds were then divided randomly into three groups of five. The experimental protocol is shown in Table 15.

TABLE 15 DOSING REGIMEN

Group	No. of Chicks	Vitamin D_3 Status	T. flavescens aqueous extracts	Sampling Time (hours post dosing)
1	5	$+D_3$	-	48
2	5	$-D_3$	-	48
3	5	$-D_3$	P.P ≡ 0.54g	48
4	5	$-D_3$	C ≡ 0.48g	48

Key: P.P. = Partially purified extract of Trisetum flavescens ("peak 4") following Sephadex chromatography.

C = Crude aqueous extract of T. flavescens.

Either 24 or 48 hours following oral dosing, blood samples (about 2mls) were taken from the wing vein of each bird and placed in clean pyrex centrifuge tubes, to which had been added 100Iu of lithium heparin. The samples were then spun on a bench centrifuge at 1156g for ten minutes. The plasma was then used for calcium and phosphate analyses, as described in the general methods section. Plasma levels of alkaline phosphatase were also measured.

RESULTS

TABLE 16 The Effects of Two Aqueous Extracts of Trisetum flavescens on Plasma Levels of Calcium, Inorganic Phosphate and Alkaline Phosphatase in Vitamin D_3-Deficient Chicks.

GROUP	TREATMENT	VITAMIN D_3 STATUS	PLASMA PHOSPHATE mg/100mls mean ± S.E	PLASMA CALCIUM mg/100mls mean ± S.E	[Ca x P_i] mean ± S.E	PLASMA ALKALINE PHOSPHATASE (I.U.) mean ± S.E***
1	-	$+D_3$	4.60 ± 0.34	10.08 ± 0.24	46.4 ± 4.9	498 ± 37.6
2	-	$-D_3$	2.07 ± 0.25	8.00 ± 0.52	17.6 ± 2.5	1154 ± 162
3	P.P* orally	$-D_3$	3.87 ± 0.38	6.94 ± 0.46	27.14± 3.7	1728 ± 206
4	C** orally	$-D_3$	5.74 ± 0.82	8.13 ± 0.85	44.8 ± 5.2	1671 ± 276

*P.P = partially purified aqueous extract of Trisetum flavescens. **C = crude aqueous extract of T. flavescens. ***= units are μ moles of paranitrophenol phosphate hydrolysed /min/litre plasma

STATISTICS - Students t-test

The following groups differed significantly.

(i) Vitamin D_3-replete v vitamin D_3-deficient

For calcium: ($p < 0.012$)

for phosphate: ($p < 0.001$)

for alkaline phosphatase: ($p < 0.004$)

for [Ca x P_i] : ($p < 0.002$)

(ii) Vitamin D_3-deficient v partially purified extract

for phosphate: ($p < 0.008$)

(iii) Vitamin D_3-deficient v crude extract

for [Ca x P_i]: ($p < 0.003$)

for phosphate: ($p < 0.006$)

There is, notably, no significant difference between the plasma calcium and phosphate levels between groups 1 and 4.

Chicks fed the vitamin D_3-deficient diet (group 2) had significantly lower plasma calcium and phosphate levels and significantly higher levels of plasma alkaline phosphatase when compared with vitamin D_3-replete chicks. Vitamin D_3-deficient chicks treated with the partially purified extract showed an average increase in plasma phosphate of 87% above the vitamin D_3-deficient controls ($p < 0.008$) whereas plasma calcium was 13% below the mean vitamin D_3-deficient control level (not significant). The levels of plasma calcium in the group treated with the partially purified extract are significantly

lower ($p < 0.001$) than those of the vitamin D_3-replete control group.

Chicks treated with the crude extract also showed a significant increase in plasma phosphate levels when compared with the vitamin D_3-deficient controls ($p < 0.006$).

DISCUSSION

The data in Table 16 suggest that the crude aqueous extract promoted an increase in plasma phosphate levels in vitamin D_3-deficient chicks resulting in a restoration of the [Ca x P_i] to levels similar to those found in vitamin D_3-replete chicks. Further, results obtained with "peak 4" indicated that only phosphataemic activity was present in this partially purified extract. Neither the crude extract nor "peak 4" had any significant effect upon alkaline phosphatase levels as might be expected if they contained a vitamin D_3-like substance.

In conclusion, there is evidence that both a crude aqueous extract of Trisetum flavescens and a partially purified aqueous extract are capable of significantly increasing the plasma phosphate levels of vitamin D_3-deficient chicks. The extracts differ in their effects upon plasma calcium (although this is not statistically significant). The partially purified extract produced a lower plasma calcium level than vitamin D_3-deficient controls. A higher level of phosphataemic activity resides in the crude aqueous extract.

Experiment 2

Evidence for the Potentiation of Biologically Active Substances by the Gut.

Introduction

The water soluble calcinogen of *Solanum malacoxylon* was shown by Ruksan and Carillo (1976) to be modified by the gut environment. Glycosidases cleaved the sugar moiety from the molecule to release calcitriol. Since the previous experiment showed that the crude aqueous extract of *Trisetum flavescens* restored the [Ca x P_i] to levels found in vitamin D_3-replete animals, an experiment was designed to test whether or not effects of *Trisetum flavescens* administration varied with dose site. To this end, a crude aqueous extract of the plant was administered via three different routes and the effects on plasma calcium and phosphate levels compared.

EXPERIMENTAL

Twenty four cock chicks (Rhode Island Red x Light Sussex) were reared to four weeks of age on a vitamin D_3-replete diet. They were then divided into four groups of six and dosed with a crude aqueous extract of *Trisetum flavescens* each dose being equivalent to one gram of dried plant leaf.

TABLE 17 DOSING REGIMEN

GROUP	NO. OF CHICKS	SITE OF ADMINISTRATION
1	6	ORAL DOSING
2	6	INTRAMUSCULAR
3	6	SUBCUTANEOUS
4	6	CONTROLS (NO DOSE)

Forty eight hours following the administration of plant extract, blood samples were taken from the wing veins for analysis of plasma calcium and phosphate.

RESULTS

TABLE 18 EFFECTS OF DOSING SITE ON PLASMA CALCIUM AND PHOSPHATE LEVELS

GROUP	MEAN PLASMA CALCIUM (± S.E.) mg/100ml	MEAN PLASMA PHOSPHATE (± S.E.) mg/100ml	DOSE ROUTE	[Ca x P_i]
1	7.17 ± 0.28	4.59 ± 0.28	ORAL	32.9 ± 2.14
2	8.57 ± 0.22	2.69 ± 0.17	INTRA-MUSCULAR	23.1 ± 1.77
3	9.15 ± 0.16	2.42 ± 0.47	SUB-CUTANEOUS	22.1 ± 3.93
4	8.11 ± 0.39	3.87 ± 0.30	CONTROLS (NO DOSE)	31.4 ± 1.67

Four groups of vitamin D_3-replete chicks were treated with a crude aqueous extract of Trisetum flavescens using different routes of administration. After forty eight hours analyses were made of plasma calcium and phosphate levels and these were compared with untreated controls.

STATISTICAL ANALYSIS - Students t-test

Significance of Differences

a) for calcium

experimental groups v control - no significant differences at 0.005 level.

oral v subcutaneous ($p < 0.001$)

oral v intramuscular ($p < 0.003$)

b) for phosphate

oral v intramuscular ($p < 0.001$)

oral v subcutaneous ($p < 0.003$)

control v intramuscular ($p < 0.007$)

control v subcutaneous ($p < 0.025$)

c) for $[Ca \times P_i]$

oral v intramuscular ($p < 0.006$)

oral v subcutaneous ($p < 0.029$)

intramuscular v control ($p < 0.006$)

subcutaneous v control ($p < 0.032$)

oral v control NS

Plasma calcium levels in chicks dosed subcutaneously or intramuscularly are elevated when compared with controls though not significantly so. The group dosed orally showed a decrease in plasma calcium, which, while not significantly lower than control levels, was significantly lower than the intramuscularly ($p < 0.003$) and the subcutaneously treated groups, ($p < 0.001$). Plasma phosphate levels in the control group are lower than in the orally dosed group, although the difference is not statistically significant. In contrast

the subcutaneously and intramuscularly treated groups show plasma phosphate levels that are significantly lower than the control group ($p < 0.025$) and ($p < 0.007$) respectively). [Ca x P_i] levels are significantly below control values in birds treated intramuscularly or subcutaneously, whereas those dosed orally show no significant change from vitamin D-deficient control values.

DISCUSSION

The results suggest that the phosphataemic factor in the crude aqueous extract is only active when the extract is introduced orally; indeed, plasma phosphate levels are depressed significantly when non-oral routes are utilised. In these circumstances the depression of plasma phosphate levels reduces the [Ca x P_i] to dangerously low levels. This may be due to either precipitation of calcium phosphate or an effect of the calcinogenic compounds themselves. When dosed orally, the extract brings about an increase in plasma phosphate and a decrease in plasma calcium without altering the [Ca x P_i].

In conclusion, a crude aqueous extract of Trisetum flavescens appears capable of exerting a phosphataemic effect only when dosed orally; when dosed at other sites there is a significant depression of plasma phosphate. There is also evidence of slight calcaemic action in groups dosed subcutaneously or intramuscularly.

Experiment 3

Ultra-Violet Spectroscopy of Aqueous Extracts of Trisetum flavescens Following Sephadex Chromatography.

Introduction

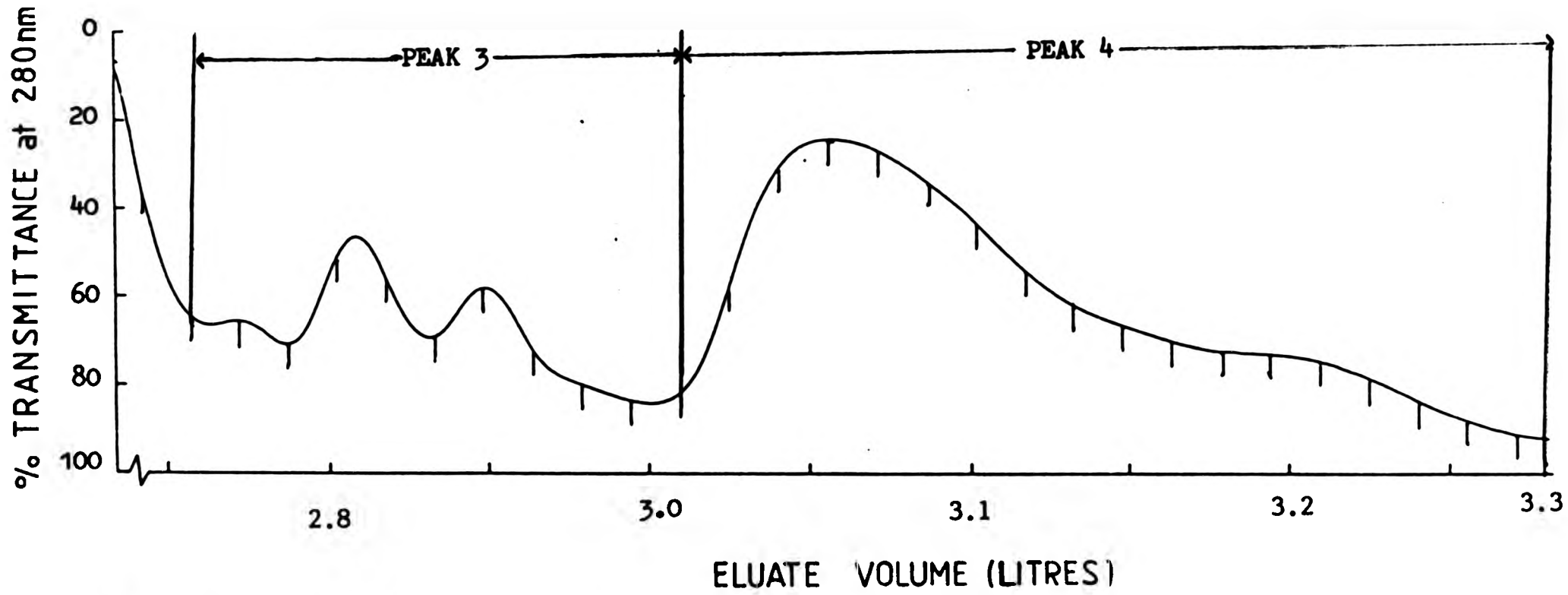
Following partial purification of the crude aqueous extract of Trisetum flavescens, the fractions obtained from the Sephadex column were routinely pooled after identification by ultra-violet spectroscopy. Use was made of the 280nm absorption profile of the eluate as a general guide to the separation efficiency of a particular run.(See figure 8).

Methodology

(i) Individual 15ml fractions eluted from the Sephadex G25 column with distilled water were routinely scanned between 400nm and 190nm using a micro-processor controlled double beam, ultra-violet/visible spectrophotometer (Perkin Elmer 552). Routinely, this procedure was confined to fractions within peaks expected to contain biologically active material. Fractions were diluted as necessary to give a suitable absorbance reading (usually one to two absorbance units). Typical absorption spectra for the biologically active regions of the elution profile are shown in figure 9. Initially the four major peaks identified from the elution profile were pooled separately and reduced in volume by either freeze drying or by evaporation under reduced pressure. The latter process was carried out at the lowest possible temperature (not more than 35°C), so as to avoid possible deterioration of the extract. Concentrated fractions containing the biologically active material were stored at -20°C for further

FIGURE 8

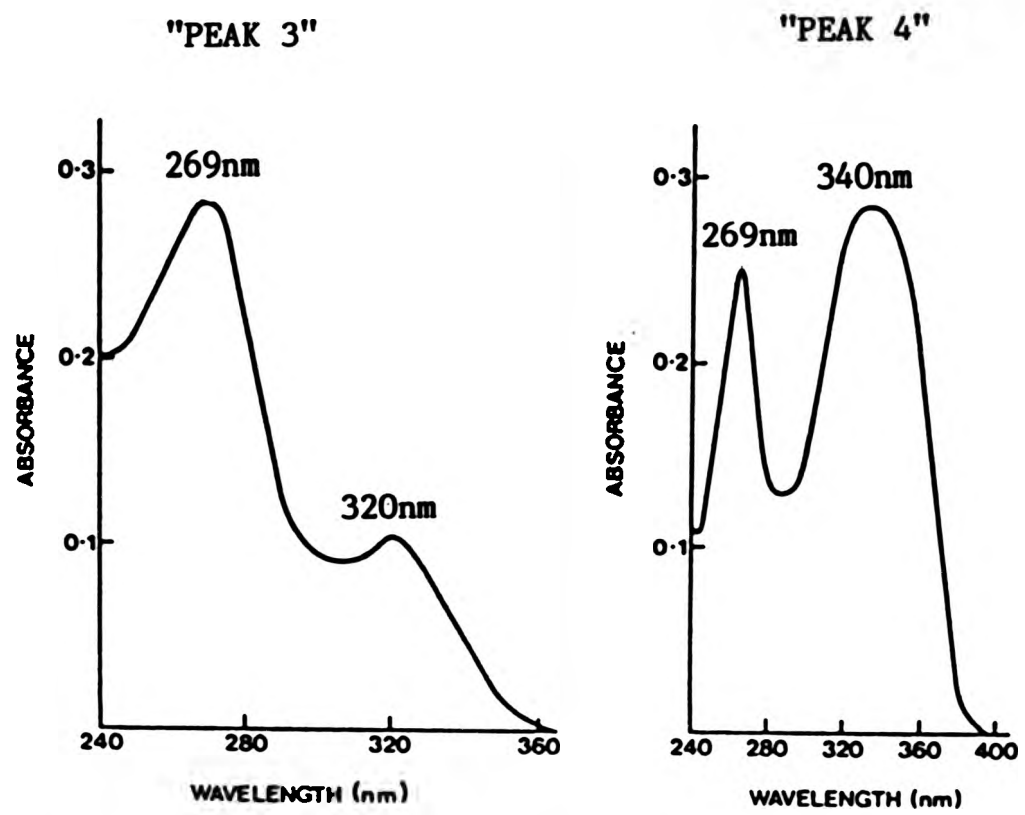
TYPICAL ELUTION PROFILE OF THE SEPARATION OF A CRUDE AQUEOUS EXTRACT OF TRISETUM FLAVESCENS, SHOWING PEAKS CONTAINING THE BIOLOGICALLY ACTIVE MATERIAL



A crude aqueous extract of Trisetum flavescens was passed through a Sephadex column and the eluate monitored for transmittance at 280nm. The peaks shown in figure 8 represent the transmittance of the last peaks to emerge from the column.

FIGURE 9

ABSORPTION SPECTRA OF FRACTIONS FROM UNDER "PEAK 3" AND "PEAK 4".



Fractions obtained from beneath either "Peak 3" or "Peak 4" (See Figure 8) were pooled and then, using a scanning ultra-violet spectrophotometer, scanned between 240nm and 360nm. The above graphs show the absorption spectra of "Peak 3" and "Peak 4" respectively.

experimental work. Fractions with very similar spectra were pooled.

(ii) After concentration by rotary evaporation, fractions within "peak 4" were subjected to further chromatography using finer grades of Sephadex (G15 and G10). The method was the same as for the runs using Sephadex G25 except that a smaller column was used (48cm x 1.5cm).

RESULTS.

(i) Fractions taken from under "peak 3" or "peak 4" had the characteristic ultra-violet absorption spectra shown in figure 9. These two elution regions were subsequently shown to contain the biologically active principles. "Peak 3", in some runs, was resolved into several smaller peaks, but their resolution was poor and they were re-pooled. Fractions from beneath "peak 3" have absorption maxima at 269nm and 320nm and absorption minima at 293nm and 227nm. Fractions constituting "peak 4" have absorption maxima at 269nm and 340nm and absorption minima at 246nm and 297nm.

(ii) No further resolution of "peak 4" was obtained on chromatography through Sephadex G10 or G15. Concentrates of both "peak 3" and "peak 4" were tested on the 'strontium chick model' (as described in experiment 4(i) chapter 4). The results are given in experiment 5.

Experiment 4

(i) The Strontium Chick Model

Introduction

Shippey et al (1922) first introduced the model of "strontium rickets", They reported that dietary stable strontium, when introduced into the diet, produced the symptoms of rickets and effectively rendered the animal vitamin D_3-deficient. Subsequent workers utilised this ability of strontium to cause rickets as a system upon which to base studies of vitamin D_3 metabolites (Omdahl and DeLuca, 1972) or calcinogenic plants (Wasserman, 1974; Zucker, Kreutzberg, Nitsche and Bittner, 1979). Thus plant substances having $1,25(OH)_2$cholecalciferol activity should be able to by pass the strontium blockage of endogenous $1,25(OH)_2$cholecalciferol production and treated animals should not show inhibition of calcium binding protein synthesis or intestinal calcium transport.

The following experiment was carried out in chicks in order to test this experimental model.

Experimental

Ten day-old cock chicks were raised to six weeks of age on a complete diet. They were then changed to a diet low in calcium (0.15%) and supplemented with 0.8% strontium (as strontium carbonate) for a further thirteen days.After eleven days the chicks were divided into two groups of five and dosed sub-cutaneously with either vitamin D_3 (100Iu/bird) or calcitriol (0.25µg/bird) forty eight hours and again at twenty four hour prior to sacrifice. Measurements of duodenal transport of radio-calcium were then made. Immediately following the fifteen minute

absorption period blood samples were taken from each wing vein and analysed for plasma strontium, calcium, phosphate and radio-calcium.

RESULTS:

TABLE 19 Effects of Vitamin D_3 and Calcitriol on several Plasma Constituents in Strontium Treated Chicks.

GROUP AND TREATMENT	PLASMA PHOSPHATE mg/100ml	PLASMA CALCIUM mg/100ml	PLASMA STRONTIUM mg/100ml	PLASMA ^{45}Ca cpm/ml	^{45}Ca (%) TRANSPORTED TO BODY	[Ca x P_i]	[(Ca +Sr) x P_i]
1 VITAMIN D_3	3.66 ± 0.13	8.67 ± 0.14	4.49 ± 0.28	7000 ± 560	21.76	31.7	48
2 CALCITRIOL	3.76 ± 0.22	8.1 ± 0.21	7.42 ± 0.34	16190 ± 2090	32.47	30.5	58

Values are mean ± S.E.

Two groups of chicks fed a strontium supplemented diet were treated with either vitamin D_3 or calcitriol. After forty eight hours each chick was tested for ^{45}calcium transport across the gut wall. Blood isotope levels were also measured as well as plasma phosphate, calcium and strontium.

STATISTICS - Students t-tests

a) for calcium: ($p < 0.05$)

b) for phosphate: (no significant difference)

c) for plasma ^{45}calcium: ($p < 0.003$)

There was little difference between plasma phosphate levels in chicks treated with either vitamin D_3 or calcitriol. There is a clear indication that calcium absorption is stimulated by calcitriol as plasma radio-calcium levels are significantly higher ($p < 0.003$) than in the vitamin D_3 treated group.

The mean level of ^{45}calcium 'transported to body' in the calcitriol treated group is approximately 50% higher than the vitamin D_3 (control) group. Plasma strontium levels in the calcitriol treated group are significantly higher than in the vitamin D_3-treated group ($p < 0.005$). There is no difference between the groups with respect to $[Ca \times P_i]$. If the relationship of $[(Ca + Sr) \times P_i]$ is considered then it is found that birds treated with calcitriol have a value of 58 as opposed to only 48 for vitamin D_3 treated birds.

DISCUSSION

The results for radio-calcium transport confirm the previously reported observation that vitamin D_3 is unable to overcome the situation of suppressed kidney 25(OH)cholecalciferol-1α-hydroxylase brought about by dietary strontium. Calcitriol, however, is able to do so as seen by the increased uptake of ^{45}calcium. There is also evidence that calcitriol is stimulating absorption of strontium in preference to calcium in the gut. (It is known that the chick, unlike other animals does not discriminate in favour of calcium at the level of intestinal

absorption (Wasserman, 1962). The fact that the [(Ca + Sr) x P_i] value for calcitriol treated birds is 58 is notable because this is the value at which the [Ca x P_i] causes precipitation in normal animals. In other words, strontium appears to be transported in the same way as calcium.

Experiment 4 (ii)

Experiment to Determine the Effects of a Crude Aqueous Extract of Trisetum flavescens in the "Strontium Chick"

Introduction

The crude aqueous extract is tested here for its ability to by-pass the strontium inhibition of the kidney L-hydroxylase, in order to determine whether calcitriol-like activity is present.

Experimental Procedure

Ten day-old cock chicks were reared to five weeks of age on a complete diet and then changed to one low in calcium (0.15%) and supplemented with 0.8% strontium (as strontium carbonate) for a further thirteen days. After eleven days the chicks were divided into two groups to form a control (undosed) and an experimental group. The latter group was treated with a crude aqueous extract of Trisetum flavescens at a level of 0.24g (original dried leaf) per bird, at forty eight hours and again at twenty four hours prior to experimentation. Forty eight hours after the administration of the first dose, calcium transport was measured. The procedure was identical to that described in experiment 4 (i), and used the radio-calcium transport technique described in the general methods section

RESULTS

TABLE 20

The Effect of a Crude Aqueous Extract on Plasma Calcium, Inorganic Phosphate and Radio-Calcium Transport

GROUP/ TREATMENT	PLASMA CALCIUM mg/100mls	PLASMA PHOSPHATE mg/100mls	[Ca x P_i] mean ± S.E.	PLASMA ^{45}CALCIUM cpm/ml	% ^{45}CALCIUM REMAINING IN LUMEN	% ^{45}CALCIUM IN GUT TISSUE
1 CRUDE EXTRACT ≡0.48g dried leaf	9.25 ± 0.14	3.01 ± 4.2	31.17 ± 3.6	18,559 ± 1649	85.95 ± 0.85	14.07 ± 0.87
2 CONTROL	9.20 ± 0.61	3.54 ± 0.46	33.08 ± 5.6	8255 ± 1493	90.03 ± 0.62	9.94 ± 0.61

Results are mean ± S.E.

Two groups of chicks fed a diet low in calcium and supplemented with strontium, were treated with either a crude aqueous extract of Trisetum flavescens or left as untreated controls. After forty eight hours, the abilities of both groups to transport ^{45}calcium across the gut wall was assessed. Levels of plasma ^{45}calcium were also measured as well as plasma calcium and phosphate levels.

STATISTICAL ANALYSIS - Students t-tests:-

(i) Plasma calcium - no significant differences

(ii) Plasma phosphate - no significant differences

(iii) [Ca x P_i] - no significant differences

(iv) for plasma ^{45}calcium - ($p < 0.002$)

(v) for % ^{45}calcium remaining in lumen - ($p < 0.004$)

(vi) for % ^{45}calcium in gut tissue - ($p < 0.004$)

The results show that while there is no significant difference between the total plasma calcium levels of the two groups, the uptake of radio-calcium by the gut is significantly stimulated by the plant extract as shown by the plasma radio-calcium levels although the detectable level of radio-calcium transferred to body is 0% for both groups. The mean level of radio-calcium remaining in the lumen of the control group is significantly higher than for the experimental group ($p < 0.004$). The control group shows a correspondingly lower level of radio-calcium in the gut tissue itself ($p < 0.004$).

DISCUSSION

The results show that the crude extract is active in promoting transport of radio-calcium across the gut as demonstrated by the much higher level of radio-calcium in the serum of experimental birds (the difference between the control and experimental groups are significant at $p < 0.002$). The gut tissue levels also support the latter observation. The ability of the plant extract to promote intestinal radio-calcium transport in the "strontium chick" suggests that it contains a substance or substances which are either water soluble forms of 1,25$(OH)_2$cholecalciferol or substances able to mimic the

intestinal actions of 1,25$(OH)_2$cholecalciferol. The chicks dosed with 0.5µg calcitriol (experiment 4(1)) show very similar levels of plasma ^{45}calcium to the chicks given the crude extract (that is, approximately double that of either control group). Provided that the dose-response of chicks given 0.5µg calcitriol is within the linear dose-response range, then the crude aqueous extract contains approximately 1µg of calcitriol/g of dried leaf material. As shown previously, (experiment 1, chapter 4) total plasma calcium levels of chicks treated with the crude extract remain similar to control values; although it might be expected that they would rise to normal physiological levels with continued administration of the extract. It is unlikely that a significant increase in overall plasma calcium levels would occur within the timespan of this experiment (where calcitriol or plant extract was dosed) particularly as the chicks were on a low calcium diet containing large amounts of strontium which would compete with calcium for transport. Restoration of normocalcaemia might therefore not be possible unless there was a large calcium contribution from the skeleton.

Experiment 5.

A Comparison of the Effects of "Peak 3" and "Peak 4" on Radio-Calcium Uptake from the Gut, in the "Strontium Chick".

Introduction.

This experiment was designed to test two partially purified aqueous extracts of Trisetum flavescens for calcitriol-like activity. Radio-calcium absorption by the duodena of chicks treated with either "peak 3" or "peak 4" were compared with untreated controls.

Methodology.

Fifteen cock chicks (RIR x LS) were reared to four weeks of age on a complete diet and then changed to one low in calcium (0.15%) and supplemented with 2.4% strontium (as strontium carbonate) for five days. This level of strontium was chosen so as to supplement strontium for calcium on a molar basis, rather than as a percentage. By this time their plasma calcium levels had dropped to about 8mg/100mls. The birds were then divided into three groups in the following manner.

TABLE 21. DOSING REGIMEN.

GROUP	TREATMENT
1	NONE (CONTROL)
2	"PEAK 3" (≡ 0.75g dried leaf, orally)
3	"PEAK 4" (≡ 0.60g dried leaf, orally)

The above test substances were administered as two half doses, forty eight and twenty four hours prior to experimentation. The usual procedure, as outlined in experiment 4(i) in this chapter

was then carried out on each chick in order to compare the effect of each treatment on calcium transport. Residual lumenal and residual gut wall isotope levels were measured.

Using the following expression it was possible to calculate the mean $\%^{45}$calcium transferred to body:-

10μCi - (dpm in lumenal fluid + dpm in gut tissue) = μCi transported to body.

RESULTS

The results are shown in tables 22 and 23.

TABLE 22 The Effects of "Peak 3" and "Peak 4" on Plasma Calcium Phosphate and Strontium Levels.

GROUP	TREATMENT	MEAN PLASMA CALCIUM (mg/100mls) ± S.E.	MEAN PLASMA PHOSPHATE (mg/100mls) ± S.E.	MEAN PLASMA STRONTIUM (mg/100mls) ± S.E.	[Ca x P_i] ± S.E.
1	CONTROL	8.29 ± 0.14	4.63 ± 0.48	3.66 ± 1.2	39.2 ± 2.2
2	"PEAK 3"	8.56 ± 0.32	4.62 ± 0.24	3.48 ± 1.0	38.6 ± 1.0
3	"PEAK 4"	7.77 ± 0.56	5.66 ± 0.49	8.09 ± 1.9	44.1 ± 2.7

Three groups of chicks were raised on a strontium supplemented diet and then treated with either of two aqueous soluble extracts of Trisetum flavescens or left as untreated controls. After forty eight hours, the chicks were tested for any changes in the levels of plasma calcium, phosphate or strontium in the blood.

TABLE 23. Table to Show the Effects of "Peak 3" and "Peak 4" on Duodenal Calcium Transport in the Chick.

GROUP	TREATMENT	MEAN % ^{45}Ca IN LUMEN ± S.E.	MEAN % ^{45}Ca IN GUT TISSUE ± S.E.	MEAN ^{45}Ca TRANSPORTED TO BODY ±S.E.
1	CONTROL	90.92 ± 4.21	9.26 ± 0.69	3.56 ± 2.18
2	"PEAK 3"	80.05 ± 2.17	9.67 ± 0.74	10.26 ± 2.06
3	"PEAK 4"	88.93 ± 5.68	10.37 ± 1.24	3.74 ± 3.74

Three groups of chicks were reared on a strontium supplemented diet and then treated with either of two aqueous soluble extracts of Trisetum flavescens. After forty eight hours each chick was tested for the ability to transport ^{45}calcium across the gut wall. From the measured values of isotope in the gut lumen and gut tissue, the level of isotope transported to the bloodstream could be calculated.

STATISTICAL ANALYSIS.

Students t-tests.

a) For plasma analyses:

(i) "Peak 3" v "peak 4"

calcium ($p<0.037$)

phosphate ($p<0.015$)

strontium ($p<0.029$)

(ii) Control v "peak 4"

phosphate ($p<0.009$)

strontium ($p<0.003$)

Otherwise differences not significant.

b) For radio-calcium analyses:

(i) Control v "peak 3"

^{45}calcium transported to body ($p<0.065$)

^{45}calcium remaining in lumen ($p<0.081$).

The results for b) (i) are not significantly different at the 0.05 level however.

Plasma total calcium was higher in "peak 3" treated birds than in any other group, although not significantly so when compared with the untreated controls. A comparison between the plasma calcium levels of group 2 and 3 reveals that "peak 3" produces a significantly higher level ($p<0.037$) of plasma calcium.

Plasma strontium levels in group 3 are significantly higher than control levels ($p<0.003$). The plasma strontium levels of group 2 are similar to control levels. The mean plasma phosphate level for group 3 is significantly higher than that of the control group ($p<0.009$) whereas for group 2 it is very similar to that of the control group. "Peak 3" had no significant effect upon plasma

phosphate levels.

The results of the radio-calcium measurements show that "peak 3" treated birds have a lower level of lumenal isotope than the controls although this is not statistically significant ($p<0.081$). "Peak 4"-treated chicks show lumenal levels of isotope very similar to control levels. Levels of isotope within the gut tissue are very similar for all three groups and none differ significantly. For levels of isotope transported to body, "peak 3"- treated chicks showed higher levels of uptake when compared with controls, although the difference was not statistically significant ($p<0.065$). "Peak 4" -treated chicks showed no evidence of isotope transport above control levels.

DISCUSSION.

The two plant extracts have contrasting effects upon the strontium treated chicks. "Peak 3" induced total plasma calcium levels slightly higher than control levels ,but not significantly so,as well as increasing radio-calcium transport, a result which is very close to being statistically significant. Plasma phosphate levels for group 2 are similar to control levels. "Peak 4", however, shows no ability to increase radio-calcium transport but produces a significant increase in plasma phosphate levels.

In conclusion, "peak 3" appears to cause responses akin to calcitriol in that total plasma calcium levels are significantly raised ($p<0.037$) and ^{45}calcium uptake from the gut is also raised, despite the strontium-induced rachitic condition of the chicks. It appears likely that the calcitriol-like effects seen in the crude aqueous extract can be ascribed to a substance contained within the partially purified extract "peak 3".

The effects upon plasma phosphate levels brought about by "peak 4"

would contribute to the higher plasma phosphate levels seen after treatment with the crude aqueous extract. Thus, two calcinogenic factors, with contrasting modes of action seem to be present in Trisetum flavescens in water-soluble form.

EXPERIMENT 6(i) .

The Effects of "Peak 4" on Plasma Calcium and Phosphate in Vitamin D_3-Deficient Chicks.

Introduction.

This experiment was designed to compare the time-course of response to "peak 4", seen in vitamin D_3-deficient chicks. Changes in plasma calcium and phosphate levels were monitored over a thirty six hour period.

Methodology.

Twenty-four chicks were reared from one day of age to four weeks on a vitamin D_3-deficient diet. They were then divided randomly into groups, as shown below, and dosed at time zero with "peak 4".

TABLE 24

Treatment of Experimental Groups.

GROUP	NO. OF CHICKS	TEST SUBSTANCE	RESPONSE TIME ALLOWED (HRS)
1	6	CONTROL	0
2	6	PEAK 4	12
3	6	PEAK 4	24
4	6	PEAK 4	36

Key: "Peak 4" was given as an oral dose in all cases,each dose being equivalent to 1g of original dried leaf material.

The control group was sacrificed at zero hours, following the removal of blood from a wing vein for measurement of plasma calcium and inorganic phosphate. Test groups were sampled at 12, 24, and 36 hours post-dosing.

RESULTS.

TABLE 25 Plasma Calcium and Phosphate Changes in Chicks Dosed with "Peak 4".

TIME POST DOSING (HRS)	PLASMA CALCIUM mg/100mls mean ± S.E.	PLASMA PHOSPHATE mg/100mls mean ± S.E.	[Ca x P_i] mean ± S.E.
0	9.06 ± 0.42	3.38 ± 0.44	30.62 ± 3.6
12	8.30 ± 0.47	3.48 ± 0.65	28.80 ± 4.6
24	7.31 ± 0.39	4.17 ± 0.41	30.48 ± 3.4
36	5.75 ± 0.32	5.32 ± 0.69	30.59 ± 2.5

Four groups of vitamin D_3-deficient chicks were dosed with plant extract at time zero and plasma minerals were tested at intervals.

STATISTICAL ANALYSIS.

The results were analysed for differences between the groups using the students t-test.

The students t-test showed significant differences between the following:

(i) Calcium

0 and 24 hours ($p<0.012$)

0 and 36 hours ($p<0.001$)

12 and 36 hours ($p<0.001$)

24 and 36 hours ($p<0.012$)

(ii) Phosphate

0 and 36 hours ($p<0.04$)

(iii) [Ca x P_i] - NS (all groups).

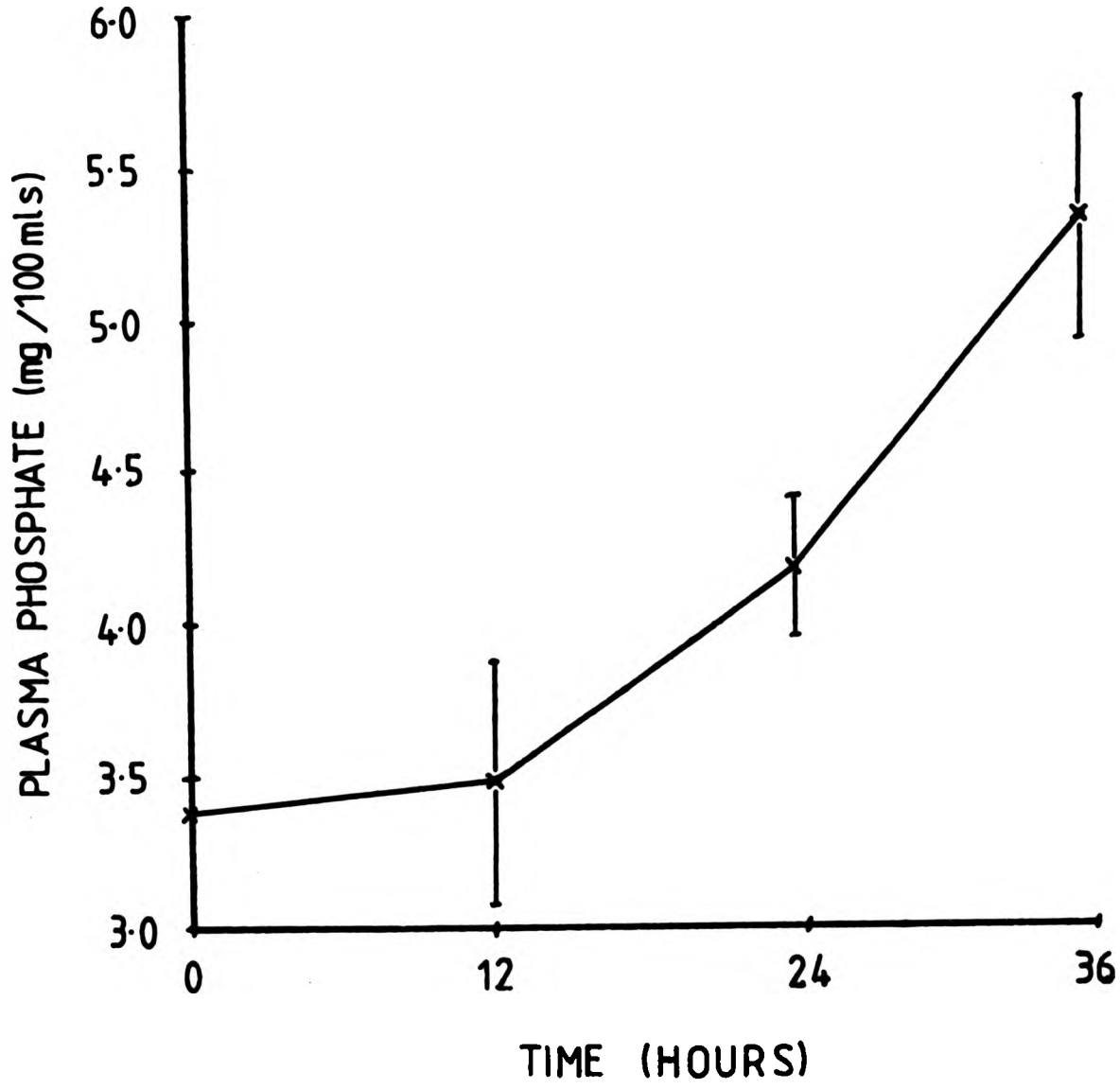
The results show that, when vitamin D_3-deficient chicks were dosed

with the partially purified extract ("peak 4") of Trisetum flavescens, there followed a dramatic decline in plasma calcium; which is significantly lower at twenty four hours post-dosing ($p<0.012$) when compared with zero time (see figure 11). Between twenty four and thirty six hours post-dosing a further significant drop in plasma calcium occurred ($p<0.012$). These changes were accompanied (figure 10) by a steady increase in plasma phosphate, which was significantly higher at thirty six hours post-dosing than at zero time ($p<0.04$)

The calcium x phosphate product levels vary very little throughout the experiment. It appears that the rise in phosphate levels is bringing about the precipitation of calcium phosphate and if this is indeed the case, then plasma phosphate levels are actually lower than they might be . The dangerous decline in plasma calcium cannot be arrested due to the vitamin D_3-deficient status of the chicks.

FIGURE 10

Graph to Show the Changes in Plasma Phosphate in Chicks Dosed with a Partially Purified Aqueous Extract of Trisetum flavescens.

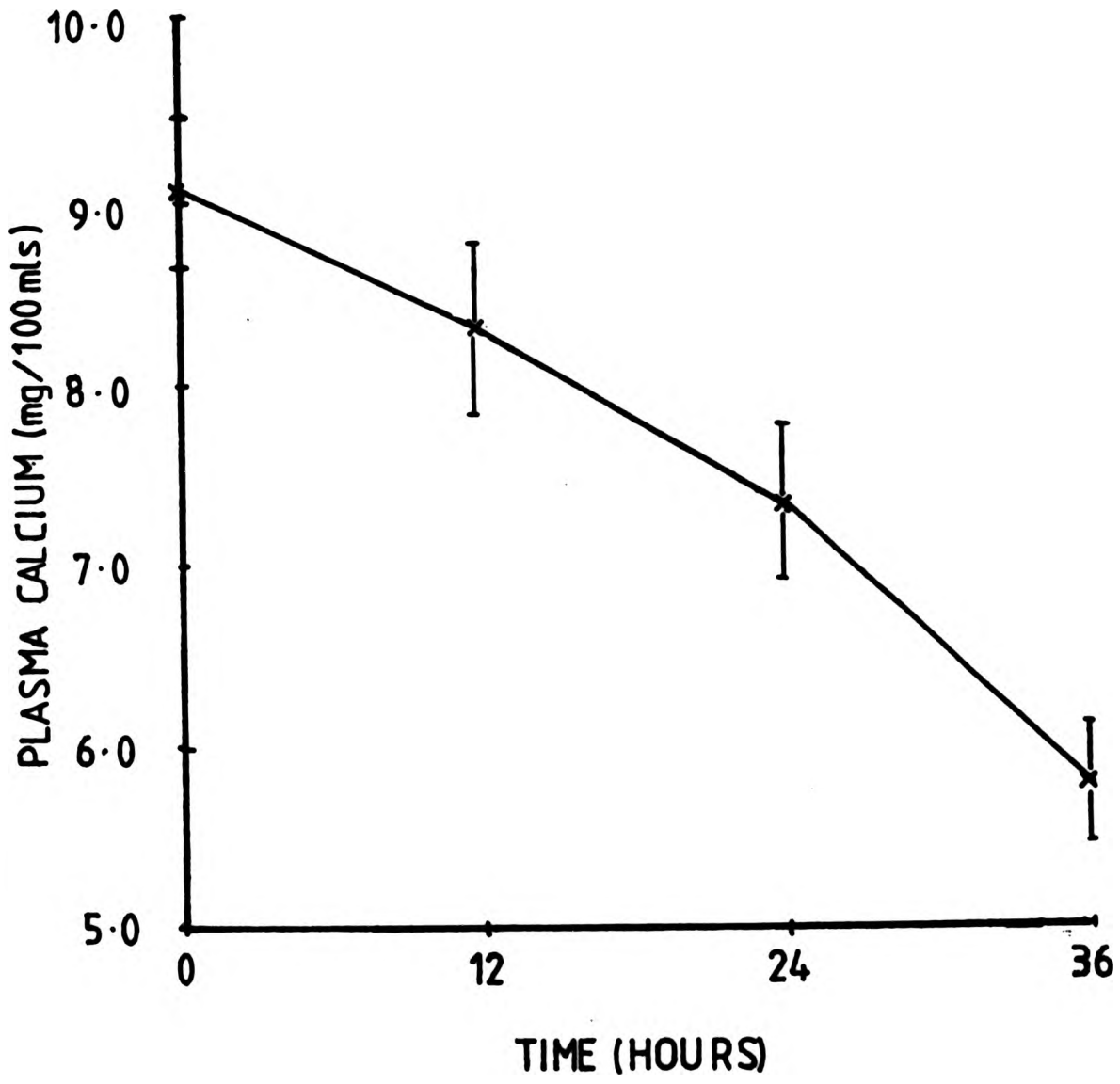


Groups of chicks dosed with partially purified Trisetum flavescens extract at twelve hour intervals, were monitored for changes in their plasma phosphate levels. The results are shown in figure 10.

The chicks were vitamin D_3-deficient.

FIGURE 11

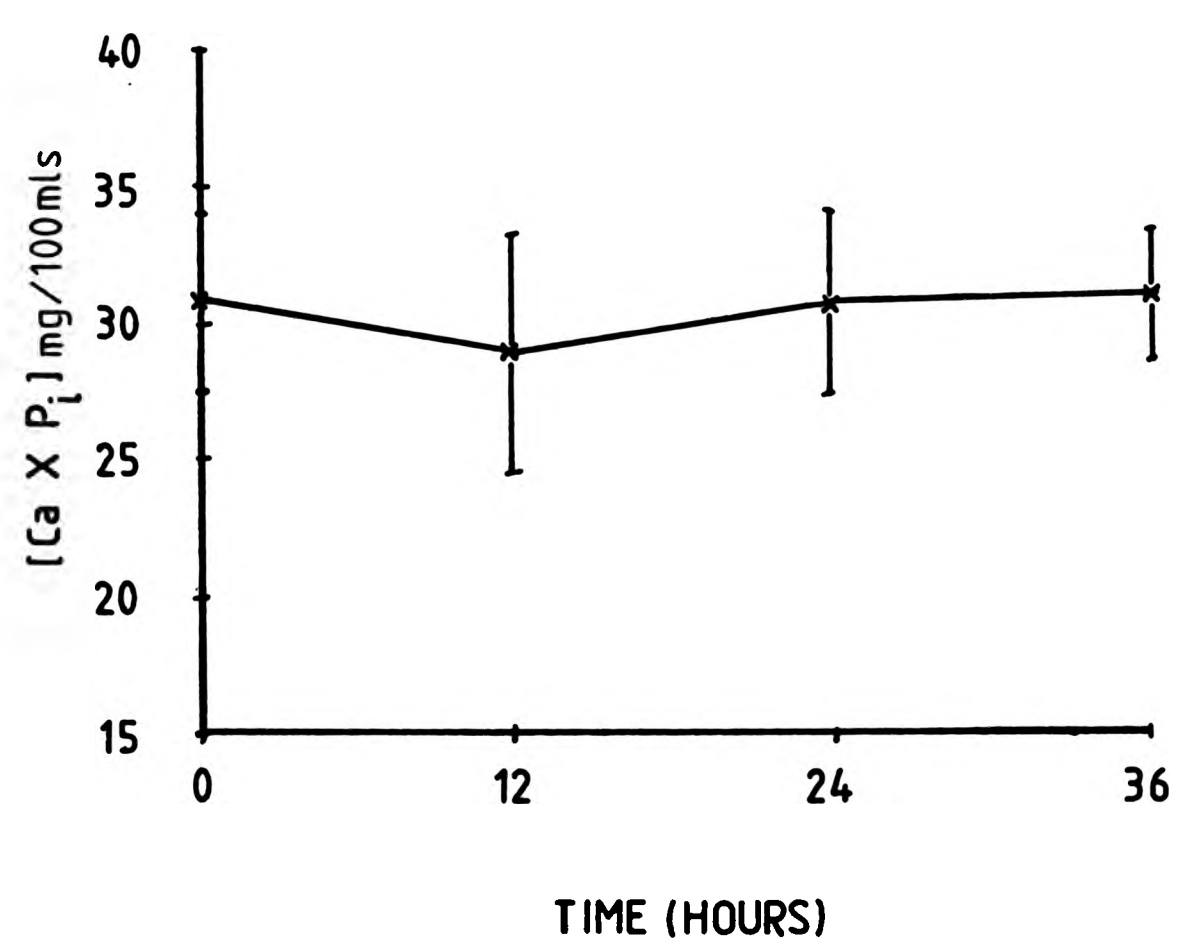
Graph to show the Changes in Plasma Calcium in Chicks Dosed with a Partially Purified Aqueous Extract of Trisetum flavescens.



Groups of chicks were dosed with partially purified Trisetum flavescens extract and monitored for changes in their plasma calcium levels at twelve hour intervals. Figure 11 shows the mean group plasma calcium levels as well as the standard error for each group. The chicks were vitamin D_3-deficient.

FIGURE 12

Graph to Show the Effects of "Peak 4" on the Calcium x Phosphate Product in Vitamin D,-Deficient Chicks.



Four groups of chicks, consisting of six chicks per group, were dosed with the partially purified aqueous extract of Trisetum flavescens at zero hours and then at twelve hour intervals. Figure 12 shows the changes in the plasma calcium and phosphate product values for each group. The standard error values for each group are also indicated.

DISCUSSION.

These results suggest strongly that the phosphataemic factor in this extract of Trisetum flavescens, exerts its effect only upon the levels of plasma phosphate, no rise in plasma calcium level is seen. The administration of "peak 4" to vitamin D_3-deficient chicks results in stability of the low [Ca x P_i] . This was not the case with vitamin D_3-deficient chicks dosed with vitamin D_3 (unpublished observations) where plasma calcium rises. "Peak 4" therefore, changes the proportions of calcium and phosphate in the blood - calcium levels decrease in direct proportion to the increasing plasma phosphate. This decrease in plasma calcium occurs because there is no way for these vitamin D_3-deficient animals to restore their plasma calcium level , therefore it may well be precipitating out as calcium phosphate as phosphate levels rise. If the latter is true, then plasma phosphate levels are actually lower than they might otherwise be.

In conclusion,"peak 4" does not reverse the rachitic state, as it shows no ability to promote uptake of calcium into the bloodstream during a thirty six hour period. Instead, "peak 4", when administered to vitamin D_3-deficient chicks appears to alter the calcium:phosphate ratio by greatly increasing the plasma phosphate level. The [Ca x P_i] remains stable at the low level brought about by the vitamin D_3-deficient status of the animals.

Therefore the biological activity of "peak 4" is not at all reminiscent of vitamin D_3 or its major hydroxylated derivatives. Following these results, a similar experimental approach was used with vitamin D_3-replete chicks.

Experiment 6(ii)

Examination of the Effect of "Peak 4" on Plasma Calcium and Phosphate in Vitamin D_3-Replete Chicks.

Introduction

Since experiment 6(i) showed a prounced decrease in the plasma calcium of chicks dosed with "peak 4" when vitamin D_3-deficient, it was decided to test the same extract on chicks reared on a vitamin D_3-replete diet. In this way, the effects of the extract could be seen in animals whose calcium homeostatic mechanisms were functioning normally. This would mimic the situation found in affected cattle, which can be presumed to be vitamin D_3-replete when they develop calcinosis.

Methodology

Twenty day-old male chicks ('Ross Brown') were raised on a complete diet to four weeks of age. The chicks were then divided into four groups housed separately and dosed orally at time zero, with "peak 4" (equivalent to 1g of original dried Trisetum flavescens). Blood samples were taken from one group successively at time zero, twelve, twenty four and thirty six hours. Each group was sacrificed immediately after the removal of blood. The treatment of the blood and analyses of plasma calcium and phosphate were carried out in the usual way.

RESULTS

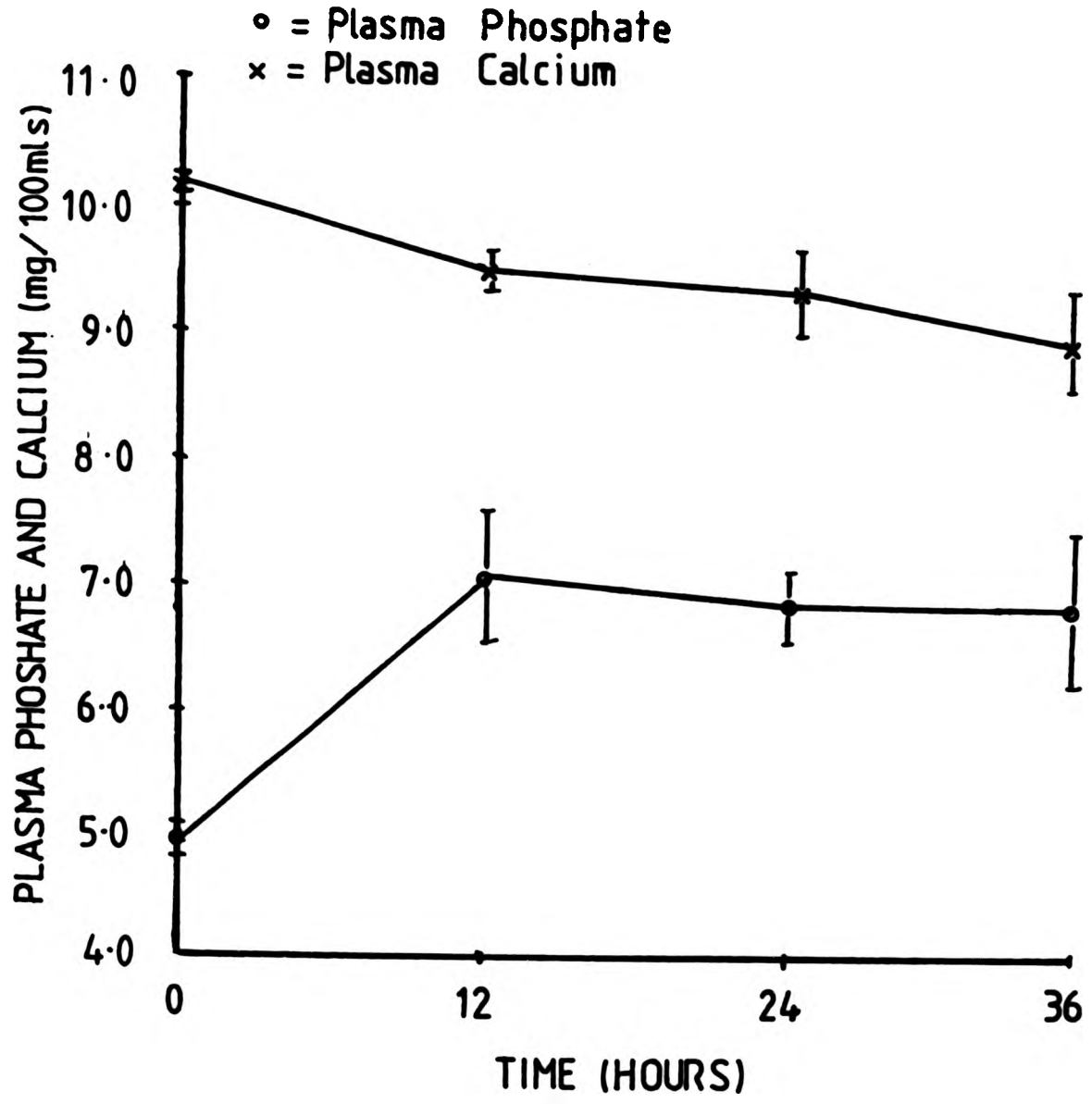
TABLE 26 Changes in Plasma Calcium and Phosphate in Response to Oral Administration of "Peak 4"

TIME POST-DOSING (HRS)	PLASMA PHOSPHATE mg/100mls mean ± S.E.	PLASMA CALCIUM mg/100mls mean ± S.E.	[Ca x P_i] mean ± S.E.
0	4.98 ± 0.18	10.88 ± 0.10	54 ± 2.1
12	7.42 ± 0.63	10.16 ± 0.19	75 ± 7.8
24	7.16 ± 0.36	9.92 ± 0.40	71 ± 5.1
36	7.13 ± 0.69	9.54 ± 0.45	68 ± 4.4

Four groups of vitamin D_3-replete chicks were treated with "peak 4" at time zero and blood samples were taken from each group after successive twelve hour intervals. Plasma levels of both calcium and phosphate were measured for each bird.

FIGURE 13

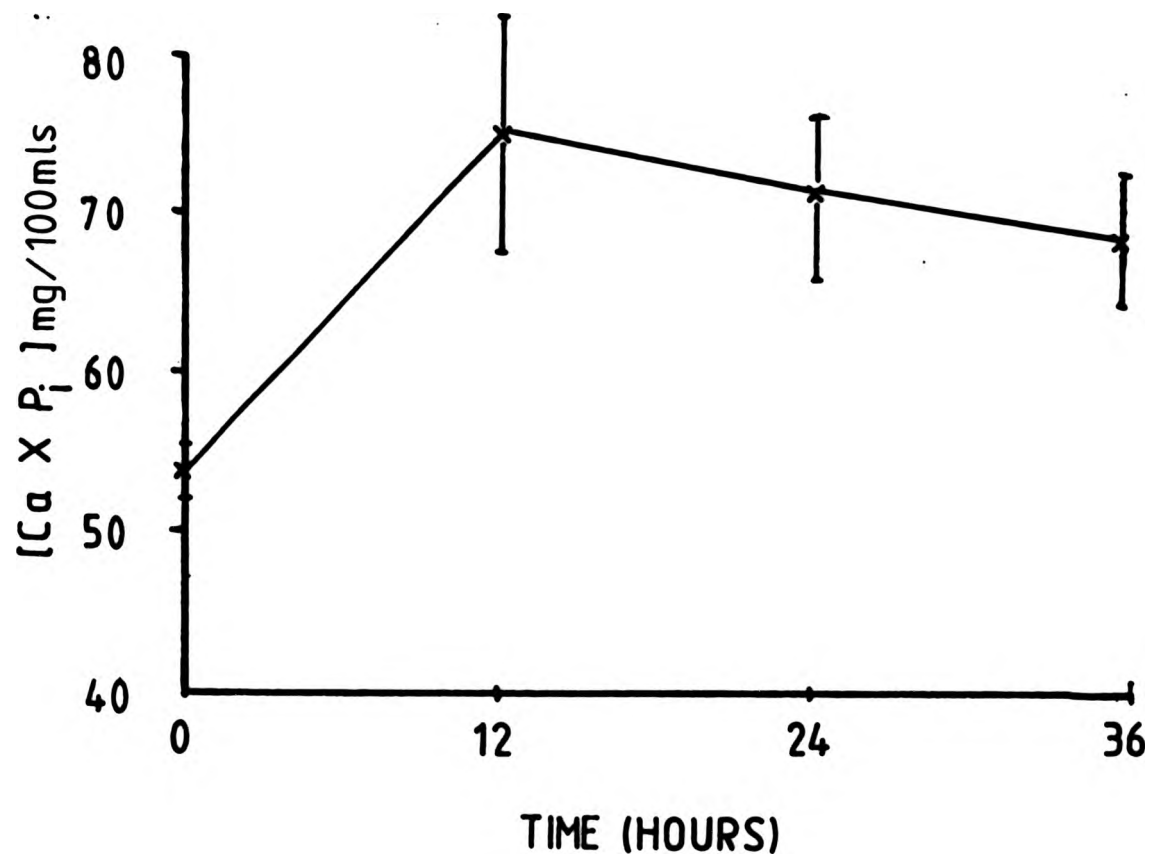
Graph to Show the Effects of a Partially Purified Aqueous Soluble Extract of Trisetum flavescens on Plasma Calcium and Phosphate.



Four groups of chicks were dosed with plant extract at time zero and blood was taken from each group at twelve hourly intervals. The above graph shows the mean plasma levels of calcium and inorganic phosphorus for each group. The chicks were vitamin D_3-deficient.

FIGURE 14

To Show the Changes in the Calcium and Phosphate Product Values of Vitamin D_3-Replete Chicks Dosed with Partially Purified Trisetum flavescens Extract.



Four groups of chicks, reared on a complete diet, were dosed with an aqueous-soluble extract of Trisetum flavescens at time zero. The three remaining groups were dosed at twelve, twenty-four and thirty-six hours respectively. The mean calcium and phosphate product values are shown in figure 14 above.

STATISTICAL ANALYSIS

The individual group results were subjected to the students t-test

(i) Calcium - all groups were compared, and the following comparisons revealed significant differences:

0 and 12 hours ($p < 0.006$)

0 and 24 hours ($p < 0.032$)

0 and 36 hours ($p < 0.012$)

(ii) Phosphate:

0 and 12 hours ($p < 0.002$)

0 and 24 hours ($p < 0.001$)

0 and 36 hours ($p < 0.01$)

Plasma phosphate levels were greatly elevated within twelve hours of administration of the extract, increasing on average by 49% to above 7mg/100mls, this being maintained throughout the experiment. Plasma calcium levels show a small decline during the course of the experiment, the greatest change being within the first twelve hours. Plasma [Ca x P_i] shows a sharp increase within twelve hours of administration of the extract, followed by a gradual decline.

DISCUSSION

When the [Ca x P_i] is greater than 58, precipitation will occur and the results showed that the [Ca x P_i] was greatly in excess of this value within twelve hours of administration of the plant extract. It can be seen that the increase in plasma phosphate in these chicks shows a very similar trend to the [Ca x P_i].

This is in contrast with the effect seen in experiment 6(i) where no increase in plasma [Ca x P_i] occurs. It appears that vitamin D_3

must be present. Without vitamin D_3 being present an increase in plasma phosphate has no effect at all upon the [Ca x P_i] because calcium is precipitated. From the results of experiment 6(ii) it can be seen that a water-soluble component (or components) of this partially purified extract of Trisetum flavescens is very likely to be causing the abnormally high [Ca x P_i] of normal animals grazing on the live plant. The effect of the partially purified extract used here appeared to be solely upon the plasma phosphate levels; calcium levels being maintained within the normal range.

These animals, of course, have a normally functioning calcium-homeostatic system. The small decline in plasma calcium during the experimental period (presumably due to precipitation of calcium phosphate in soft tissue) suggests that the homeostatic mechanisms are being hard-pressed to maintain normo-calcaemia.

Unlike the chicks in the previous experiment, where a continuing decline in plasma calcium would have occurred, these chicks could be expected to develop calcinosis but survive for a considerable time.

The results of both experiment 6(i) and 6(ii) suggest that the partially purified aqueous extract ("peak 4") acts as a calcinogen by increasing blood phosphate levels. The source of the high levels of blood phosphate is not likely to be the bone because calcium would necessarily be released with the phosphate. Therefore either the intestine (increased phosphate absorption), or the kidney (increased renal retention) are likely to be the source of the phosphate. Increased renal retention is probably not the cause, since Dirksen et al (1974) reported elevations in the urinary phosphate of affected cattle. The ability of "peak 4" to increase intestinal phosphate absorption was investigated in experiment 7.

Experiment 7

An Investigation into the Relative Effects of "Peak 4" and Vitamin D_3 on Ileal Radio-Phosphate Uptake.

Introduction

The ability of "peak 4" to stimulate intestinal phosphate transport was investigated. This was necessary in order to determine whether the elevations in plasma phosphate seen in earlier studies were the result of increased uptake at the intestinal site.

Experimental

Thirty 'Ross Brown' chicks were reared to four weeks of age on a vitamin D_3-deficient diet. They were then randomly assigned to five groups of six birds and dosed with either vitamin D_3 or "peak 4". Half the dose was administered forty-eight hours and half twenty-four hours prior to estimation of ^{32}phosphate transport.

TABLE 27 DOSING REGIMEN

	GROUP				
	1	2	3	4	5
TREATMENT	VEHICLE (Propylene Glycol)	VITAMIN D_3 500 Iu sub-cutaneously	VITAMIN D_3 500 Iu orally	PEAK 4 ≅ 1g orally	PEAK 4 ≅ 1g sub-cutaneously

Doses of "Peak 4" were equivalent to 1 gram of dried leaf material per bird

RESULTS

TABLE 28

The Effects of Vitamin D_3 and "Peak 4" upon Plasma Calcium, Plasma Phosphate and Radio-Phosphate Absorption

GROUP	MEAN PLASMA CALCIUM (mg/100mls)	MEAN PLASMA PHOSPHATE (mg/100mls)	MEAN PLASMA ALKALINE PHOSPHATASE*	% ^{32}P IN LUMEN	% ^{32}P IN GUT TISSUE	% ^{32}P TRANSFERRED TO BODY	[Ca x P_i] mean ± S.E.
1 CONTROLS	7.93 ± 0.4	4.19 ± 0.23	1503 ± 313	90.60 ± 4.9	10.76 ± 2.08	-1.36 ± 5.24	33.23
2 VITAMIN D_3 S.C.	8.90 ± 0.24	3.83 ± 0.32	1259 ± 104	62.49 ± 15.9	33.32 ± 8.8	4.18 ± 7.17	34.09
3 VITAMIN D_3 ORALLY	9.10 ± 0.11	4.26 ± 0.26	1273 ± 298	80.21 ± 6.06	21.04 ± 3.9	-1.25 ± 2.34	38.77
4 PEAK 4 S.C.	6.88 ± 0.63	5.57 ± 0.40	1592 ± 350	79.62 ± 8.6	10.21 ± 3.0	10.14 ± 11.1	38.32
5 PEAK 4 ORALLY	7.00 ± 0.39	5.19 ± 0.64	1798 ± 270	86.7 ± 3.56	10.89 ± 1.94	2.41 ± 4.26	36.33

* Units are μ moles of para-nitrophenol phosphate hydrolysed per minute per litre of plasma.

S.C. refers to sub-cutaneous injection.

Five groups of vitamin D_3-deficient chicks were treated with either "peak 4" or vitamin D_3, plasma minerals and intestinal ^{32}phosphate transporting ability were measured after forty eight hours.

RESULTS

TABLE 28 The Effects of Vitamin D_3 and "Peak 4" upon Plasma Calcium, Plasma Phosphate and Radio-Phosphate Absorption

GROUP	MEAN PLASMA CALCIUM (mg/100mls)	MEAN PLASMA PHOSPHATE (mg/100mls)	MEAN PLASMA ALKALINE PHOSPHATASE*	% ^{32}P IN LUMEN	% ^{32}P IN GUT TISSUE	% ^{32}P TRANSFERRED TO BODY	[Ca x P_i] mean ± S.E.
1 CONTROLS	7.93 ± 0.4	4.19 ± 0.23	1503 ± 313	90.60 ± 4.9	10.76 ± 2.08	-1.36 ± 5.24	33.23
2 VITAMIN D_3 S.C.	8.90 ± 0.24	3.83 ± 0.32	1259 ± 104	62.49 ± 15.9	33.32 ± 8.8	4.18 ± 7.17	34.09
3 VITAMIN D_3 ORALLY	9.10 ± 0.11	4.26 ± 0.26	1273 ± 298	80.21 ± 6.06	21.04 ± 3.9	-1.25 ± 2.34	38.77
4 PEAK 4 S.C.	6.88 ± 0.63	5.57 ± 0.40	1592 ± 350	79.62 ± 8.6	10.21 ± 3.0	10.14 ± 11.1	38.32
5 PEAK 4 ORALLY	7.00 ± 0.39	5.19 ± 0.64	1798 ± 270	86.7 ± 3.56	10.89 ± 1.94	2.41 ± 4.26	36.33

* Units are μ moles of para-nitrophenol phosphate hydrolysed per minute per litre of plasma.

S.C. refers to sub-cutaneous injection.

Five groups of vitamin D_3-deficient chicks were treated with either "peak 4" or vitamin D_3, plasma minerals and intestinal ^{32}phosphate transporting ability were measured after forty eight hours.

Significance of Differences - Students t-test

a) For calcium

group 1 v group 2 ($p < 0.06$)

group 1 v group 3 ($p < 0.027$)

group 2 v group 5 ($p < 0.002$)

group 3 v group 5 ($p < 0.001$)

group 2 v group 4 ($p < 0.015$)

gropp 3 v group 4 ($p < 0.013$)

b) For phosphate

group 3 v group 4 ($p < 0.031$)

group 1 v group 4 ($p < 0.014$)

group 2 v group 4 ($p < 0.007$)

c) For alkaline phosphatase

No significant differences

d) Levels of ^{32}phosphate in gut tissue

group 1 v group 2 ($p < 0.023$)

group 1 v group 3 ($p < 0.037$)

group 1 v group 4 (not significant)

group 1 v group 5 (not significant)

e) Levels of residual ^{32}phosphate in lumen

no significant differences.

f) Levels of ^{32}phosphate transferred to body

no significant differences.

Only significant differences are reported.

The results for plasma calcium levels show that vitamin D_3 administered by either route, causes a significant rise in plasma calcium ($p < 0.06$ for sub-cutaneous administration and $p < 0.027$ for orally administered vitamin D_3). Vitamin D_3 caused no significant change in plasma phosphate when administered by either route when compared with control animals.

Both groups treated with plant extract show plasma calcium levels significantly lower than the vitamin D_3-treated groups. The plasma calcium levels of extract treated groups are also lower than the control group although not significantly so.

The plant extract treated groups show plasma phosphate levels that are higher than the other groups; group 4 is significantly higher than vitamin D_3-treated groups or the controls ($p < 0.014$, $p < 0.007$ and $p < 0.031$ respectively). Plasma alkaline phosphatase, as expected, has dropped below control levels in vitamin D_3-treated birds (although not significantly); in both plant extract treated groups there is a slight (not significant) increase in plasma alkaline phosphatase levels.

Levels of radio-phosphate in the gut tissue are higher in vitamin D_3-treated chicks than in the control and plant extract treated groups. The latter are very similar to control levels; the vitamin D_3-treated groups are significantly higher than other groups at the 0.05 level. Residual lumenal isotope levels show that all treated groups have lower levels of radiophosphate in the lumen than the control group; "peak 4" dosed sub-cutaneously shows comparable residual isotope levels to vitamin D_3 dosed orally. These differences are not statistically significant however. The estimated levels of isotope transported to the bloodstream for each group reveal that, with the

surprising exception of group 3, there is stimulation of isotope transport taking place. Variations in transport varied considerably within each group, as indicated by the standard errors, but overall, both plant extract treated groups and the vitamin D_3 subcutaneously treated group show a generalised uptake of isotope when compared to control levels. Again, the levels of variation precluded a statistically significant result.

DISCUSSION

The plasma analyses confirm the results of previous experiments in that administration of an aqueous soluble extract of Trisetum flavescens to vitamin D_3 deficient chicks brings about an elevation in plasma phosphate with an accompanying decrease in plasma calcium. Vitamin D_3, of course, raises calcium levels and has little effect on plasma phosphate levels. The isotope transport results show surprisingly high levels of ^{32}phosphate within the gut tissue of vitamin D_3 treated groups and relatively low transport to body. Groups 4 and 5 however, show definite evidence of transfer of the isotope into the bloodstream with no build up of ^{32}phosphate within the gut tissue itself. This was the picture presented by the organic soluble extracts (Chapter 3, experiment 1) of Trisetum flavescens in a similar experimental situation.

In conclusion it appears that the activity of "peak 4" is influencing the mechanism which transfers phosphate from the gut tissue to the bloodstream, this was also concluded for the organic-soluble phosphataemic factor (chapter 3, experiment 1). The vitamin D_3-treated birds experience a build up of phosphate within the gut tissue cells and a lower rate of transfer to the bloodstream.

In conclusion, it appears that the "peak 4 " phosphataemic factor

does not effect phosphate uptake by the gut in the same way as vitamin D_3 and must be influencing a different phosphate transport mechanism. This mechanism may possibly be the para-cellular route by which phosphate can cross the gut wall, and is thought to be "the only route for phosphate entry in vitamin D_3-deficient animals" (Fuchs and Peterlik,1979 ; Birge and Avioli 1981). If this is indeed the case, then the active factor in "peak 4" is the first yet found that influences para-cellular phosphate transport.

Experiment 8

The Effect of Partially Purified Aqueous Extracts of Trisetum flavescens on Bone Cultured In Vitro

Introduction

Having established the action of crude and partially purified aqueous extracts of Trisetum flavescens upon intestinal calcium and phosphate transport, the effects of the two active partially purified extracts were examined using the bone culture model previously described.

Experimental

Twenty four mouse calvariae from six-day old mice were utilised, fourteen of them were from mice pre-labelled at one day of age with 5μCi ^{45}calcium. The remaining bones were unlabelled. Following pre-incubation of all bones, the bones were transferred to fresh medium and treated with either "peak 3" or "peak 4" according to the following regimen

TABLE 29 DOSING REGIMEN

GROUP	CULTURE NUMBERS	TREATMENT	WEIGHT OF DRY PLANT MATERIAL/ CULTURE (mg)	WHETHER PRE-LABELLED WITH ^{45}CALCIUM
1	1 - 7	"PEAK 4"	4.4	5μCi/MOUSE
2	8 - 14	NONE	-	5μCi/MOUSE
3	15 - 17	"PEAK 3"	5.7	NO

The fourteen pre-labelled bones were sampled at various times throughout their experimental incubation period by removing 20µl aliquots of culture medium. This was then added to 200µl of 100% formic acid for solubilisation. 150µl of this solution was then added to 10mls of Amersham triton scintillant prior to counting in the Beckman LS7500 scintillation counter. Aliquots of culture medium were taken at the following times for analysis.

TABLE 30 Sampling Times of Tissue Culture Medium.

CULTURE NUMBERS	SAMPLING TIMES (HOURS)					
1-14	2	4	6	8	24.3	48

Analyses of stable calcium, phosphate, radio-active calcium and glucose utilisation were carried out at forty-eight hours post addition of test substances.

RESULTS

TABLE 31 Table to Show the Effects on Medium Calcium and Phosphate Levels of "Peak 3" and "Peak 4" and Glucose Utilisation

ANALYSES OF TISSUE CULTURE MEDIUM AT 48 HOURS.				
GROUP	CULTURE NUMBERS	CALCIUM (mg/100mls)	PHOSPHORUS mg/100mls)	% AVAILABLE GLUCOSE UTILISED
1(T)	1-7	8.02 ± 0.57	3.97 ± 0.37	30%
2(C)	8-14	7.25 ± 0.25	3.79 ± 0.53	30%
3(T)	15-17	8.02 ± 0.24	2.88 ± 0.06	25%

STATISTICAL ANALYSIS

Students t-tests

a) For calcium:

group 2 v group 3 ($p < 0.01$)

b) For phosphate:

group 1 v group 3 ($p < 0.02$)

group 2 v group 3 ($p < 0.014$)

Otherwise differences not significant.

TABLE 32 **The Effects of "Peak 4" Upon Radio-calcium Release from Pre-labelled Bones**

CULTURE NUMBERS	TIME POST ADDITION OF TEST MATERIAL (HOURS)					
	2	4	6	8	24.3	48
1-7(T)	0.64 ± 0.11	1.11 ± 0.13	1.46 ± 0.09	2.24 ± 0.14	5.85 ± 0.6	9.47 ± 0.54
8-14(C)	0.73 ± 0.11	1.28 ± 0.14	1.59 ± 0.1	2.35 ± 0.19	5.77 ± 0.3	9.81 ± 0.41

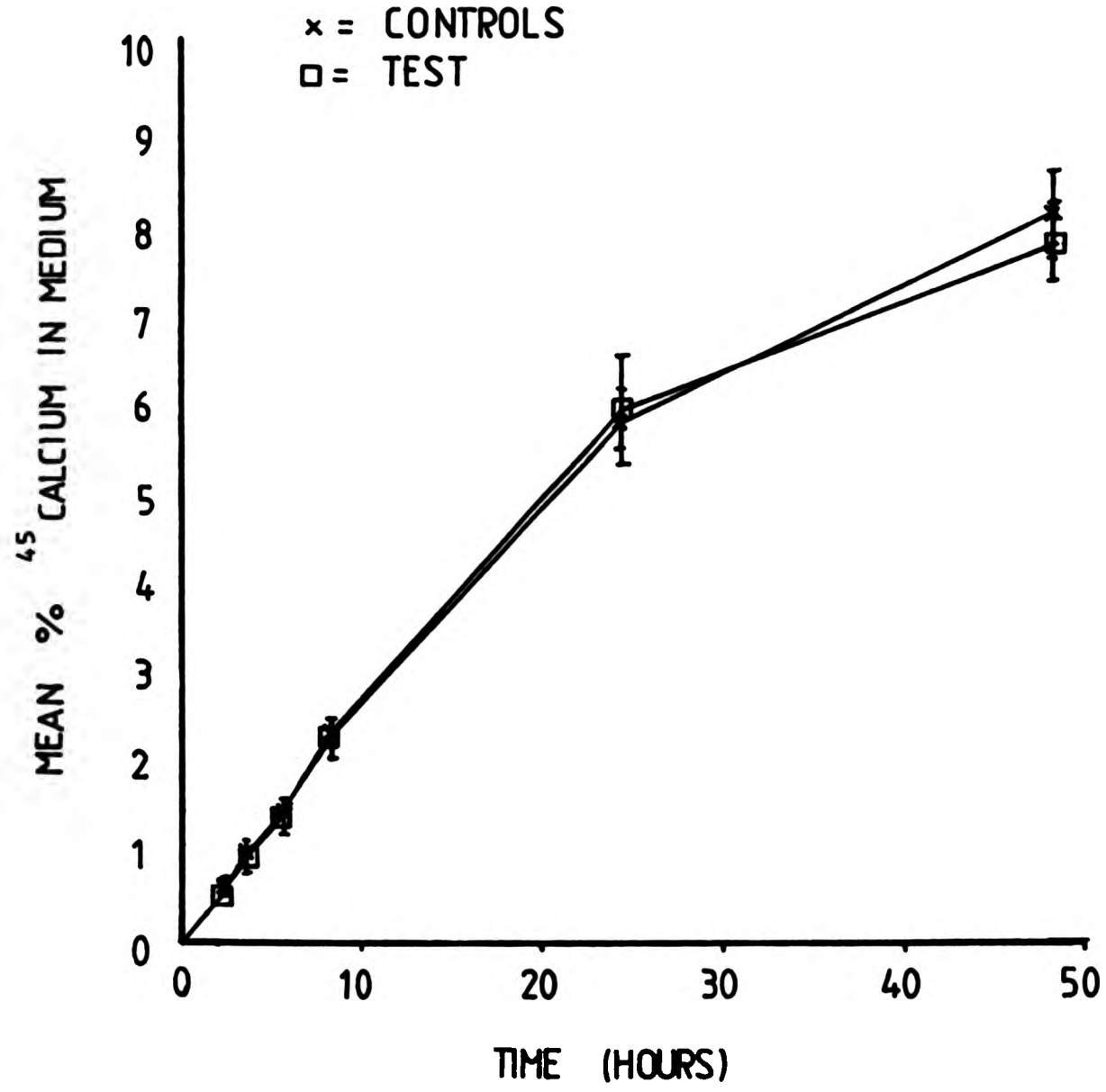
Note:

values represent % total radiocalcium released to culture medium, group average with standard error. (T)= test cultures, (C)= control cultures.

Two groups of mouse calvariae wereeither treated with a partially purified aqueous soluble (T) extract of Trisetum flavescens or were left as untreated controls(C)At intervals during a forty eight hour period, samples of the medium were removed for analysis of ^{45}calcium levels. The calvariae had been pre-labelled with isotope.

FIGURE 15

The Effect of "Peak 4" on ^{45}Calcium Release from Pre-Labelled Bone *in vitro*.



The experimental group of ^{45}calcium labelled bones cultured *in vitro* were treated with the partially purified aqueous extract of *Trisetum flavescens.* The control group was untreated. During a forty-eight hour culture period samples of culture medium were removed at various times for measurements of isotope levels to be taken.

The results show that pre-labelled bones treated with "peak 4" material (group 1), show a very similar trend of radio-calcium release into the medium when compared to control bones. This is despite a significantly higher level of total calcium in the medium of treated bones after forty-eight hours. Medium phosphate levels of groups 1 and 2 are very similar (no significant difference). Group 3 (unlabelled bones treated with "peak 3") show a significant increase in medium calcium when compared with controls ($p < 0.01$) and have a significantly lower level of medium phosphate ($p < 0.014$)

DISCUSSION

The virtually identical pattern of ^{45}calcium release into the medium, seen in both control and "peak 4" treated bones suggests that no bone resolving compound such as calcitriol is present in the plant extract. The significant increase in medium calcium levels produced by treatment of the bones with "peak 3" suggests that a vitamin D_3-like metabolite may be present in this extract. "Peak 4" however has no detectable effect on bone resorption.

CHAPTER 5

PARTIAL PURIFICATION AND CHARACTERISATION OF THE AQUEOUS SOLUBLE PHOSPHATAEMIC PRINCIPLE

CHAPTER 5

PURIFICATION AND CHARACTERISATION OF THE AQUEOUS SOLUBLE PHOSPHATAEMIC PRINCIPLE

Introduction

Investigations into the purity and composition of the phosphataemic fraction obtained by Sephadex chromatography were carried out using a number of techniques.

Initially, the non-purity of the biologically active fractions was determined, followed by attempts at isolation of the components and general characterisation using, mostly, physical analytical techniques.

Experiment 1

Evidence That The Biologically Active Eluate From The Liquid Chromatography Column Was Not Pure

Introduction

Because of the relatively wide band of fractions pooled under the phosphataemic peak of the eluate, it was reasonable to expect that the phosphataemic fractions were not of uniform composition. This is particularly so because the starting material was the crude aqueous extract. Nevertheless, examination of the eluate in the region in question using ultra-violet spectroscopy (as was routinely carried out on the fresh eluate) always yielded a predictable pair of peaks in the ultra-violet range and none at all in the visible range.

Methodology

Evidence that more than one substance was present in the phosphataemic fractions was obtained from four main sources which were as follows:

1 Measurements of the relative intensities of the peak heights in the ultra-violet absorption spectrum.

2 Fluorescence Spectroscopy.

3 Thin-Layer Chromatography.

4 Polarimetry.

1:1 Examination of the Ultra-Violet Absorption Spectrum

Following Sephadex chromatography, examination of the ultra-violet absorption spectrum of the pooled fractions obtained by Sephadex chromatography, revealed that consecutive fractions varied in the relative height of the two absorption maxima. The wavelengths of the absorption maxima did not vary however.

If only one substance were present in the solution, then the relative heights of individual peaks would not be expected to vary, if conditions were uniform. Typical differences were detected among phosphataemic fractions obtained from a Sephadex column containing Sephadex grade G15 (column dimensions 48 cm x 1.5 cm) and used for attempting to separate the constituents of pooled phosphataemic fractions obtained from Sephadex (grade G25) chromatography of the crude aqueous extract.

When individual fractions of the eluate from this column were examined at constant wavelength (269 nm) only one absorption maximum was obtained (i.e. no separation of individual substances had occurred) but measurements of the relative intensity of the peak heights of these fractions (following scanning of each fraction in the ultra-violet range 400 nm to 190 nm) showed that variations existed in the relative heights of the two peaks.

The differences between the relative peak heights of consecutive fractions are shown in Table 33.

Further confirmation of these observations was obtained when the relative intensities of the peak heights of consecutive fractions obtained from under "peak 4" of the elution profile, were scanned between 190 nm and 400 nm.

Results

TABLE 33

Table to show the Ratios of Absorption Maxima Intensities of fractions obtained from a Sephadex G15 column and a sample of Partially Purified Trisetum flavescens Extract containing the Phosphataemic Principle.

Fraction No.	Ratio of Peaks At 270nm : 345nm	Absorbance At 270nm
72	1.47	0.10
74	1.41	0.14
76	1.37	0.18
78	1.33	0.21
80	1.31	0.24
82	1.27	0.28
84	1.30	0.30
86	1.27	0.31
88	1.26	0.30
90	1.26	0.29
92	1.31	0.26
94	1.31	0.22
96	1.27	0.19

TABLE 34

Table to Show Relative Peak Heights of Fractions Obtained from a Preparative Sephadex G25 Column, in the Region of the Phosphataemic Principle

Fraction No.	Ratio of Peak 270nm : 345nm	Absorbance Of Peak Maxima	
		270nm	345nm
27	5.73	0.315	0.055
29	4.62	0.37	0.08
30	2.32	0.545	0.235
32	1.82	0.565	0.31
33	1.52	0.63	0.415
35	1.37	1.22	0.89
37	1.23	1.08	0.88
40	1.15	0.46	0.4

Conclusions

These observations suggested that gel filtration was not a suitable method for separating the constituents.

The finest grades of Sephadex (Sephadex G10 and G15) failed to yield more than one absorption maximum, on examination of the eluate but the observations made on the differences in the ratio of peak heights led to the use of other techniques to detect and characterise the constituents.

Experiment 1:2

Fluorescence Spectroscopy

Introduction

This technique was chosen to examine the biologically active phosphataemic fractions of the plant, because fluorimetry allows a fluorescent material in the presence of a non-fluorescent material to be scanned independently of a contaminant present in the solution. If one of the constituents of the extract were fluorescent, the excitation spectrum would be similar to its ultra-violet absorption spectrum in that one is usually a mirror image of the other (Pesce, Rosen and Pasby, 1971).

Method

A dry sample of the aqueous soluble phosphataemic extract ("peak 4") was placed in a cuvette, dissolved in water and scanned in a Pye-Unicam Luminescence spectrophotometer for the presence of absorption and emission spectra. The results are shown in Figures 16 and 17.

Results

The results of the scan (see Figure 16) revealed that there was a fluorescent compound present in the extract, with an absorption maximum of 346 nm - identical to one of the two peaks seen in ultra-violet absorption spectroscopy. The ultra-violet spectrum of the fluorescent compound shows no absorbance at 270 nm. Hence it seems likely that the peak corresponding to the phosphataemic factor contains two substances, one with an ultra-violet maximum of 346 nm (pH dependent) and the other with a maximum of 270 nm.

FIGURE 16

Absorption Spectrum of Fluorescent Component of "Peak 4".

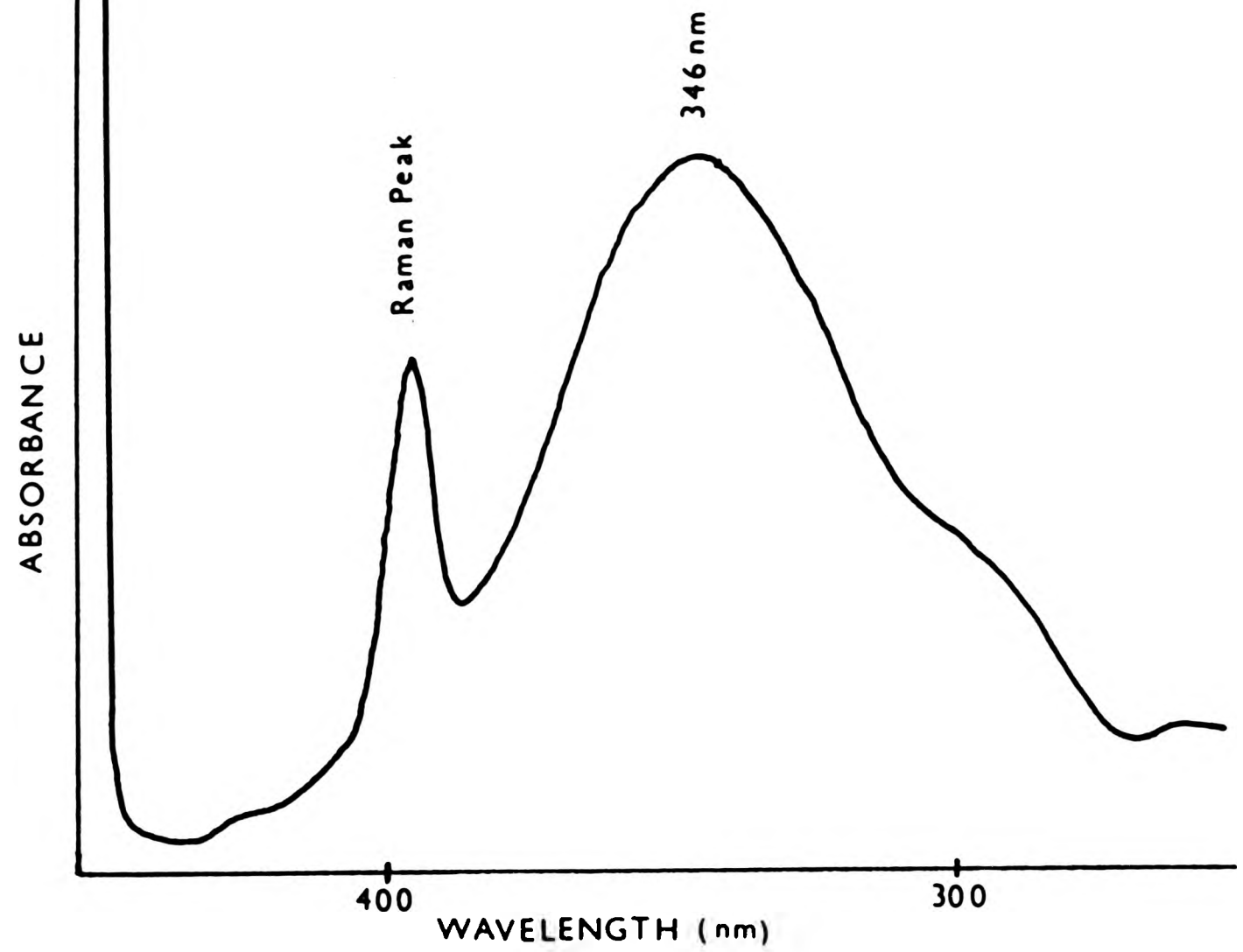
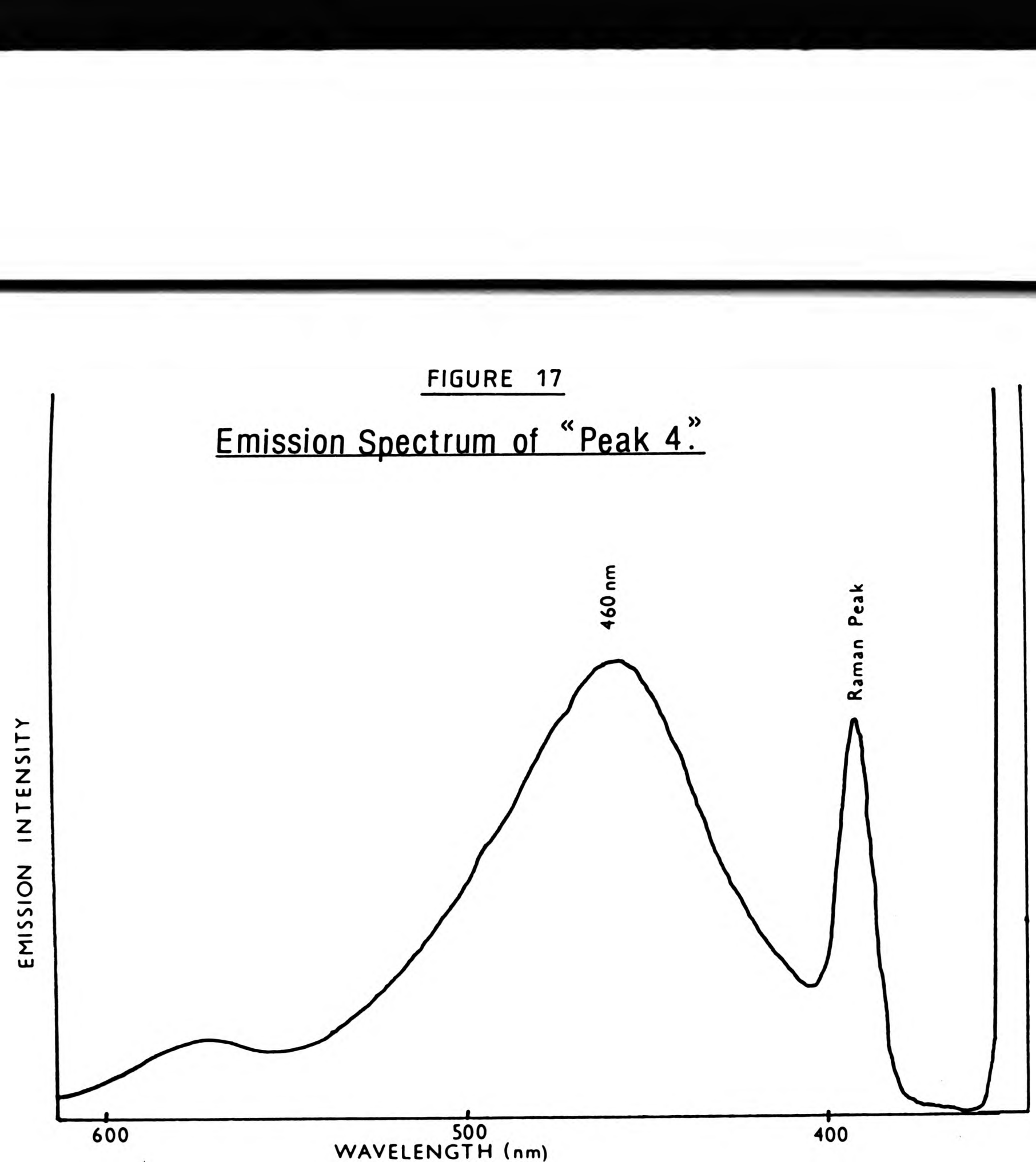


FIGURE 17

Emission Spectrum of "Peak 4."



The emission maximum of the fluorescent compound peaks at 458 nm (the emission maximum of a fluorescent compound is always higher than that of the ultra-violet absorption spectrum; this is known as the 'Stokes shift'). This is strong evidence that two compounds were present in the solution.

Experiment 1:3

Thin Layer Chromatography

Introduction

Because of the suspected presence of more than one compound in the Sephadex fractions with phosphataemic activity ("peak 4") and the failure of liquid chromatography to achieve a separation, thin layer chromatography was used in an attempt to separate the compounds. This technique is very adaptable to quick trials with different stationary and mobile phases allowing many combinations of the system to be tried with minimum loss of plant extract and time. A similar procedure was used for Solanum malacoxylon (Peterlik and Wasserman, 1975).

Methodology

Selection of a Suitable System for Separation of the Aqueous Soluble Constituents

The solid 'peak 4' material was moderately soluble in alcohol as well as water and so various alcohol/water mobile phases were tested with either cellulose or silica gel G stationary phases. Visualisation of the components of the extract was possible using Ehrlich's reagent (see below) or Potassium ferricyanide/ferric chloride.

reagent. The following table shows the results of using various combinations of stationary and mobile phases:-

Results

TABLE 35

Choice of a Suitable Mobile and Stationary Phase

Mobile Phase	Stationary Phase	Comments
1. Water	Cellulose	Very slow rate of travel.
2. Butanol/Acetic Acid/Water 4:1:1	Cellulose	Slow travel - no evidence of separation of the compounds.
3. n-pentanol/ Water 1:1	Silica Gel G	Travels well - but no evidence of separation.
4. 100% Methanol	Silica Gel G	Good rate of travel and spray reagents revealed two separate spots.

100% methanol with Silica gel G was chosen as the most suitable and was used for all subsequent studies on "peak 4". It was necessary for the solvent front to travel 25 - 30 cms to achieve separation of the compounds and so 20 cm x 40 cm plates (Camlab Ltd.)were used with analytical grade methanol (BDH chemicals). The extract was dried on to the base of the plate in a current of air and allowed to run in an ascending tank for 3-4 hours. Subsequently, after brief drying, suitable chromogenic reagents could be used. The

reagent. The following table shows the results of using various combinations of stationary and mobile phases:-

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100% methanol with Silica gel G was chosen as the most suitable and was used for all subsequent studies on "peak 4". It was necessary for the solvent front to travel 25 - 30 cms to achieve separation of the compounds and so 20 cm x 40 cm plates (Camlab Ltd.)were used with analytical grade methanol (BDH chemicals). The extract was dried on to the base of the plate in a current of air and allowed to run in an ascending tank for 3-4 hours. Subsequently, after brief drying, suitable chromogenic reagents could be used. The

ineffectiveness of aqueous solvents with Silica gel G is because of its structure. It is a dehydrated product of polysilicic acid with surface pores and hydroxyl groups which hydrogen bond with polar molecules. Hence an aqueous solvent prevents the polar constituents of the extract from reaching the gel surface, thus affecting the residing time and convective velocity of the sample. Pure alcohol was therefore chosen as an effective compromise.

Testing of Various Spray Reagents as an aid to Characterisation of the Components.

The various spray reagents used are shown in the following table together with their colour reactions with the fraction components. (Reagent compositions are according to Stahl (1969) and are detailed in Appendix 3).

TABLE 36

Reagents Used In The Characterisation Of "Peak 4" Fractions

	REAGENT	TARGET COMPOUNDS	COMMENTS
1.	Ferric Chloride/ Potassium Ferricyanide	Amines and reducing compounds.	POSITIVE - Two dark blue spots indicate Aromatic Amines.
2.	4-Dimethylaminobenz aldehyde (Ehrlich's Reagent)	Amines, Hydroxy-skatoles, Sulphon-amides.	One purple and one yellow spot. Positive
3.	Silver Nitrate	Phenols and Aroma-tic Amines	Positive - Brown/ Black, similar to Amidol standard.
4.	Formaldehyde /HCL (Prochazka Reagent)	Indoles and their derivatives.	Substance at Rf 0.68 fluoresces - implies indole structure.
5.	Dimethylaminocinnam - aldehyde	Indoles	Two yellow patches on orange background.
6.	1, 2 Naphthoquinone - 4 - sulphouic acid	Aromatic Amines.	Test gives limited reaction colour but pale yellow at Rf. 0.76.

Contd/...

TABLE 36 Contd/...

	REAGENT	TARGET COMPOUNDS	COMMENTS
7.	Fluorescein/ Ammonia	Purine, Pyrimidines Barbiturates.	Substance at Rf 0.76 absorbs strongly as do nucleotide bases.
8.	Perchloric Acid/ Ferric Chloride	Indole Derivatives	Indeterminate result.
9.	Iodine/Potassium Iodide	Alkaloids	Negative
10.	Bismuth Trichloride	Sterols	Negative.
11.	2, 6 dichlorophenol - indophenol.	Organic Acids	Negative
12.	Antimony Trichloride	Vitamin D_3	Negative

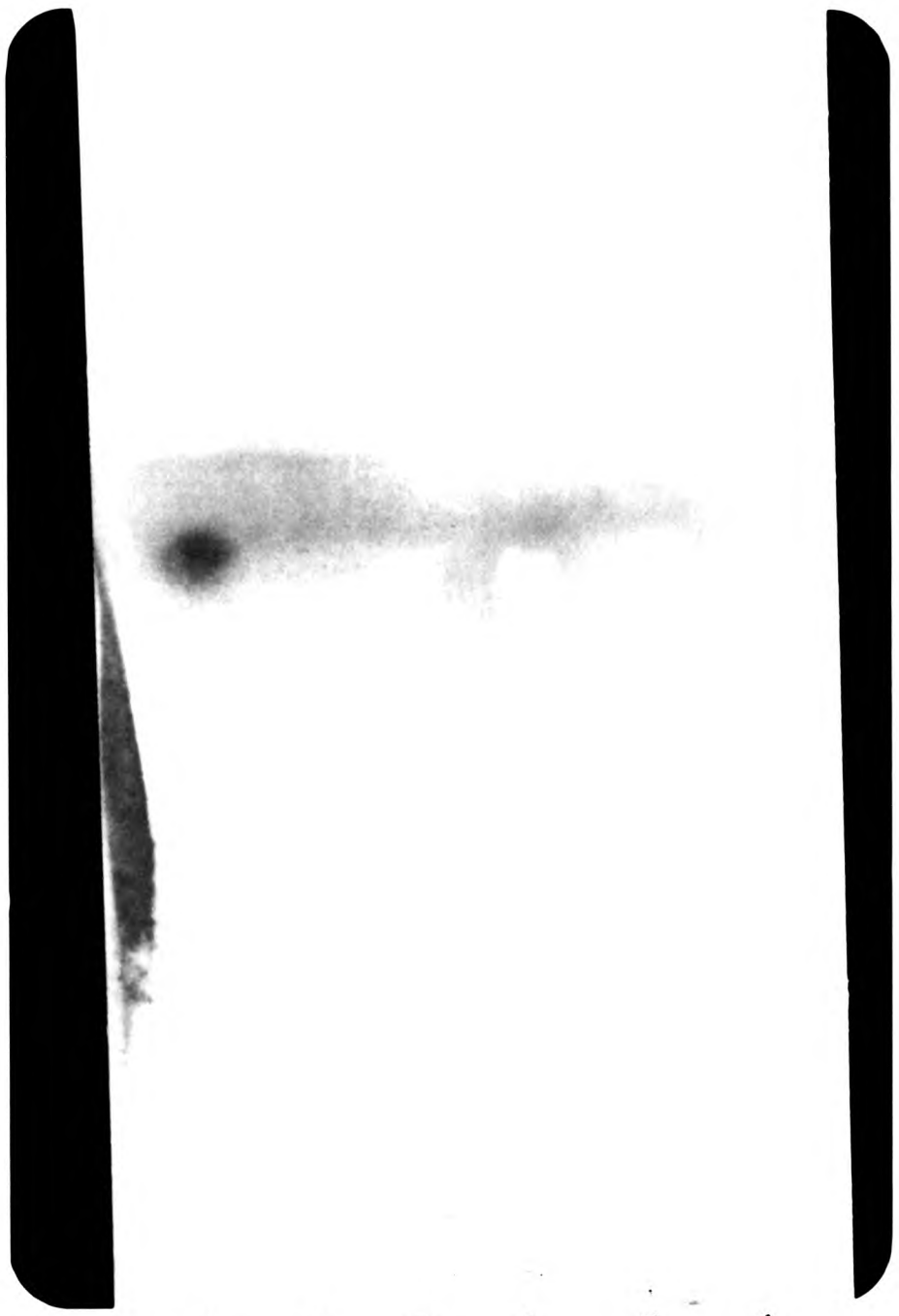
See Appendix 3 for reagent composition.

DIMETHYLAMINOCINNAMALDEHYDE REAGENT.

a

This reagent is a test for indoles. Pure indole (position 1) shows up as blue. Positions 2 and 3 are from later fractions of "peak 4" and positions 4 and 5 are from earlier "peak 4" fractions.

DIMETHYLAMINOCINNAMALDEHYDE REAGENT.



a

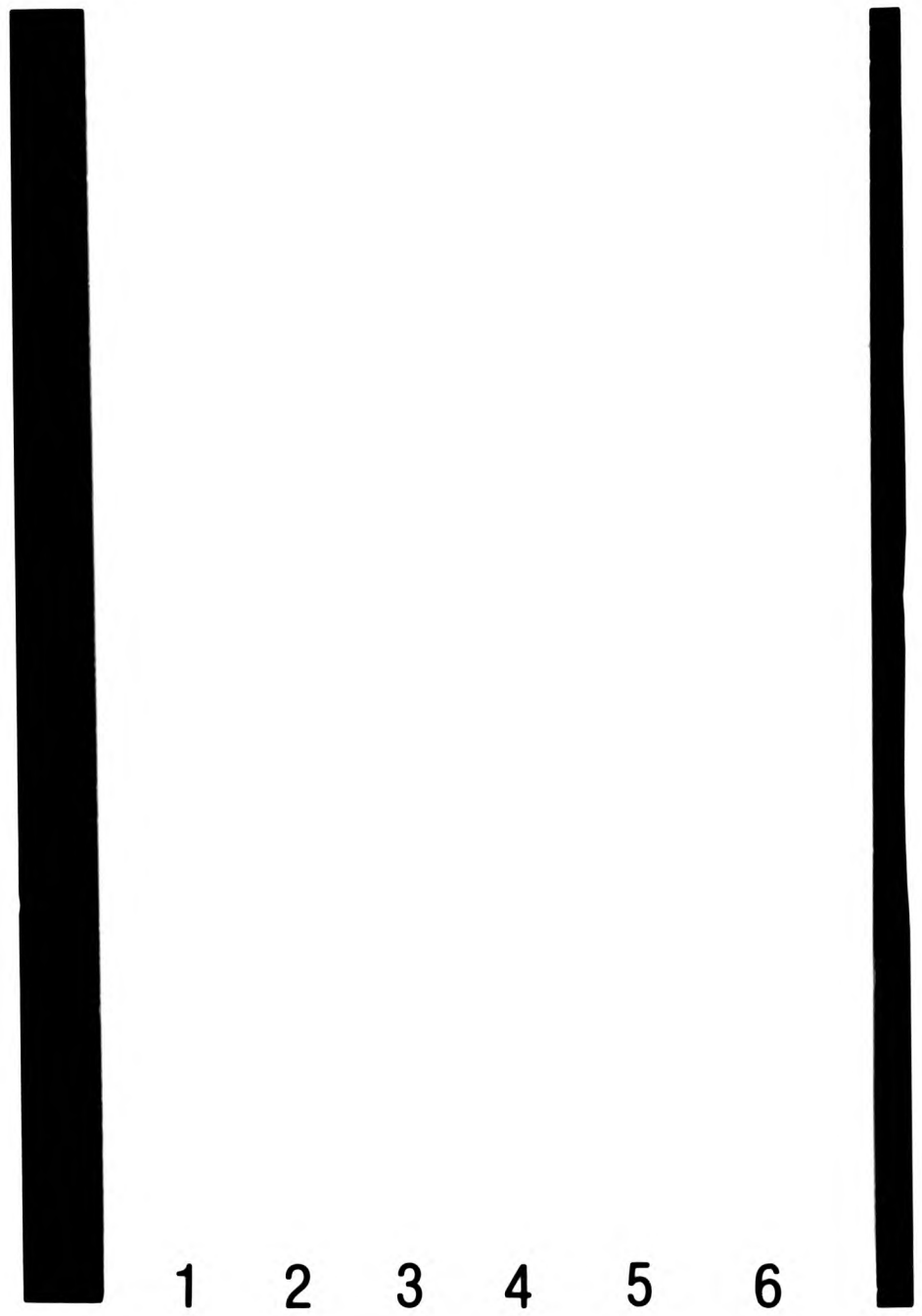
This reagent is a test for indoles. Pure indole (position 1) shows up as blue. Positions 2 and 3 are from later fractions of "peak 4" and positions 4 and 5 are from earlier "peak 4" fractions.

IODINE/POTASSIUM IODIDE REAGENT.

b

This reagent detects alkaloids. Position 1 shows a positive reaction with the alkaloid atropine. Positions 2, 4 and 5 were treated with "peak 4" material. Positions 3 and 6 were treated with "peak 3" material. Plate 'b' shows no positive reaction for any of the samples tested.

IODINE/POTASSIUM IODIDE REAGENT.



b

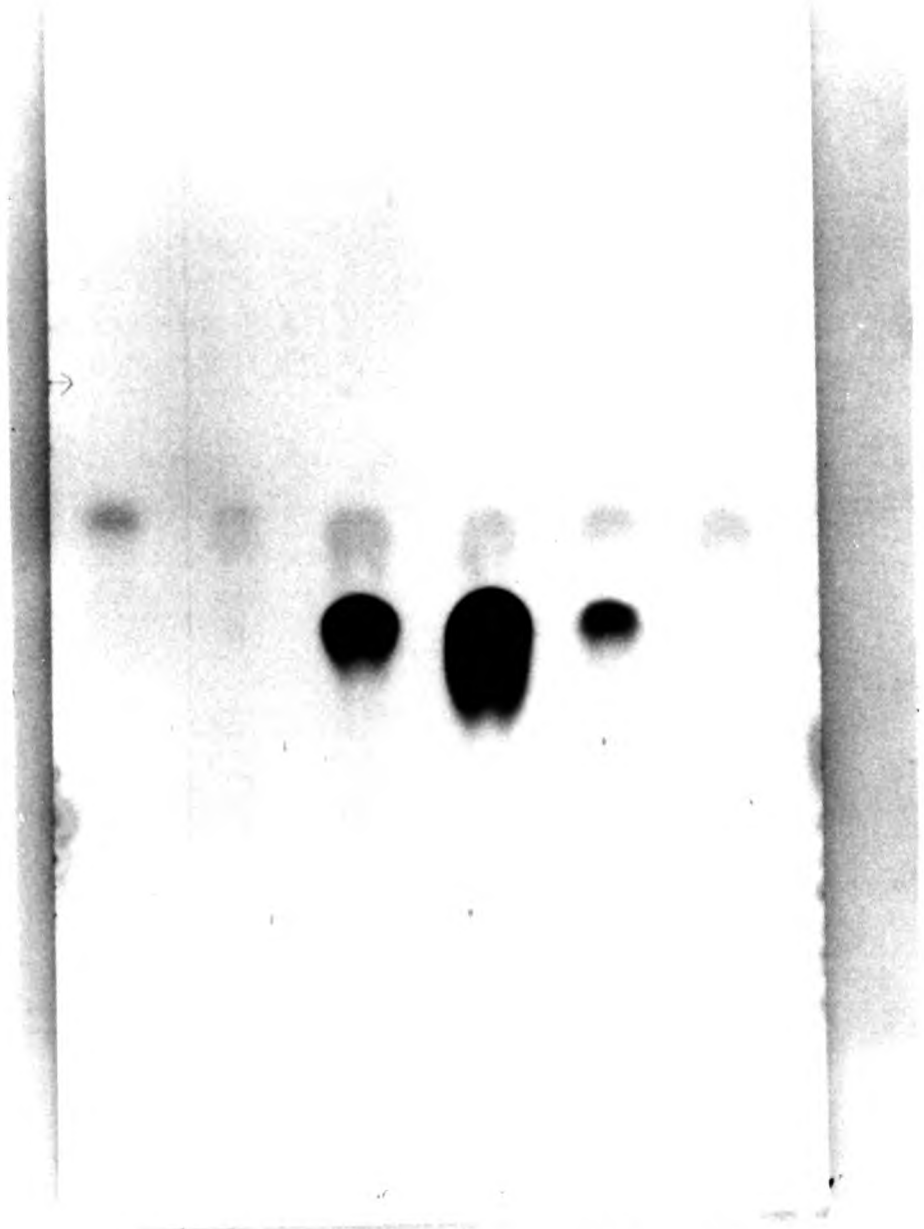
This reagent detects alkaloids. Position 1 shows a positive reaction with the alkaloid atropine. Positions 2, 4 and 5 were treated with "peak 4" material. Positions 3 and 6 were treated with "peak 3" material. Plate 'b' shows no positive reaction for any of the samples tested.

4-DIMETHYLAMINOBENZALDEHYDE (EHRLICHS') REAGENT.

c

This reagent reacts with amines, hydroxyskatoles and sulphonamides. Samples at positions 1-6 are consecutive fractions from beneath "peak 4" as detailed in table 37. Two distinct compounds have been separated with Rf values of 0.76 (yellow spots) or 0.68 (blue spots).

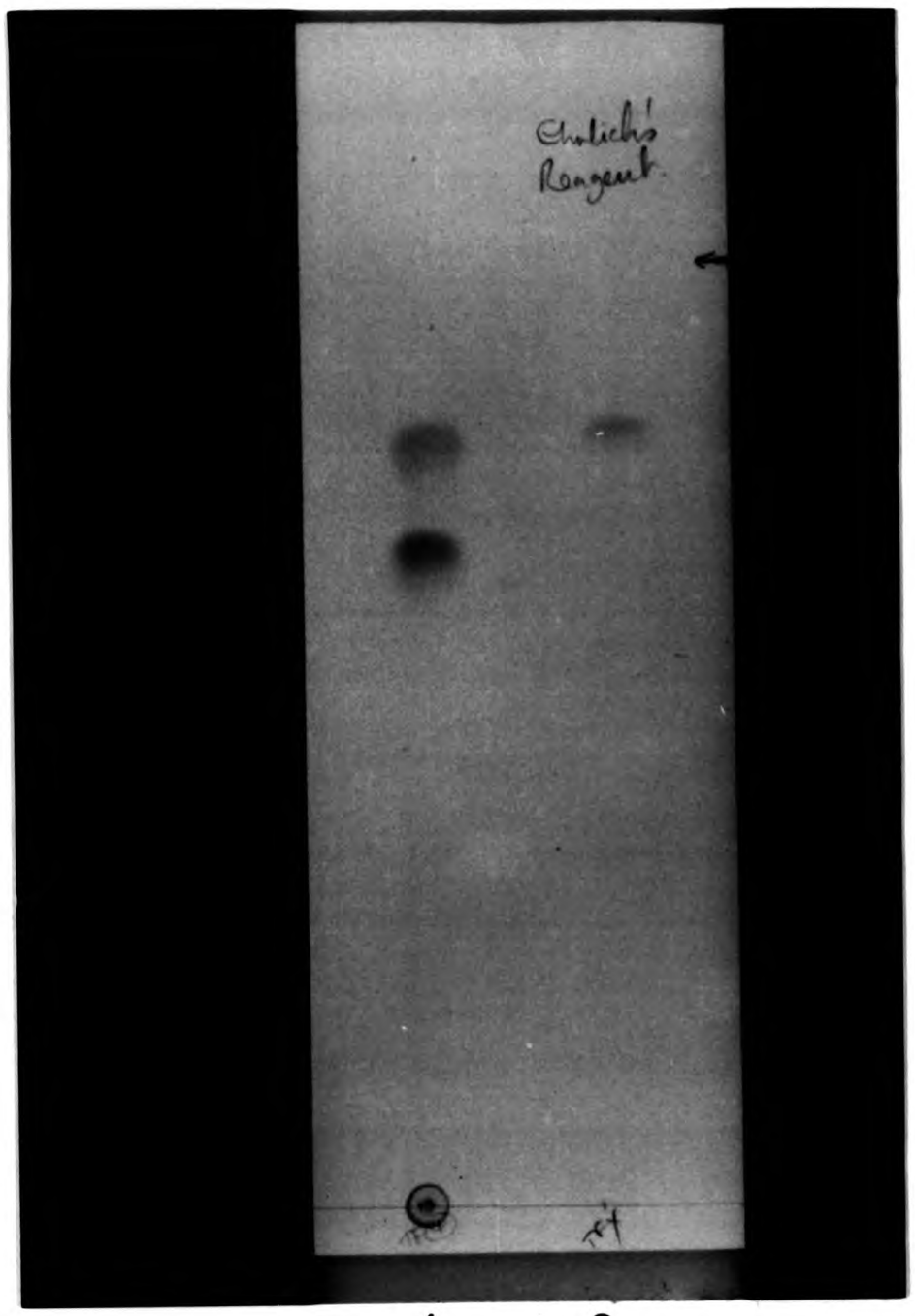
4-DIMETHYLAMINOBENZALDEHYDE (EHRLICHS') REAGENT.



c

This reagent reacts with amines, hydroxyskatoles and sulphonamides. Samples at positions 1-6 are consecutive fractions from beneath "peak 4" as detailed in table 37. Two distinct compounds have been separated with Rf values of 0.76 (yellow spots) or 0.68 (blue spots).

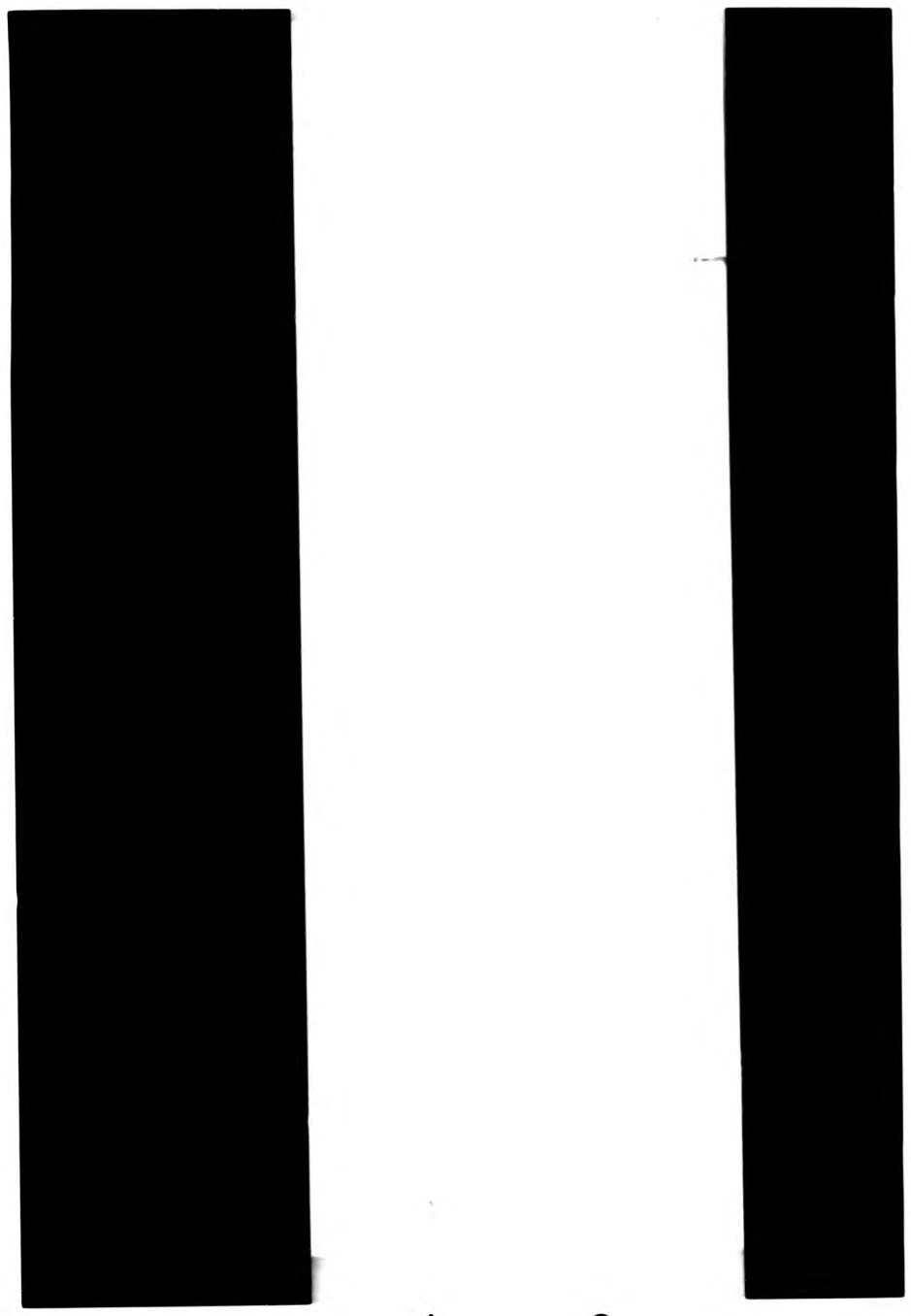
4-DIMETHYLAMINOBENZALDEHYDE (EHRLICHS') REAGENT.



d

This plate shows the clear separation of the two compounds found under "peak 4". The sample at position 2 was taken from the final fractions of "peak 4".

4-DIMETHYLAMINOBENZALDEHYDE (EHRLICHS') REAGENT.



1 2

d

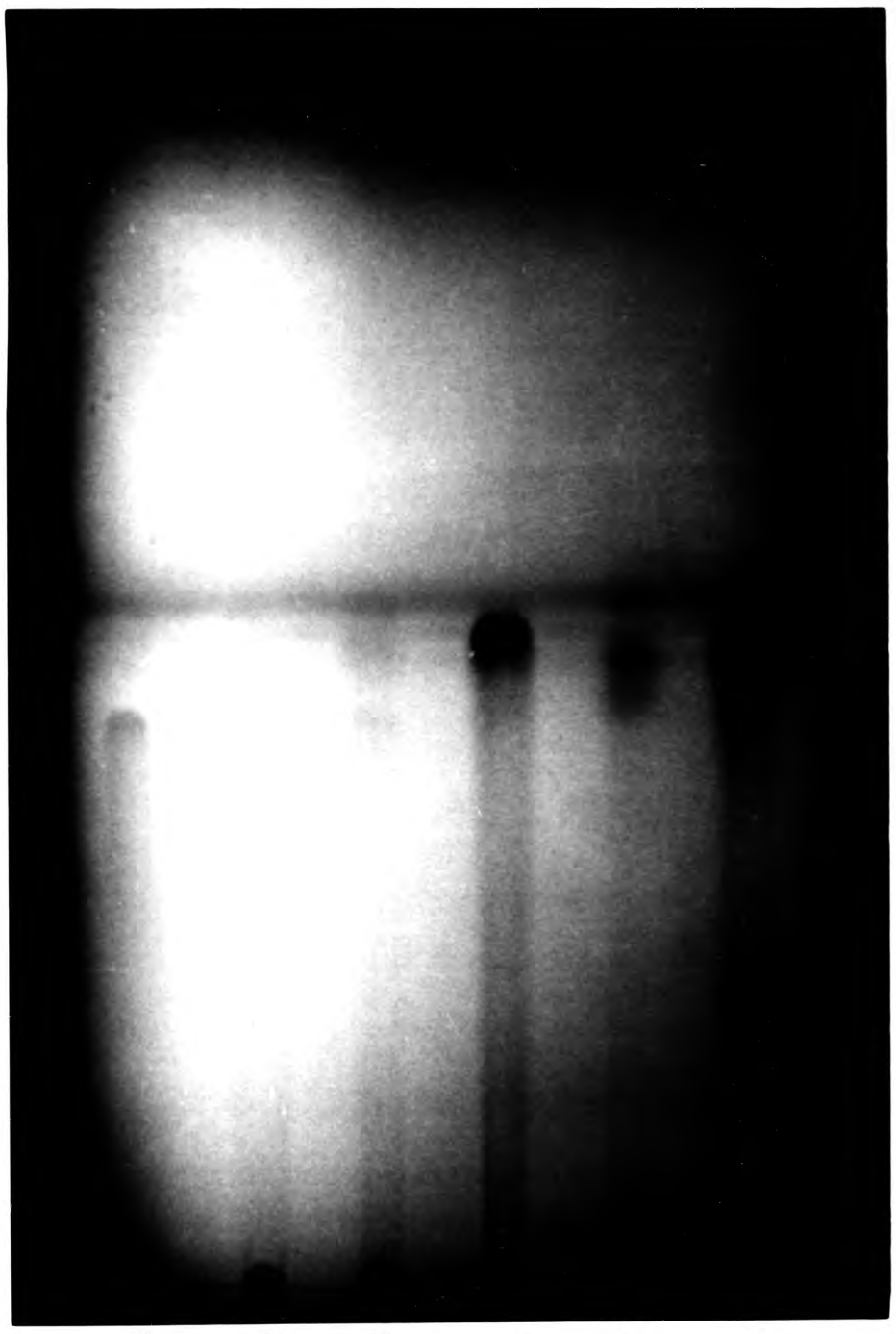
This plate shows the clear separation of the two compounds found under "peak 4". The sample at position 2 was taken from the final fractions of "peak 4".

FLUORESCEIN/AMMONIA REAGENT.

e

This reagent detects purines and pyrimidines. The samples at positions 4 and 6 absorb most strongly (a positive reaction) and they are derived from "peak 4 " material, having an Rf of 0.76. The samples at positions 1 and 2 are standards, a purine and pyrimidine.

FLUORESCEIN AMMONIA REAGENT.



e

This reagent detects purines and pyrimidines. The samples at positions 4 and 6 absorb most strongly (a positive reaction) and they are derived from "peak 4 " material, having an Rf of 0.76. The samples at positions 1 and 2 are standards, a purine and pyrimidine.

ALKALINE SILVER NITRATE REAGENT.

f

This reagent reacts with phenols and aromatic amines. There is a positive reaction to "peak 4" material (positions 3 and 5). Position 4 is material from "peak 3" and responds negatively to this reagent. Position 1 is the standard , amidol.

ALKALINE SILVER NITRATE REAGENT.

f

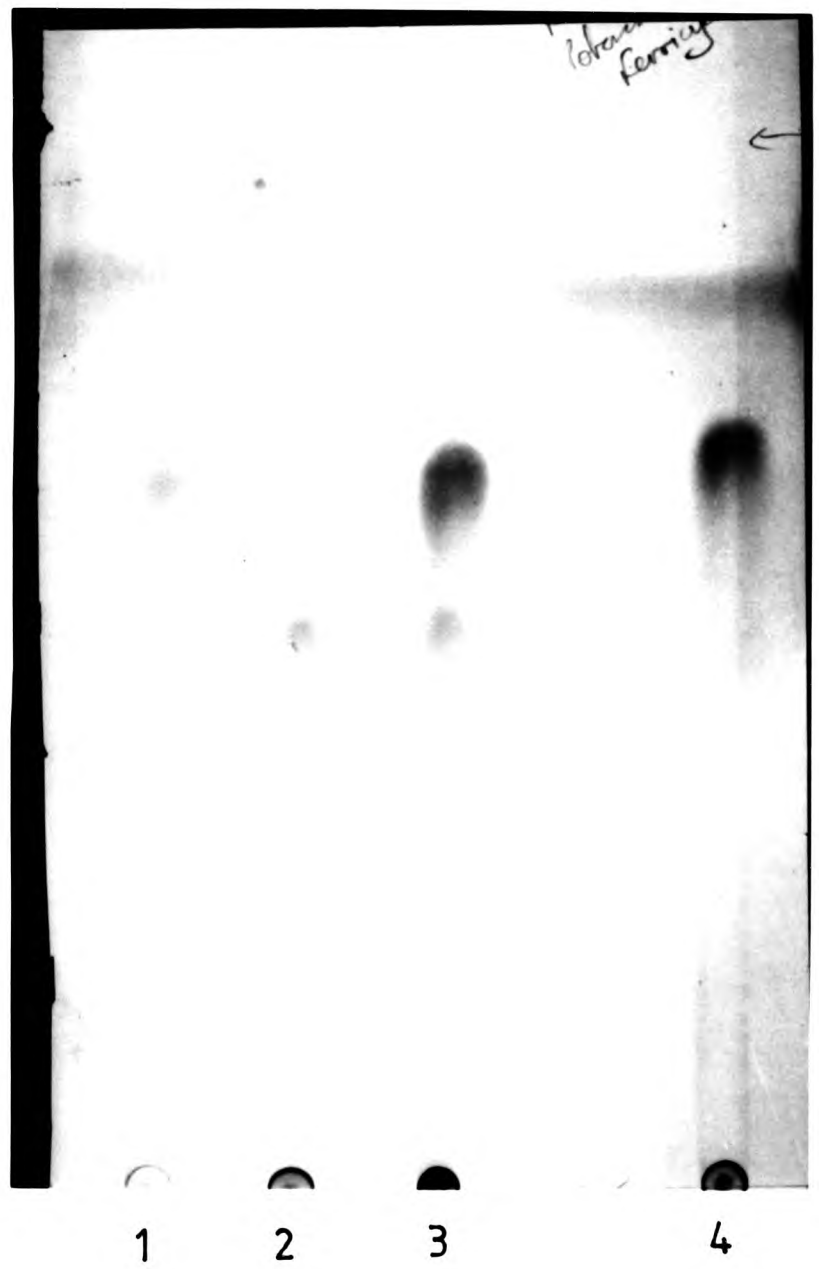
This reagent reacts with phenols and aromatic amines. There is a positive reaction to "peak 4" material (positions 3 and 5). Position 4 is material from "peak 3" and responds negatively to this reagent. Position 1 is the standard , amidol.

FERRIC CHLORIDE/POTASSIUM FERRICYANIDE REAGENT.

8

This reagent detects aromatic amines. This plate shows the reaction produced with various fractions from beneath "peak 4". Both the compounds, at Rf 0.76 and Rf 0.68 produce a dark blue colouration indicating that they are possibly aromatic amines.

FERRIC CHLORIDE/POTASSIUM FERRICYANIDE REAGENT.



g

This reagent detects aromatic amines. This plate shows the reaction produced with various fractions from beneath "peak 4". Both the compounds, at Rf 0.76 and Rf 0.68 produce a dark blue colouration indicating that they are possibly aromatic amines.

Photographs taken of the most informative TLC plates, soon after the spray reagents were used,are shown. Each plate was run with a suitable standard that could be expected to react with the spray reagent chosen.

Key to Plates.

a = Dimethylaminocinnamaldehyde reagent. Sample at position 5 shows two compounds coloured yellow. This sample is from the central fractions of "peak 4" indicating separation from the substance with a lower Rf value. As this reagent is a test for indoles, both substances may be indolic. Position 1 shows the colour reaction of pure indole with this reagent.

b = Iodine/potassium iodide reagent for alkaloids. The sample at position 1 is a standard alkaloid (atropine). No evidence is shown for the presence of an alkaloid in the test substances.

c = 'Ehrlichs'' reagent. This plate shows the effect of Ehrlichs' reagent on consecutive fractions of "peak 4". From left to right fractions applied to the plate are shown in table 37.

Table 37

Sample Positions.

Sample Position	Fraction Numbers
1	25-26
2	27-28
3	29-30
4	41-42
5	43-44
6	45+

It can be seen that the purple coloured component of lower Rf. value is only present in fractions 29 - 44.

d The sample at position 2 again shows reaction of Ehrlich's reagent with "peak 4" material. Position 1 is a fraction obtained from the final column fractions.

e Fluorescein/Ammonia spray for the detection of purines and pyrimidines. Sample at position 6 shows that only the "peak 4" compound with the highest Rf value absorbs ultra-violet light (photograph taken under ultra violet light). The sample at position 4 is pure material from the far right of "peak 4". Samples at positions 1 and 2 are pure nucleotide bases, run as standards.

f Alkaline silver nitrate solution. The sample in position 3 is material from the centre of "peak 4". Position 1 is a standard, amidol.

g Ferric chloride/Potassium ferricyanide for aromatic amines.

The sample at position 3 shows material from the centre of "peak 4". Position 4 (far right) shows material from the final fractions and position 2 shows material from the earliest fractions of "peak 4".

Discussion

The use of the ferric chloride/potassium ferricyanide reagent resulted from infra-red spectrophotometric evidence that a secondary amine was present; the ferric chloride reagent is able to react with aromatic amines. The reagent gave an immediate dark blue colouration with both the substances within "peak 4", thus indicating that both substances may be aromatic amines.

Ehrlich's reagent indicated that the two substances were structurally dissimilar, giving a yellow spot at Rf 0.76 which is indicative of an amine or a sulphonamide structure (Clark and Humphreys, 1970; Kirchner 1978). The substance at Rf 0.68 reacted with Ehrlich's reagent to give a deep purple colouration which is indicative of an indolic structure, eg 5 - hydroxyskatole. The substance with Rf 0.68 also showed fluorescence, when treated with Prochazka reagent, which is characteristic of indoles.

With alkaline silver nitrate solution the substance with Rf value 0.76 yielded brown spots which are characteristic of arylamines or phenols, (Smyth and McKeown, 1964). The plate is shown in photograph f.

Sprays used to test for sterols, organic acids and alkaloids all yielded negative results.

Conclusions

Thin layer chromatography indicated that the substance at Rf 0.76 had the characteristics of an aromatic amine, and possibly also a phenol. The lemon yellow colour obtained with Ehrlich's reagent allows the possibility of a sulphonamide structure.

The substance at Rf 0.68 gave strong indications of being an indole derivative, in that it reacts as an aromatic amine with ferric chloride/potassium ferricyanide, and shows purple colouration with Ehrlich's reagent and fluorescence under long wavelength ultra-violet light. The latter two reactions are typical of indole derivatives.

EXPERIMENT 1:4

Polarimetry

Use of a polarising microscope on a dried sample of the "peak 4" extract revealed that 90% - 95% of the crystals were yellow and did not rotate the plane of polarised light. The remaining crystals were tiny and did show optical rotation, indicating a dissymetric molecule. Unfortunately the polarimeter used was not sensitive enough for an accurate measurement of the angular rotation.

Discussion

The observations and results detailed above revealed the presence of two substances within the partially purified phosphataemic extract.

Fluorescence spectroscopy showed that the substance with absorption maximum 270nm does not fluoresce, whereas the peak at 345nm does fluoresce. When this is considered with the results of the thin - layer chromatography and polarimetry it is certain that two substances are present in the extract.

The results for thin layer chromatography indicate that either of the two compounds could be aromatic amines. The compound with Rf 0.68 may be an indole.

EXPERIMENT 2

Evidence That The Phosphataemic Fraction Contains No Substances Structurally Similar to Vitamin D_3

2:1 Water Solubility

Vitamin D_3 itself is organic soluble, hence the phosphataemic fraction cannot contain pure vitamin D_3. The dried phosphataemic fraction was completely insoluble in organic solvents such as chloroform and hexane.

2:2 Mass Spectrometry

If the phosphataemic fraction contained any vitamin D_3 metabolites that were rendered aqueous soluble by substituents or side chains, (as in Solanum malacoxylon) then analysis by Mass Spectroscopy would reveal breakdown products with identical molecular weights to fragments of vitamin D_3 breakdown products. Typical peaks occur at m/e 416 (Holick, Schnoes, DeLuca, Suda and Corradino, 1971) m/e 134 and m/e 380 for calcitriol and these are not seen in the mass spectrum of the two compounds.

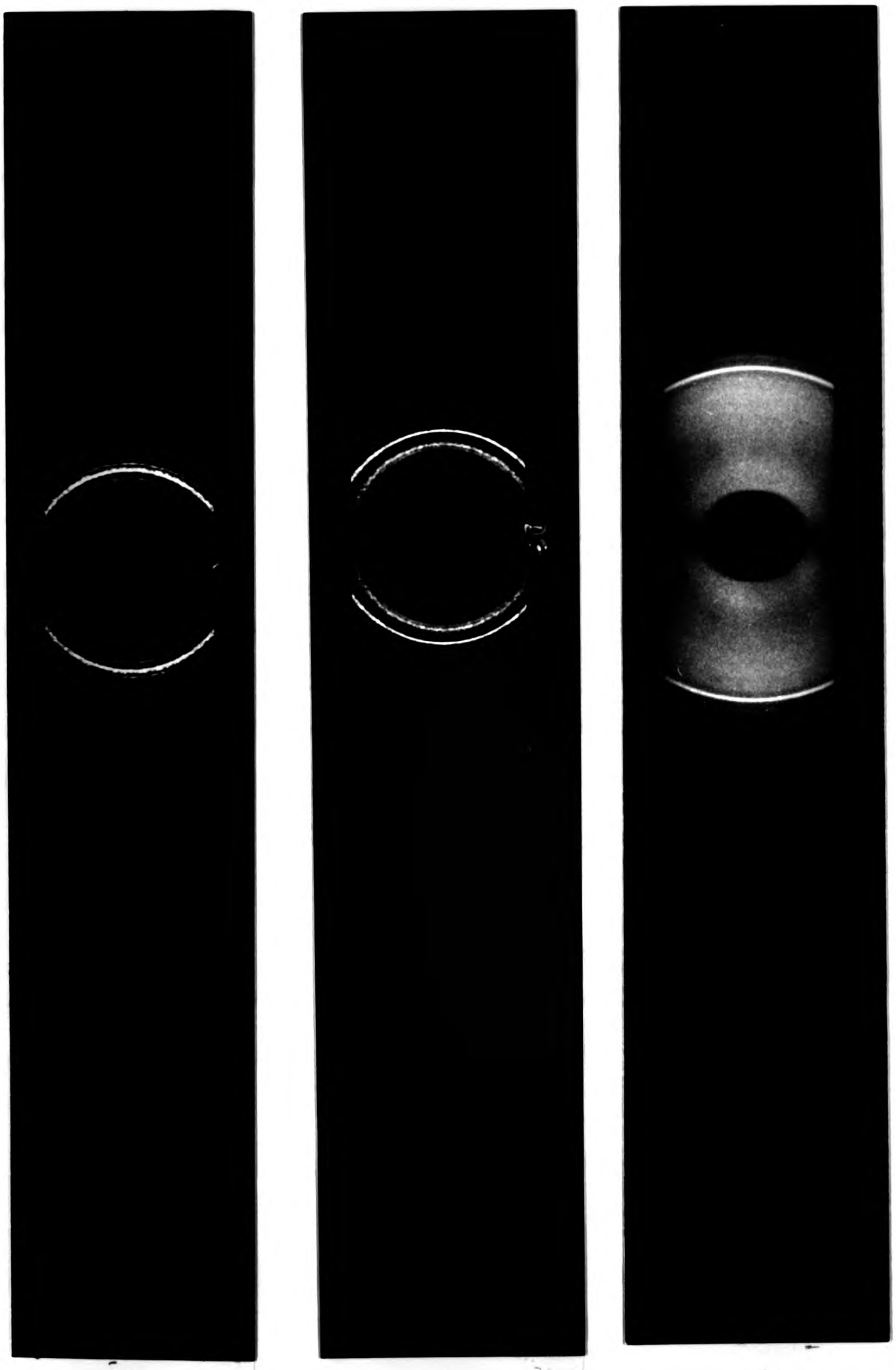
This suggests that neither compound is structurally related to vitamin D_3.

2:3 Comparison Between Vitamin D_3 And The Phosphataemic Fraction Using X-Ray Diffraction

The plates show the results of X-ray Powder Diffraction Analysis of both vitamin D_3 and the dried phosphataemic fraction (h - k).

PHOTOGRAPHS OF THE POWDER X-RAY DIFFRACTION PATTERNS

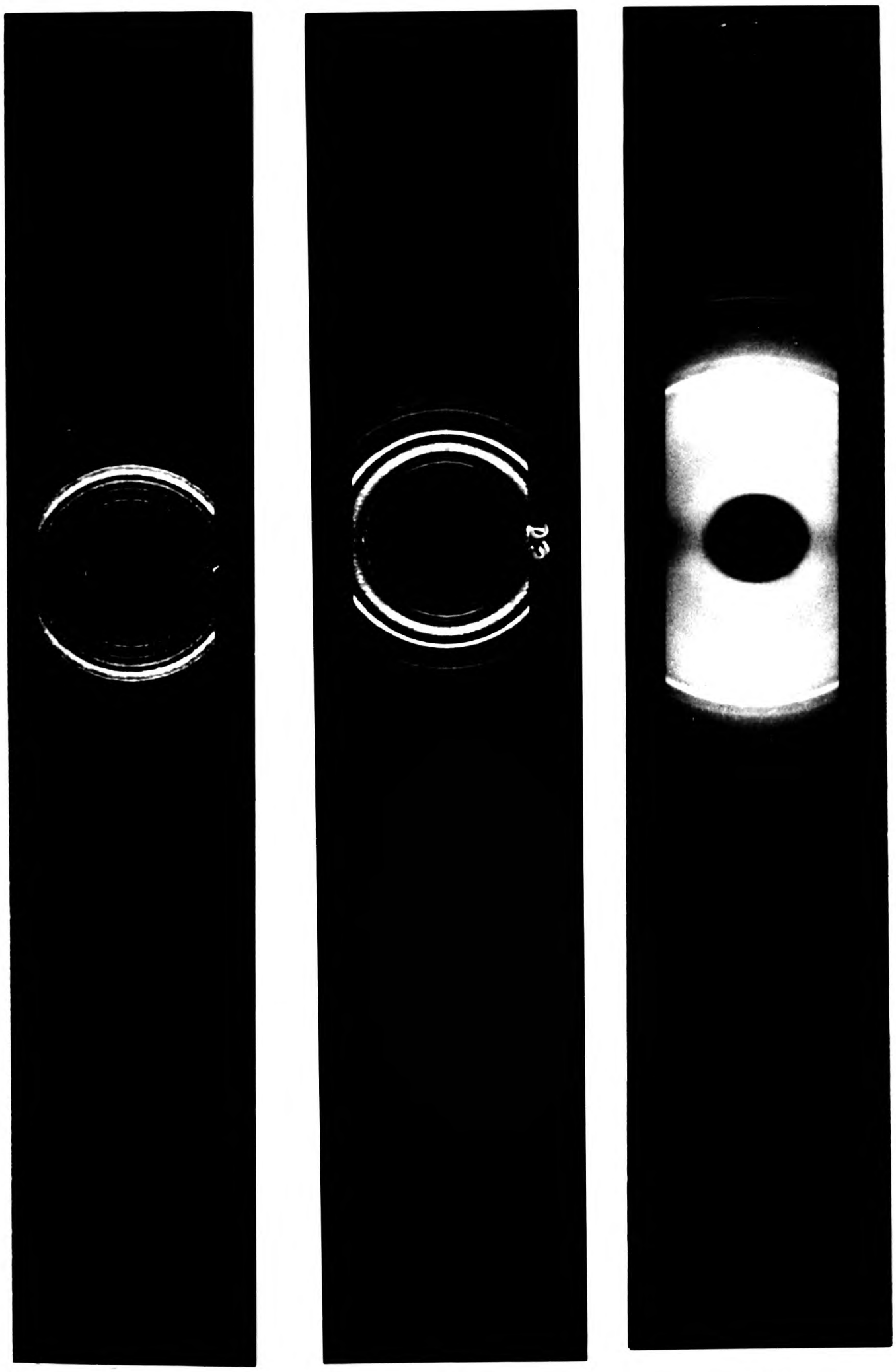
OF VITAMIN D_2, VITAMIN D_3 AND "PEAK 4".



h j k

PHOTOGRAPHS OF THE POWDER X-RAY DIFFRACTION PATTERNS

OF VITAMIN D_2, VITAMIN D_3 AND "PEAK 4".



X-ray diffraction is commonly used as a confirmatory technique in the analysis of the composition and stereochemistry of complex molecules. Powdered or crystalline material is capable of scattering incident X-radiation in a way dictated by the relative angles of the planes of the crystal lattice. The radiation is reflected as light on a mirror, and the resultant photograph of the diffracted X-rays gives a reliable fingerprint of the substance. If a mixture of substances is present then the two will produce superimposed photographic images. (Lipson and Steeple, 1970).

Therefore, the X-ray diffraction pattern for the phosphataemic fraction cannot be used to identify the structure of either compound but can be used to determine whether or not rings are comparable with those of vitamin D_3. It is clear that the two diffraction patterns are very different and thus vitamin D_3 is not present in the phosphataemic fraction. The above plates show the powder X-ray diffraction photographs of vitamin D_3, vitamin D_2 and "peak 4" material (plates j, h and k respectively).

2:4 Thin Layer Chromatography

The Carr-Price reagent is used to identify vitamin D_3 in thin-layer chromatography. When the extract was tested the reaction was negative - no orange reaction product was revealed. (See Appendix 3 for reagent composition).

2:5 Ultra-violet Spectroscopy

The ultra-violet absorption spectrum of 1,25$(OH)_2$ cholecalciferol has an absorption maximum of 265 nm in absolute ethanol. When

dissolved in pure ethanol "peak 4" has two absorption maxima. These are at 345 nm and 270 nm, which is similar to those obtained in aqueous solution. Vitamin D_3 has absorption maxima at 265 nm when dissolved in absolute ethanol.

Hence, it appears that the plant extract components have different structures and compositions from either of these vitamin D_3 compounds. It is likely that the two peaks of the plant fraction correspond to each of the compounds separated by thin layer chromatography in Experiment 1:3.

3. CHARACTERISATION OF THE "PEAK 4" MATERIAL

3:1 Confirmation of the Presence of a Secondary amine within "Peak 4"

The presence of an amine may be confirmed by using the nitrous acid test. Nitrous acid is liberated from the test substance by hydrochloric acid and sodium nitrate. The nitrous acid is then detected by starch-iodide paper. (Vogel,1978).

Method

A small amount of freeze dried "peak 4" material was dissolved in 0.5 ml 2M HCl, cooled in ice and then 0.2 ml ice-cold 10% aqueous sodium nitrite was slowly added.

Result

The solution gave an immediate positive result with starch-iodide paper in that the colour of the solution changed to orange/brown.

Conclusion

The "peak 4" material contains an amine.

Determination of the Type of Amine Present in "Peak 4"

(i) The "peak 4" material was subjected to more specific tests for aliphatic and aromatic primary amines which proved negative.

(ii) Similarly a test for tertiary aliphatic amines proved negative.

(iii) The presence of the orange-brown colour following the nitrous acid test was indicative of a secondary amine. This was confirmed by the Liebermann nitroso reaction. The nitrosamine (formed by the nitrous acid test) is warmed with phenol and concentrated sulphuric acid. The latter liberates nitrous acid from the nitrosamine and this reacts with the phenol to form p-nitrosophenol The latter can then be combined with another molecule of phenol to give red indophenol.

Method

The oil from the nitrous acid test was extracted with ether and washed with water, dilute sodium hydroxide and again water. The ether was then evaporated. The nitroso compound was added to phenol, warmed, then cooled and concentrated sulphuric acid was added. The solution then turned purple and became red on addition of the solution to an excess of cold water.

Conclusion

The "peak 4" material contains a secondary amine.

3:2 The Effect of Solvent pH on the Partially Purified Material from "Peak 4"

Introduction

The effects of solvent pH on the ultra-violet absorption spectrum of the extract, in aqueous solution, was examined.

Methodology

An aqueous solution of the material from "peak 4" was placed in a cuvette and the absorption spectrum scanned between 200 nm and 500 nm. The resultant absorption profile was drawn out on a chart recorder. The pH of the solution was then carefully varied and the absorption profile at each pH drawn superimposed on previous spectra. Sodium hydroxide or hydrochloric acid was used to raise or lower the pH respectively.

Results

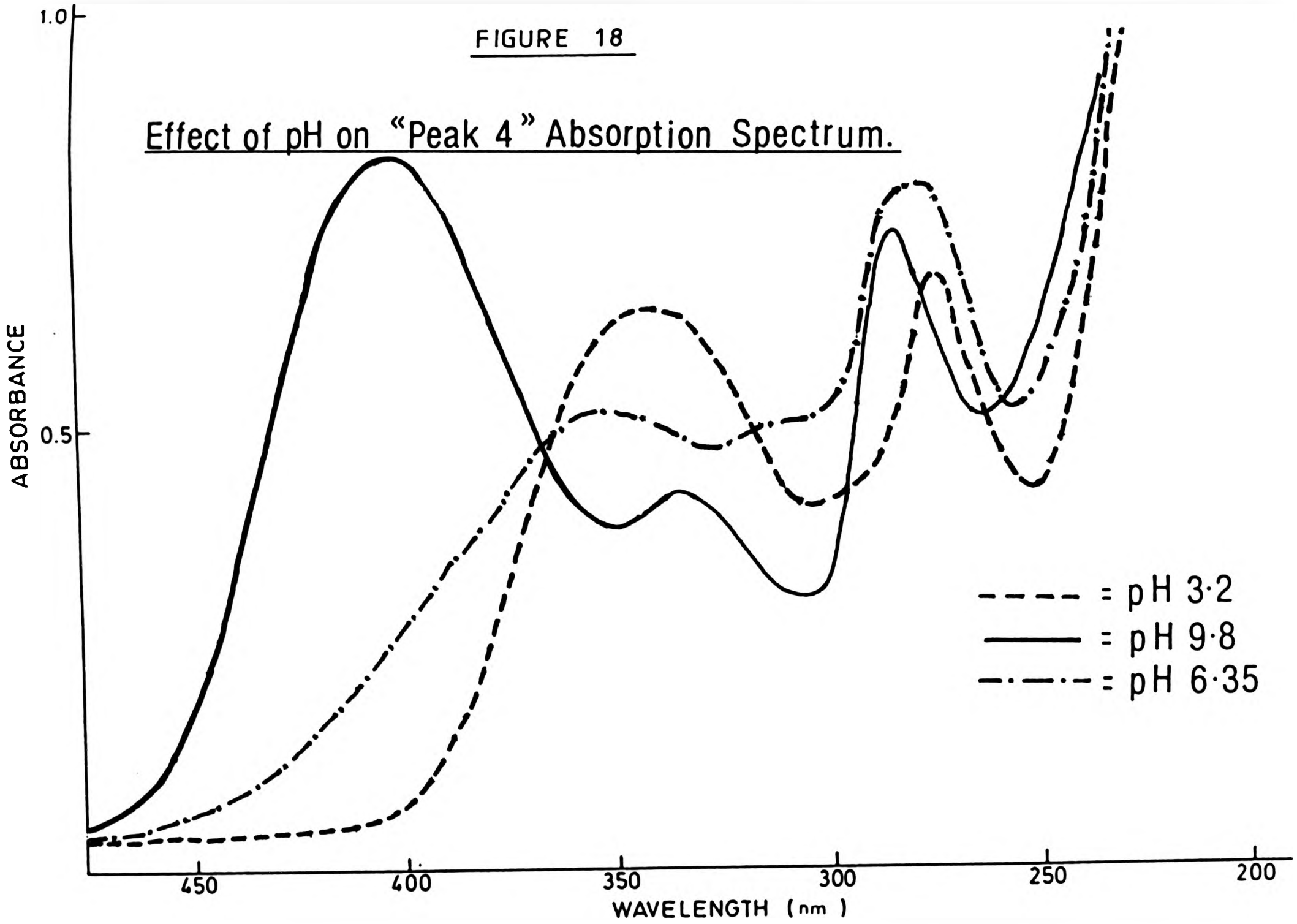
It was found that the solution became a deep yellow colour at high pH values. Below pH 6.0 the solution became colourless. The different spectra are shown in the following Figure 18.

Discussion

The results of previous experiments have shown that two substances are present in the partially purified phosphataemic extract, in particular fluorescence spectroscopy indicated that the peaks

FIGURE 18

Effect of pH on "Peak 4" Absorption Spectrum.



(at neutral pH) at 270 nm and 345 nm belonged to separate compounds. This experiment also suggested that two individual absorption spectra are present because the peak at pH 6.35 is at 350 nm and this shifts to 400 nm at pH 9.8. The 270 nm peak only shifts to 283 nm. Similarly, the 350 nm peak at pH 6.35 shifts to 340 nm at pH 3.2, while the 270 nm peak is unchanged. The increase in absorption maxima under higher pH conditions is termed a'batho-chromic shift'and is often accompanied by an increase in the intensity of absorption as the result of a substituent attaching to the molecule. (Dawber and Moore, 1973). There is evidence of an increase in the intensity of the peak at 400 nm with increasing pH, which is indicative of a 'bathochromic shift'; the 270 nm peak, however, shows little variation in intensity with changing pH.

These observations indicate that the substance with absorption maximum 350 nm (pH 6.35) becomes hydroxylated at higher pH values and that the hydroxyl group(s) is lost at lower pH.

The deepening in colour of an alkaline solution (to yellow) is typical of primary and secondary aliphatic nitro compounds and also where there is a nitro group substituted in the 'o' and 'p' positions of an aromatic phenol. The latter observation would best fit the compound under study. It may be that the hydroxy group is incorporated as the result of raising the pH and the nitro-groups were part of the original molecule. The hydroxyl group would then be lost on acidification (generating a clear solution).

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6

GENERAL DISCUSSION

The speed with which Trisetum flavescens could induce the clinical signs of calcinosis in domestic ruminants (Simon, Daniel, Hanichen and Spiess, 1978) was an indication that a potent calcinogen was present. Researchers had found calcinogenic activity in organic extracts of the plant which produced significant elevations in the plasma phosphate levels of rats (Zucker, Kreutzberg and Rambeck , 1977) and thus it was clear that the calcinogenic activity of Trisetum flavescens was not immediately comparable with that of Solanum malacoxylon which possesses a water soluble calcinogen. Furthermore, Rambeck, Oesterhelt, Vecchi and Zucker (1979) were unable to detect significant amounts of vitamin D_3-related substances in organic extracts of Trisetum flavescens.

The presence of a phosphataemic principle in organic extracts is confirmed in Chapter 3 (Experiment 1) where the organic extract is reported to promote intestinal phosphate transport in vitamin D_3-deficient chicks. That this is not likely to be caused by vitamin D_3 or $1,25(OH)_2D_3$ is suggested by a) the findings of Rambeck et al (1981) that only 0.1 ppm vitamin D_3 and no $1,25(OH)_2D_3$ is present in the organic fractions. These workers used lyophilised Trisetum flavescens for their bioassays for vitamin D_3 - potency of the plant and it was recognised that the organic fractions might not possess all of the calcinogenic activity; and b) the findings of Zucker, Kreutzberg, Nitsche and Bittner (1979) who showed that organic extracts of the plant were unable to overcome strontium inhibition of the renal

25(OH) cholecalciferol - 1α - hydroxylase.

The results of the present study (chapter 3, experiment 2) which indicate that organic extracts are not able to promote intestinal calcium transport in vitamin D_3- deficient birds are in accordance with Zucker's results. The results reported in chapter 3 (experiment 1) indicate that the increase in intestinal phosphate transporting ability of vitamin D_3- deficient birds given a single dose of an organic extract (equivalent to 1.5 g dried plant) is in excess of that in birds fed a vitamin D_3- replete diet. On the other hand, vitamin D_3- deficient birds given 500 iu vitamin D_3 and subsequently tested for radiophosphate transport (chapter 4, experiment 7) showed a low net transfer of phosphate combined with much higher levels of phosphate 'trapped' in the gut tissue when compared with either controls or "peak 4" treated birds. Vitamin D_3 - deficient controls have both a low level of radiophosphate 'trapped' in the gut tissue and a low net transfer to the body.

Thus, there appears to be a difference in the 'pattern' of phosphate transport induced by vitamin D_3 and organic extracts of the plant. The rate limiting step in the transfer of phosphate from the gut lumen to the bloodstream is the transfer out of the gut tissue and into the blood (Wasserman and Taylor, 1972). These workers thought that this process was vitamin D_3 - dependent although later workers have thought that only diffusion is involved. Peterlik and Wasserman (1980) and Fuchs and Peterlik (1979) state that vitamin D_3 only affects phosphate uptake from the mucosal side, by increasing the Vmax of the reaction.

There appear to be two D_3-dependent trans-cellular mechanisms for phosphate transfer across the mucosal surface - one Na^+-dependent and the other Ca^{2+} -dependent - and a non-vitamin D_3-dependent paracellular route. Very little is known about the transfer of phosphate to the bloodstream, except that a Na^+ K^+ ATPase is involved (Taylor, 1974) with the sodium dependent process. The Na^+ K^+ ATPase is the means by which the sodium ion gradient is formed and maintained (Matsumoto, Fontaine and Rasmussen, 1980). Later research has shown that the Ca^{2+} -dependent phosphate transport process is probably associated with an intestinal calcium-dependent alkaline phosphatase, which in turn is stimulated by calcium and magnesium ions. The calcium-dependent alkaline phosphatase could be acting as an ATPase (Birge and Avioli, 1981). It is not possible to tell which of the transport mechanisms is being influenced by the organic extracts of Trisetum flavescens but it is most likely to be the paracellular pathway.

Experiment 2 (chapter 3) reveals the non-identity of the final purified extract used in Experiment 1 with vitamin D_3; a higher dose level was used (= 2.7g) and yet vitamin D_3(200Iu/chick) stimulated the transport of significantly higher levels of radio-calcium into the bloodstream. This again suggests that the plant extracts influence phosphate transport and do not affect vitamin D_3-dependent calcium transport.

In addition, biologically active fractions of the organic extract of Trisetum flavescens show little variation from control values in their effects upon the time course of radiocalcium release from

bone tissue cultured *in vitro*. The lack of evidence for calcium release from bones treated with organic extracts, together with the fact that there is no significant release of phosphate from bones cultured *in vitro* suggests that the high plasma phosphate levels of animals treated with these extracts result from increased intestinal transport and not bone resorption. An increase in the renal retention of phosphate is also a possibility but it was not possible for this to be investigated in this project. Dirksen has reported elevations in urinary phosphate (Dirksen, Plank, Dammrich and Hanichen, 1970) in cattle with *Trisetum flavescens*-induced calcinosis and thus increased renal reabsorption of phosphate is not likely to be the cause of the phosphataemia.

Thus the organic soluble phosphataemic factor is not directly comparable with either vitamin D_3 or calcitriol and yet has a potent effect upon the transfer of phosphate to the bloodstream. It could therefore be influencing the, as yet unidentified, mechanism (s) for phosphate uptake by the bloodstream from the gut.

The technique of strontium inhibition of the kidney 25(OH) cholecalciferol-1α-hydroxylase as a model for testing calcitriol-like effects of calcinogenic plants has been used in investigations into the activity of purified organic extracts of *Trisetum flavescens*. The results showed that no reversal of the strontium-induced vitamin D_3-deficiency occurred (Zucker at al 1979) and yet when whole lyophilised leaves of the plant were used (Peterlik, Regal, Kohler, 1977) it was found that there was a reversal of the effects of strontium. Similarly, workers found a high level of vitamin D_3

potency in lyophilised plant material used in the 'Japanese Quail Egg-Shell Test' Rambeck, Kreutzberg, Bruns-Droste and Zucker (1981), and yet it was only the organic extracts that were examined for vitamin D_3 content. These observations suggested that vitamin D_3-like activity might reside in aqueous soluble extracts of the plant.

Investigations into the activity of aqueous soluble extracts of the plant, as described in Chapter 4, have shown this to be the case. Activity found in a crude aqueous extract of the plant was quickly shown to be unlike that of Solanum malacoxylon. Feeding an active aqueous extract of Solanum malacoxylon to vitamin D_3-deficient rats caused an increase in plasma calcium after twenty-four hours, following an initial decline in the levels, which reached a maximum at forty-eight hours. Plasma phosphate, in the same animals, showed a decline at twenty-four hours after an initial transient increase (Campos, Ladizesky and Mautalen, 1973). In contrast, a crude aqueous extract of Trisetum flavescens given to vitamin D_3-deficient chicks caused a significant increase in plasma phosphate at forty-eight hours, and little change in plasma calcium. This compares well with the situation seen in cattle suffering from Trisetum flavescens-induced calcinosis where plasma phosphate levels are persistently above normal.

There is evidence (chapter 4, experiment 2) that the phosphataemic effect of the crude aqueous extract of Trisetum flavescens is only active when dosed orally. This is surprising, since the purified organic extract (chapter 3, experiment 1) and the partially purified aqueous soluble extract ("peak 4": chapter 4, experiment 7) are

both shown to increase plasma phosphate levels when dosed sub-cutaneously. Also, the crude aqueous extract (chapter 4, experiment 1) and whole lyophilised plant will raise plasma phosphate when given orally and so the decline in both plasma phosphate and [Ca x P_i] when the crude aqueous extract is dosed non-orally, is difficult to interpret. It is possible that the aqueous soluble calcemic and the phosphataemic factors complex when dosed in this fashion.

The evidence showing that the crude aqueous extract, when dosed sub-cutaneously or intra-muscularly causes a rise in plasma calcium levels (significantly higher than in orally dosed birds) suggested that the crude aqueous extract possessed a vitamin D_3-like substance capable of stimulating calcium transport. To determine the ability of the crude aqueous extract to promote intestinal calcium transport, use was made of the 'strontium chick model' (experiment 4(i) chapter 4). As shown by Peterlik at al (1977), who used whole lyophilised plant leaf, the crude aqueous extract was found to be able to promote intestinal calcium transport. Therefore the contradictory results of Peterlik at al (1977) and Zucker at al (1979) can be explained in the light of these results, namely, that a phosphataemic factor, not similar to calcitriol, is present in organic soluble fractions of Trisetum flavescens, whilst an aqueous soluble constituent is present which resembles calcitriol, at least in its action. Thus only the use of whole lyophilised plant material or aqueous extracts can result in the reversal of strontium inhibition of calcium transport.

Evidence for a third aqueous soluble calcinogenic factor in the

plant was found when partially purified material obtained from under the final major peak ("peak 4"), following Sephadex chromatography (See Figure 8) was tested in the strontium chick model and compared with the previous major peak ("peak 3"). This revealed that "peak 4" could significantly raise plasma phosphate levels but had no significant effect upon plasma calcium or calcium transport levels. "Peak 3", however, did significantly increase intestinal calcium transport and plasma total calcium levels were above those of control birds although not significantly so. It can therefore be presumed that the stimulation of calcium uptake seen in chicks having strontium-induced vitamin D_3-deficiency, and dosed with the crude aqueous extract, is brought about by the activity found in "peak 3" material. Further, this active fraction, in order to overcome strontium inhibition, must be akin to a calcitriol analogue or a substance able to mimic the effects of calcitriol, or calcitriol itself linked to a water-soluble constituent as seen in the sterol-glycoside of Solanum malacoxylon. This would explain the sizeable increase in $[Ca \times P_i]$ since plasma phosphate is also raised when vitamin D_3-deficient chicks are given a crude aqueous extract of Trisetum flavescens, as well as the stability of plasma calcium levels in affected cattle where plasma calcium is maintained at a little above normal despite large increases in plasma inorganic phosphate.

Investigations into the mode of action of the aqueous soluble phosphataemic factor ("peak 4") showed that it raised plasma phosphate levels in chicks with strontium-induced vitamin D_3 deficiency, dietary vitamin D_3 deficiency as well as in vitamin D_3-replete birds (experiments 5, 6(i) and 6(ii) respectively, chapter 4).

The increase in plasma phosphate levels appears to reach a 'ceiling' only in the vitamin D_3-replete birds. This, I would suggest, was due to the maximal solubility product being exceeded after a twelve hour period leading to precipitation of calcium and phosphate and resulting in the slow decline in the $[Ca \times P_i]$. It therefore appears that this aqueous soluble phosphataemic factor is operative in both vitamin D_3-replete and vitamin D_3-deficient animals and appears to affect only plasma phosphate directly.

Comparison of the effects of vitamin D_3 and "peak 4" upon ileal radio-phosphate transport (experiment 7, chapter 4) reveals a great dissimilarity between the effects of the two treatments. Vitamin D_3 and "peak 4" had opposite effects upon plasma calcium, phosphate and alkaline phosphatase levels although the latter were not significantly different from vitamin D_3-deficient controls. "Peak 4" had a greater effect upon radio-phosphate transport and showed only control levels of isotope within the gut tissue, but higher levels of isotope transported into the bloodstream. "Peak 4" can be seen to differ from calcitriol as it does not increase calcium transport in chicks with strontium-induced vitamin D_3-deficiency (experiment 5, chapter 4). These observations suggest that the factor under "peak 4" is able to influence one of the mechanisms involved in intestinal phosphate uptake. Furthermore, the phosphataemic factor will only bring about an increase in the $[Ca \times P_i]$ in vitamin D_3-replete chicks; in vitamin D_3-deficient chicks it produces a steep decline in calcium levels which is accompanied by a stable $[Ca \times P_i]$. In other words, calcium is being displaced by phosphate in the $[Ca \times P_i]$ equilibrium presumably because of calcium phosphate

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deposition in soft tissues, and of course the calcium homeostatic system is not able to maintain normo-calcaemia (experiment 6(i) chapter 4) because the animals are vitamin D_3-deficient.

This is very illustrative of the concept that phosphate transport is here being stimulated independently of the vitamin D_3-endocrine system. It is widely accepted that phosphate uptake is independent of calcium absorption by the gut, and in this instance it appears that the aqueous soluble phosphataemic factor stimulates a phosphate transport mechanism without having any effect upon calcium metabolism. The pathway of phosphate transport here is possibly the paracellular route which is independent of vitamin D_3 and is thought to be the... "only possible route for phosphate transport in vitamin D_3-deficient animals" (Fuchs and Peterlik, 1979).

It is possible that the aqueous soluble phosphataemic factor increases the permeability of the intestinal epithelium in the regions of these paracellular pathways. Hence it may be concluded that Trisetum flavescens contains three distinct biologically active factors that are able to influence calcium and phosphate metabolism in animals,(four if the low level of vitamin D_3 is included, although the level of 4 Iu/g dry Trisetum flavescens of vitamin D_3 per se would not produce a significant response in chicks at the dosages used).

One of these three major calcinogens is organic soluble and influences intestinal phosphate transport. It is able to raise the plasma [Ca x P_i] of vitamin D_3-deficient chicks and lower plasma alkaline

phosphatase. The two latter effects are indicative of the presence of vitamin D_3. However, the organic soluble factor does not stimulate intestinal calcium transport in vitamin D_3-deficient chicks.

Aqueous soluble extracts contain two factors, one of which is similar to that found in Solanum malacoxylon in that it appears to act in the manner of a water-soluble form of calcitriol. Finally there is a potent phosphataemic factor in the aqueous extract that has effects upon the levels of plasma phosphate similar to those of the organic soluble phosphataemic factor. The major difference between the modes of action of the two phosphataemic substances is that the aqueous soluble one causes a dramatic decline in plasma calcium levels in vitamin D_3-deficient animals whereas the organic soluble one does not, this is possibly due to contamination with vitamin D_3.

Rambeck has found sizeable amounts of vitamin D_3 in the milk of lactating ewes fed Trisetum flavescens (Rambeck, Elmer, Dirksen, Krausslich and Zucker, 1980) which suggests that more than the estimated 4 Iu/g of vitamin D_3 may be present in the plant.

Investigations into the physico-chemical properties of the aqueous soluble phosphataemic factor ("peak 4", chapter 5)revealed the presence of two substances within the fraction, one with fluorescent properties and one without. Analysis of this mixture using X-ray diffraction and Mass Spectrometry techniques gave strong evidence that neither substance was structurally similar to vitamin D_3. The two compounds could be separated by thin layer chromotography, but time did not permit the use of preparative TLC in order to further test these substances in vivo. Evidence was obtained

that one substance was a secondary aromatic amine, and the other possibly had an indolic structure.

The mixture, when dissolved in water, showed pronounced changes in colour and ultra-violet absorption profile at different pH values. This is indicative of the presence of a nitro compound. Fluorescence spectroscopy revealed that the two peaks seen in the ultra-violet absorption belonged to each of these two constituents.

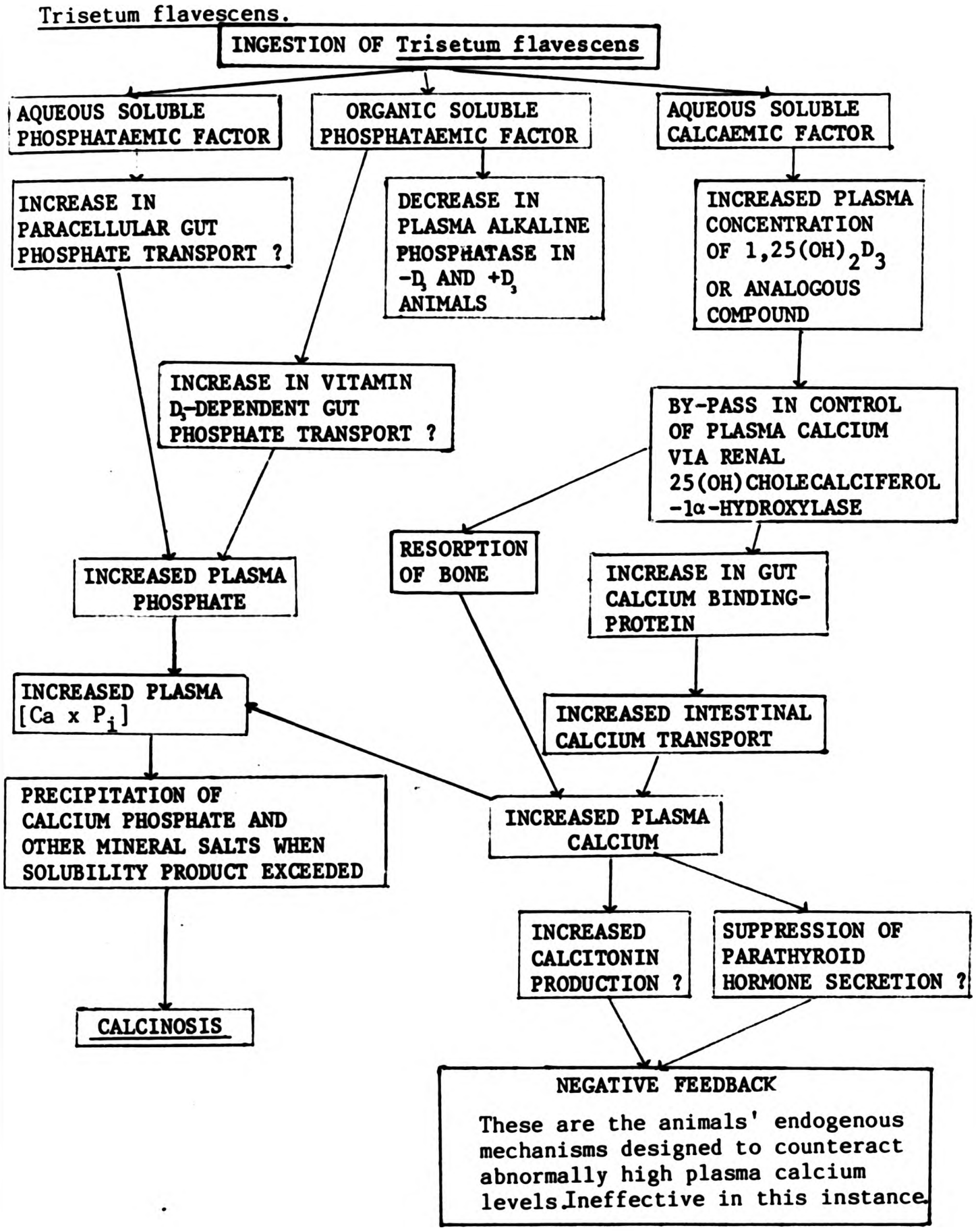
TABLE 38

Comparison of the Physiological Effects of the Three Calcinogenic Factors of Trisetum flavescens with Whole Lyophilised Plant Leaf.

		TRISETUM FLAVESCENS EXTRACT		
PARAMETER	WHOLE LYOPHILISED PLANT LEAF (according to other workers)	ORGANIC SOLUBLE EXTRACT	AQUEOUS SOLUBLE EXTRACT"(PEAK 3)"	AQUEOUS SOLUBLE EXTRACT"(PEAK 4)"
PLASMA PHOSPHATE	INCREASED	SIGNIFICANTLY INCREASED IN VITAMIN D_3-DEFICIENT CHICKS	UNCHANGED IN VITAMIN D_3-DEFICIENT(STRONTIUM FED) CHICKS	SIGNIFICANTLY INCREASED IN BOTH VITAMIN D_3-DEFICIENT AND REPLETE CHICKS
PLASMA CALCIUM	SLIGHT INCREASE	SLIGHT INCREASE IN VITAMIN D_3-DEFICIENT CHICKS(NOT SIGNIFICANT)	SLIGHT INCREASE IN STRONTIUM FED CHICKS (NOT SIGNIFICANT)	FALLS SHARPLY IN VITAMIN D_3-DEFICIENT CHICKS. SMALL DECLINE IN VITAMIN D_3-REPLETE CHICKS.
$[Ca \times P_i]$	INCREASED	SIGNIFICANT INCREASE IN VITAMIN D_3-DEFICIENT CHICKS	NO SIGNIFICANT INCREASE - UNCHANGED IN STRONTIUM FED CHICKS	INCREASED IN VITAMIN D_3-REPLETE CHICKS. UNCHANGED IN VITAMIN D_3-DEFICIENT CHICKS
PLASMA ALKALINE PHOSPHATASE	DECREASED IN VITAMIN D_3-REPLETE ANIMALS	DECREASED IN VITAMIN D_3-DEFICIENT CHICKS (NOT SIGNIFICANTLY)	UNCHANGED	UNCHANGED IN VITAMIN D_3-DEFICIENT CHICKS
INTESTINAL CALCIUM TRANSPORT	STIMULATES CALCIUM TRANSPORT IN STRONTIUM-INDUCED VITAMIN D_3-DEFICIENCY	NO SIGNIFICANT INCREASE IN VITAMIN D_3-DEFICIENT CHICKS	SIGNIFICANTLY INCREASED IN THE "STRONTIUM CHICK"	NO EFFECT IN STRONTIUM FED CHICKS
INTESTINAL PHOSPHATE TRANSPORT	NOT TESTED	SIGNIFICANTLY INCREASED IN VITAMIN D_3-DEFICIENT CHICKS	NOT TESTED	INCREASED IN VITAMIN D_3-DEFICIENT CHICKS
BONE RESORPTION	NO EFFECT OF ORGANIC SOLUBLE EXTRACT ON BONE	NO EFFECT	SIGNIFICANTLY INCREASES MEDIUM ^{40} CALCIUM LEVELS	NO EFFECT

FIGURE 19

Diagrammatic Representation of Proposed Mechanism of action of Trisetum flavescens.



SUMMARY

1. Studies have been conducted on the calcinogenic plant Trisetum flavescens in order to determine the physiological actions and chemical nature of the toxic substance or substances.

2. An organic soluble substance was extracted and purified using HPLC and shown to promote intestinal transport of radio-phosphate, increase plasma levels of inorganic phosphate, reduce plasma alkaline phosphatase; but to have no effect on plasma total calcium, calcium transport across the gut or bone resorption when studied in vitro.

3. An aqueous soluble substance was extracted and partially purified on Sephadex columns, which when administered to both vitamin D_3-deficient and vitamin D_3-replete animals raised plasma inorganic phosphate levels but unlike the organic soluble extract, caused a dramatic decline in plasma total calcium. Intestinal radio-phosphate transport was stimulated supposedly by the paracellular pathway of phosphate transport.

4. A second aqueous extract was shown to have no effect on plasma phosphate, but significantly increased intestinal transport of calcium and strontium in chicks with a metabolic deficiency of vitamin D_3 caused by the inclusion of strontium in the diet. This substance appears to be able to mimic the actions of 1,25 $(OH)_2$cholecalciferol.

5. The potent aqueous soluble phosphataemic factor is not related chemically to vitamin D_3 but has the characteristics of an aromatic indole or amine with nitro groups.

6. The organic soluble phosphataemic factor is apparently not vitamin D_3 since it did not promote intestinal transport of calcium in chicks with a privational deficiency of vitamin D_3.

7. It is concluded that the actions of these three substances in raising plasma [Ca x P_i] chiefly by increasing intestinal absorption of phosphate, results in calcinosis.

APPENDICES

APPENDIX 1

ANIMAL HUSBANDRY

Day-old cock chicks were obtained from hatcheries and housed in large steel cages (1m square) on sterile sawdust. They were kept warm using powerful white-light lamps (Philips). The varieties used were either 'Ross-Brown' or "RIR x LS'. They were fed and watered ad-lib and separated into smaller groups as they grew to avoid overcrowding.

DIETS

The basic diet used was always obtained from the University of Reading and had the following compositions:

INGREDIENTS OF BASIC DIET.

Ingredient	% Composition
Ground Maize	48
Soyabean Meal	44
Soyabean Oil	4
Limestone Granules	0.8
Sodium Chloride	0.54
D-L Methionine	0.06
Dicalcium Phosphate	2.6

(N.B. Limestone granules were omitted from diets in which strontium was supplemented, giving a calcium content of 0.15%).

A vitamin/trace element mix was obtained from Beecham Pharmaceuticals. This was in a finely powdered form suitable for addition to animal feeds, and was carefully added to the basic diet to give the following composition:

BEECHAM VITAMIN/TRACE ELEMENT MIX

COMPONENT	CONCENTRATION/KG BASIC DIET
Vitamin A	8000 units
Vitamin B_{12}	5µg
Vitamin B_2	6.4mg
Vitamin E	10 Iu
Vitamin K	1.6mg
Pantothenic Acid	8.0mg
Nicotinic Acid	14.4mg
Folic Acid	1.0mg
Choline	250mg
Copper	4.8mg
Manganese	64.0mg
Iodine	1.6mg
Selenium	0.1mg

Vitamin D_3 was supplied separately for addition to the vitamin D_3-replete diets. The supplement was added to the diet at a level of 2000 Iu/Kg.

APPENDIX 2

FORMULATION OF MEDIUM BGJ_b (Fitton-Jackson, modification)

(Supplied by GIBCO Life Technologies Inc.)

COMPONENT	CONCENTRATION mg/l
INORGANIC SALTS	
$NaH_2PO_4H_2O$	90.0
$MgSO_4\ 7H_2O$	200.0
KCl	400.0
KH_2PO_4	160.0
$NaHCO_3$	3,500.0
NaCl	5,300.0
OTHER COMPONENTS	
Calcium lactate	555.0
Glucose	10,000.0
Phenol red	20.0
Sodium acetate (anhyd)	50.0
AMINO ACIDS	
L - Alanine	250.0
L - Arginine	175.0
L - Aspartic acid	150.0
L - Cysteine HCL-H_2O	101.0
L - Glutamine	200.0
Glycine	800.0
L - histidine	150.0
L - Isoleucine	30.0
L - Leucine	50.0
L - lysine	240.0

AMINO ACIDS Contd/...

L - Methionine	50.0
L - Phenylalanine	50.0
L - Proline	400.0
L - Serine	200.0
L - Threonine	75.0
L - tryptrophan	40.0
L - tyrosine	40.0
DL - Valine	65.0

VITAMINS

Alpha Tocopherol phosphate (disodium salt)	1.0
Ascorbic acid	50.0
Biotin	0.2
D - Calcium Pantothenate	0.2
Choline chloride	50.0
Folic acid	0.2
i - inositol	0.2
Nicotinamide	20.0
Para-aminobenzoic acid	2.0
Pyridoxal phosphate	0.2
Riboflavin	0.2
Thiamine hydrochloride	4.0
Vitamin B_{12}	0.04

FORMULATION OF MEDIUM 199 (Hanks' Salts)

COMPONENT	CONCENTRATION (mg/l)
INORGANIC SALTS	
$CaCl_2$ (anhyd.)	140.0
$Fe(NO_3)_3.9H_2O$	0.72
KCl	400.0
KH_2PO_4	60.0
$MgSO_4.7H_2O$	200.0
NaCl	8,000.0
$NaHPO_4.H_2O$	90.0
OTHER COMPONENTS	
Adenine sulphate	10.0
Adenosinetriphosphate (disodium salt)	1.0
Adenylic acid	0.2
Cholesterol	0.2
Deoxyribose	0.5
Glucose	1,000.0
Glutathione	0.05
Guanine (free base)	0.3
Hypoxanthine	0.3
Phenol red	20.0
D-ribose	0.5
Sodium acetate	50.0
Thymine	0.3
Tween 80	20.0
Uracil	0.3
Xanthine	0.3

AMINO ACIDS	CONCENTRATION (mg/l)
DL - Alpha - Alanine	50.0
L - Arginine HCl	70.0
DL - Aspartic acid	60.0
L - Cysteine - $HCl.H_2O$	0.1
L - Cysteine	20.0
DL - Glutamic acid	150.0
Glycine	50.0
L - Histidine $HCl.H_2O$	20.0
L - Hydroxyproline	10.0
DL- Isoleucine	40.0
DL - Leucine	120.0
L - Lysine HCl	70.0
DL - Methionine	30.0
DL - Phenylalanine	50.0
L - Proline	40.0
DL - Serine	50.0
DL - Threonine	60.0
DL - Tryptrophan	20.0
L - Tyrosine	40.0
DL - Valine	50.0

Contd/...

VITAMINS	
Ascorbic acid	0.05
Alpha tocopherol phosphate (disodium salt)	0.01
Biotin	0.01
Calciferol	0.1
D - Ca pantothenate	0.01

VITAMINS Contd/...	CONCENTRATION (mg/l)
Choline chloride	0.5
Folic acid	0.01
i - inositol	0.05
Menadione	0.01
Niacin	0.025
Niacinamide	0.025
Para - aminobenzoic acid	0.05
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.01
Thiamine HCl	0.01
Vitamin A	0.1

The pH of the medium is 7.4.

APPENDIX 3

COMPOSITION OF TLC SPRAY REAGENTS

1. Ferric chloride/potassium ferricyanide reagent.

a) A 1% aqueous solution of potassium ferricyanide.

b) 2% aqueous ferric chloride. These solutions are mixed together in equal volumes just prior to use.

2. 4 - dimethylaminobenzaldehyde (Ehrlich's reagent).

1g 4 - dimethylaminobenzaldehyde in a mixture of 25ml 36% HCl and 75ml ethanol.

3. Alkaline silver nitrate for phenols and aromatic amines.

Spray 1

1 ml of a saturated solution of aqueous $AgNO_3$ was added, with stirring to 20ml acetone then this was treated dropwise with water until the precipitated $AgNO_3$ had just dissolved.

Spray 2

The plate was treated finally with a solution of 0.5% sodium hydroxide in 80% ethanol.

Arylamines + phenols give brown/black spots.

4. Formaldehyde and HCl (Prochazka reagent).

The following solutions are mixed together:

i) 10mls 35% formaldehyde solution

ii) 10mls 25% HCl v/v

iii) 20mls ethanol.

The plate is sprayed with the above mixture, then heated in an oven at 100°C. The plate is then examined in long wave ultra-violet light.

5. Dimethylaminocinnamaldehyde Reagent.

2g of this is added to 100mls 6N HCl and 100ml ethanol. This is then diluted with four times its volume to produce the spray reagent.

6. 1,2 Naphthoquinone-4-sulphonic acid (sodium salt) 5ml acetic acid added to 0.5g 1,2 naphthoquinone-4-sulphonate in 95ml water. Reaction occurs within 30 minutes.

7. Fluorescein and ammonia Reagent. A 0.005% solution of fluorescein in 0.5M ammonium hydroxide. Inspect in long and short wave ultra-violet light.

8. Perchloric acid/ferric chloride Reagent. 100mls of 5% aqueous perchloric acid is mixed with 2ml of 0.05M ferric chloride solution prior to spraying.

9. Iodine and potassium iodide Reagent. 1g of iodine and 10g of potassium iodide are dissolved in 50ml water by warming. 2mls of acetic acid was added and then the solution is made up to 100mls with water.

10. Bismuth Trichloride Reagent. A 33% ethanolic solution of $BiCl_3$, is sprayed onto the plate, which is then heated at 110°C until there is optimum fluorescence of the spots in long wavelength ultra-violet light.

11. 2,6 dichlorophenol - indophenol (sodium salt) Reagent. A 0.1% ethanolic solution of the sodium salt of 2,6 dichlorophenol-indophenol. Red spots indicate a positive reaction with organic acids.

12. Carr Price Reagent for vitamin D. A saturated solution of antimony III chloride in chloroform is sprayed onto the plate and the plate is then heated at 100°C for 10 minutes. Orange spots indicate a positive result.

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EVIDENCE FOR AQUEOUS SOLUBLE VITAMIN D-LIKE SUBSTANCES IN THE CALCINOGENIC PLANT, TRISTETUM FLAVESCENS

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SUMMARY

A crude aqueous extract of the leaves of T. flavescens when administered orally to vitamin D-deficient chicks produced significant increases in plasma phosphate but had little effect on plasma calcium. When chicks, fed a high strontium diet to inhibit endogenous 1,25$(OH)_2$ vitamin D_3 production and intestinal calcium transport, were dosed with the extract or synthetic 1,25$(OH)_2D_3$ ^{45}Ca absorption from the duodenum in vivo was stimulated, whereas vitamin D_3 was ineffective. Partial purification of the crude extract on a Sephadex GH25 column yielded two factors, one of which mimicked 1,25 $(OH)_2D_3$ activity in chicks fed the high strontium diet while the other produced a significant increase in plasma phosphate. The presence of these substances, together with previously demonstrated organic solvent soluble vitamin D-type activity, may account for the calcinogenic nature of the plant.

Cattle grazing on the alpine pastures of Austria and Germany develop calcinosis, a disease characterised by elevated levels of plasma calcium and phosphate, soft tissue calcification, lameness and emaciation (1,2). The disease, which is reminiscent of vitamin D intoxication, is caused by the ingestion of Trisetum flavescens L. (Golden oat grass) (3) which has been shown to possess antirachitic activity when fed to vitamin D-deficient chicks (4). The leaves posses vitamin D_3 (5) but at a concentration (0.1 ppm) which would not be calcinogenic. The solanaceous plants Solanum malacoxylon Sendtner and Cestrum diurnum L. are calcinogenic (6,7) and the causative agent has been identified as 1,25$(OH)_2$ vitamin D_3 (1,25$(OH)_2D_3$) (8,9) which occurs as a glycoside. It has been suggested that the calcinogenic principle in T. flavescens is also 1,25$(OH)_2D_3$ (10). Since organic solvent extracts have been reported not to contain this metabolite (11) we have examined aqueous extracts of the leaves of the grass for vitamin D and 1,25$(OH)_2D_3$-like activity.

MATERIALS AND METHODS

Animals and diets

Day old Rhode Island red x White Leghorn cock chicks were used. They were reared on a diet based on maize and soya bean and supplemented with minerals and vitamins with the exception of vitamin D. The diet contained 0.95% calcium and

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0.46% available phosphorus. To produce a D-replete control diet 1200 IU vitamin D_3/kg were added. These diets were fed for three and a half weeks prior to the commencement of the experiments.

To produce a high strontium diet supplemental calcium as the carbonate was replaced on a molar basis by strontium as the carbonate. Thus, this diet contained 1.74% Sr. and 0.15% Ca. The incorporation of stable stronium into the diet produces rickets (12). Strontium inhibits vitamin D_3-induced calcium binding protein (CaBP) production and the intestinal calcium absorptive mechanism (13) by blocking the conversion of 25(OH) vitamin D_3 to $1,25(OH)_3$ by the kidney (14).

Preparation of T. flavescens extracts

Finely ground dried leaves of the grass, harvested from a monoculture in early July, were extracted with chloroform (10 g leaf in 150 ml chloroform) for two hours. Chloroform soluble material was discarded and 10 g of the drief leaf residue stirred for 20 hours in 100 ml distilled water. The extract was separated from insoluble material by filtration and freeze-dried. Freeze-dried material was taken up in distilled water, so that 1.0 ml contained the equivalent of 2.0 g of original leaf powder, to provide a crude aqueous extract.

Chromatography

1.5 ml aliquots of the crude aqueous extract were applied to 40 x 2.8 cm columns packed with Sephadex G25 (fine) which had been equilibrated with distilled water. The columns were eluted with distilled water (2.0 ml/min.) and 7.5 ml fractions collected. The chromatographic profile of the eluent was followed by determining the UV absorbance at 267 nm (Fig.1). Fractions under the peaks were bulked and freeze-dried to provide six partially purified extracts (I-VI) for biological testing. Prior to testing the extracts were taken up in distilled water so that 1.0 ml contained the equivalent of 2.0 g of starting material.

Chemical determinations

Plasma calcium and strontium were measured by atomic absorptiometry and plasma inorganic phosphorus (Pi) by a commercial method (Boehringer Kt No. 124974). Radioactivity was measured in a Beckman LS7500 liquid scintillation counter, corrected for quench and efficiency and is expressed as disintegrations per minute (dpm).

Experiment 1

The crude aqueous extract was screened for vitamin D and $1,25(OH)_2$ vitamin D-type activity. To test for vitamin D-type activity four groups of five chicks were used; group 1 was vitamin D-replete, groups 2,3 and 4 vitamin D-deficient. After 28 days group 3 received one oral dose of crude extract (≡ 0.5 g dried leaf) at time 0 and plasma Ca and Pi were estimated 24 hours later. Group 4 was similarly dosed at 0 and 24 hours and plasma Ca and Pi measured at 48 hours: at this time plasma Ca and Pi were also assayed in groups 1 and 2.

In order to test the crude extract for $1,25(OH)_2D_3$-type activity four groups, each of five chicks, were transferred from the D-replete/Ca-replete diet to the D-replete high Sr. diet at 23 days of age. On day 28 and again on day 29 the groups were dosed with either 0.2 ml propylene glycol (control) vitamin D_3 (100 IU/dose), $1,25(OH)_2D_3$(0.25 µg/dose) or 0.25 ml crude aqueous extract of T. flavescens (≡ 0.5 g dried leaf powder/dose). On day 30 the chicks were

anaesthetized with sodium pentabarbitone (3.5 mg/100 g body weight). The duodenum was exteriorized and an 8 cm segment ligated to form a sac. 1.0 ml of a solution, at 40°C, containing 1 mg of calcium as calcium acetate and 10µCi ^{45}Ca was injected into the sac (15). The duodenum was returned to the body cavity, the wound closed by clips and a fifteen minute absorption period allowed. At the end of this time, radio-calcium, total calcium and strontium were measured in blood samples taken from the wing vein.

Experiment 2

Following Sephadex G25 chromatography material under peaks I-VI (Fig.1) was screened for vitamin D and $1,25(OH)_2D$-type activity in a pilot experiment. Biological activity appeared to be associated with peaks III and IV and these were subjected to further examination. The experimental protocols were those adopted in experiment 1, except that at the end of the fifteen minute absorption period the duodenum was removed and residual radioactivity determined in the ligated segment. The absorption data are expressed as percentage of administered dose.

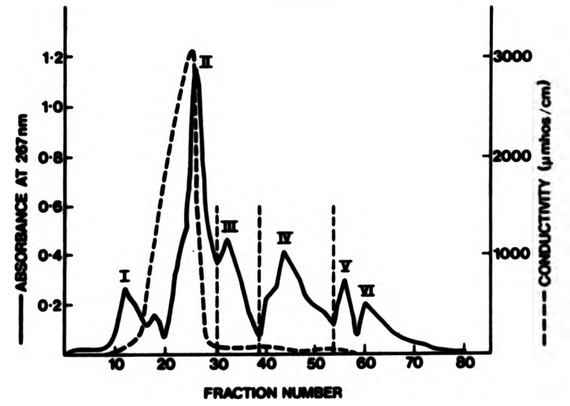

FIG. 1

Partial purification of an aqueous extract of T-flavescens on a Sephadex G25 column. Chromatographic conditions are described in the text. Biological activity resides in fractions under peaks III and IV. Conductivity measurements indicated that fractions under these peaks were essentially salt-free.

RESULTS

Experiment I

Plasma phosphate levels in vitamin D-deficient chicks given oral doses of a crude aqueous extract of the grass were significantly greater than in untreated vitamin D-deficient chicks. Following a single dose of extract phosphate levels were significantly elevated twenty-four hours later. The mean plasma phosphate level in the group given two doses of extract was greater than for vitamin D-replete chicks, but not significantly so. The extract was without significant effect on plasma calcium levels (Table 1).

TABLE 1

Plasma Phosphate and Calcium in Vitamin D-deficient chicks following the oral administration of a crude aqueous extract of T. flavescens (TFE).

Group	Treatment (oral doses)	Plasma levels (mean ± SEM) Pi	Ca
1	D-replete	4.60 ± 0.33	10.10 ± 0.24
2	D-deficient	2.06 ± 0.25	8.00 ± 0.69
3	D-deficient + TFE (24h)	3.08 ± 0.28	8.36 ± 0.12
4	D-deficient + TFE (48h)	5.74 ± 0.82	8.12 ± 0.85

Significance of differences (t-test)

For Pi: Group 1 v 2, $P < 0.001$
2 v 3, $P < 0.025$
2 v 4, $P < 0.001$
1 v 4, NS

For Ca: Group 1 v 2, $P < 0.025$
2 v 3, NS
2 v 4, NS

Chicks were dosed orally with a crude aqueous extract of T. Flavescens (TFE) equivalent to 0.5 g dried leaf per dose. Group 3 chicks were given one dose and plasma Ca and Pi estimated 24 hours later. Group 4 chicks were dosed at 0 hours and 24 hours and plasma Ca and Pi measured at 48 hours. Values are mg/100 ml plasma ± S.E.M.

In chicks fed the high strontium diet the crude extract was without significant effect on plasma calcium or strontium although the mean value of the latter was considerably greater than that for the control group. Absorption of radio-calcium from the duodenum was significantly increased by this treatment. When compared with the control group $1,25(OH)_2D_3$ prompted uptake into the plasma of duodenally administered ^{45}Ca, significantly increased plasma strontium, but was without significant effect on plasma calcium levels. Vitamin D_3 was without effect on any of these variables (Table II).

Experiment 2

The partially purified extracts, peaks III and IV, were without significant effect on plasma calcium and this was also true for $1,25(OH)_2D_3$ and vitamin D_3. When compared with the control group plasma phosphate was significantly reduced by $1,25(OH)_2D_3$ but significantly increased by peak IV. Vitamin D_3 and peak III were without effect on this variable. Duodenal transport of ^{45}Ca was not influenced by vitamin D_3 or peak IV but was significantly increased by $1,25(OH)_2D_3$ and peak III (Table III).

TABLE II

Effect of a Crude Aqueous Extract of T. flavescens (TFE), $1,25(OH)_2D_3$ and Vitamin D_3 on plasma calcium and strontium levels and on duodenal transport of ^{45}Ca to the plasma of chicks fed a High Strontium diet.

Group	Treatment (oral doses)	Plasma levels (mean ± SEM) Ca (mg/100ml)	Sr (mg/100ml)	^{45}Ca (dpm/100µl)
1	Propylene glycol	9.20 ± 0.25	3.66 ± 0.54	1169 ± 225
2	Vitamin D_3 (200 IU)	8.67 ± 0.06	4.49 ± 0.29	954 ± 68
3	$1,25(OH)_2D_3$ (0.5µg)	8.69 ± 0.10	7.42 ± 0.56	2181 ± 281
4	TFE (≡ 1.0g dried leaf)	9.25 ± 0.16	6.09 ± 0.94	2558 ± 222

Significance: (t-test)
Calcium: Differences between groups are not significant (NS)
Strontium: Gp.1 v Gp.2, NS: Gp.1 v Gp.3, $P < 0.005$: Gp.1 v Gp.4, NS
^{45}Ca: Gp.1 v Gp.2, NS: Gp.1 v Gp.3, $P < 0.025$: Gp.1 v Gp.4, $P < 0.005$

Chicks were fed a diet in which supplemental calcium (0.8%) was replaced on a molar basis by strontium (1.74%) in order to inhibit endogenous production of $1,25(OH)_2D_3$ and consequently intestinal calcium transport. On days 5 and 6 of this regimen chicks were dosed as indicated and plasma measurements made on day 7. ^{45}Ca was introduced into a ligated duodenal segment and a fifteen minute absorption period allowed.

TABLE III

The effect of two Partially Purified Aqueous Extracts of T. flavescens (peaks III and IV) on the plasma levels of Calcium and Phosphate and on Duodenal ^{45}Ca transport in chicks fed a High Strontium diet.

Group	Treatment (oral doses)	Plasma levels (mean ± SEM) Ca (mg/100ml)	Pi (mg/100ml)	Duodenal ^{45}Ca transport (% dose transferred to body during 15 min absorption period)
1	Propylene glycol	8.29 ± 0.06	4.63 ± 0.21	1.20 ± 0.42
2	Vitamin D_3 (200IU)	8.29 ± 0.32	4.92 ± 0.45	4.68 ± 1.62
3	$1,25(OH)_2D_3$ (0.5µg)	8.50 ± 0.29	3.77 ± 0.22	22.12 ± 3.30
4	Peak III (≡ 1.0g dried leaf)	8.58 ± 0.19	4.62 ± 0.14	9.78 ± 1.40
5	Peak IV (≡ 1.0g dried leaf)	7.80 ± 0.22	5.66 ± 0.22	0.85 ± 0.30

Significance: (t-test)

Calcium: Differences between groups are not significant (NS)

Phosphate: Gp.1 v Gp.2, NS: Gp.1 v Gp.3, $P < 0.05$: Gp.1 v Gp.5, $P < 0.01$: Gp.4 v Gp.5, $P < 0.025$

^{45}Ca: Gp.1 v Gp.2, NS: Gp.1 v Gp.3, $P < 0.001$: Gp.1 v Gp.4, $P < 0.01$: Gp.4 v Gp.5, $P < 0.001$

The experimental protocol was that outlined beneath Table II except that at the end of the 15 minute absorption period the duodenum was removed and residual radioactivity determined in the ligated segment.

DISCUSSION

These experiments have revealed the presence of water-soluble vitamin D-like substances in the leaves of the calcinogenic plant T. flavescens. The observation that the extract was greatly effective in increasing plasma phosphate levels but had little effect on plasma calcium is in accord with the reported levels of these ions in the blood of animals affected by calcinosis. Such animals have plasma calcium levels in the upper part of the physiological range whereas plasma phosphate levels may be twice normal (16). Since the crude aqueous extract was able to overcome the inhibitory effect of the high stable strontium diet on calcium absorption from the duodenum, this provides evidence for the presence of $1,25(OH)_2D_3$ or a substance able to mimick its actions. In the vitamin D-deficient chick the crude extract produced very small increases in plasma calcium suggesting that the grass contains a low $1,25(OH)_2D_3$-like activity.

Following Sephadex G25 chromatography a partially purified factor was isolated which overcame the strontium inhibition of duodenal calcium absorption, thus confirming the $1,25(OH)_2D_3$-like action of the crude extract. The presence of this $1,25(OH)_2D_3$-like substance in a water-soluble form would explain the induction of calcium binding protein (CaBP) in chicks fed a high strontium diet to which lyophilized leaves of T. flavescens had been added (10). It also affords an explanation for the failure of Zucker and colleages (11) to overcome strontium inhibition of CaBP synthesis with the plant and for the failure of Rambeck and colleages (5) to identify the presence of $1,25(OH)_2D_3$ by gas chromatographic/mass spectrometric analysis, since in both cases diethyl ether extracts were used.

The administration of material from a second peak (peak IV) was ineffective in overcoming strontium inhibition of calcium absorption but did increase plasma phosphate. Hence, it appears that the vitamin D-like activity of the crude aqueous extract is due to the presence of at least two substances, one having a $1,25(OH)_2D_3$-like effect, the other having a potent phosphataemic action. It may be that this latter substance is the major causative principle in T. flavescens-induced calcinosis.

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