The distribution of vitamin D between the blood and the liver in the pig, and observations on the pathology of vitamin D toxicity

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The distribution of vitamin D in the body has not been studied thoroughly because of its normally very low concentration and difficulty of assay. Most estimations have been made on blood but, if vitamin D is concentrated in the liver in the same way as the fat-soluble vitamins A and E, estimation of the level in blood cannot give an indication of the total amount of the vitamin in the body. That such concentration of vitamin D can occur under certain circumstances has been shown in rats by Kodicek (1958). He gave rats 1 mg ergocalciferol by mouth and found, 24 h later, 5·7 % of the dose as biologically active vitamin D in the liver and 1·2 % in the blood. The vitamin D content of the bones and intestines was similar to that of the blood. A similar distribution was found when the rats were given only 10 μg (400 i.u.) of the vitamin (Kodicek & Ashby, 1960). Earlier work from the same laboratory (Cruickshank & Kodicek, 1953) showed that the recovery of vitamin D from liver and intestinal tissue decreased considerably during the first 2 days after the dose was given but the recovery from the rest of the body remained more constant. Thus the percentage of the dose recovered from the liver after each of the first 4 days was 3·7, 1·2, 1·0 and 1·0, that from intestinal tissue was 0·6, 0·2, 0·2 and 0·1, and that from the rest of the body was 1·7, 1·6, 1·9 and 1·3. A similar distribution of vitamin D among the tissues 24 h after a relatively large dose had been given to rats and Rhesus monkeys was found by Blumberg, Aebi, Hurni & Schönholzer (1960). It is possible that in these experiments the relatively higher levels of vitamin D found initially in liver and intestinal tissue may have been a consequence of a relatively large dose of the vitamin or a transitory result independent of the dose. Evidence with other animals is scanty, but it has been shown in calves that were given vitamin D supplements, or whose dams were given supplements before parturition, that the liver levels of the vitamin were always about the same as or lower than the blood levels (Eaton, Spielman, Loosli, Thomas, Norton & Turk, 1947 *a, b*; Guerrant, Morck, Bechdel & Hilston, 1938). Guerrant *et al.* (1938) estimate that the total amount of vitamin D in the blood is about four times that in the liver. The antirachitic activity in brain, heart and kidney was too small to estimate. Other investigations of the problem of vitamin D distribution in sheep (New Zealand Department of Agriculture, 1949–50) failed to reveal any concentration of the vitamin in any of thirty-five organs or eight fat depots at 1 and 4 months after a subcutaneous injection. These tissues contained only traces of vitamin D compared with the blood.

It is important to know how the distribution of the vitamin changes when the intake is excessive, normal or very low, both in order to understand its metabolism and to select the best tissue for estimation of the vitamin D status of an animal.

In this experiment we have studied pigs given for a month a basic diet deficient in vitamin D or the same diet adjusted to give either near-normal or toxic levels of vitamin D intake. The vitamin D supplements were then withheld, so that all the groups received the deficient diet. At suitable intervals pigs were killed and changes in the levels of vitamin in the blood and liver were studied by biological assay. The opportunity was taken also to study histologically those animals that had received the toxic dose in order to correlate the natural history of the lesions, the calcium content of the tissues and the blood levels of vitamin D.

EXPERIMENTAL

Animals and their management

Seven Large White sows were mated during the 1st week in May and about 4 weeks later were put in concrete pens and screened completely from sunlight by sheets of sacking. They were given a conventional diet (Lodge & McPherson, 1961) but with a reduced vitamin D content of 110 i.u./kg diet. The piglets born were farrowed and reared in the absence of sunlight. Before weaning at 8 weeks the piglets received the starter and follower diets of Lodge & McPherson (1961), the former containing no vitamin D supplement and the latter containing 165 i.u. vitamin D/kg. At weaning the piglets, sixty-two in all, were distributed according to sex, weight and litter into five groups of six, twelve, ten, fifteen and nineteen animals. The distribution of the animals among the groups was not random but was arranged as far as possible so that members of one litter given differing levels of vitamin D were killed together. The first group of six pigs was killed at weaning and blood and liver were taken for saponification, extraction and biological estimation of vitamin D activity. The other groups were given a diet of barley 55%, middlings 25%, white fish meal 10%, grass meal 5%, decorticated groundnut meal 5%, chalk 1·5%, salt 0·5%, Aurofac 2A (Cyanamid of Great Britain Ltd) 0·3%, riboflavine 3·1 mg/kg and vitamin A (Rovimix A; Roche Products Ltd) 885 i.u./kg. An analysis of the diet gave calcium 0·63% and phosphorus 0·66%. For 30 days this diet was supplemented as necessary with vitamin D sufficient to provide each animal in the four groups with, respectively, 0, 90, 350 or 250000 i.u./day in addition to the small amount of vitamin D that may have been in the basal diet. (These groups will be referred to as the '0 i.u.', '90 i.u.', '350 i.u.' and '250000 i.u.' groups.)

The supplement of 90 i.u. vitamin D a day provided less than the requirement generally accepted ((USA) National Research Council, 1959) of pigs of the weight of those in this experiment at any time during the 4-week period of vitamin D supplementation. The supplement of 350 i.u. a day provided more than this requirement. A dose rate of 250000 i.u./day was chosen for the fourth group as a result of earlier trials with various levels of vitamin supplementation in which weanling pigs given 500000 i.u. or more daily ceased eating or died in less than a month.

Since it was desirable that the vitamin D preparation used should be the same for all three groups in the extreme range of quantities required and in a form suitable for mixing uniformly with the diet, a water-miscible preparation was used which contained 5000 i.u. cholecalciferol/ml (Roche Products Ltd). When small volumes of this preparation were required they were diluted with water before being mixed with the daily feed.

The pigs in each group were kept and fed together in the same pen. The 0 i.u. group was fed *ad lib.* but the amount of food the other groups were given each day was always a little more than that eaten on the previous day. Supplementation was continued for 1 month and at the end of this time ('time 0') six pigs from each group were killed and the remainder were thereafter given the same diet with no cholecalciferol supplement and kept as before out of sunlight. Representative pigs from each group were killed 1, 4, 12 and 24 weeks later.

Analytical methods

At all killings enough blood was taken to provide two 500 g samples of pooled blood from each group. The livers were weighed and those from each group pooled and minced; two 500 g samples were taken for vitamin D assay. The blood was saponified with alcoholic potash (50 g KOH and 250 g ethanol to 500 g blood) but the liver required stronger alkali (200 g KOH, 350 ml ethanol and 150 ml water to 500 g liver) and longer heating than blood before it was completely digested. The unsaponifiable fraction was extracted with diethyl ether, the solution washed free of alkali with water, dried over sodium sulphate, reduced to about 5 ml by distillation and evaporated to dryness under nitrogen. The residue was ground with cottonseed oil and stored at $-20°$ under nitrogen until required for vitamin D assay. The curative, radiographic assay with rats used to estimate vitamin D and the method used for the statistical assessment of the measurements have been described by Dalgarno, Hill & McDonald (1962).

Calcium was measured in serum by the method of Clark & Collip (1925) and inorganic phosphate in whole blood by the method of Fiske & Subbarow (1925). Citrate was measured in blood samples by the method of McArdle (1955) and haemoglobin by that of Nicholas (1951). The left radius and ulna were removed from each pig, cleaned, extracted with acetone, dried at 105°, weighed and radiographed. Samples of tissue weighing 5–10 g were taken at the first two killings from the pyloric region of the greater curvature of the stomach and from the kidney, peripheral parts of the lungs, aortic arch, duodenum, jejunum and ileum near the ileocaecal valve. At later killings samples were taken only from kidney, lung and duodenum. The samples were dried at 105°, weighed, ashed and dissolved in 2 ml N-HCl. This solution was diluted to about 25 ml, made alkaline with 12 ml N-NaOH and titrated with ethylenediamine tetraacetic acid with Calcon (Eriochrome Blue-Black R) as indicator.

The histological studies were confined to the pigs of the 250000 i.u. group, all of which had shown signs of vitamin D toxicity, and to pigs of the 350 i.u. group, which were used to supply histological control material. Tissues were taken from all the pigs of the former and from two or three pigs at each killing of the latter group.

Histological examination

From each pig pieces of tissue were selected from each chamber of the heart, from the aortic arch, lungs and kidney. The tissues were fixed in phosphate buffer, pH 7 with 4 % (v/v) formaldehyde and processed in the usual way for the preparation of paraffin sections. Sections from each block were stained by some or all of the following methods: the von Kossa and the alizarin red methods for calcium salts; haematoxylin and eosin; Van Gieson's picro-fuchsin method for collagen; the Weigert–Sheridan method for elastic tissue and the periodic acid Schiff and haemalum method for the study of vascular lesions.

It was not intended that the histological observations should stand alone or constitute a full morphological study of vitamin D poisoning in the pig. The histological picture from each slaughter-group was considered as a whole, with particular attention to the stage of evolution or involution of the lesions found. Because of the consistency of the histological findings within the 250000 i.u. group at each stage, this approach was satisfactory in practice and resulted in a convincing histological sequence of injury, resorption and repair.

RESULTS

Vitamin D assays, chemical and physical observations

The results of the assays for vitamin D activity are given in Table 1 for blood and in Table 2 for liver. They have been published in part by Quarterman, Dalgarno & Adam (1963).

Table 1. *Vitamin D activity (i.u./100 g) in the blood of pigs at various times after a diet supplemented with different amounts of cholecalciferol was changed to one with no supplement*

Time after withdrawal of supplement (weeks)	0 i.u. group*	90 i.u. group*	350 i.u. group*	250000 i.u. group*
0	26	33	52	2337
1	—	—	25	1062, 1005†
4	28	22	37, 29†	204
12	8	32	31‡	64
24	—	—	5, 11†	12, 18†

The mean 95 % fiducial limits for these assays are 46–270 % of the estimates.

* Diet provided 0, 90, 350 or 250000 i.u./pig daily. The number of pigs in a group at the different times is given in Table 3.

† Duplicate assays of the same material.

‡ In the assay on this sample and three listed in Table 2 three out of eight litters of rats were eliminated because they showed irregular healing.

Before cholecalciferol dosing began the level of vitamin D in the pooled blood sample from the six pigs killed was 21 i.u./100 g and the liver level 13 i.u./100 g. (These figures are not shown in the tables.) At the killing immediately after the 1-month period of cholecalciferol supplementation (time 0), the blood of the animals receiving 350 i.u. daily had a higher vitamin D concentration than the blood of those

from both the o and 90 i.u. groups. Blood vitamin D levels had fallen below 12 i.u./ 100 g 12 weeks later in the pigs receiving no supplement and by 24 weeks in those receiving 350 i.u. daily. The liver values did not differ significantly among these three groups and were generally lower than the blood values. In the group that received 250000 i.u. daily the blood and liver values were about the same at the first sampling, but thereafter the concentration of vitamin D in the liver decreased more rapidly than in the blood.

At the beginning of the supplementation period the pigs consumed a mean of o·23 kg food/day; at the end the pigs in the 250000 i.u. group ate o·77 kg each and those in the 350 and 90 i.u. groups ate 1·13 kg each. On the 15th and 21st days of the supplement period the pigs in the 250000 i.u. group ate very little and cholecalciferol was withheld on the day following refusals.

Table 2. *Vitamin D activity (i.u./100 g) in the liver of pigs at various times after a diet supplemented with different amounts of cholecalciferol was changed to one with no supplement*

Time after withdrawal of supplement (weeks)	0 i.u. group*	90 i.u. group*	350 i.u. group*	250000 i.u. group*
0	9	7	21†	2710
1	—	—	10†	528, 450‡
4	14, 16‡	12, 22‡	7,† 14‡	89
12	9, 13‡	9	9	15
24	—	—	5	9

The mean 95 % fiducial limits for these assays are 36–214 % of the estimates.
* Diet provided 0, 90, 350 or 250000 i.u./pig daily. The number of pigs in a group at the different times is given in Table 3.
† See Table 1, footnote ‡.
‡ Duplicate assays of the same material.

At the end of the period of cholecalciferol supplementation the body-weight tended to increase with the intake at the three lowest levels but was least of all in the 250000 i.u. group (Table 3). The 350 i.u. group was significantly heavier than the others at 3 months after the end of the period of supplementation. The 250000 i.u. group was always the lightest. The liver weights and the weights of the radius and ulna showed the same trend as the body-weights. The blood citrate content was higher in the 250000 i.u. group at the first killing, but thereafter there were no significant differences. The content of blood inorganic phosphate was higher in the 350 i.u. group than in the other three groups at the first killing and higher than in the 250000 i.u. group at the second killing. There were no significant differences among the groups in serum calcium levels or radius and ulna lengths. At the second killing, a week after the end of cholecalciferol supplementation, the mean blood haemoglobin levels of the 250000 and 350 i.u. groups were 10·5 and 11·2 g/100 ml, respectively. This difference is statistically but probably not physiologically significant.

At the first killing the mean calcium contents of the lung, kidney, duodenum, ileum and stomach of the 250000 i.u. group were respectively 10·00, 2·49, 1·53, 0·59 and 0·49 mg/g dried tissue, and were significantly higher than those of the same tissues

Table 3. *Means of results obtained for the four groups of pigs killed* 0, 1, 4, 12 *and 24 weeks after the cholecalciferol supplements* (0, 90, 350 *and* 250000 *i.u./pig daily*) *were withheld*

Time of killing (weeks after supplement withdrawn)	Level of supplement (i.u./day)	No. of pigs in group	Live wt (kg)	Wt of liver (g)	Wt of radius and ulna (dry, fat-free) (g)	Length of radius and ulna (cm)	Serum cacium (mg/ 100 ml)	Blood in-organic phos-phate (mg/ 100 ml)	Blood citrate (mg/ 100 ml)
0	250000	6	19·5	477	33·8	13·1	13·4	6·5	4·1
	350	6	25·9	> 486*	43·4	13·4	12·3	7·6	2·5
	90	6	24·5	783	41·4	13·5	12·3	6·2	2·8
	0	6	22·3	675	37·6	13·5	12·0	6·4	3·2
1	250000	3	23·6	887	49·5	13·8	12·4	4·9	2·8
	350	3	28·6	940	43·7	13·8	11·4	6·8	2·3
4	250000	3	27·7	844	41·7	14·1	13·5	8·0	2·6
	350	2	38·6	1271	55·7	15·2	13·8	8·3	2·4
	90	2	34·5	1051	50·3	14·7	14·0	8·0	3·4
	0	3	35·0	974	49·5	14·5	14·3	7·9	3·4
12	250000	3	79·5	1456	83·0	19·1	11·1	9·8	—
	350	2	91·3	1555	88·2	19·4	10·7	9·5	—
	90	2	87·2	1475	76·0	18·7	11·1	8·5	—
	0	3	84·9	1588	86·4	19·3	11·0	10·1	—
24	250000	4	116·7	1629	117·8	21·3	12·2	8·2	—
	350	2	136·7	2328	144·2	21·4	11·8	8·1	—

* A portion of the liver removed for pathological inspection was not weighed.

from the 350 i.u. group which were 0·39, 0·23, 0·48, 0·34 and 0·28 mg/g. The two groups were not different in tissue calcium content at any other killing or in the other tissues sampled at the first killing.

Pathological observations

These observations refer to pigs which had been given 250000 i.u. cholecalciferol/day.

The aorta was the only organ that showed macroscopic lesions. These were infrequent and took the form of small, irregular, raised areas, roughly elliptical in shape and each with a slightly depressed centre. The vascular endothelium remained smooth and the lesions evidently subintimal.

Microscopic lesions

Kidney. All the kidneys from pigs killed at time 0 showed pathological depositions of calcium salts in the cortex (Pl. 1a) and in the medulla (Pl. 1b). Numerous small foci were present, most commonly in or adjacent to the epithelial cells of the renal tubules. Areas of calcification in the wall of a tubule were commonly overgrown by epithelial cells of normal appearance, resulting in the development of knob-like protuberances into the lumen. There was calcification of glomerular capillaries, of the epithelium of Bowman's capsule and also in the interstitial tissues. There were foci of calcification in the walls of the larger renal arteries (Pl. 1c) which showed local necrosis of smooth muscle cells in the vessel wall in the affected areas.

The non-calcified tubular epithelium appeared normal, but cystic dilatation of the renal tubules was common and there was considerable disorganization of the renal cortex. There were occasional groups of mononuclear cells and a general enlargement of interstitial cells of fibroblast type. Sections stained by Van Gieson's method showed an early, diffuse fibrosis.

More disorganization and scarring were evident than might have been expected from the observed extent of calcification, suggesting that marked toxic effects had been experienced earlier in the treatment and that the tissues were undergoing repair. At 1 week after the period of supplementation the size and number of areas of calcification were greatly reduced, but there was extensive scarring of the renal cortex (Pl. 1d) and diffuse fibrosis of the renal medulla. Dilated or cystic tubules occurred frequently in the cortex. The next killing (4 weeks) revealed a few very small calcium deposits in the renal cortex of one animal only. Much mature collagen was present in the renal cortex (Pl. 1e). There were degenerative changes in some of the renal tubules enveloped in fibrous connective tissue and many glomeruli were undergoing fibrous replacement. No deposits of calcium salts remained at 12 and 24 weeks, but there were further thickening and contraction of collagen fibres, with dilatation of renal tubules. Occasional foci of mononuclear cells were present in the renal cortex.

Kidneys from the control animals were normal but for one at the 24-week killing. In this kidney there were groups of mononuclear cells in the cortex, fibrosis and tubular dilatation. These lesions were less severe than those found in the group receiving cholecalciferol, but their existence suggests that the histological picture may be complicated by low-grade renal infections. It might be supposed that calcification of the kidney would predispose to episodes of renal infection.

There was a broad agreement between the pathological findings and the results of calcium determination in kidneys from the same animals.

Lung. All the pigs, including the controls, showed pneumonic changes suggestive of virus infection. These consisted of perivascular and peribronchial cuffing and thickening of interstitial tissues between the alveoli resulting in alveolar collapse and considerable reduction in the respiratory surface area.

In all the pigs that received cholecalciferol supplements there were at time 0 small foci of calcification in the bronchial cartilage, the bronchial mucosa and the bronchial smooth muscle (Pl. 2a). In cartilage the process of mineralization involved both the cartilage cells and the matrix. The calcification of smooth muscle was associated with local necrosis and disruption. There was commonly a giant-cell reaction round the damaged tissue. The bronchial epithelium was normal and the calcification occurred in the deeper parts of the bronchial mucosa. The alveolar tissues were not affected except in one pig in which there was calcification of those areas affected by the pneumonic process, giving a striking regional distribution (Pl. 2b). Not all the thickened areas were calcified. In the affected areas the alveolar epithelium, the interstitial tissues and the walls of blood vessels were involved in the process of mineralization. There was a very rapid resolution of all the calcified lesions, and at 4 weeks lesions due to vitamin D toxicity were found only in one pig in which there were small calcified areas in the bronchial cartilage and in which resorbing lesions were

found in the smooth muscle of the bronchi. Giant cells were present in the bronchial lesions. At 12 weeks small, localized deposits of calcium salts were found in the bronchial cartilage of two pigs, and calcified lesions were absent at 24 weeks.

The calcium content of lung tissue decreased rapidly from the high values at time 0 to normal values a week later. This decrease can be correlated with the rapid resolution of the calcified lesions found, which after the first killing were greatly reduced and largely confined to small areas in the bronchial cartilage. It appears that the results of a calcium determination would depend to some extent on the amount of bronchial tissue in the sample taken, and possibly on the extent of incidental virus pneumonia.

Aorta. Only two of the six pigs that had received vitamin D showed lesions in the aorta at time 0. The lesions consisted of foci of calcification in the vessel wall (Pl. 2c). The mineral deposits were subintimal. They caused distortion of the wall and bulging of the intima but the endothelium was unaffected. In the lesions there was destruction and calcification of elastic tissue, which was fragmented with individual fibres separated by calcified ground-substance. This mineralized tissue between the elastic fibres and the surface of the fibres themselves gave a positive staining reaction with Schiff reagent. The lesions varied in severity and small lesions were present in which there was little calcium but increased fibrous connective tissue (Pl. 2c). Here the elastic tissue was thickened and frayed. At 1 week similar lesions were found in one of three pigs. There was an increase in fibrous connective tissue in and round the lesion. At 4 weeks lesions that were severe but showed signs of repair were found in two of the three pigs. There was a distinct fibroblast, macrophage and giant-cell reaction (Pl. 2d) in and round the calcified area. The elastic tissue in the lesion was fragmented (Pl. 2e). Mild, non-calcified lesions were present in which there was fibrosis (Pl. 2f). In two pigs at 12 weeks there were numerous healing lesions which consisted of a core of dense fibrous connective tissue interspersed with delicate elastic fibres and containing fragments of calcified elastic tissue. At the periphery were distorted, eosinophilic elastic fibres.

There was further evidence of repair at 24 weeks when there remained scarred areas in which were embedded remnants of the calcified, fragmented elastic tissue (Pl. 2g and 2h). Apart from these fragments, the calcium deposits had undergone complete resorption. In one animal a cellular plaque was found in the intima of the aorta somewhat similar to the late lesion found by Gillman & Gilbert (1956a) in the root of the rat aorta after ergocalciferol intoxication.

Heart. Four of the six pigs that had received vitamin D showed cardiac damage at time 0. The damage was in the form of multiple lesions in the coronary arteries in all chambers of the heart. The auricles were affected most severely and the left ventricle least severely.

In one pig only were the lesions heavily calcified (Pl. 1f). The lesions were all centred on the smooth muscle of the arterial walls which were necrotic and swollen. The adjacent internal elastic membrane was destroyed. The vascular endothelium was intact but was sometimes separated from the underlying muscle by an oedematous or gelatinous substance. The lumen of the affected vessels was greatly reduced, but complete occlusion or thrombosed arteries were not found. Fibrous replacement of

the myocardium was seen in the right auricle of one pig (Pl. 1*g*) in which there were also areas of subendocardial calcification. The lesions in the arterial walls were usually multiple (Pl. 1*h*), and many extended through the full thickness of the muscle of the arterial wall; others were slight and were detected only by the periodic acid Schiff stain. The damaged areas were strongly Schiff positive. In addition, metachromasia was demonstrable in these non-calcified lesions after staining by toluidine blue, and in many instances Perls's test gave a positive granular reaction for iron. There was no increase in fibrous connective tissue in the damaged blood vessels. There was appreciable mineralization in the blood vessels of only one heart and some of the vascular lesions in other specimens contained a few granules of calcium salts.

Vascular injury unaccompanied by calcification has been observed in the coronary arteries of the rat under somewhat similar circumstances (Gillman & Gilbert, 1956*a*) but such lesions due to direct vitamin D toxicity in the rat usually involve the intima or, at their most severe, consist of panarteritis with a variable degree of periarteritis (Gillman & Gilbert, 1956*a*, *b*).

Healing was rapid, and at 1 week slight, non-calcified lesions were found in the coronary arteries of only one of the pigs. These were characterized by the aggregation of darkly staining nuclei of smooth-muscle cells in the arterial wall. They were not regularly orientated and gave the impression of a nodule of new growth. The intima was normal. Vascular lesions were not present in specimens from the later killings. Healing of the coronary arteries appeared to be by smooth-muscle regeneration, and the delayed vascular sclerosis found in rats after administration of excess ergocalciferol (Gillman & Hathorn, 1957; Gillman, Grant & Hathorn, 1960) was not observed in our material. Two pigs showed diffuse fibrosis of the right auricle. Thickening of the endocardium was not seen.

DISCUSSION

In this experiment the vitamin D intake of the pigs was planned to range over the widest limits possible, from what was present in the unsupplemented diet to the highest the animals would tolerate. With two exceptions, in every group of animals killed the level of vitamin D in the blood was greater than it was in the liver. One exception was in the 0 i.u. group when the levels were very low, but the other exception was that in which the conditions were most nearly like those in the experiments of Kodicek (1958) with rats and of Blumberg *et al.* (1960) with rats and Rhesus monkeys, that is the animals had received a relatively high dose of vitamin D in the 24 h immediately before slaughter. In contrast to their results, however, the total amount of vitamin D found in the blood of these pigs was three times as great as the amount found in the liver. (For this calculation the blood volume values used were those given by Bush, Jensen, Cartwright & Wintrobe, 1955). In every other instance but one the ratio of total vitamin D in blood to that in the liver was greater. Table 4 shows that the ratio may be as high as 14. In these experiments the pigs were hung and bled for a few minutes before the livers were removed and minced. The livers contained some blood, how much it is difficult to estimate, and the ratios of total blood vitamin D to total liver vitamin D must be greater than the figures given.

When the liver level of the vitamin was as high as the blood level, as in the 250000 i.u. group, its rate of decrease after withdrawal of cholecalciferol from the diet was more rapid than was that of the blood level. Kodicek (1958) has suggested that vitamin D, when present in excessive amounts, is broken down in the liver, and this finding is compatible with that suggestion. In the 250000 i.u. group the highest blood level recorded was comparable with that found in human adults given a similar oral dose of the vitamin (Warkany, Guest & Grabill, 1942). In children less than 5 years old, similar in body-weight to the pigs used in our experiments, serum levels of over 200 i.u./100 ml have been reported with daily doses of only 1250 or 4400 i.u. vitamin D (Fellers & Schwartz, 1958; Smith, Blizzard & Harrison, 1959).

Table 4. *Ratios of total blood vitamin D to total liver vitamin D in pigs at various times after a diet supplemented with different amounts of cholecalciferol was changed to one with no supplement*

Time after withdrawal of supplement (weeks)	0 i.u. group*	90 i.u. group*	350 i.u. group*	250000 i.u. group*
0	7·8	12·0	10·5	3·0
1	—	—	6·0	4·6
4	5·1	3·2	7·0	6·0
12	2·4	12·6	12·0	14·3
24	—	—	5·3	6·7

* Diet provided 0, 90, 350 or 250000 i.u./pig daily.

Most of the blood levels, except those found in the 250000 i.u. group, were near a mean value of 29 i.u./100 g (ranging between 22 and 37). The blood levels found in the 350 i.u. group were close to this figure between 1 week and 12 weeks after the end of the period of supplementation, but by 6 months the level was considerably lower. The 90 i.u. group also maintained this value for 3 months. In the 0 i.u. group the blood levels had fallen 3 months after the end of the supplementation period, that is after 4 months on the unsupplemented basal diet. Thus there was a period of about 3 months during which, even though the animals received little or no vitamin D and their weight increased fourfold, the vitamin D level in the blood remained fairly constant. The same considerations apply to the liver vitamin D levels which were steady during this period about a mean of 12 (range from 7 to 22) i.u./100 g. There must therefore be a store in some tissue or tissues which we have not identified from which the vitamin D content of the blood and liver is maintained. As a method of diagnosis of vitamin D status an assay of the vitamin in the blood will indicate whether the intake has been much above requirements, but not necessarily how soon a decrease to very low levels is to be expected. This period of stability of blood vitamin D levels is similar to that observed in sheep during the winter months of vitamin D deprivation (Quarterman *et al.* 1964). Johnson & Palmer (1939) found that it can take as long as 4 months for pigs on a vitamin D-deficient diet to develop signs of a deficiency. It seems that the blood level of vitamin D is influenced only slightly by dietary levels

ranging from almost none up to an amount which is in excess of the currently recommended allowance.

The slower rate of growth of pigs in the 250000 i.u. group was probably a result of their reduced food intake. Gillman *et al.* (1960) demonstrated inappetence and reduced rate of growth in rats suffering from ergocalciferol intoxication. Pair-fed control animals showed a similar reduction in growth rate.

The toxicity of vitamin D in the 250000 i.u. group was shown by the periods of inappetence, the relatively poor growth rate and by the elevated blood citrate and possibly serum calcium levels. In addition, morphological changes were demonstrated histologically in various tissues from the animals in this group. The most striking feature of the histological findings was the rapidity with which the lesions resolved as the levels of circulating vitamin D declined towards the levels found in the other groups. In some instances resorption of pathological calcium deposits and tissue repair were already in progress at the end of the period of cholecalciferol supplementation. It is possible that partial recovery might have resulted from a lower cholecalciferol intake during the periods of inappetence and that by this means the toxicity was self-limiting at the dietary levels used.

In spite of much past work, the pathogenesis of vitamin D toxicity is not fully understood. It seems probable that there is a primary direct toxic effect on the smooth muscle of blood vessels, and that the damaged tissues then become calcified. It is clear, however, that the toxicity of ergocalciferol can be influenced by the amount of calcium in the diet (Duguid, 1930). Ham & Lewis (1934) believe that the vascular lesions are produced by hypercalcaemia *per se*. Gillman & Gilbert (1956a) believe that tissue damage and tissue calcification may be dissociated, and point out that pathological calcification may follow the mobilization of calcium from the skeleton when the diet is low in calcium.

In our animals with high levels of vitamin D in blood and liver there was hypercalcaemia, and fully calcified, partially calcified and non-calcified vascular lesions were present. It is possible that the non-calcified lesions contained local accumulations of calcium salts which were not detected by our staining methods. Mucopolysaccharide and iron salts were present, both of which may be associated with calcium deposition in tissues.

SUMMARY

1. Weanling pigs were given for 4 weeks a diet supplemented with 0, 90, 350 or 250000 i.u. vitamin D/day and then the same diet with no vitamin D supplement. Some were killed when the supplement was withdrawn and some 1 week, 1 month, 3 months or 6 months later. At each killing blood and liver were assayed for vitamin D activity.

2. The concentration of vitamin D in the blood was always about the same as or greater than that in the liver, and when the vitamin D supplement was withdrawn the concentration in the liver decreased more rapidly than did that in the blood.

3. The total quantity of the vitamin in the blood was between 2·4 and 14·3 times as great as the quantity in the liver.

4. The blood level of vitamin D did not fall below about 20 i.u./100 g until more than 3 months after the pigs had been put on a diet free from the vitamin.

5. Pigs fed at the highest level of vitamin D intake showed signs of intoxication, and pathological calcification was found in several organs. There was evidence of rapid recovery as the blood content of the vitamin declined to the levels found in the other groups.
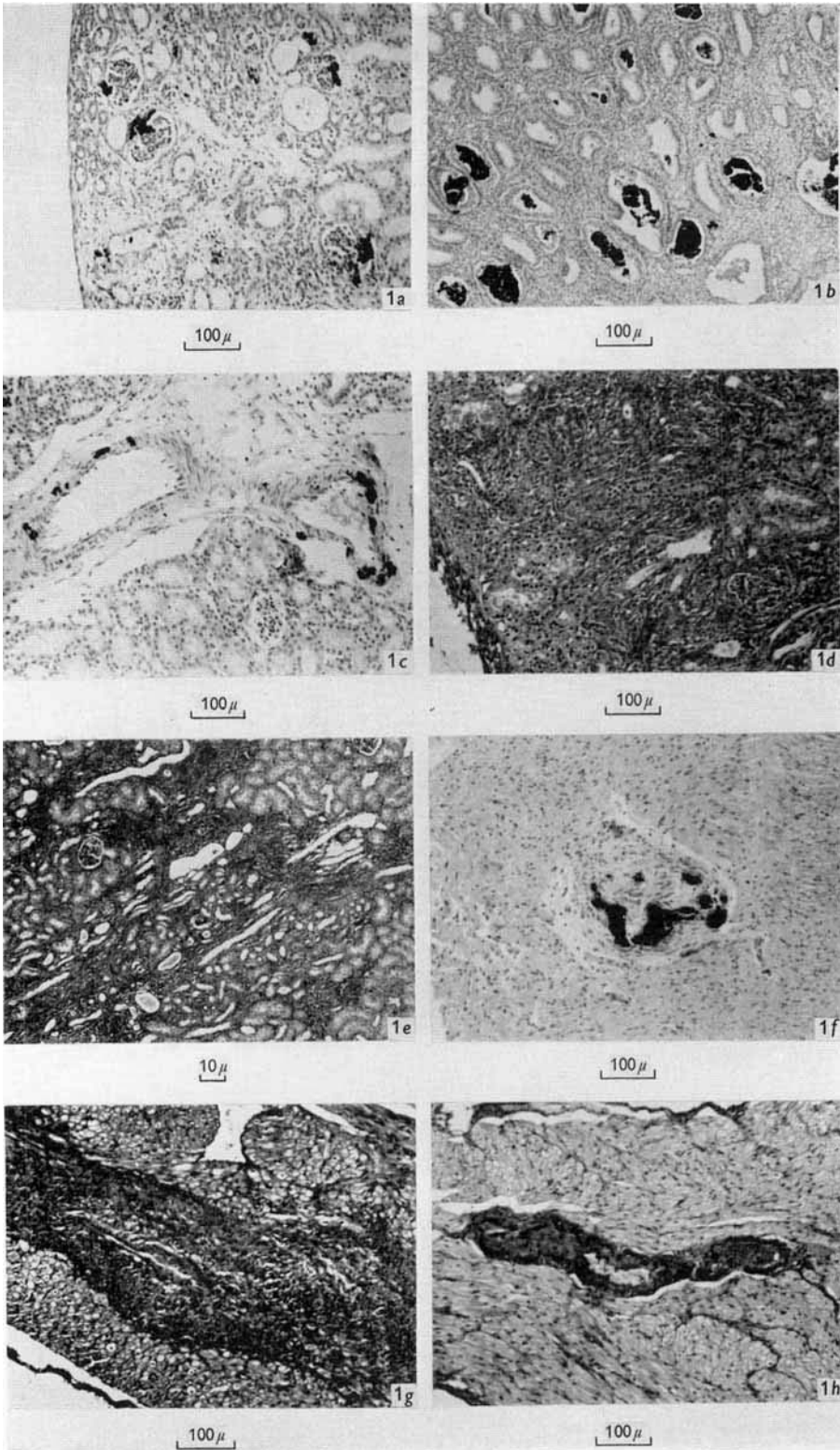
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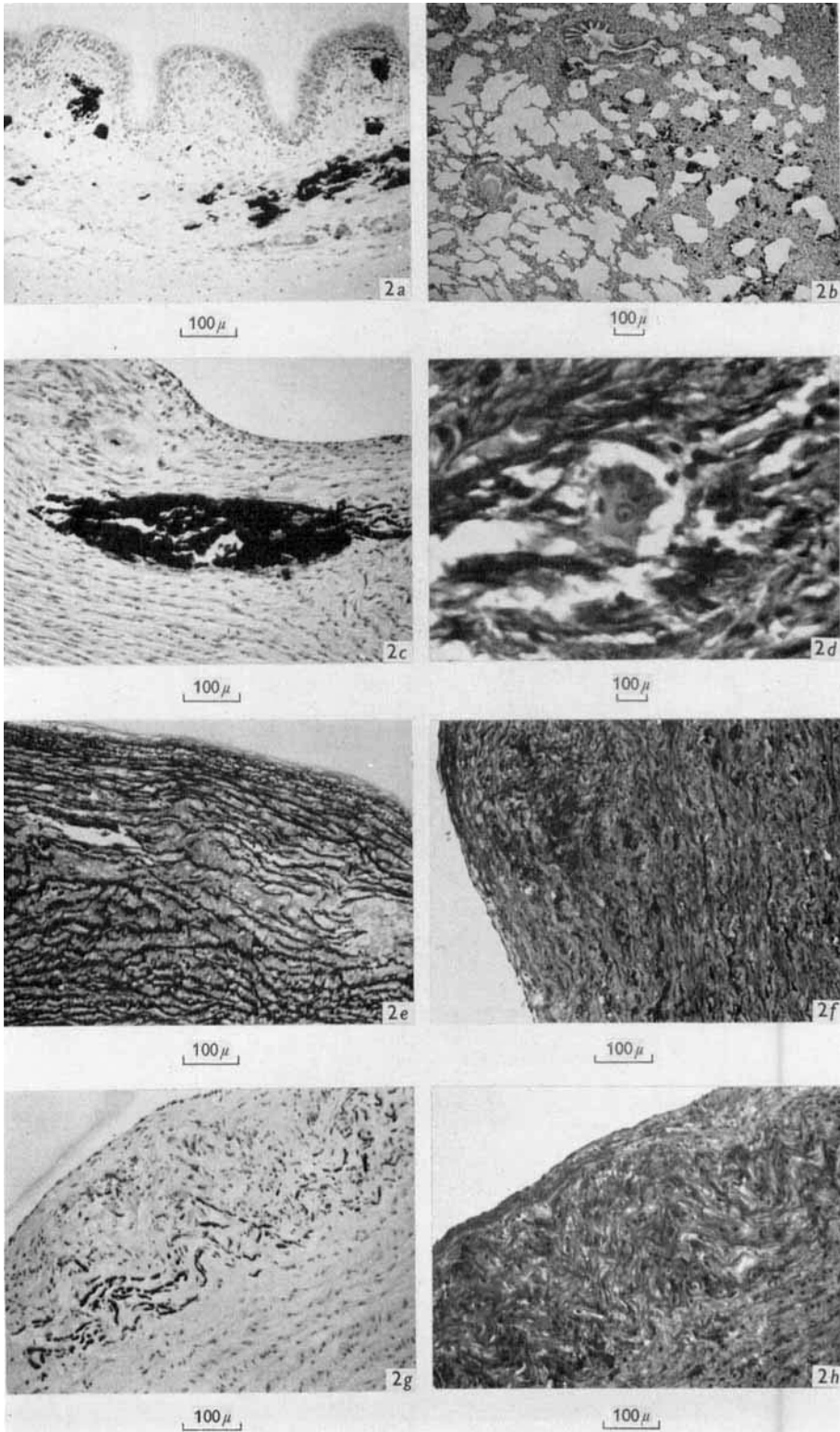
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EXPLANATION OF PLATES

PLATE I

a Calcium deposits in the renal cortex of a pig killed immediately after the period of cholecalciferol supplementation (time 0). von Kossa.

b Calcified casts in the renal pelvis (time 0). von Kossa.

c Foci of calcification in the wall of a renal artery (time 0). von Kossa.

d Scarring of the renal cortex 1 week after the end of cholecalciferol supplementation. Van Gieson.

e Intensification of the scarring at 1 month. Van Gieson.

f Calcification of the wall of a coronary artery at time 0. von Kossa.

g Myocardial lesion in the right auricle at time 0. Van Gieson.

h Coronary artery cut longitudinally. Non-calcified lesions at time 0. Periodic acid Schiff and haemalum.

PLATE 2

a Calcification of the bronchial mucosa and smooth muscle (time o). von Kossa.

b Calcification of pulmonary tissue affected by a pneumonic process (time o). von Kossa.

c Calcification in the wall of the aorta at time o. Both severe and mild lesions are shown, the latter with minimal calcification and a fibrous connective tissue reaction. von Kossa.

d Wall of aorta, 1 month after the period of cholecalciferol supplementation. Giant-cell reaction in the calcified tissue. Van Gieson.

e As *d*, showing fragmentation of elastica. Weigert–Sheridan stain.

f Wall of aorta 1 month after cholecalciferol supplementation. Non-calcified scar. Van Gieson.

g Wall of aorta 6 months after cholecalciferol supplementation, showing calcified remnants of elastic tissue. von Kossa.

h Scarring of the same area. Van Gieson.