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REVERSIBLE UREMIA WITH HYPERCALCEMIA DUE TO VITAMIN-D INTOXICATION*

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CHRONIC renal disease with uremia is usually an irreversible process. A case of reversible uremia is therefore reported as a reminder that the diagnosis is simple if the cause is present and is thought of but is often overlooked because its possibility is not considered. If it is recognized early, cure can be expected.

CASE REPORT

A 40-year-old advertising executive was first seen on February 28, 1950, because of progressive weakness, fatigability, dryness of skin and mouth, excessive thirst and polyuria. His past history was irrelevant except for the following: At the age of 14, during a routine insurance examination, albumin was found in his urine. For the three subsequent years, with a diagnosis of nephritis, he was under the care of an able internist who kept him on a low-protein, low-salt diet. At the end of three years the situation was regarded as cleared. He was able to obtain life insurance, although there was a slight mark-up of the premium rate. He remained well until the age of 20, when albumin was again discovered at an insurance examination. For the two subsequent years he was under the care of the same physician, again on restricted salt and protein.

After presumable recovery from this episode, he remained perfectly well until the onset of his present illness. He was able to obtain several more life-insurance policies at normal premium rates and was even able to have the mark-up on the first policy removed. His systolic blood pressure was never over 120; the diastolic pressure was not over 90, except during the two attacks of nephritis, when he thought the readings were somewhat higher.

In November, 1949, at the age of 40, four months prior to our first examination, he developed what he and his

TABLE 1. *Urine.*

DATE	FLUID BALANCE: DAY	FLUID BALANCE: NIGHT	FLUID BALANCE: TOTAL	ALBUMIN	SPECIFIC GRAVITY: HIGHEST CONCENTRATION	PHENOLSULFONPHTHALEIN EXCRETION: 15 MIN.	30 MIN.	1 HR.	2 HR.	TOTAL	CALCIUM EXCRETION (SULKOVITCH)
	cc.	cc.	cc.			%	%	%	%	%	
March 1-5 (average)	In 4120 Out 2930	In 700 Out 1490	In 4820 Out 4400	0-1+	1.010	15	5	10	10	40	3+
April 26	In 1600 Out 1140	In 300 Out 780	In 1900 Out 1920	1+	1.007	6	8	7	3	24	—
September 29	In 1320 Out 1170	In 900 Out 990	In 2220 Out 2160	1+	1.018	23	10	8	6	47	Normal

TABLE 2. *Blood.*

DATES	HEMOGLOBIN	WHITE-CELL COUNT	BLOOD FILM	SERUM NONPROTEIN NITROGEN	SERUM CALCIUM	SERUM POTASSIUM
	gm./100 cc.	x10³		mg./100 cc.	mg./100 cc.	milliequiv./liter
March 1-5	9	7.5	Normocytic, mild hypochromic anemia	50	14.1	4.8
April 26	10.1	8.6	Same	33	10.1	—
September 29	12.5	9.0	Normal	23	9.8	—

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physician thought was typical grippe. He had a running nose, cough and bone aches; his temperature rose to 100°F., but no higher. He was treated with penicillin and was absent from work for only one week. However, after returning to his office in mid-November, he did not feel entirely well. The first symptom was persistent fatigue. He could accomplish very little work in the afternoon, and arrived home each evening so exhausted that he found it necessary to retire immediately. During this same period, he occasionally felt a little chilly, as if he might have a slight fever; he noticed some stuffiness of the nose, which could be controlled by one or two aspirin tablets. These latter symptoms alone were never sufficient to interfere with work; fatigue was the predominant problem. He also developed some trouble in controlling his urine — that is, when his bladder became moderately full he would have to void immediately or lose control. There was no nocturia. When he stopped drinking his customary 6-8 daily cups of coffee, the urgency promptly ceased. Because withdrawal of the coffee appeared to cause constipation he resumed his previous intake, and the urgency reappeared. Meanwhile, in mid-January, about six weeks prior to his first visit, he developed persistent unquenchable thirst and resultant polyuria. His fluid intake rose to more than a gallon a day and he voided large amounts approximately every two hours, day and night. There were no other urinary symptoms. His skin and mouth became dry; distressing generalized pruritus set in. There was no appreciable loss of weight or other symptoms except greater fatigability.

The patient looked worn and pale. The skin and mucous membranes were dry. The blood pressure was 140/80. There were no other pertinent abnormal physical signs. His prostate was normal in size; its secretion showed no evidence of infection. A single specimen of urine passed in the office was normal except for a specific gravity of 1.008. Examination of the blood revealed a hemoglobin of 9 gm., a red-cell count of 3,460,000 and a white-cell count of 10,300, with a normal differential count. The stained film showed evidence of normocytic, slightly hypochromic anemia. A blood Hinton test was negative. The nonprotein nitrogen was 61 mg. per cent.

The patient was promptly admitted to the Massachusetts General Hospital for further investigation. Initially it was thought that he had either chronic glomerular nephritis, hyperparathyroidism or perhaps pure diabetes insipidus, although it was recognized that the last-named would not account for the elevated nonprotein nitrogen and the anemia. A decided source of confusion was the normal blood pressure in the presence of what appeared to be severe chronic renal impairment.

On admission, physical examination was unchanged except for a blood pressure reading of 160/90. Typical band keratitis was later found in both eyes.

The results of pertinent laboratory studies are noted in Tables 1 and 2.

The glucose tolerance test showed a mild diabetic curve; a bromsulfalein test showed only 2 per cent retention of dye. No evidence of intrinsic bone change was found on x-ray films of skull, spine and pelvis. The lamina dura around all teeth was intact. No trace of calcium deposit in the region of the kidneys was seen in a plain film of the abdomen. An electrocardiogram was normal except for a short QT interval, which is reported to be common in hypercalcemia.

Because the case obviously did not conform to the pattern of any of the usual forms of renal disease or to hyperparathyroidism or pituitary diabetes insipidus, and because the diagnosis appeared obscure, Dr. George W. Thorn was consulted. In addition to the diagnoses mentioned above, he raised the question of three other possibilities: chronic pyelonephritis with hypercalcemia and alkalosis, as described by Burnett and his co-workers[1]; metastatic disease such as myeloma; vitamin-D intoxication superimposed on chronic nephritis.

Further questioning of the patient brought out the fact that he had been taking 200,000 units of vitamin D daily for approximately six months. It had been prescribed by another physician for pain in the shoulder. The diagnosis was thus established.

After six days of hospitalization, the patient was discharged on a low-calcium diet and a regimen of forced fluids. During the subsequent seven months he has shown continued clinical improvement. On September 29, 1950, he reported that he felt very well and that since June he had been able to perform a full day's work without difficulty. Fatigue, thirst, polyuria and all other symptoms had entirely disappeared. His blood pressure was 120/70. The band keratitis had also cleared. This observation was confirmed by an ophthalmologic consultant.

The progress of pertinent laboratory findings is shown in Tables 1 and 2.

The manifestations of vitamin-D intoxication, as recently summarized by Chaplin, Clark and Ropes, are indicated below.[2] Not all these are necessarily present in a single case.

General: weakness, fatigue, weight loss.

Gastrointestinal: nausea, vomiting, diarrhea, abdominal cramps.

Neuropsychiatric: headache, paresthesia, vertigo, variable mental symptoms (depression, mild psychosis, coma).

Hematologic: normocytic, normochromic anemia.

Urologic: red cells, casts, albumin in the urine, with progressive impairment in concentrating ability and symptomatic frequency and nocturia.

Chemical: elevated serum calcium and phosphorus, with normal or slightly elevated alkaline phosphatase; progressive nitrogen retention.

Roentgenologic: diffuse demineralization of the bones in advanced cases; periarticular soft-tissue calcifications.

Pathologic: scattered calcium deposition in heart, kidney, blood vessels, stomach, lung, thyroid, pancreas and periarticular soft tissues.

Ophthalmologic: band keratitis.

The case reported above showed weakness and fatigue, band keratitis, mild anemia that was slightly hypochromic as opposed to the normochromic anemia usually reported, polyuria, impairment of urine concentration, slight albuminuria without abnormal solid elements, nitrogen retention and elevated blood calcium. Blood phosphorus was normal in contrast to the slightly elevated reading usually reported. The normal blood phosphorus and phosphatase and the absence of

TABLE 2 (*Concluded*).

Dates	Serum Phosphorus (Inorganic)	Serum Alkaline Phosphatase	Serum Sulfate	Serum Chloride	Serum Carbon Dioxide Content	Serum Cholesterol	Total Serum Protein
	mg./100 cc.	*units/100 cc.*	*milliequiv./liter*	*milliequiv./liter*	*milliequiv./liter*	*mg./100 cc.*	*gm./100 cc.*
March 1-5	5.0	3.	147	100	53	198	7.5
April 26	4.0	3.6	—	—	—	—	—
September 29	5.3	7.8	139	97	31.8	—	—

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any bone changes at x-ray examination tended to exclude hyperparathyroidism. The result of simple therapy, notably cessation of the vitamin-D intake, low calcium intake and increased fluids, is evident from the protocol.

Summary

A case of vitamin-D intoxication is reported. The outstanding features were increased fatigue and weakness, polydipsia and polyuria, nitrogen retention and a high blood calcium with relatively normal levels of other chemical elements. Improvement since the omission of vitamin D has been most satisfactory. This case is reported in order to emphasize the danger of large doses of vitamin D and the necessity of considering overdosage of this vitamin as a possible cause of uremia and hypercalcemia.

References

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(From the Histological Department of the University of Upsala. Principal: Professor Erik Agduhr, M.D.)

Z. Für Krebsforschung 54: (1935)

Ergosterol increases the prolific capacity of the experimental animals, and normal sexual functions intensify their resisting power against the toxicity of the ergosterol.

By Erik Agduhr.

(Received for publication, October 25th 1934)

From earlier published investigations [1] [2] it appears that in white mice receiving a basal diet consisting of unskimmed milk, bread and oats repeated gravidities have caused a rather important increase in the weight of the females. This increase in weight of the mice was not due to an increased fatty tissue but was to the greater part caused by a real increase of a number of organs inter alia also the skeleton.

Later on these investigations were continued also on rabbits and human being [3] and as far as I can judge at present the results are also positive. Of course one and another negative result can be met with — also in mice a few such negative results are reached when experimenting with members of a new stock — but these

negative results have only occasionally occurred, and as a rule they could be ascribed to distinct factors.

When performing these investigations with mice two remarkable observations were made which deserve a closer examination with the aid of continued experiments. The one observation was a conformation of my earlier discovery [1] [2], namely

that the irradiated ergosterol highly increases the prolific capacity of the mice, and the second bears upon the fact,

that the resisting power of mice against the toxicity of large quantities of irradiated ergosterol increases considerably when normal sexual functions occur in comparison with that which happens when such functions do not occur.

Below will be given an orientation of results from experiments performed in order to control these observations.

Besides controls the experiments include 97 white mice (52 males and 45 females), which form the series A, B and C. To each series belong a number of groups of mice.

All these mice received a basal diet composed of unskimmed milk, bread and oats parts of which were given ad libitum. This basal diet has proved itself to give the experimental animals a greater resisting power against the toxicity of large quantities of irradiated ergosterol than such as e. g. the Drummond's diet (Agduhr) and the Mc Collum's diet (Hauch).

The animals of the series A, B and C received ergosterol activated through irradiation by ultra-violet light (with the mercury-lamp Hanau for 20 minutes at a distance of 50 cm.) of a fixed quantity solved in ether and the solution included in a U-formed and flattened quartz-tube. In that way it was possible to irradiate the solution with the absence of air. When the ether had been volatilized completly the irradiated ergosterol was solved in refined soy-oil to such a quantity that one drop ($^1/_{50}$ cc) of the solution contains 100 µg ergosterol *).

Each mouse of the series A and B received a daily quantity of 200 µg ergosterol, and those of the series C received each 100 µg daily. All administration of ergosterol has been made per os and with the aid of calibrated pipets.

As is seen from the table the grouping of the mice has been of that kind in the series A and C so that normal sexual intercourse between mice of different sexes was excluded, while in the series B the mice were kept in pairs with a male and a female together in the same room of a cage.

In the few control-groups recorded below (Series D) the mice were kept in pairs for the making of normal sexual functions possible.

In all groups where the females gave birth to young ones these were taken away and immediately killed after birth in order to release the female from the exertion of the organism a lactation might give rise to.

The results as to the numbers of the gravidities appear perspicuously in the table but here they will be somewhat dealt more closely with.

In the group 238 A the one male died after 114 days of expe-

* When later on as hier the word „ergosterol" is written, it is always meant irradiated „ergosterol".

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riments — the female of the same room of the cage showed two gravidities only, while the other female of the same group had ten gravidities. That is why the average value of the number of the gravidities in this group is 6 only; but nothing contradicts the opinion that the average value here should instead have been ten for the whole group when both the males were alive and capable of fertilizing during the whole time of experiment. It may be also tath in the groups 237 A, 238 B and 239 A (see the table) and on account of ergosterol-lesions some males have been less capable of fertilizing than those of the remaining groups of the series B. But not considering these possibilities and counting with the number of gravidities really existing we reach the result that during 285 days of experiment with mice 30 days old at the start the average number of gravidities occurring has been 8,7. The control-animals showed an average of 5.2 gravidities during a corresponding time. In these experiments the large daily quantity of ergosterol added has increased the number of gravidities occuring with 67 per cent.

Of course some contingencies may act and unfavourably influence upon a small material, and one could regard the control-material recorded in the table as rather small to found something on. If, however, I examine my whole material of white mice where the numbers of gravadities and the number of young ones of the different births are followed for several years — a material now including more than four hundred groups — I find that the value 5 births is rather good for the average number of gravidities occurring in mice of up to 10 months of age when receiving the basal diet in question. In these mice, it is true, some groups showed an average number of gravidities of 8, but such groups were met with where the average value of the number of gravidities did not exceed 2 or 3 during the time mentioned.

On the other hand the ergosterol-group did not show any breeding-group, where a smaller average number of gravidities than 6 had occurred when an age of 10 months was reached. In return many of these groups are met with where the females at a corresponding age had passed an average of 11 gravidities. No doubt contingencies have caused in these groups (Series B) the average number of the gravidities to be somewhat too small rather than the reverse. At any rate these experiments demonstrate very well that circumstance where the irradiated ergosterol highly furthers the prolific capasity in the experimental-animals employed. How are things managed then if the ergosterol works most stimulating on the sexual functions of the males or on those of the females; it might also be as stimulating on the sexual functions of both the sexes; these different possibilities will be further discussed below.

The pure vitamin D has given results which on principle agree with what is written about the ergosterol concerning its influence on the sexual functions etc. (see also A g d u h r [1]).

The results as to the number of deaths occurring are also perspicuously presented in the table.

The *series A* includes 17 males and 13 females. During the time of experiment 11 males and 1 female or 64,7 and 7,7 per cent respectively died. The males died almost in the middle of the time of experiment, whereas the female got liver-necroses and died already after 13 days of experiment. Judging from the lesions the death of the males must be ascribed to the toxicity of the ergosterol. Any obvious difference in the number of deaths occurring cannot be found when a comparison is made between mice which are kept isolated from each others and such which are kept together with others of the same sex. The female which died after 13 days of experiment belonged, however, to a group where the different mice were kept isolated from each other. *These experiments show that the males are much more susceptible to the toxicity of the ergosterol than are the females when both the sexes are shut off from sexual intercourse.* Now, however, objection can be made that when only one female has died and thereto already after 13 days of experiment — although the female died from typical ergosterol-lesions, namely liver-necroses — the difference of both the sexes as to the resisting power against the toxicity of the ergosterol is, in reality, as yet, much greater than these experiments seem to show.

In order to illustrate these conditions it may be of interest to study the number of deaths occuring in the mice of the series C. The animals of these series received only 100 µg ergosterol per mouse per day. The series include 21 males and 18 females, and of those 10 males and 2 females, 47.6 and 11,1 per cent respectively, died during the time of experiment. Also here the ergosterol-lesions (see A g d u h r [1]) occurring were of such a kind that the toxicity of the ergosterol can be regarded as being the cause of death. Both the females of these series died after 161 and 225 days of experiment respectively. These results support the idea that the quantities of irradiated ergosterol added to the mice of these series, especially those of 200 µg, show the limit of what females can endure when they are shut out of normal sexual functions.

The smaller number of deaths about 17 per cent shown by the males of the series C when compared with that of the series A is probable, even if someone could have expected a still greater difference because the dose of ergosterol in series A which was twice as large as that of the series C. The results reached are, however, in accordance with the increase of the number of deaths as well as with that increase of organic lesions which have occurred at the doubling

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of the injurious substances added in some other experiments (Agduhr[1]) concerning the toxicity of different constituent parts of cod liver oil. Concerning the number of deaths in the females, on the other hand, the results reached cannot possibly form the base for a general estimation as to the lethal effect of a quantity of 100 µg or 200 µg daily of ergosterol. Obviously these doses are too small for getting a more general lethal effect in females which are shut off from sexual intercourse. Consequently they kill only the most susceptible females which can be thought to vary very much as to their occurrence in an animal material of so relatively small size as this nevertheless is.

Examining the number of deaths in the series B we find that only one male and no female died during the whole time of experiment. The experiments with the mice of these series are quite similar to those of the series A with the one exception that the mice of these series B were suitable kept for practising normal sexual intercourse.

When comparing the results reached as to the number of deaths occurring in the series B with those found in the series A we find a most interesting condition, namely that the only varying factor, the occurrence (Series B) resp. the non-occurrence (Serie A) of normal sexual intercourse has diminished the numbers of death to a ninth and resp. increased them ninefold as far as the males are concerned. Among females of the series B deaths on the whole did not occur. These remarkable conditions do not lessen in interest the fact that the mice of the series B at the same time also showed advanced organic lesions, described more in detail in my paper; Studies etc. Part II (see Agduhr[1] 1935). In spite of these great organic lesions the mice showed this high prolific capacity described above and shown in the table.

Summary and discussion.

Briefly summarised these investigations on mice have shown

1. *that* the males are much more susceptible to an overdosing of irradiated ergosterol than are the females;
2. *that* a daily quantity of 200 µg irradiated ergosterol administered to mice shut off from normal sexual intercourse caused 64.7 per cent of deaths in males, while a decrease of the ergosterol-dose to 100 µg has decreased the number of deaths to 47,6 per cent;
3. *that* a keeping of the experimental animals in pairs in order to make normal sexual intercourse possible has decreased the number of deaths in males from 64,7 to about 7 per cent and in females from about 8 to 0 per cent;

4. *that* an administration of 200 µg irradiated ergosterol to mice kept in pairs with one female and one male together in the same room of a cage has increased the numbers of gravidities considerably in spite of the occurrence of important organic lesions in the mice of the both sexes.

Concerning the last statement or the capability of the irradiated ergosterol to increase the number of the gravidities considerably I already in 1931[1], p. 88, expressed my opinion about the mechanism when writing: «Gewisse Beobachtungen sprechen dafür, dass das Ergosterin für das innersekretorische System die Rolle eines Irritaments gespielt hat».

In the summary of my work 1932[2] I wrote inter alia (p. 78): «The treatment with ergosterol in females has accelerated the increase in weight of the pituitary gland. In the males such an increased weight is not proved statistically — in pars anterior D (the difference) is even negative.

The females show in the pars anterior an increase in number of the glandular epithelium cells by about 40 %, in the mass of the glandular epithelium by about 70 % and in the secreting surface by about 60 %. Among the glandular epithelium-cells those with eosinophil granules show the greatest augmentation in number etc. From page 79 we cite: «With increased number of the gravidities in «the ergosterol-series» — the series which besides basal diet also received irradiated ergosterol — the increase in weight of pars anterior showed a fairly small augmentation. On the other side the increase in weight of pars intermedia is retarded and that of pars posterior was hardly effected at all.

The increased number of the gravidities and the treatment with irradiated ergosterol in co-operation have augmented the increase in weight of the whole pituitary body and especially of its pars anterior to a greater extent than either of these factors separately, but the results reached are far from being as great as the sum of the results reached when both the factors affected the organism

*1 In this last work quantitative micro-morphologic examinations are described for numbers of organs belonging to groups of white mice were the females had been the subjects of an increased number of gravidities and where some groups of mice had received the basal diet only while others in addition also received a daily quantity of 200 µg irradiated ergosterol from about 30 days of age and 7 to 8 months onwards. The results of the quantitative examinations are treated statistically. In the paper of 1932 as well as in this these examinations are based on the assumption that factors which during the time of post natal developpement are stimulating an endocrine gland to increased or to decreased development and function will cause an increase or a checking of the normal growth of the gland in question. The endocrine glands which are examined quantitatively up to now are the hypophysis cerebri, the thyroid and the parathyroid glands.

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separately. In the pars intermedia and the pars posterior the augmentation of the increase in weight is very small, if there is any at all etc.

The thyroid and the parathyroid glands are evidently influenced by the irradiated ergosterol (see page 80 of my paper 1932). My statements 1931 [1] are demonstrated in my histo-experimental studies of 1932 [2]. Afterwards these things have also been confirmed biochemically. For example we can read in Annual Review of Biochemistry [3] p. 89. 1934. that Rosenheim and King found the somewhat unexpected fact now emerges that an apparently unconnected series of physiologically highly active substances, such as calciferol (crystalline vitamin D) oestrogenic hormones, carcinogenic hydrocarbons, cardiac glucosides (strophanthin, etc.), and probably also toad poison (bufotoxin) have a phenanthrene nucleus in common." the relation of the sterols to oestrogenic hormones is clearly expressed in the formulation of the latter, based on the new cholane structure, and is emphasised by the discovery that ergosterol, calciferol, and especially neoergosterol possess oestrogenic activity [3]. Also in the Annual Reports of the Progress of chemistry 1933. Vol. 30. p. 216 and 340 etc. we can read about these problems. And from this place I content myself by citing: In addition to the compounds already mentioned, neoergosterol, calciferol, and ergosterol also exhibit some oestrogenic action, diminishing in the order cited."

Oestrin causes heat but no ovulation in rabbits (personal information of Axel Westman). Here it consequently does not stimulate pars anterior of the pituitary gland, which regulates the ovulation, but gives only the heat-effect otherwise regulated from the genital glands. That the irradiated ergosterol also stimulates the pars anterior of the pituitary gland which also has been examplified above is clearly demonstrated in my earlier work (1932). *This stimulating influence on the endocrinal system of glands stretches itself also beyond the pituitary and the genital glands, and probably we have here to look for the cause why these experimental animals are able to show such a great prolific capacity in spite of the rather severe ergosterol-lesions appearing in different organs at the same time.*

The first point above emphasizes that the males are much more susceptible to overdosing of irradiated ergosterol than are the females when both sexes are shut off from normal sexual intercourse. This condition seems to be as interesting as unexpected, and beforehand it might not be easy to say which causes are at work. When, however, the results from the quantitative micro-morphological examinations are looked over carefully we find that everything goes

to prove that also this mechanism is to be looked for in the sphere of the endocrinal glands. The influence of the ergosterol leads to widely varying results in the endocrinal glands of the two different sexes. These different results of the added ergosterol as to the pituitary gland have been somewhat mentioned above. In this gland the dosed ergosterol causes an increase of the weight of pars anterior in females while such a change did not occur in males *).

In the thyroid glands of males the irradiated ergosterol (200 µg per day per mouse) dosed has caused an important retardation of the postnatal increase of the whole gland but above all of its epithelium cells, while in females such a retardation was not settled or made probable statistically.

In mice receiving the basal diet only and shut off from normal sexual intercourse the weight of the parathyroid glands was more than twice as large in males as in females. When adding ergosterol in a quantity of 200 µg per mouse per day for eight months a very important retardation of the post-natal development of this gland occurred. The average weights of these glands are decreased by almost two thirds in the males and a little more than the half in the females. The parathyroid glands varied much more in size from animal to animal in these ergosterol-males than was the case with the corresponding females, and in those males where the parathyroids showed especially low weights highly atrophied glandular epithelium cells occurred and many of these atrophied cells also showed pycnotic nuclei. At least in some part of the glands all these males show atrophied glandular epithelium cells.

In one of these dead males no trace of the parathyroid glands was met with although they were carefully looked for in an unbroken series of sections from a large part of the throat-jaw-region. In the females on the other hand the parathyroids showed a very small variation in size and they had an almost similar locality in the different individuals.

Other endocrinal glands of my material have not yet been examined by quantitative micro-morphological methods, but already, the results related seem to me to evidently support the opinion that the greater mortality in males is due to the more severe endocrinal disturbances occurring here, and which further decrease the resisting power of the males than that of the females against the toxicity of the irradiated ergosterol.

As is already put forward in the second point above the decrease in the number of deaths in males from 64,7 per cent to 47,6 per

*) For the technic used when examining the organs quantitatively see: Agduhr, Erik: Beiträge zur Technik der quantitativen mikro-morphologischen Bestimmungen. Verh. d. Anat. Gesellsch. 41. Vers. Lund, 1932.

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cent when the daily dose of ergosterol is diminished from 200 µg to 100 µg is quite in accordance with what has appeared in similar experiments with other toxic substances where a 50 % decrease in the size of the toxic substances occurred.

The third of the four points in the summary above remains to be discussed. Now it is a question of trying to penetrate the problem why normal sexual intercourse is able to decrease the number of deaths so considerably as is the case in males as well as in females. We shall also try to see to what extent the results of the quantitative micro-morphological analysis of the endocrinal glands (1932) can explain these remarkable conditions. In the females we find that the weight of the whole pituitary gland which was already considerably increased on account of the irradiated ergosterol received is still further increased on account of the gravidities passed through, and this increase is especially due to the pars anterior.

In males, on the other hand, the increased sexual intercourse has caused a little decrease of the pituitary gland mostly depending on a decrease of the pars anterior.

In the thyroid glands of the ergosterol-males shut off from sexual intercourse, as we have seen, an important retardation was shown as well what concerns the gland in its entirety as also the quantity of its glandular epithelium-cells. Among ergosterol-mice, on the other hand, which were kept in pairs (one male and one female), and where subsequent normal sexual intercourse occurred, the males have shown a considerably increased thyroid gland — an increase which was mostly due to an increase of the quantity of the glandular epithelium cells at the same time as the colloid-content was diminished absolutly. The value of the relation $\frac{\text{Epithel}}{\text{Colloid}}$ has been especially high in these males. In the same direction but still more pronounced are the changes of the thyroid glands of those females receiving the corresponding quantity of irradiated ergosterol and having normal sexual intercourse.

The parathyroid glands show particularly interesting conditions also. In both sexes but mostly in the males is the parathyroid retarded in its development on account of the quantity of ergosterol added. At a maximal number of gravidities (sexual functions) does the parathyroid gland increase considerably in weight in the females; the males, on the other hand, show a decrease in weight of the parathyroid glands which are somewhat below the decreased size already caused by the irradiated ergosterol administered. At the same time, however, the parathyroid glands become more constant in their size — they did not vary much in size from mouse

to mouse as when shut off from sexual intercourse, and any especially small parathyroids with highly atrophic cells were not seen in these males which was the case in several of those males completely shut off from sexual intercourse.

Thus in males *not* receiving ergosterol normal sexual functions cause a decrease in the weight of the parathyroid glands a decrease which is absolutely larger than that caused by normal sexual functions in males which at the same time received 200 µg irradiated ergosterol daily.

CONCLUSIONS.

The males are much more susceptible than the females to an overdosing of irradiated ergosterol when received under the postnatal development and afterwards.

My results published in 1931 and 1932 and showing inter alia that a quantity of 200 µg *irradiated ergosterol considerably increase the prolific capacity of the animals* have been further confirmed.

The irradiated ergosterol not only causes heat but it also influences the postnatal development of several endocrinal glands promoting or checking — unequal in the different sexes.

A keeping of the animals in pairs in order to make *normal sexual intercourse possible increases considerably the resisting power of the mice against the toxicity of the irradiated ergosterol* — the number of deaths in about ten month of dosing (200 µg per day per animal) decreases in males from 65 to 7 per cent and in females from 8 to 0 per cent.

Everything seems to speak in favour of the explanation of these phenomena being in the sphere of the system of the endocrinal glands, as in the stimulating to increased development and function or decreased function and checking in development of a number of endocrinal glands (up to now only the hypophysis cerebri, the thyroid and the parathyroid glands are examined with my quantitative micromorphological method) caused by these large doses of the irradiated ergosterol. In addition one has also to reckon with direct ergosterol-lesions in the endocrinal glands, which were retarded in their post natal development.

ZUSAMMENFASSUNG.

Ergosterol vermehrt die Fortpflanzungsfähigkeit von Versuchstieren und die normalen Sexualfunktionen erhöhen die Widerstandskraft gegen die toxische Wirkung des Ergosterols. Von Erik Agdahr.

Männliche Mäuse sind viel empfindlicher gegen Überdosierung des bestrahlten Ergosterols als weibliche. Die früheren Ergebnisse des Verfassers, dass eine Dose von 200 µg bestrahlten Ergosterols die Fortpflanzungsfähigkeit der Tiere beträchtlich vermehrt, konnten weiter bestätigt werden. Die bestrahlte

Number of the group	Transferred, irradiated with ultra-violet light: mg per 100 g per day	Date of the irradiation	Number of mice in the group ♂	♀	Age at the beginning of the experiment (Days)	Length of the experiment (Days)	Bodyweights, gr. at the start of the experiment	at the end of the experiment	at the middle of the experiment time	Basal diet: Female maintained with oats	Beginning of the experiment
233	200	2000	1		32	286	13,2	26	21	1	16. 11. 31
234	200	2000	3		36	152	10,9	20,1	17,2	1	16. 11. 31
235	200	2000		5	36	265	11	22,4	19,6	1	16. 11. 31
236	200	2000	4		32	258	6,5	23,2	20,2	1	23. 11. 31
243	200	2000	6		39	290	10,1	26,7	25,4	1	29. 12. 31
244	200	2000		1	35	296	12,1	21,9	20,5	1	12. 1. 32
245	200	2000		4	32	272	11,8	24,7	22,2	1	19. 1. 32
237 A	200	2000	2	2	32	310	9,7	26,7	23,9	1	23. 11. 31
237 B	200	2000	2	2	32	316	10,6	31,3	27,1	1	23. 11. 31
238 A	200	2000	2	2	32	270	11,1	26,5	23,2	1	7. 12. 31
238 B	200	2000	2	2	32	270	10,1	27,8	26	1	7. 12. 31
239 A	200	2000	2	2	36	280	11,2	27	25,5	1	7. 12. 31
239 B	200	2000	2	2	36	280	11,1	28,7	26,1	1	7. 12. 31
249	200	2000	2	2	36	272	10,6	28,6	26,2	1	2. 2. 32
228	100	1000	6	2	40	270	15,2	25,5	23,3	1	30. 9. 31
231	100	1000		1	39	283	11,2	22,5	20,6	1	25. 10. 31
232	100	1000	5		32	286	11,2	25,8	23,6	1	25. 10. 31
246	100	1000	4		34	278	12,9	32,3	29,6	1	25. 1. 32
247	100	1000		4	36	272	11,9	27,7	22,9	1	25. 1. 32
248	100	1000	6		36	272	12,5	25,5	24,4	1	25. 1. 32
251 A	100	1000		4	36	272	10,2	22,2	21,8	1	2. 2. 32
251 B	100	1000		4	36	272	8,8	26,1	27,1	1	2. 2. 32
Controls on the basal diet only:											
320	—		2	2	30	320	8,1	30,4	25,9	1	8. 3. 33
321	—	—	2	2	30	314	11	26,1	25,7	1	15. 4. 33
328	—	—	2	2	32	303	8,9	25,5	24,3	1	22. 4. 33
331	—	—	2	2	34	303	10,9	28,6	26,2	1	23. 4. 33
Controls on the basal diet and on the solvent:											
144		—	2	2	30	220	12,6	26,6	25,6	1	2. 1. 31
159	—	—	2	2	31	244	14,2	25,6	25,2	1	2. 1. 31
144 b / 159 b	—	—	8		30	240	11,3	27,8	26,4	1	10. 1. 32

Animals diet: Number of the mice ♂	♀	Average days of the experiment ♂	♀	Average number of the arachides per female	Male and female together in pairs in different rooms of the same cage	Separated from each other in different rooms of the same cage	Together in the same cage	Quantity solvent (refined sesame oil) per day per house etc.	Percentage of deaths: Males	Females	Series and its total number of mice ♂	♀
2		106				1		1/25				
3		128				1		1/25				
	1		13			1		1/25				
3		167				1		1/25	64,7	7,7	27	13
3		169					1	1/25				
—							1	1/25				
—						1		1/25				
—	—			8	1			1/25				
—	—			11	1			1/25				
1		114		6	1			1/25				
				7,5	1			1/25	7,14	0	14	14
				8,5	1			1/25				
				9	1			1/25				
				11	1			1/25				
2		11				1		1/500				
	1		161			1		1/500				
5		132				1		1/500				
2		176				1		1/500				
	1		212			1		1/500	17,6	11,1	21	18
1		225				1		1/500				
						1		1/500				
						1		1/500				
						1		1/500				
				6,5	1							
				6,5	1							
				4	1							
				1	1							
											20	12
				4	1			1/500				
				6,5	1			1/500				
						1		1/500				

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Ergosterol erzeugt nicht nur Brunst, sondern es beeinflusst auch die postnatale Entwicklung mehrerer endokriner Drüsen, teils fördernd, teils hemmend, ungleich bei den verschiedenen Geschlechtern. Die Möglichkeit normalen Sexualverkehrs erhöht die Widerstandskraft der Mäuse gegenüber der toxischen Wirkung des bestrahlten Ergosterols. Die Letalität sinkt bei den Männchen von 65 auf 7 und bei den Weibchen von 8 auf 0 %. Alles scheint dafür zu sprechen, dass sich das daraus erklärt, dass die endokrinen Drüsen teils zu erhöhter Tätigkeit angeregt, teils in ihrer Entwicklung und Funktion gehemmt werden. Bisher wurden nur die Hypophyse, die Schilddrüse und die Nebenschilddrüsen mit der quantitativen mikromorphologischen Methode des Verfassers untersucht. Ausserdem muss man noch mit direkten Ergosterolschäden an denjenigen endokrinen Drüsen rechnen, welche in ihrer postnatalen Entwicklung gehemmt sind.

RÉSUMÉ.

L'ergostérol augmente la puissance de reproduction chez les animaux servant aux expérimentations, et les fonctions sexuelles normales accentuent la force de résistance à l'action toxique de l'ergostérol lui-même. Par Erik Agduhr.

Les souris mâles sont beaucoup plus sensibles que les femelles à un fort dosage d'ergosterol irradié. Les observations antérieures de l'auteur — qu'une dose de 200 µg d'ergostérol irradié augmente considérablement la puissance de reproduction des animaux — ont encore pu être confirmées dans la suite. L'ergostérol irradié ne provoque pas seulement de rut, mais exerce aussi une influence sur le développement postnatal de plusieurs glandes endocrines, et en favorise ou en ralentit l'action de façon inégale selon le sexe. La possibilité des fonctions sexuelles normales accroît la force de résistance des souris à l'action toxique de l'ergostérol irradié. La mortalité qui en résulte diminue de 65 à 7 chez les mâles et de 8 à 0 % chez les femelles. Tout semble concourir à expliquer ainsi que l'activité des glandes endocrines soit stimulée, pour les unes, ou entravée, chez les autres, dans leur développement et leurs fonctions. Jusqu'alors on n'avait expérimenté la méthode micrologique quantitative de l'auteur que sur les hypophyses, les glandes thyroïdes et para-thyroïdes. En outre, on doit encore tenir compte des inconvénients directs de l'ergostérol dans les glandes endocrines, qui se trouvent gênées dans leur développement postnatal.

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ON THE NUTRITIONAL REQUIREMENTS OF YOUNG CHILDREN WITH PARTICULAR REFERENCE TO CALCIFICATION[1]

By Thelma Allen, Ada V. MacLeod, and E. Gordon Young

Abstract

Two nutritional surveys have been carried out on 158 children of one to six years in Halifax, including dietary records, anthropometric measurements, roentgenograms of the bones of the hand, and laboratory tests. Observations were repeated after six months. Deficiencies in calories, calcium, and vitamin D occurred most frequently on the basis of the Canadian dietary standard. Seventy-eight per cent of the children received less than 1 gm. of calcium per day. The ratio Ca : P averaged 0.77. The consumption of vitamin D varied widely and was less than 400 I.U. in 47% in the first survey and 84% in the second. The average intake was less than 200 I.U. per day. From data of repetitions it was apparent that half of the children showed adequate growth and calcification on substandard diets while 30% did not. It was evident that normal maturation of bone could take place on intakes of vitamin D of 25 to 150 I.U. and of calcium of less than 1 gm. Calcium was adequate at 0.2 to 0.7 gm. per day in some cases but not in others.

Introduction

This study was commenced in 1945 when the scientific literature contained relatively few papers on the dietary requirements of infants and children. The baby under one year of age has been very carefully studied. Extensive anthropometric measurements of growing children are available but in few instances related to diet (1, 3, 9, 28, 33, 35). Within the past decade moderately accurate methods of analysis for vitamins have been developed so that an assessment of diet in relation to growth in children is now possible.

Surveys based on questionnaires or the use of 'average' portions cannot be considered as contributing reliable *quantitative* data to our knowledge of dietary requirements for normal growth. The classical work of Holt and Fales (13) on a selected group of 100 children between one and 16 years of age has provided data in terms of energy, protein, fat and carbohydrate. These results have been extended in nutritional surveys of wider scope by Youmans, Patton, and Kern (37) in Tennessee, by Beal, Burke, and Stuart (2) in Boston, and by Scobie, Burke, and Stuart (26) in Newfoundland. The most extensive survey of children's diet has however been published by Widdowson (36) in England. In this study 916 children between 1 and 18 years of age were examined and dietary records during one week were obtained between 1935 and 1939. A résumé of the evidence contributing to the selection of levels of requirements is already available (19). Many of the values set down as recommended dietary allowances are without adequate scientific foundation, particularly for vitamins and especially for vitamin D. While 400 to 800 I. U. per child per day are recommended the factual basis for these figures is meager (14, 19, 23, 30).

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The purpose of the surveys to be described was to obtain dietary data for young children from one to six years of age and to correlate these findings with the physical condition and rate of growth by repeating the examination at intervals of approximately six months. Anthropometric and radiographic measurements and a limited number of laboratory tests were also made. From the data it was hoped to be able to deduce levels indicative of amounts of essential nutrients which would or would not permit of normal growth, ossification, and good physical condition.

Procedure

Two nutritional surveys have been made on children of preschool age, between one and six years. The first was carried out between 1945 and 1947 on 55 children from the wealthier class in Halifax, N.S. The second was conducted between 1949 and 1951 on 101 children from the poorest class in the same city with a population of approximately 100,000.

The food consumed by the children was weighed on standard dietetic scales. The investigator made three visits to the family during the course of the week. The child then came to the Dalhousie Public Health Clinic as soon as possible. Physical examinations were made by a pediatrician and records kept on standard forms. Anthropometric measurements included body weight, standing height, pelvic girth, and chest circumference by the methods of Vickers and Stuart (33). Chest circumference was measured in the sitting position. X-radiographs of the bones of the wrist were taken in the Department of Anatomy on many of the children to determine age of maturation by the method of Todd (32).

A specimen of urine was tested routinely for the presence of protein and of reducing sugar. Drops of blood were taken for red and white cell counts and for the estimation of hemoglobin by the Newcomer method.

The data on food consumption were calculated in terms of calories, protein, fat, carbohydrate, calcium, phosphorus, iron, and vitamins (A, B_1, B_2, C, D, and niacin), from standard tables (5, 8). They are expressed as the mean of the consumption for one day from the observations during one week. Differences between sexes are not statistically significant up to the age of seven in any category according to the evidence of Widdowson (36) and we have confirmed this conclusion. No differentiation has therefore been made between boys and girls in our calculations. Meredith (18) has made a careful analysis of stature in this regard.

The distribution of the children in the two surveys in terms of age and sex is shown in Table I. The mean values for the anthropometric measurements of the two groups are listed in Table II. It is apparent that the children in group II were smaller than those in group I. The dimensions in group I are almost identical with the values obtained by Vickers and Stuart (33), taken at the half-year, for children of North European stock in Boston. They are slightly lower than the values for weight and height in Cleveland, Ohio, of

TABLE I

DISTRIBUTION OF CHILDREN IN RELATION TO AGE AND SEX

Age, yr.	Survey 1		Survey 2		Total
	♂	♀	♂	♀	
1 – 2	4	10	7	7	28
2 – 3	7	6	6	5	24
3 – 4	8	5	10	14	37
4 – 5	2	5	9	7	23
5 – 6	5	3	9	10	27
6 – 7	—	—	11	8	19
Totals	26	29	52	51	158

TABLE II

ANTHROPOMETRIC MEASUREMENTS

Age, yr.	Weight, kgm.			Height, cm.			Chest circumference, cm.			Pelvic girth, cm.		
	1	2	S*	1	2	S*	1	2	S*	1	2	S*
1 – 2	11.5	10.6	*11.5*	79.5	78.3	*81.9*	48.4	48.1	*49.9*	15.4	12.0	*14.0*
2 – 3	14.5	12.5	*13.7*	91.5	85.5	*91.1*	50.7	49.5	*51.7*	15.0	13.5	*15.1*
3 – 4	15.2	14.4	*15.7*	95.5	93.8	*99.5*	51.6	51.1	*53.1*	16.0	14.7	*16.3*
4 – 5	17.3	17.0	*17.6*	104.0	101.9	*105.2*	52.0	53.4	*54.2*	17.0	15.5	*17.1*
5 – 6	19.9	19.5	*19.7*	112.5	108.7	*112.0*	55.4	53.4	*55.6*	18.4	15.0	*18.1*
6 – 7	—	20.8	*21.7*	—	116.3	*117.8*	—	49.1	*57.1*	—	15.9	*19.1*

1 – *First survey.*
2 – *Second survey.*
* *Vickers and Stuart (33).*

Simmons (28) and rather irregular as compared with the values given by Widdowson (36) for English children. There are no comparable figures for Canadian children of this age.

Hematological values are presented in Table III. The average for the hemoglobin levels of 55 children on initial examination was 12.4 gm. per 100 ml. but if subsequent repetitions are included this becomes 12.7 gm. from 92 analyses. The range was from 10.6 to 14.1. A comparable series of observations was made on 319 children, one to six years of age, in various

parts of Canada by Pett and Ogilvie (24). The average was 12.5 and the approximate range 10 to 15. Figures obtained by Widdowson (36) in England on a similar group of 25 children averaged 12.5. There is thus remarkably close agreement. The red cell counts are slightly higher than those usually quoted in textbooks on pediatrics at 4.5 to 4.8 × 10^6. The white cell counts in Table III are slightly lower than those currently quoted at 8 to 9 × 10^3 (4).

TABLE III

HEMATOLOGICAL VALUES—SURVEY I

Age, yr.	Hemoglobin, gm./100 ml.	R.B.C., cells × 10^{-6}/mm.^{3}	W.B.C., cells × 10^{-3}/mm.^{3}
1 - 2	12.5	5.0	7.5
2 - 3	12.1	5.0	7.2
3 - 4	12.4	5.2	6.7
4 - 5	12.4	5.0	7.0
5 - 6	12.9	5.4	6.0
Av.	12.4	5.1	7.0

The consumption of food in terms of essential nutrients for both surveys is presented as averages per child per day in Table IV, together with requirements as calculated from the current Canadian dietary standard (6). It is apparent that the children of survey I were consuming more food per child than those in survey II. Their greater weight and height are shown in Table II. As compared with the standards, deficiencies in calories, calcium, and vitamin D are evident in most categories according to age. The intakes of other essential nutrients are close to, or far in excess of the standards. If compared with the recommended dietary allowances of the U.S.A., which bracket children of one to three years and four to six years, it is frequently apparent that the consumption of the children in the lower year is less than that recommended, about equal in the intermediate year and higher in the upper year. Intakes lower than recommended are indicated in calcium and occasionally in calories, phosphorus, iron, thiamine, ascorbic acid, niacin, and vitamin D. This applies particularly to the children in survey II. The intakes of children in survey I were deficient only in calcium and occasionally in calories and niacin.

The variation in the levels of intake is frequently very wide both for a particular group and a single child. In order to present this fact in the most useful form, the variation, inclusive of 80% of the children in each group, selected towards establishing normal distribution, is shown in Table V for calories, calcium, phosphorus, and vitamin D. It is evident that the widest variation occurs in the consumption of vitamin D and the second in calcium.

TABLE IV

AVERAGE CONSUMPTION OF ESSENTIAL NUTRIENTS PER CHILD PER DAY CANADIAN STANDARD BASED ON AVERAGE WEIGHTS FOR AGE CATEGORIES

Age, yr.	No. of survey and children	Cal.	Protein, gm.	Fat, gm.	Carbo., gm.	Ca, gm.	P, gm.	Fe, mgm.	Vitamins: A, I.U.	B1, mgm.	B2, mgm.	C, mgm.	D, I.U.	Niacin, mgm.
1 - 2	I - 14	992	38	36	121	0.90	0.92	12.4	13,100	0.77	1.57	51	1,525	4.6
	II - 14	735	30	30	90	0.47	0.61	5.6	5,380	0.45	0.80	34	1,310	3.3
	S	1150	36	32		1.0	1.0	6	700	0.35	0.58	30	400	3.5
2 - 3	I - 13	1,251	42	46	166	0.82	0.81	9.9	10,350	0.79	1.45	58	1,107	6.1
	II - 11	952	36	41	108	0.64	0.73	4.5	5,126	0.54	1.17	40	365	4.7
	S	1350	37	36		1.0	1.0	6	1000	0.40	0.67	30	400	4.0
3 - 4	I - 13	1,187	49	51	190	0.83	1.06	10.1	7,840	0.81	1.44	65	352	5.8
	II - 24	1,172	38	47	143	0.53	0.83	6.4	3,187	0.52	0.95	31	164	5.5
	S	1380	38	42		1.0	1.0	6	1100	0.45	0.75	30	400	7.5
4 - 5	I - 7	1,291	48	56	164	0.84	1.06	8.8	4,030	0.81	1.35	89	586	6.8
	II - 16	1,211	42	46	151	0.54	0.95	6.1	4,544	0.52	0.93	42	165	6.1
	S	1500	40	47		1.0	1.0	6	1100	0.50	0.80	30	400	5.0
5 - 6	I - 8	1,518	52	55	210	0.83	1.14	9.4	12,910	0.93	1.55	82	1,010	6.9
	II - 19	1,527	50	58	194	0.46	1.00	6.5	5,308	0.45	1.16	37	283	7.3
	S	1670	42	47		1.0	1.0	6	1500	0.52	0.85	40	400	5.2
6 - 7	II - 19	1,586	53	64	195	0.82	1.10	8.1	3,885	0.75	1.41	64	334	7.1
	S	1800	44	50		1.0	1.0	6	1500	0.55	0.90	30	400	5.5

TABLE V

VARIATION INCLUSIVE OF 80% OF CHILDREN

Nutrient	Survey	Age, yr. 1–2	2–3	3–4	4–5	5–6	6–7
Calories	I	739 – 1104	828 – 1457	1023 – 1525	1139 – 1469	1076 – 1668	—
	II	617 – 1112	765 – 1159	531 – 1503	938 – 1425	933 – 1938	1217 – 1974
Calcium (gm.)	I	0.55 – 1.30	0.35 – 0.90	0.53 – 1.13	0.57 – 0.90	0.43 – 1.06	—
	II	0.24 – 0.83	0.23 – 0.89	0.23 – 0.99	0.21 – 0.80	0.25 – 1.03	0.56 – 1.16
Phosphorus (gm.)	I	0.64 – 1.22	0.50 – 1.27	0.68 – 1.48	0.77 – 1.30	0.84 – 1.29	—
	II	0.48 – 1.05	0.47 – 0.84	0.28 – 1.28	0.50 – 1.00	0.52 – 1.33	0.83 – 1.31
Vitamin D (I.U.)	I	85 – 2734	36 – 1332	26 – 521	21 – 1516	25 – 1665	—
	II	27 – 119	13 – 343	15 – 153	32 – 184	20 – 425	32 – 1468

Calories

The results are expressed graphically in Fig. 1 in which it may be seen that the levels of energy of the food consumed by children in survey I corresponded closely to the standards and were definitely above those of survey II by 200 Cal. or more, in the lower categories. At one to two years there is an apparent departure from the linear relationship of age to calories. This coincides with the normal curve of growth. The results of Virtue and McHenry (34) on about 400 children in Toronto, Ont., are plotted as they also represent a level of intake lower than the standards. The results of Beal, Burke, and Stuart (2) in Boston and those of Scoble, Burke, and Stuart (26) for Newfoundland are higher than ours at all ages.

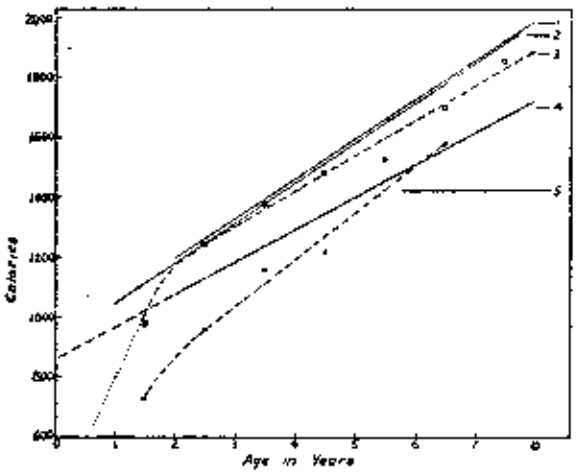

FIG. 1. Calories in relation to age: (1) U.S.A. recommended dietary allowances, (2) Canadian dietary standard, (3) our results in survey 1, (4) results of Virtue and McHenry (24), (5) our results in survey 2.

Protein

The consumption of protein in survey I was slightly higher than in survey II but both are in astonishingly close agreement with previous results in England (36) and in Newfoundland (26). They tend to be a few grams higher than the Canadian standards. A departure from a linear relationship to age is notable at one to two years. Protein accounted for between 13 and 16% of the total calories.

Fat

Fat consistently provided between 34 and 36% of the total calories and was consumed in amounts somewhat above the standards, based on 25% of the total calories.

Calcium and Phosphorus

As compared to the present standard of one gram the intake of calcium in both surveys was definitely low. This was particularly true for the children

in the second group at all ages. The intake of phosphorus was also lower than the standard at most ages in the second group. These facts are most clearly shown in the variations listed in Table V. The ratio Ca : P varied between 0.73 and 1.0 in the first group and between 0.64 and 0.89 in the second group, with an over-all average of 0.77. The percentage of the total number receiving less calcium than the standard was 78. This fact is of considerable importance in the consideration of the degree of maturation from the roentgenograms discussed below. In all comparable groups studied by Widdowson (36) and by Scobie *et al.* (26) the average intakes were less than one gram of calcium.

Iron

The consumption of iron may be considered as adequate in relation to the standard with few exceptions and in no individual was it less than 5 mgm. in survey I. It was notably high in children of one to three years because of the use of special foods rich in iron. In survey II 50% of the children were ingesting less than 6 mgm. and it frequently fell to 4 mgm. or less. Unfortunately we have no values for levels of hemoglobin in this group.

Vitamin A

There is a notably high consumption of this vitamin regardless of the factor adopted for the equivalence of β-carotene and vitamin A. In the children of survey I this was due to the frequent use of fish liver oils. The range of individual intake was very wide. Our figures are higher than those of the English and Newfoundland surveys.

Vitamin B_1

Based on a standard allowance of 0.3 mgm. per 1000 Cal., no deficiency in either group was apparent in the averages although about 25% of the children in survey II were substandard in their intakes.

Vitamin B_2

On the basis of a standard allowance of 0.5 mgm. per 1000 Cal., the same adequacy of intake as for B_1 is evident. Nevertheless about 20% of the children in survey II were below the standard.

Vitamin C

The agreement of the averages in survey II with the standard is close in several categories and a substandard consumption occurred in 36% of the children of this group.

Vitamin D

The sharpest contrast between our two surveys is to be found in this vitamin. This is attributable to the general use of fish liver oils in group I and the lack of them in group II except for infants of one to two years. The individual variation was very wide as can be seen in Table V. Children receiving this

supplement often ingested over 1000 I. U. per day while children without it frequently ingested less than 100 I. U. In survey I 47% were receiving less than 400 I. U. and in survey II it was 84%. On a distribution curve the great mass of children were below an intake of 200 I. U. This fact, especially when considered in relation to growth and calcification, is probably the most important finding of the survey. It agrees with the results of Widdowson (36) for English children, although our averages in group II are somewhat higher than hers. Few surveys have thus far included this vitamin and these facts may be of help in establishing a proper physiological level as a standard. It is significant that no case of active rickets was observed during physical examination.

Niacin

The average consumption was adequate based on a standard of 3.0 mgm. per 1000 Cal. Of the children in survey II, 36% were below the standard.

Nutrient Intake per Kgm. of Body Weight

Consumption of the essential nutrients in both surveys has been calculated per kilogram of body weight, except for vitamins B_1, B_2, and niacin which have been expressed per 1000 Cal., as listed in Table VI. The results for the children in group I are still somewhat higher than those of group II especially for calcium, iron, vitamins A, B_1, B_2, C, and D. It is evident that the high nutritional plane of the first year of life is carried over into the second. No particular drift of any nutrient is evident with increasing age, although this has already been established for calories and protein by Holt and Fales (13). Our results with protein suggest that 3.0 gm. per kgm. is adequate at one to three years and 2.5 gm. per kgm. at four to six years, in exact agreement with the current Canadian standard (6).

The intake of fat at approximately 3.1 gm. at all ages examined is close to the value of 3.3 to 3.6 gm. recorded by Holt and Fales (13).

The consumption of calcium was 46 ± 8 mgm. and of phosphorus 61 ± 10 mgm. per kgm. The former value is to be compared with the level of 50 mgm. proposed as desirable by Sherman and Hawley (27), of 45 to 58 mgm. found by Daniels *et al.* (7), and of 32 to 44 mgm. determined by Outhouse *et al.* (21).

Iron at 0.55 ± 15 mgm. is close to the figure of 0.50 mgm. found by MacKay (16) and Stearns and Stinger (31). Other values in the literature range from 0.32 to 0.60 mgm. (14).

The consumption of vitamin A was high in all subgroups and well above recommended allowances.

Relative to the Canadian standard for B_1, B_2, and niacin at 0.3, 0.5, and 3.0 mgm. per 1000 Cal. respectively, the average intakes of the Halifax groups were more than adequate.

Vitamins C and D have not been correlated with either body weight or the energy value of the diet. For C there appears to be a certain uniformity, i.e.

TABLE VI

INTAKE OF ESSENTIAL NUTRIENTS PER KGM. OF BODY WEIGHT OR PER 1000 CAL.*

Age yr.	Survey	Cal.	Protein, gm.	Fat, gm.	Ca, mgm.	P, mgm.	Fe, mgm.	Vitamins: A, I.U.	B1*, mgm.	B2*, mgm.	C, mgm.	D, I.U.	Niacin*, mgm.
1–2	I	86	3.3	3.2	74	81	1.08	1135	0.76	1.59	4.4	134	4.6
	II	71	2.8	3.0	46	58	0.53	548	0.62	1.09	3.3	134	4.3
2–3	I	85	2.9	3.2	45	55	0.62	747	0.63	1.18	4.0	82	4.9
	II	78	3.0	3.4	53	59	0.37	422	0.56	1.21	3.2	39	4.0
3–4	I	91	3.3	3.3	54	74	0.60	407	0.58	1.05	4.1	23	5.4
	II	82	3.1	3.3	37	58	0.43	290	0.45	0.83	2.7	6	3.4
4–5	I	75	2.8	3.1	50	64	0.51	531	0.63	1.09	5.1	30	5.3
	II	71	2.5	2.9	32	51	0.36	295	0.48	0.77	2.5	10	5.0
5–6	I	76	2.6	2.7	40	56	0.51	616	0.61	1.02	3.9	49	4.5
	II	67	2.8	3.3	38	57	0.56	305	0.42	0.77	2.2	16	4.8
6–7	II	76	2.6	3.1	40	53	0.39	174	0.47	0.91	3.0	16	4.6
Av.			2.9	3.1	46	61	0.55	506	0.57	1.05	3.5	48	4.7

weight at 3.5 ± 1.0 mgm. The variation in D on this basis is very wide. Assuming a figure of 350 I. U. for the infant (14), this would be distributed as follows on a basis of per kgm.

100 I. U./kgm. at birth		
34	kgm.	1 year
23	kgm.	3 years
18	kgm.	5 years

Our results show definite inadequacy in some subgroups on this basis and a more detailed analysis is given below. Glasser *et al.* (11) found that 100 I. U. were sufficient for normal growth and that 200 I. U. were as efficacious as 400 I. U. in promoting retention of calcium in premature infants. On the basis 200 I. U. the distribution per kilogram of body weight would be

17 I. U. for 1 – 2 yr.
13 I. U. for 3 – 4 yr.
10 I. U. for 5 – 6 yr.

Radiological Examinations

Roentgenograms of the bones of the wrist of some of the children in the first survey and most of the children in the second survey were taken by the method of Todd (32). One person, with considerable experience in this field, made the assessment of degree of maturation against the standards of Greulich and Pyle (12) without knowledge of the chronological age. On first examination of the 56 children, 68% were below the standard densities by 2 to 35 months and 30% were above by 1 to 49 months. The average number of months for the subnormal group was − 9.5 and for the supernormal group + 9 months. The latter figure is affected by one particularly high value of + 49. Deleting this value, the average becomes + 6.

The extent of what may be termed normal discrepancy between chronological and assessed age is undetermined at present. For middle and later childhood Todd (32) was inclined to regard a discrepancy of more than six months as indicative of retarded or accelerated maturation. Accepting this figure tentatively, we find 29% of our group retarded and 14% accelerated. On second examination nine children of 32 examined changed their relative rating by ± 3 months or more; six lost an average of five months and three gained an average of five months. This group will receive closer analysis in relation to diet below.

Repetitions

For the reason that the children in survey I were better fed than in survey II and few radiological examinations were made on them, analysis of the repetitions has been confined to the second survey.

There were 39 repetitions of the standard procedures of the survey done on the group of 63 children (i.e. 62%), after the lapse of approximately six months.

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This group provides a better criterion of the adequacy of the diets in comparison with the standards. It furnishes data on which to check the standards themselves in relation to growth and development. The record—physical, dietetic, and radiographic—of each child must be considered individually. Physical development and state of health have been assessed from the records.

In only about one fifth of the children was the usual standard of 1 gm. of calcium attained, whereas about 60% reached this level for phosphorus, and about 70% the standard intake for iron. About one third ingested 400 I. U. or more of vitamin D during one or other period of observation. In the group of 39, about 15 showed a delayed state of maturation beyond the six months allowed for normal variation as indicated above (i.e. about 40%).

Certain important conclusions may be drawn from the details of individual cases as analyzed. Of the 39 cases, 3 should be deleted for various reasons. Of the remaining 36, only 6 conformed to normal standards of diet and growth. Some 17 showed adequate calcification and growth on substandard diets. Some 11 showed inadequate calcification or growth on substandard diets. One showed markedly retarded calcification on an apparently adequate diet. One showed a mixed picture of retarded development on a substandard diet but normal calcification over the period of observation.

It is evident that normal maturation of bone may proceed on intakes of vitamin D at 25 to 150 I. U., and of calcium and phosphorus at less than one gram. Calcium may be adequate at 0.2 to 0.7 gm. in some cases but it is apparently not adequate in others.

Discussion

Several possible sources of error enter into the validity of the data presented and these have been discussed by McHenry *et al.* (15). The period of one week (21 consecutive meals) for dietary records has been generally adopted by workers in the field of nutrition. Longer periods would be more desirable but present several added difficulties. Repetitions with the Halifax children have helped to check the accuracy of these observations, but the inherent potential errors of this type of observation remain a factor of "indeterminacy" in nutrition. It is of importance to mention that seasonal variation, especially in the lower income groups, has been found to be remarkably small in the Canadian urban population (10).

McHenry *et al.* (15) found this variability was greatest for protein, iron, thiamine, and ascorbic acid from observations on a small, selected group of adults. The correspondence between the actual amounts of the nutrients ingested and those calculated from the tables is another important aspect of all dietary observations. Young and McHenry (38) have checked this correspondence for ascorbic acid as the nutrient most susceptible to error because of destruction during preparation and serving. The particular table of values used made a considerable difference but in the great majority of meals so assayed the actual ascorbic acid ingested was *less* than that calculated. Agreement with the food tables was good in the case of calories, fair for protein

and poor for fat (22). Deviation in the case of calcium indicated that the actual content of the food ingested was from 86 to 94% of the calculated values (29). Our results may therefore be as much as 15% too high. For iron the actual intakes were probably fairly close to the calculated values (39). The latter tend to be greater than the actual values by about 25%.

The reciprocal relationships between calcium, phosphorus, vitamin D, and sunlight have long been recognized. The individual efficiency in the absorption and retention of calcium differs markedly. Retentions of 15 to 25% have been shown to occur on an intake of 0.9 gm. when the vitamin D supply was adequate. Retention was 40 to 90% on one of 0.2 gm. when all but a very small amount of vitamin D was obtained through the direct action of the sun's rays on the skin (7, 17, 20, 21). Normal calcification and growth of Halifax children on substandard intakes of calcium cannot be accounted for by the ingestion of standard amounts of vitamin D because this occurred in only two of 17 children in this category. The time of exposure of the children to sunlight was not known but was probably not great because of the character of their living conditions. It is more probably explained by adaptation to low intake with greater efficiency of absorption and retention of calcium phosphate. The Ca : P ration was never greater than one and never so much less than one as to be considered a factor which might affect the calcium balance.

It is recognized that the assessment of degree of maturation of the wrist bones by roentgenograms still requires further investigation and trial. Nevertheless it furnishes a useful tool, supplementary to anthropometric measurements, with which to judge the normality of growth and, indirectly, the adequacy of the diet.

The work described has been carried out as a contribution to the establishment of the range of essential nutrients which do or do not lead to "normal" growth and development. If figures can be found which lead to standards which can be applied to the feeding of the nation's children, improvement in the national health and welfare will become possible on a more scientific basis.

Summary and Conclusions

Nutritional surveys have been carried out on two groups of children, aged one to six years, in Halifax, N.S., during 1945-47 and 1949-51. Food consumption was determined by individual weighings. Roentgenograms were taken of the bones of the hand and assessed as to age of maturation. Observations were repeated after six months, as an assessment of dietary adequacy.

The most important observations and deductions are as follows:

1. The level of hemoglobin in the blood of children in the first survey averaged 12.7 gm. per 100 ml. and the range was 10.6 to 14.1.
2. Deficiencies in calories, calcium, and vitamin D occurred most commonly in comparison with current Canadian standards.
3. Protein accounted for between 13 to 16% of the total calories and fat between 34 and 36%.

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4. The ratio of calcium to phosphorus averaged 0.77 and varied between 0.64 and 1.0. Seventy-eight per cent of the children received less calcium than the standard.
5. Variation in the consumption of vitamin D was very wide depending on whether a supplementary feeding of fish liver oil was taken. In the first survey 47% received less than 400 I. U. and in the second survey it was 84%. The average intake was less than 200 I. U. No case of active rickets was noted in physical examination.
6. Essential nutrients were calculated per kilogram of body weight or per 1000 Cal. Our results confirm the standards at 3.0 gm. of protein per kgm. at one to three years and 2.5 gm. at four to six years. Fat was ingested at 3.1 gm. per kgm. at all ages. Other figures per kgm. were 46 ± 8 mgm. for calcium, 61 ± 10 mgm. for phosphorus, 0.55 ± 15 mgm. for iron. ?

From the repetitions it was apparent that about half of the children showed adequate growth and calcification on substandard diets while 30% of them did not. It was evident that normal maturation of bone *may* proceed on intakes of vitamin D of 25 to 150 I. U. and of calcium and phosphorus of less than 1 gm. Calcium *may* be adequate at 0.2 to 0.7 gm. in some cases but not in others.

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British Medical Journal

Vitamin D Intoxication, with Hypernatraemia, Potassium and Water Depletion, and Mental Depression

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Summary: Two elderly patients suffering from manic-depressive psychosis/depressive reaction had concurrently hypercalcaemia from vitamin D intoxication. They developed hypernatraemia with severe potassium and water depletion. Hypercalcaemia was pronounced, but both patients recovered quickly and their depressive symptoms resolved following water and potassium repletion and corticosteroid therapy.

Introduction

It is easy to overlook vitamin D intoxication (Chaplin *et al.*, 1951) unless the plasma calcium is regularly estimated in patients receiving more than a few thousand units of vitamin D per day. The two cases of vitamin D intoxication reported here are of practical interest. Both patients were receiving large doses of vitamin D for metabolic bone disease and previously had been treated for depression. Recurrence of depressive symptoms appeared to be the initial manifestation of vitamin D intoxication. The diagnosis of vitamin D intoxication was made later when both patients had marked water depletion with hypokalaemia, hypernatraemia, and azotaemia.

Case 1

A 65-year-old woman was admitted to a psychiatric hospital on 8 June 1966, following a suicidal attempt, suffering from manic-depressive psychosis—depressive reaction (I.C.D. 301.1) of two months' duration. The previous year she had attempted suicide after her husband's death and later had undergone partial gastrectomy for a duodenal ulcer. In January 1966 she developed severe back pain; x-ray examination showed her bones to be thin, so calciferol 6,000 i.u. daily was prescribed for probable osteomalacia. On admission in June 1966 she weighed only 60½ lb. (27.4 kg.). Two weeks after admission amitriptyline 25 mg. t.d.s. and calcium Sandoz 3 g. daily were prescribed, and calciferol was increased to 1.25 mg. t.d.s. (150,000 i.u. daily). Her depression lifted, she gained 20 lb. (9 kg.) in weight, and after eight weeks amitriptyline was discontinued.

Three weeks later, however, her depressive symptoms returned and she continued to deteriorate despite further treatment with amitriptyline. She complained later of weakness in the legs and difficulty in walking. An electroencephalogram was grossly abnormal, showing generalized slow activity. She refused food but continued to drink; she became polyuric, was incontinent of urine, and gradually developed severe fluid depletion and a toxic confusional state. The electrocardiogram showed S–T depression with prominent U waves. On 12 October she became deeply unconscious, with sunken eyes and marked loss of skin turgor; blood pressure was 120/70 mm. Hg and pulse rate 76/min. Plasma values and urine ratios before treatment are shown in the Table. Hypernatraemia and hypokalaemia were evident 14 days before vitamin D intoxication was diagnosed. She was treated with intravenous 5% dextrose, 4 litres in 24 hours, containing 50 milliequivalents of potassium chloride. Hydrocortisone and later prednisolone plus ampicillin and penicillin were given. Vitamin D was stopped.

Response to Treatment.—There was a dramatic improvement in her physical state within 24 hours and she rapidly regained consciousness. This coincided with a rapid fall in plasma calcium and correction of hypernatraemia and of water depletion (Fig. 1). She remained hypokalaemic for two weeks following correction of the hypercalcaemia, despite ingestion of a normal diet. Urine potassium rapidly fell to low levels. The plasma potassium level rose after oral potassium chloride was added and steroids were discontinued; plasma urea fell to normal. Eleven days after the start of treatment she was fully mobile, felt very well, and was not depressed. She remained well during two months of follow-up.

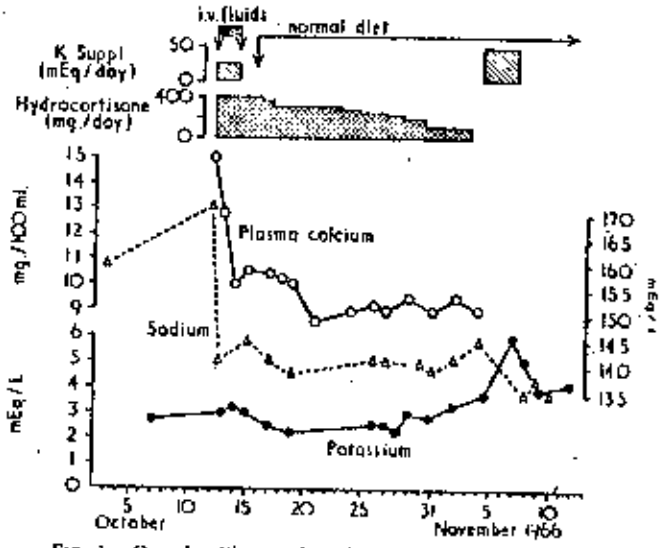

Fig. 1.—Case 1. Plasma electrolyte changes with treatment.

Case 2

A 73-year-old man was admitted to a psychiatric hospital in September 1966 suffering from manic-depressive psychosis/depressive reaction (I.C.D. 301.1) in a setting of cerebral arteriosclerosis (I.C.D. 306). His medical history included partial gastrectomy for gastric ulcer in 1954, a depressive psychosis in 1961, epilepsy due to cerebral arteriosclerosis since 1963, and severe osteomalacia treated for two years with calcium gluconate 600 mg. b.d. and calciferol 100,000 i.u. daily. The calcium and calciferol were continued after his admission. Though initially he became spontaneously less depressed, he relapsed late in November and imipramine was prescribed. On 15 December imipramine was withdrawn when, following a syncopal attack, he was found to be shrunken, with dry tongue, loss of skin turgor, and a parotid abscess. His blood pressure was 128/75 mm. Hg, pulse rate 110/min. Plasma values and urine ratios are shown in the Table. The diagnosis of vitamin D intoxication was first considered because these results were strikingly similar to those seen in Case 1.

He was treated with hydrocortisone and was given 3 litres of 5% dextrose and 1 litre of 1/6 molar sodium lactate with 100 milliequivalents of potassium chloride in the first 24 hours; ampicillin and later penicillin and streptomycin were given. Calciferol and calcium gluconate were discontinued.

Response to Treatment.—Rapid improvement in his physical state was accompanied by electrolyte changes similar to those shown by

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Plasma Values and Urine Ratios Before Treatment

Case No.	Date	Plasma Urea and Electrolyte Values							Urine Electrolyte/Creatinine Ratios		
		Urea (mg./100 ml.)	Na (mEq/l.)	K (mEq/l.)	Cl (mEq/l.)	HCO_3 (mEq/l.)	Ca (mg./100 ml.)	P (mg./100 ml.)	Ca/Cr* (mg./mg.)	K/Cr (mEq/mg.)	Na/Cr (mEq/mg.)
1	22/10/66	216	174	3·0	122	35	15	2·3	0·186	0·14	0·03
2	13/12/66	126	153	2·8	108	23	14·8	3·0	0·27	0·124	0·151

Normal Ca/Cr ratio = 0·03–0·28 (Nordin, 1959).

Case 1 (Fig. 2), and a fall in plasma urea to normal, closely followed by disappearance of his depression and hypochondriacal delusions. He was discharged home well but still moderately demented after two months' observation.

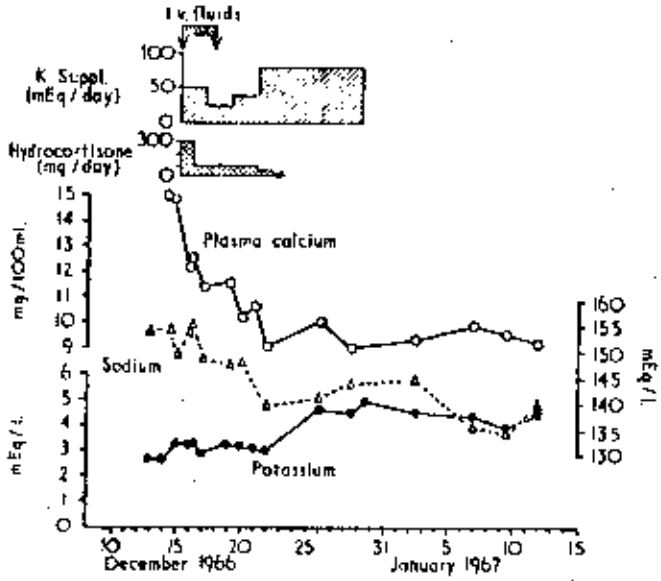

FIG. 2.—Case 2. Plasma electrolyte changes with treatment. Steroid continued only while calcium was raised.

Discussion

Depression

Vitamin D intoxication may be associated with an organic mental syndrome (Lehrer and Levitt, 1960), but we have found only one previous report of its association with mental depression (Chaplin *et al.*, 1951). Our two patients are known to have previously suffered from manic-depressive psychosis, but the return of their depressive symptoms shortly before the appearance of physical symptoms of vitamin D intoxication, together with the rapid improvement in their depression after treatment of the metabolic upset, suggests that vitamin D intoxication may have been important in precipitating and maintaining the depression to which they were both predisposed.

Depression has been described in hypercalcaemic states, notably in primary hyperparathyroidism (Henson, 1966–7), and is known to be a feature of potassium deficiency (Mayer-Gross *et al.*, 1960). However, it is probably unjustifiable to speculate on the mechanism of production of depression in these two patients, since the electrolyte and metabolic changes were so profound, though in depressive illness alterations in sodium and potassium distribution (Coppen and Shaw, 1963; Shaw and Coppen, 1966) and calcium balance (Flach, 1964) have been reported.

Electrolyte Disturbances

Among other renal effects hypercalcaemia may lead to a state resembling diabetes insipidus that is pitressin-resistant (Cohen *et al.*, 1957; Epstein, 1960; Gill and Bartter, 1961), and may also cause potassium depletion (Huth *et al.*, 1959; Ferris *et al.*, 1961; Sanderson, 1967; Schwartz and Relman, 1967). Our two patients had hypokalaemia and there was a relatively high urine potassium, which fell along with the plasma calcium level during recovery (Fig. 3). Potassium depletion alone causes an effect similar to that of hypercalcaemia on water excretion (Epstein, 1960); our patients therefore showed a double reason for a renal water-losing state, and when they were too ill to maintain an adequate fluid intake they became hypernatraemic.

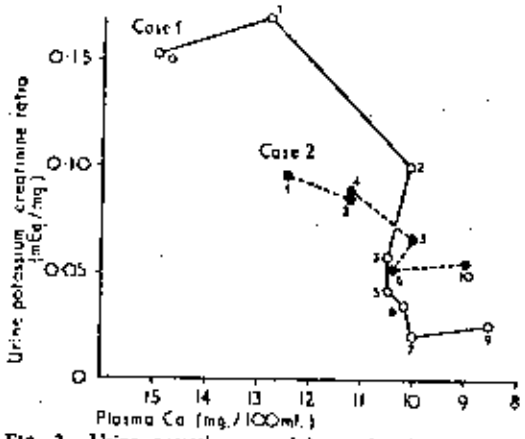

FIG. 3.—Urine potassium:creatinine ratio plotted against plasma calcium level. Continuous lines (Case 1) and dotted lines (Case 2) connect consecutive results; small numbers adjacent to points indicate number of days after start of therapy.

Hypernatraemia has only rarely been reported complicating hypercalcaemia; two other patients with vitamin D intoxication had plasma sodium levels of 150 and 156 mEq/l. (Verner *et al.*, 1958), and Guyer (1965) reported a case of thyrotoxicosis with hypercalcaemia and vomiting who had a serum sodium of 178 mEq/l. That hypernatraemia is unusual may be because hypercalcaemia can also impair the ability of the kidney to conserve sodium, at least when azotaemia has developed (Epstein, 1960); in contrast, potassium depletion may cause sodium retention (Black and Milne, 1952; Wolff *et al.*, 1968). Our Case 2 was losing more sodium than potassium when hypercalcaemic, though Case 1 was losing very little. Clinically, the diagnosis of hypernatraemic dehydration is easily missed, since the circulation is well maintained even in advanced fluid depletion. It is worth noting in contrast that hypernatraemia from other causes is often associated with hypocalcaemia (Finberg and Harrison, 1955).

These two cases emphasize the importance of adequate water and electrolyte repletion to correct the secondary disorders caused by hypercalcaemia; short-term steroid therapy is also of importance in vitamin D intoxication. Other authors (Kenny and Holliday, 1964; Sterling and Rupp, 1967) have used isotonic sodium sulphate infusions in vitamin D intoxication to lower the plasma calcium, but since this treatment may itself cause hypernatraemia (Heckman and Walsh, 1967) and it increases potassium excretion (Chakmakjian and Bethune, 1966) we think that it is dangerous. Our results demonstrate that sodium sulphate and sodium phosphate infusions are not necessary in treating hypercalcaemia from this cause.

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We are grateful to Professor P. D. Griffiths and his staff for the biochemical estimations, to Dr. W. K. Stewart for permission to publish these cases, and to Dr. P. G. Aungle and Professor I. R. C. Batchelor for their helpful comments.

Requests for reprints should be sent to Dr. D. C. Anderson.

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Proposed Rule Making

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Food and Drug Administration

[21 CFR Parts 3, 15, 16, 17, 18, 45, 121, 125]

VITAMIN D

Status as Special Dietary Food, Drug, Food Additive, and as Optional Ingredient in Standardized Foods

The need for vitamin D in human nutrition and the importance of maintaining a daily intake sufficient to protect infants and growing children from developing rickets is well established. It is widely recognized that a daily intake of 400 U.S.P. units of vitamin D is ample to prevent vitamin D deficiencies and is adequate for the supplementation of the diet of infants, children, and pregnant and lactating women. It is recognized also that vitamin D, when ingested daily in excessive amounts, is toxic. The question of possible excessive amount of vitamin D in the diet of the public was raised by Dr. Robert Cooke of Johns Hopkins University, Baltimore, Md. He expressed his concern that this may be [illegible] important factor in the development [illegible] infantile hypercalcemia. In doing so, [illegible] called attention to the fact that there [illegible] presently marketed a number of vitamin preparations labeled for special dietary use or with directions for use in self-medication as well as a number of foods with added vitamin D, which together may supply a substantially greater dosage of vitamin D than is necessary to accomplish its useful purpose.

In the interest of fully evaluating this problem, the Commissioner of Food and Drugs requested the Committee on Nutrition of the American Academy of Pediatrics and an ad hoc committee of the Council on Drugs and the Council on Foods and Nutrition of the American Medical Association to consider the possibility of hazard that may confront the American public in the consumption of currently available foods and vitamin supplements containing added vitamin D.

The Committee on Nutrition of the American Academy of Pediatrics has submitted a report containing the following recommendations:

1. The Committee wishes to reiterate the statement made in its published memorandum with respect to vitamin D fortification of foods other than milk or infant formula products: "* * * the present practice of enriching foods other than milk and infant formula products is not justified, and discontinuation of this practice is to be recommended."

The addition of vitamin D to fluid [illegible] evaporated milk or to special milk products and milk substitutes designed for consumption by infants is desirable and should continue. However, the vitamin D fortification should be carefully controlled so that in normal usage the total daily consumption will not be more than 400 units (i.e. 400 units/quart of fluid milk, or 400 units per reconstituted quart of formula products), which is the present practice in the United States. The addition of vitamin D to other foods may serve only to increase the intake by infants, children and pregnant women to levels beyond requirement.

3. Special purpose foods, designed to serve as the total daily source of nutrient or calories of infants, children or adults, may properly contain vitamin D. The vitamin should be added in an amount that does not exceed 400 units in the total daily intake.

4. At the present time, various vitamin preparations provide different amounts of vitamin D, some considerably in excess of 400 units in the recommended daily dosage. The Committee suggests that no preparations sold over the counter should provide more than 400 units of vitamin D in the amount of the product designated on the label as the recommended daily intake. Since it is the practice to dispense vitamin preparations designed for use by infants in liquid form, adjustment of intake from supplements to levels below 400 units/day remains feasible if consideration of total vitamin D available to the infant makes such reduction desirable. The Committee suggests that products containing amounts of vitamin D in excess of 400 units in the recommended daily dosage be restricted to sale only by prescription.

5. If in the future an unambiguous causal relation between vitamin D intake of the pregnant woman or the infant and the severe form of the hypercalcemic syndrome can be proved, the Committee would favor labeling of all vitamin products containing vitamin D so as to indicate potential toxicity if taken in excess of the recommended daily dosage. In addition, the user should be advised to seek medical advice before administering greater amounts of the product.

6. Before prescribing vitamin D supplements for any patient, the physician should assess the total intake of vitamin D from other sources, and unless high doses of vitamin D are required to treat specific disturbances of calcium and phosphorus metabolism, he should attempt to establish an intake of vitamin D of approximately 400 units per day from all sources. This applies for pregnant and lactating women as well as for infants and children. Normal adults living under ordinary conditions do not require supplementary vitamin D.

The joint ad hoc committee of the Council on Drugs and the Council on Foods and Nutrition of the American Medical Association has submitted a report with the following recommendations:

1. The addition of vitamin D to fluid, evaporated and dry whole milk, fluid skim milk, and nonfat dry milk should be permitted and encouraged but the quantity added should not exceed 400 USP units per quart of fluid or reconstituted milk.

2. The addition of vitamin D to milk substitutes and milk-based infant formulas which provide the sole or major source of calories should be encouraged. The vitamin D supplement should provide no more than 400 USP units in one quart of fluid when prepared for use.

3. Vitamin D supplementation of other food items should be prohibited.

4. Supplementary vitamin preparations available to the public for purchase without prescription should contain no more than 400 USP units of vitamin D in the suggested daily dose.

5. All vitamin preparations containing more than 400 USP units of vitamin D in suggested daily dosage should be prohibited from over-the-counter sale and should be available only by prescription.

6. Consumers should be advised that there is no advantage to the daily intake of more than 400 USP units of vitamin D and that the continued ingestion of amounts much in excess of that may be hazardous.

Copies of these reports are on file with the Hearing Clerk of the Department of Health, Education, and Welfare, and may be consulted by any interested person.

The Commissioner of Food and Drugs has reached the conclusion on the basis of these reports and on other relevant data and information available to him, including the abundant scientific evidence that an excessive intake of vitamin D is of no value, that it is prudent to issue a proposal which reflects the recommendations of these groups.

Therefore, pursuant to the provisions of the Federal Food, Drug, and Cosmetic Act (secs. 401, 403(j), 409(d), 502(f), 503(b)(4), 701 (a), (e); 52 Stat. 1046, 1048, 1051, 1052, 1055; 70 Stat. 919; 72 Stat. 1787; 21 U.S.C. 341, 343(j), 348(d), 352(f), 353(b)(4), 371 (a), (e)) and under the authority delegated to him by the Secretary of Health, Education, and Welfare (21 CFR 2.90) the Commissioner proposes to amend Title 21, Chapter I, of the Code of Federal Regulations in the following respects.

1. By adding to Part 3 the following new section:

§ 3.... Vitamin D (vitamin D_2 (calciferol, ergocalciferol); vitamin D_3 (activated 7-dehydrocholesterol, cholecalciferol)).

(a) Vitamin D from any source, natural or synthetic, alone, or as a component of a food, for supplementing the human diet is regarded as a food additive subject to the provisions of section 409 of the Federal Food, Drug, and Cos-

metic Act and regulations promulgated thereunder.

(b) Any preparation containing more n 400 U.S.P. Units of vitamin D per sage unit or any preparation supplying more than 400 U.S.P. Units per day when used in accordance with its directions for use is regarded as a drug that by reason of its toxicity or other potentiality for harmful effect, or the collateral measures necessary to its use, is not safe for use except under the supervision of a practitioner licensed by law to administer drugs. The label of any such article shall bear the statement "Caution: Federal law prohibits dispensing without prescription" and its labeling shall bear adequate information for its use by a practitioner licensed by law to administer it.

(c) Any drug containing vitamin D whose labeling bears directions for use in self-medication of vitamin D deficiency in recommended dosages greater than 400 U.S.P. Units per day will be regarded as misbranded within the meaning of sections 502 (a), (f), and (j) and 503(b)(4) of the act.

(d) Preparations supplying 400 or less U.S.P. Units of vitamin D represented or intended for use as drugs for the treatment of vitamin D deficiency by the lay public may be regarded as misbranded under sections 502 (a), (f), and (j) and 503(b) of the Federal Food, Drug, and Cosmetic Act, since adequate directions for safe and effective use for the self-ment of vitamin D deficiency by the r without medical supervision cannot written.

2. By deleting from paragraph (b) of § 2.17 *Labeling of oleomargarine or margarine*, subparagraph 7, which is reserved, and by deleting from paragraph (d) of this section the phrase "vitamin D, or both".

3. By revoking paragraph (b) of § 15.10 *Enriched flour* * * *, which is reserved, and by changing the last sentence in the section to reflect the deletion.

4. By revoking paragraph (b) of § 15.60 *Enriched self-rising flour* * * *, which is reserved, and by changing the last sentence in the section to reflect the deletion.

5. By revoking paragraph (a)(2) of § 15.140 *Enriched farina* * * *, which is reserved; by deleting the next to the last sentence in paragraph (a), and by changing the last sentence of this paragraph to eliminate the reference to subparagraph (2).

6. By revoking paragraph (a)(2) of § 15.513 *Enriched corn meals* * * *, which is reserved, and by changing the third sentence in subparagraph (3) to reflect this deletion.

7. By revoking paragraph (a)(2) of § 15.514 *Enriched corn grits* * * *, which is reserved, and by changing the fourth sentence of subparagraph (3) to reflect the deletion.

8. By revoking paragraph (a)(2) of § 15.525 *Enriched rice* * * *, which is reserved, and by deleting the references to the revoked subparagraph (2) from paragraphs (a)(3), (b), and (c).

9. By revoking paragraph (a)(2) of § 16.9 *Enriched macaroni products* * * *, which is reserved, and by deleting the references to subparagraph (2) from paragraph (a)(5) and the last sentence of paragraph (a).

10. By revoking paragraph (a)(2) of § 16.10 *Enriched noodle products* * * *, which is reserved, and by deleting from paragraph (a)(5) and the last sentence of paragraph (a) the references to the revoked subparagraph.

11. By revoking paragraph (a)(2) of § 17.2 *Enriched bread and enriched rolls* * * *, which is reserved, and by deleting from the last sentence in paragraph (a) the reference to the revoked subparagraph.

12. By deleting the phrase "not less than" from paragraph (a)(2) of § 18.520 *Evaporated milk* * * *.

13. By deleting the parenthetical phrase "(with or without any accompanying vitamin D and with or without vitamin D concentrate)" from paragraph (a)(3)(iii) of § 45.1 *Oleomargarine, margarine* * * *.

14. By deleting the items "Vitamin D_2" and "Vitamin D_3" from the list in paragraph (d)(5) of § 121.101 *Substances that are generally recognized as safe.*

15. By adding to Subpart D of Part 121 the following new section:

§ 121.____ Vitamin D (Vitamin D_2, Vitamin D_3).

Vitamin D may be safely used in food in accordance with the following prescribed conditions:

(a) It is added to fluid milk, fluid nonfat milk, dry whole milk,[1] evaporated milk, concentrated milk, or infant-feeding formulations used in place of milk in an amount, in the case of fluid milk, fluid nonfat milk, dry whole milk, evaporated milk, or concentrated milk to provide 400 U.S.P. Units per quart of milk or reconstituted milk and, in the case of infant-feeding formulations used in place of milk in an amount to provide 400 U.S.P. Units per quart of such food as customarily or usually prepared for consumption; or

(b) It is a vitamin supplement or a component of a vitamin supplement that when ingested according to its labeled directions for use furnishes not more than 400 U.S.P. Units per day; and

(c) The food is labeled with a statement that vitamin D has been added; a statement of the number of U.S.P. Units of vitamin D in a specified quantity of the food; and in case it is an article covered by paragraph (b) of this section, it bears a label statement warning that unless prescribed by a physician the article should not be used to supplement the diet in excess of 400 U.S.P. Units per day from all sources.

16. By deleting the last sentence of paragraph (a)(1) of § 125.3 *Label statements relating to vitamins* and by deleting the last sentence of § 125.5 starting with the words, "The provisions of paragraph (c) * * *."

Any interested person may, within 60 days from the date of publication of this notice in the FEDERAL REGISTER, file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 5440, 330 Independence Avenue SW., Washington, D.C., 20201, written comments, preferably in quintuplicate, on this proposal. Comments may be accompanied by a memorandum or brief in support thereof.

Dated: August 24, 1965.

GEO. P. LARRICK,
Commissioner of Food and Drugs.

[F.R. Doc. 65-9124; Filed, Aug. 27, 1965; 8:45 a.m.]

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[1] Nonfat dry milk not included due to its statutory definition (Public Law 78-244, 58 Stat. 108, 70 Stat. 486; 21 U.S.C. 321c).

GPO : 1965 O—795-591

1967 No. 1867

FOOD AND DRUGS

COMPOSITION AND LABELLING

The Margarine Regulations 1967

Made - - -	14*th December* 1967
Laid before Parliament	21*st December* 1967
Coming into Operation	4*th January* 1971

The Minister of Agriculture, Fisheries and Food and the Minister of Health, acting jointly, in exercise of the powers conferred on them by sections 4, 7, 123 and 136 (2) of, and paragraph 2(2) of Schedule 12 to, the Food and Drugs Act 1955(a) and of all other powers enabling them in that behalf, hereby make the following regulations after consultation with such organisations as appear to them to be representative of interests substantially affected by the regulations and reference to the Food Hygiene Advisory Council under section 82 of the said Act (insofar as the regulations are made in exercise of the powers conferred by the said section 7):—

Citation and commencement

1. These regulations may be cited as the Margarine Regulations 1967; and shall come into operation on 4th January 1971.

Interpretation

2.—(1) In these regulations, unless the context otherwise requires—

"the Act" means the Food and Drugs Act 1955;

"container" includes any form of packaging of food for sale as a single item, whether by way of wholly or partly enclosing the food or by way of attaching the food to some other article, and in particular includes a wrapper or confining band;

"food" means food intended for sale for human consumption and includes drink, chewing gum and other products of a like nature and use, and articles and substances used as ingredients in the preparation of food or drink or of such products, but does not include—

(*a*) water, live animals or birds,

(*b*) fodder or feeding stuffs for animals, birds or fish, or

(*c*) articles or substances used only as drugs;

"food and drugs authority" has the meaning assigned to it by section 83 of the Act;

(a) 4 & 5 Eliz. 2. c. 16.

"... an c... ...mptio... ...ncludes use in the preparation of food for human consumption;

"margarine" means the food usually known as margarine, b... plasticised emulsion of edible oils and fats with water or skimmed milk, with or without the addition of vitamins A and D, sodium chloride, sugars and other minor ingredients and permitted additives;

"permitted additive" means any colouring matter, antioxidant, emulsifier, stabiliser, preservative or solvent, as the case may be, inasmuch as its use in margarine is permitted by the appropriate regulations mentioned in Schedule 1 to these regulations;

"sell" includes offer or expose for sale or have in possession for sale, and "sale" and "sold" shall be construed accordingly;

"sell by retail" means sell to a person buying otherwise than for the purpose of re-sale, and "sold by retail" shall be construed accordingly;

"vitamin A" means vitamin A present as such or as its esters and includes beta-carotene on the basis that 0.75 micrograms of beta-carotene shall be regarded as equivalent to 1.0 international unit of vitamin A;

"vitamin D" means the anti-rachitic vitamins;

AND other expressions have the same meaning as in the Act.

(2) The Interpretation Act 1889(a) shall apply to the interpretation of these regulations as it applies to the interpretation of an Act of Parliament, and as if these regulations and the orders and regulations hereby revoked or partially revoked, as the case may be, were Acts of Parliament.

(3) All percentages mentioned in these regulations are percentages calculated by weight.

(4) Any reference in these regulations to a label borne on a container shall be construed as including a reference to any legible marking on the container however effected.

(5) For the purposes of these regulations, the supply of food, otherwise than by sale, at, in or from any place where food is supplied in the course of a business shall be deemed to be a sale of that food, and a reference to purchasing and purchaser shall be construed accordingly.

(6) Any reference in these regulations to any other regulations shall be construed as a reference to such regulations as amended by any subsequent regulations.

Exemptions

3. The following provisions of these regulations shall not apply in relation to any margarine—

(*a*) sold, consigned or delivered for exportation to any place outside the United Kingdom; or

(*b*) supplied under Government contracts for consumption by Her Majesty's Forces or supplied for consumption by a visiting force within the meaning of any of the provisions of Part I of the Visiting Forces Act 1952(b); or

(*c*) sold, consigned or delivered to a manufacturer for the purposes of his manufacturing business or to a caterer for the purposes of his catering business.

(a) 1889 c. 63.

(b) 1952 c. 67.

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Continuation of Part IVA of the Labelling of Food Order 1953

4. U[illegible]rt IVA of the Labelling of Food Order 1953(a) as amended (b) is revoke[illegible] accordance with regulation 14(2) of these regulations, regulations 8 and 10 of these regulations, insofar as they relate to a label, ticket or notice, shall not apply in relation to any label, ticket or notice which conforms to the requirements of the provisions of the said Part IVA.

Composition of margarine

5.—(1) Any margarine sold, consigned or delivered—

(*a*) shall contain not less than 80 per cent. of fat of which not more than one tenth calculated by weight may be fat derived from milk, and

(*b*) shall not contain more than 16 per cent. of water.

(2) No person shall sell, consign or deliver, as the case may be, any margarine in contravention of this regulation.

Vitamin content of margarine

6.—(1) Any margarine sold by retail shall contain in every ounce of such margarine—

(*a*) not less than 760 international units and not more than 940 international units of vitamin A, and

(*b*) not less than 80 international units and not more than 100 international units of vitamin D,

and a proportionate amount in any part of an ounce.

(2) No person shall sell by retail any margarine in contravention of this regulation.

General requirements as to composition and description

7.—(1) No person shall sell any food under such a description as to lead an intending purchaser to believe that he is purchasing any margarine for which compositional requirements are specified in these regulations unless the food complies with the appropriate compositional requirements set out in these regulations in relation to that margarine.

(2) Where a person sells any food to a purchaser in response to a request for any kind of margarine for which compositional requirements are specified in these regulations, he shall be deemed to sell margarine of that kind and conforming to the compositional requirements for margarine of that kind which are specified in these regulations in relation to that margarine, unless he clearly notifies the purchaser at the time of sale that the food is not of that kind.

Labelling and advertisement of margarine

8. No person shall give with any margarine sold by him any label, whether attached to or borne on the container or not, or display with any margarine offered or exposed by him for sale any ticket or notice, being a label, ticket or notice, as the case may be, which—

(a) S.I. 1953/536 (1953 I, p. 665).
(b) The relevant amending instrument is S.I. 1955/1900 (1955 I, p. 930).

[illegible] bear [illegible] incl[illegible] any [illegible]ripti[illegible] [illegible]me, [illegible] bran[illegible] des-criptive name or any pictorial device which, in any such [illegible] refers to, or is suggestive of, butter or of anything connected with th[illegible] interest;

(*b*) claims that the margarine contains or is made with cream or milk unless—

(i) such claim is made in the following words (and no others), that is to say:

"made with (X), milk-fat equivalent to (Y)% of butter" inserting at (X) the word "cream" or the word "milk", as the case may be, and at (Y) the figure which represents the minimum percentage of butter calculated on the amount of milk-fat introduced by the cream, or the milk, as the case may be, and

(ii) such claim is made in letters or figures all of which, other than initial letters, are of equal size;

(*c*) claims that the margarine contains butter unless—

(i) such claim states the minimum percentage of butter which the margarine contains, and

(ii) the letters of the word "butter" contained in any such claim are not taller than the letters of the word or the figures, as the case may be, which indicate such minimum percentage of butter;

(*d*) bears or includes any brand or descriptive name unless the word "margarine" appears in immediate proximity to such brand or descriptive name at least once, in accordance with the requirements set out in Schedule 2 to these regulations;

(*e*) bears or includes the word "butter", "cream" or "milk", or any word suggestive of butter, cream or milk, or any brand or descriptive name derived from the words "butter", "cream" or "milk" in letters taller than those used for the word "margarine":

Provided that the provisions of sub-paragraphs (*a*) to (*c*) of this regulation shall not apply as respects any list of ingredients of the margarine appearing on a label attached to or borne on the container thereof in accordance with the provisions of regulation 5 of the Labelling of Food Regulations 1967(a).

9.—(1) No person shall publish, or be a party to the publication of any advertisement for margarine unless—

(*a*) when it is presented visually, the word "margarine" appears clearly and prominently at least once;

(*b*) when it is presented orally, the word "margarine" is spoken audibly and clearly at least once.

(2) No person shall publish, or be a party to the publication of any advertisement for margarine which includes any brand or descriptive name unless—

(*a*) when it is presented visually, the word "margarine" appears in accordance with the requirements set out in paragraph 2 of Schedule 2 to these regulations, at least once, immediately before or immediately after such brand or descriptive name;

(a) S.I. 1967/1864.

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(b) when it is presented orally, the word "margarine" is spoken audibly and at least once, immediately before or immediately after such brand or descriptive name.

(3) No person shall publish or be a party to the publication of any advertisement for margarine which—

(a) includes any description of margarine, any brand or descriptive name or any pictorial device which, in any such case, refers to, or is suggestive of, butter or of anything connected with the dairy interest;

(b) claims that the margarine contains or is made with cream or milk unless—

(i) such claim is made in the following words (and no others), that is to say;

"made with (X), milk-fat equivalent to (Y)% of butter" inserting at (X) the word "cream" or the word "milk", as the case may be, and at (Y) the figure which represents the minimum percentage of butter calculated on the amount of milk-fat introduced by the cream, or the milk, as the case may be, and

(ii) such claim, if presented visually, is made in letters or figures all of which, other than initial letters, are of equal size;

(c) claims that the margarine contains butter unless—

(i) such claim states the minimum percentage of butter which the margarine contains, and

(ii) the letters of the word "butter" contained in any such claim, if presented visually, are not taller than the letters of the word or the figures, as the case may be, which indicate such minimum percentage of butter;

(d) includes the word "butter", "cream" or "milk", or any word suggestive of butter, cream or milk, or any brand or descriptive name derived from the words "butter", "cream" or "milk" in letters taller than those used for the word "margarine".

10. No person shall—

(a) give with any food sold by him, any label, whether attached to or borne on the container or not, or display with any food offered or exposed by him for sale, any ticket or notice, or

(b) publish, or be party to the publication of any advertisement for any food,

being a label, ticket, notice or advertisement, as the case may be, which bears or includes the word "margarine" unless—

(i) such food complies with the appropriate compositional requirements specified in regulations 5 and 6 of these regulations, or

(ii) the word is used in such a context as to indicate clearly that the margarine to which it relates is an ingredient, with one or more others, of such food and such margarine complies with the compositional requirements specified in regulation 5 of these regulations, or

the is in si con as to cate icitly by clear implication that such food is not margarine and does not include any.

Penalties and enforcement

11.—(1) If any person contravenes or fails to comply with any of the foregoing provisions of these regulations he shall be guilty of an offence and shall be liable to a fine not exceeding one hundred pounds or to imprisonment for a term not exceeding three months, or to both, and, in the case of a continuing offence, to a further fine not exceeding five pounds for each day during which the offence continues after conviction.

(2) Each food and drugs authority shall enforce and execute such provisions in their area.

(3) The requirement of section 109(3) of the Act (which requires notice to be given to the Minister of Agriculture, Fisheries and Food of intention to institute proceedings for an offence against any provisions of these regulations made under section 7 of the Act) shall not apply as respects any proceedings instituted by a council for an offence against any such provisions of these regulations.

Defences

12.—(1) In any proceedings for an offence against these regulations in relation to the publication of an advertisement, it shall be a defence for the defendant to prove that, being a person whose business it is to publish or arrange for the publication of advertisements, he received the advertisement for publication in the ordinary course of business.

(2) In any proceedings against the manufacturer or importer of any margarine or of any food containing margarine for an offence against these regulations in relation to the publication of an advertisement, it shall rest on the defendant to prove that he did not publish, and was not a party to the publication of, the advertisement.

Applications of various sections of the Act

13.—(1) Sections 108(3) and (4) (which relate to prosecutions), 110(1), (2) and (3) (which relate to evidence of analysis), 112 (which relates to the power of a court to require analysis by the Government Chemist), 113 (which relates to a contravention due to some person other than the person charged), 115(2) (which relates to the conditions under which a warranty may be pleaded as a defence) and 116 (which relates to offences in relation to warranties and certificates of analysis) of the Act shall apply for the purposes of these regulations as if references therein to proceedings, or a prosecution, under or taken or brought under the Act included references to proceedings, or a prosecution as the case may be, taken or brought for an offence under these regulations and as if the reference in the said section 112 to subsection (4) of section 108 included a reference to that subsection as applied by these regulations.

(2) Paragraph (b) of the proviso to section 108(1) of the Act shall apply for the purposes of these regulations as if the reference therein to section 116 of the Act included a reference to that section as applied by these regulations.

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Revocation

14.—(1) T[illegible]od Standards (Margarine) Order 1954(a) and the Food Standards (B[illegible] and Margarine) Regulations 1955(b) are hereby revoked.

(2) The definitions of margarine and margarine cheese in article 2(1), and the whole of Part IVA, of the Labelling of Food Order 1953(c) as amended (d) and the Labelling of Food (Amendment) Regulations 1955(e) shall be revoked on 5th July 1971.

In Witness whereof the Official Seal of the Minister of Agriculture, Fisheries and Food is hereunto affixed on 8th December 1967.

(L.S.) *Frederick Peart,*
Minister of Agriculture, Fisheries and Food.

Given under the official seal of the Minister of Health on 14th December 1967.

(L.S.) *Kenneth Robinson,*
Minister of Health.

SCHEDULE 1

Regulation 2(1)

Regulations	References
The Colouring Matter in Food Regulations 1966	S.I. 1966/1203 (1966 III, p. 3203).
The Antioxidant in Food Regulations 1966	S.I. 1966/1500 (1966 III, p. 4140).
The Emulsifiers and Stabilisers in Food Regulations 1962	S.I. 1962/720 (1962 I, p. 729).
The Preservatives in Food Regulations 1962	S.I. 1962/1532 (1962 II, p. 1655).
The Solvents in Food Regulations 1967	S.I. 1967/1582 (1967 III, p. 4385).

SCHEDULE 2

Regulations 8(*d*) and 9 (2)(*a*)

Requirements as to marking on labels on containers, on tickets and notices and in advertisements

1.—(1) Where the word "margarine" appears on any label on a container, or on any ticket or notice, as the case may be, pursuant to regulation 8(d) of these regulations every letter of such word shall—

(*a*) be clearly legible;

(a) S.I. 1954/613 (1954 I, p. 804).
(b) S.I. 1955/1899 (1955 I, p. 915).
(c) S.I. 1953/536 (1953 I, p. 665).
(d) The relevant amending instrument is S.I. 1955/1900 (1955 I, p. 930).
(e) S.I. 1955/1900 (1955 I, p. 930).

[illegible] app[illegible] a pr[illegible]ent position on the label, ticket or notice;

(*c*) appear in a dark colour upon a light coloured ground or [illegible]ght colour upon a dark coloured ground;

(*d*) be of uniform colour and size, save that the initial letter of such word may be taller than any other letter therein;

(*e*) be of such size that the height of the shortest letter shall be not less than one half the height of the tallest letter, other than an initial letter, in any word of more than one letter appearing on any label on that container, or on that ticket or notice, as the case may be.

(2) For the purposes of this paragraph the height of any lower case letter shall be taken to be the x-height thereof, disregarding any ascender and descender thereof.

2. Where the word "margarine" is included in any advertisement pursuant to regulation 9(2)(*a*) of these regulations—

(*a*) such word shall appear substantially as prominently as the brand or descriptive name to which it is in immediate proximity, and

(*b*) every letter in such word shall be of uniform size and colour on a ground of contrasting colour:

Provided that the initial letter of such word may be taller than any other letter therein.

EXPLANATORY NOTE

(*This Note is not part of the Regulations.*)

These regulations, which apply to England and Wales only, supersede the Food Standards (Margarine) Order 1954, the Food Standards (Butter and Margarine) Regulations 1955 (insofar as they relate to margarine) and Part IVA of, and supporting definitions in, the Labelling of Food Order 1953, as amended.

These regulations come into operation on 4th January 1971. The revocation of the Part IVA of, and the supporting definitions in, the Order of 1953 as amended is, however, postponed until 5th July 1971 and until that date regulations 8 and 10 of these regulations, insofar as they relate to any label, ticket or notice, will not apply to any label, ticket or notice which conforms to the requirements of the said Part IVA (regulations 4 and 14(2)).

The regulations—

(*a*) specify requirements as to the fat, water, and vitamin content of margarine (regulations 5 and 6);

(*b*) specify requirements as to the wording of labels and on tickets and notices displayed with margarine and in advertisements for margarine (regulations 8 and 9 and paragraph 1 of Schedule 2);

(*c*) restrict the use of the words "butter", "cream" and "milk" on labels, tickets, notices and advertisements relating to the sale of margarine (regulations 8 and 9);

(*d*) specify requirements as to the use of the word "margarine" in advertisements for margarine (regulation 9 and paragraph 2 of Schedule 2).

The regulations do not apply to margarine sold for export or supplied for consumption by Her Majesty's Forces or a visiting force or sold to a manufacturer for the purposes of his manufacturing business or to a caterer for the purposes of his catering business (regulation 3).

Printed in England by McCorquodale & Co. Ltd., London
and published by
Her Majesty's Stationery Office: 1967
(*Second impression 1968*)
1s 0d net

[illegible] K8 2/68 McC.

0165

Reprinted from Federal Register of July 6, 1968; 33 F.R. 9783)

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Food and Drug Administration

[21 CFR Parts 3, 15, 16, 17, 18, 45, 121, 125]

VITAMIN D

Withdrawal of Proposed Rulemaking

In the matter of amending Title 21, Chapter I, with reference to vitamin D intake:

A notice of proposed rulemaking in the above-identified matter was published in the FEDERAL REGISTER of August 28, 1965 (30 F.R. 11140), proposing a policy statement on vitamin D and changes in references to vitamin D in various food-standard, food-additive, and food-for-special-dietary-uses regulations.

In response to the proposal, over 90 comments were filed which with minor exceptions raised objections; however, the recommendations made in the comments were widely divergent.

In view of the time that has elapsed since the proposal was published and the comments were received, the Commissioner of Food and Drugs concludes that it should be withdrawn. Therefore, pursuant to the provisions of the Federal Food, Drug, and Cosmetic Act (secs. 401, 403(j), 409(d), 502(f), 503(b)(4), 701 (a), (e), 52 Stat. 1046, 1048, 1051–52, 1055, 70 Stat. 919, 72 Stat. 1787; 21 U.S.C. 341, 343(j), 343(d), 352(f), 353(b)(4), 371 (a), (e)) and under authority delegated to the Commissioner (21 CFR 2.120), the proposed rulemaking published in the FEDERAL REGISTER of August 28, 1965 (30 F.R. 11140–41), regarding vitamin D is hereby withdrawn.

This action is entirely separate from and independent of the orders published in the FEDERAL REGISTER of June 18, 1966 (31 F.R. 8521, 8524, 8525), and the subsequent proceedings concerning foods for special dietary uses and dietary supplements.

Dated: May 14, 1968.

JAMES L. GODDARD,
Commissioner of Food and Drugs.

Presse Medicale, 78:556, 1970

Abusive Prescriptions of Vitamin D

Abusive prescriptions of vitamin D may lead to poisoning characterized by digestive disorders: anorexia, nausea, vomiting, constipation, severe thirst, by nervous disorders: depression, irritability, and by general weakening of the health with loss of weight. Hypercalcemia, exceeding 125mg/l hyperazotemia with or without hyperchloremic acidosis, proteinuria with hypercalciuria and cylindruria constitute the biological syndrome. Renal or cerebral lesions may irreversibly lead to death, but usually a cure can be obtained by a suppression of the vitamin supply, a low calcium diet and corticotherapy. An abusive prescription of vitamin D is not always the fault of the doctor, but of the mother who forces doses as "fortifiers", or of several doctors consulted successively who are not advised of previous prescriptions. Contra-indications for vitamin D are very few. These include exceptionally severe cases of hypercalcemia, paralyzed children or those being treated for orthopedic reasons in whom a de-mineralization syndrome with hypercalcemia and hypercalciuria can be observed. In children afflicted with severe vitamin-resistant rickets, the transition from an insufficient dosage to a toxic dosage can easily be made. (A. Hennequet, Concours med., 91(45): 8158-8167, 1969). It should be noted that a dosage of 400 to 800 units per day administered in the form of drops is very advisable until the end of the third month of life. Beyond this, a single dose of two milligrams every four to six months may be prescribed for reasons of convenience up until 12 or 18 months, prescriptions of vitamin D must be systematically present in the medical care of the child.

Presse Medicale. 78:556, 1970

DOSES ET RÉACTIONS THÉRAPEUTIQUES INHABITUELLES

LES PRESCRIPTIONS ABUSIVES DE VITAMINE D peuvent aboutir à une intoxication caractérisée par des troubles digestifs : anorexie, nausées, vomissements, constipation, soif vive, par des troubles nerveux : tristesse, irritabilité, et par une atteinte de l'état général avec amaigrissement. L'hypercalcémie, dépassant 125 mg/l, l'hyperazotémie, avec ou sans acidose hyperchlorémique, la protéinurie avec hypercalciurie et cylindrurie forment le syndrome biologique. Des lésions rénales ou cérébrales irréversibles peuvent conduire à la mort, mais on obtient d'habitude la guérison avec la suppression de l'apport vitaminé, un régime pauvre en calcium et une corticothérapie. La prescription abusive de vitamine D n'est pas toujours le fait du médecin traitant, mais de la mère qui force les doses en pensant donner un « fortifiant », ou de plusieurs médecins, consultés successivement et qui ne se sont pas enquis des ordonnances antérieures. Les contre-indications de la vitamine D sont peu nombreuses. Elles sont représentées par les exceptionnelles affections avec hypercalcémie, par l'immobilisation des enfants paralysés ou suivant une cure orthopédique, chez qui on peut observer un syndrome de déminéralisation avec hypercalcémie et hypercalciurie. Chez les enfants atteints de l'exceptionnel rachitisme vitamino-résistant, on peut enfin passer facilement d'une posologie insuffisante à une posologie toxique (A. HENNEQUET, *Concours méd.*, 1969, 91, n° 45, 8159-8167). Rappelons qu'une posologie de 400 à 800 unités par jour, administrée sous forme de gouttes, est très souhaitable jusqu'à la fin du troisième mois de la vie. Au-delà, on peut prescrire tous les quatre à six mois une dose unique de deux milligrammes pour des raisons de facilité. Jusqu'à douze ou dix-huit mois, les prescriptions de vitamine D doivent apparaître systématiquement sur le dossier médical de l'enfant.

1970 No. 400

FOOD AND DRUGS

The Labelling of Food Regulations 1970

Made - - -	*12th March* 1970
Laid before Parliament	*25th March* 1970
Coming into Operation—	
Regulations 1, 2, 33, 34, 35, *Schedule* 6	*31st March* 1970
Remainder	*1st January* 1973

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30. Penalties and enforcement.
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PART VII

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33. Amendment of the Labelling of Food Order 1953.
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Schedule 4. Vitamins and minerals.
Schedule 5. Manner of marking or labelling.
Schedule 6. Orders and regulations revoked.

The Minister of Agriculture, Fisheries and Food and the Secretary of State for Social Services, acting jointly, in exercise of the powers conferred upon them by sections 7, 123 and 136(2) of, and paragraph 2(2) of Schedule 12 to, the Food and Drugs Act 1955(a), as read with the Secretary of State for Social Services Order 1968(b), and of all other powers enabling them in that behalf,

(a) 4 & 5 Eliz. 2. c. 16.

(b) S.I. 1968/1699 (1968 III, p. 4585).

hereby make the following regulations after consultation with such organisations as appear to them to be representative of interests substantially affected by the regulations and reference to the Food Hygiene Advisory Council under section 82 of the said Act :—

PART I

PRELIMINARY

Citation and commencement

1. These regulations may be cited as the Labelling of Food Regulations 1970, and shall come into operation as follows :—

(*a*) this regulation, regulations 2, 33, 34 and 35 and Schedule 6 shall come into operation on 31st March 1970, and

(*b*) the remaining provisions of these regulations shall come into operation on 1st January 1973.

Interpretation

2.—(1) In these regulations, unless the context otherwise requires—

"the Act" means the Food and Drugs Act 1955 ;

"appropriate designation" has the meaning assigned to it by regulation 3 ;

"artificial sweetening tablet" has the meaning assigned to it by the Artificial Sweeteners in Food Regulations 1969(a) ;

"biscuits" includes wafers, rusks, crispbreads, oatcakes, matzos and chocolate-coated, chocolate-filled or chocolate-flavoured biscuits ;

"carbohydrate" means any neutral polyhydroxy alcohol containing carbon, hydrogen and oxygen in which the hydrogen and oxygen occur in the same proportion as in water, but does not include any polysaccharide which is not metabolised by man ;

"chocolate confectionery" means any solid or semi-solid product complete in itself and suitable for consumption without further preparation or processing, of which the characteristic ingredient is chocolate or cocoa, with or without the addition of nuts or fruit ; and includes any kind of chocolate and products made by enrobing, coating or embedding sugar confectionery or other ingredients in chocolate, but does not include chocolate-coated, chocolate-filled or chocolate-flavoured biscuits, flour confectionery, any kind of ice-cream including chocolate ice-cream, or pharmaceutical products ;

"container" includes any form of packaging of food for sale as a single item, whether by way of wholly or partly enclosing the food or by way of attaching the food to some other article and in particular includes a wrapper or confining band, but does not include any crimp case used to support the base or the base and sides of flour confectionery, sugar confectionery or chocolate confectionery or any similar product partly or wholly encased in pastry if such a product has been subject to a baking or cooking process ;

"fish" includes edible molluscs and crustacea ;

"flavouring" includes flavouring essence and flavouring extract and means any product consisting of a flavouring agent and such other substances, if any, the use of which in food is not forbidden and which are reasonably necessary to produce a solid, a solution or an emulsion, but no other ingredient or ingredients ;

"flavouring agent" means any sapid or odorous substance capable of imparting and primarily intended to impart a specific and distinctive taste

(a) S.I. 1969/1817 (1969 III, p. 5638).

show [illegible] litre, the statement may be expressed in terms of the calorie content of 100 millilitres of that food ;

(iii) as to the nature or effect of such food suggesting either directly or indirectly that such food is a source of protein unless at least 12 per cent. of the calorie content of such food is provided by protein :

Provided that the provisions of this sub-paragraph shall not apply—

(*aa*) as respects any words, device or description to which regulation 21 of the Bread and Flour Regulations 1963 applies ;

(*ab*) if no claim is made on the label or in the advertisment other than a statement, without further elaboration, that the food contains protein if such statement is accompanied by a true statement of the minimum protein content or a mention of protein in a list of ingredients in compliance with the provisions of regulation 5 or a statement of the minimum protein content as part of a statement of the total nutrient content of such food ;

(*ac*) to any milk food intended for consumption by babies, being a food to which the Dried Milk Regulations 1965(a) or the Condensed Milk Regulations 1959(b), as amended (c), or the Skimmed Milk with Non-Milk Fat Regulations 1960(d), as amended (e), apply and the protein content of which is approximately similar to the protein content of human milk.

(2) For the purposes of this regulation and of regulation 24(1)(i)(*ac*) and 25(i), the following factors shall be employed in calculating the calorie content of a food, namely :—

(*a*) 1 gramme of carbohydrate shall be deemed to contribute 3·75 kilocalories ;

(*b*) 1 gramme of glycitol shall be deemed to contribute 3·75 kilocalories ;

(*c*) 1 gramme of protein shall be deemed to contribute 4 kilocalories ;

(*d*) 1 gramme of alcohol shall be deemed to contribute 7 kilocalories ; and

(*e*) 1 gramme of fat shall be deemed to contribute 9 kilocalories.

Claims as to vitamins and minerals

22. Subject to the provisions of regulation 27, no person shall—

(*a*) give with any food sold by him any label, whether attached to or borne on the container or not, or display with any food offered or exposed by him for sale any ticket or notice, or

(*b*) publish, or be a party to the publication of, any advertisement for food, which bears or includes any words, device or description—

(i) which constitute or constitutes a general claim that vitamins or minerals are present in that food unless that food contains, in the case of a claim as to vitamins, one or more of the substances specified in column 1 of the table in, or in paragraph 1 of, Part I of Schedule 4 or, in the case of a claim as to minerals, one or more of the substances specified in column 1 of Part II of that Schedule and the words, device or description, as the case may be, are or is accompanied by a true statement, expressed in the manner prescribed in the said

(a) S.I. 1965/363 (1965 I, p. 1079).
(b) S.I. 1959/1098 (1959 I, p. 1305).
(c) S.I. 1962/1287 (1962 II, p. 1378).
(d) S.I. 1960/2331 (1960 II, p. 1463).
(e) S.I. 1962/1287, 1968/1474 (1962 II, p. 1378; 1968 III, p. 4214).

Sche[illegible], of the minimum quantity of every such substance contained in [illegible]unce, fluid ounce, hundred grammes or hundrd millilitres of the [illegible]: so however that where the minimum quantity of the food in the container is stated by weight the minimum quantity of each such substance per ounce, or per hundred grammes if the minimum quantity of the food in the container is stated in grammes or kilogrammes, shall be specified and where the minimum quantity of the food in the container is stated by volume the minimum quantity of every such substance per fluid ounce, or per hundred millilitres if the minimum quantity of the food in the container is stated in millilitres or litres, shall be specified ;

(ii) which constitute or constitutes any claim or in any way suggest or suggests that any particular substance or substances specified in column 1 of the table in, or in paragraph 1 of, Part I of Schedule 4 or in column 1 of Part II of that Schedule is or are present in that food unless the words, device or description, as the case may be, are or is accompanied by a true statement, expressed in the manner prescribed in the said Schedule, of the minimum quantity of every such substance contained in each ounce, fluid ounce, hundred grammes or hundred millilitres of the food, so however that where the minimum quantity of the food in the container is stated by weight the minimum quantity of each such substance per ounce, or per hundred grammes if the minimum quantity of the food in the container is stated in grammes or kilogrammes, shall be specified and where the minimum quantity of the food in the container is stated by volume the minimum quantity of every such substance per fluid ounce, or per hundred millilitres if the minimum quantity of the food in the container is stated in millilitres or litres, shall be specified :

Provided that the requirements of this sub-paragraph shall not apply in relation to any substance specified in Schedule 4 if the only claim or suggestion that the substance is present in a food is contained in a statement of the ingredients of that food made on a label on the container thereof in order to comply with the provisions of regulation 5 ;

(iii) which constitute or constitutes any claim or in any way suggest or suggests that any particular vitamin or mineral not specified in Schedule 4 is present in that food.

Slimming claims

23. No person shall—

(*a*) give with any food sold by him any label, whether attached to or borne on the container or not, or display with any food offered or exposed by him for sale any ticket or notice, being a label, ticket or notice which bears any words, device or description calculated to indicate either directly or indirectly that the food has specific weight reducing properties, or

(*b*) publish, or be a party to the publication of, any advertisement for any food, being an advertisement which includes any such words, device or description as aforesaid, or

(*c*) use on, or in connection with, the sale of any food any such words, device or description as aforesaid.

list of appropriate designations of ingredients or constituents required by regulation 5(2) or 24(1)(i)(*ac*) and the word or words heading or [illegible]ding it in accordance with regulation 5(5), any statement required by re[illegible]on 7 or 9, any statement required by regulation 15, 21, 22, 24, 25 or 26 appearing elsewhere than in an advertisement, the words required by regulation 12(*b*), any declaration required or permitted by regulation 16(4) or (5), any indication of the country or locality of origin of intoxicating liquor, not included in the appropriate designation of such liquor, in accordance with regulation 16(2)(*b*) or (3)(*b*) and the word required by regulation 18(2).

(2) If any food is pre-packed in more than one container, any label which by virtue of any provision of these regulations is required to be marked on or securely attached to the container shall either be marked on or securely attached to the outermost container or shall be readily discernible and easily read through, or notwithstanding, that outermost container.

Use of trade marks

29. Nothing in these regulations shall prohibit the use on any label on a container or on any ticket or notice of any registered trade mark registered and used in relation to the food in the container, or to which the ticket or notice applies, before 1st January 1968.

Part VI

Legal Proceedings

Penalties and enforcement

30.—(1) If any person contravenes or fails to comply with any of the foregoing provisions of these regulations he shall be guilty of an offence and shall be liable to a fine not exceeding one hundred pounds or to imprisonment for a term not exceeding three months, or to both, and, in the case of a continuing offence, to a further fine not exceeding five pounds for each day during which the offence continues after conviction.

(2) Each food and drugs authority shall enforce and execute such provisions in their area.

Defences

31.—(1) In any proceedings for an offence against these regulations in relation to the publication of an advertisement, it shall be a defence for the defendant to prove that, being a person whose business it is to publish or arrange for the publication of advertisements, he received the advertisement for publication in the ordinary course of business.

(2) In any proceedings against the manufacturer or importer of food for an offence against these regulations in relation to the publication of an advertisement, it shall rest on the defendant to prove that he did not publish, and was not a party to the publication of, the advertisement.

(3) In any proceedings for an offence against regulation 21(1)(*b*)(ii), 22(*b*)(i) or (ii), 24(1)(*b*)(i)(*ac*) or (*ad*), 25(*b*)(i) or 26(*b*)(i) or (ii) in respect of a failure to include in an advertisement for any food the required statement or to specify therein the required particulars, it shall be a defence for the defendant to prove that he took all reasonable steps to secure that the food when sold pre-packed would not be so sold without a label bearing or including the required statement or particulars, as the case may be.

plic of s s of Act

32.—(1) ns 108(3) and (4) (which relate to prosecutions), 110(1), (2) and (3) h relate to evidence of analysis), 112 (which relates to the power of a c t to require analysis by the Government Chemist), 113 (which relates to a contravention due to some person other than the person charged), 115(2) (which relates to the conditions under which a warranty may be pleaded as a defence) and 116 (which relates to offences in relation to warranties and certificates of analysis) of the Act shall apply for the purposes of these regulations as if references therein to proceedings, or a prosecution, under or taken or brought under the Act included references to proceedings, or a prosecution as the case may be, taken or brought for an offence under these regulations and as if the reference in the said section 112 to subsection (4) of section 108 included a reference to that subsection as applied by these regulations.

(2) Paragraph (*b*) of the proviso to section 108(1) of the Act shall apply for the purposes of these regulations as if the reference therein to section 116 of the Act included a reference to that section as applied by these regulations.

Part VII

Amendments and Revocations

Amendment of the Labelling of Food Order 1953

33. The Labelling of Food Order 1953(a), as amended (b), shall be further amended by adding to Table A of Schedule 1 thereto immediately after item 7 the following item :—

" Column 1	Column 2	Column 3
7A. Permitted artificial sweetener as defined in the Artificial Sweeteners in Food Regulations 1969, when forming an ingredient of some other food.	Article 4(3)	As respects any sale or display for sale by retail during the period from 31st March 1970 to 31st December 1970 inclusive, exempt to the extent that it may be designated as 'permitted artificial sweetener' or 'artificial sweetener' or by its appropriate designation without, in any case, further specification as to composition. "

Amendment of the Fish and Meat Spreadable Products Regulations 1968

34. The Fish and Meat Spreadable Products Regulations 1968(c) shall be amended by substituting in regulation 8 thereof for all the words following sub-paragraph (*b*) the words "and such description, name or word shall appear as or as part of, or in immediate proximity to, the name of the paste and shall appear conspicuously and legibly in a dark colour upon a light coloured ground or in a light colour upon a dark coloured ground.".

(a) S.I. 1953/536 (1953 I, p. 665).
(b) The relevant amending instruments are S.I. 1955/1889, 1955/1900, 1959/471, 1967/1864 (1953 I, p. 685; 1955 I, p. 930; 1959 I, p. 1326; 1967 III, p. 5013).
(c) S.I. 1968/430 (1968 I, p. 1130).

oca and itio ovis

35.—(1) The orders and regulations specified in column 1 edule 6 shall be revoked to the extent and on the dates respectively s d in relation thereto in columns 3 and 4 of that Schedule.

(2) Until revoked, any provision of the Labelling of Food Order 1953(a), as amended (b), shall not apply in relation to any food, label, statement, advertisement or invoice which complies with a corresponding provision of these regulations.

In Witness whereof the Official Seal of the Minister of Agriculture, Fisheries and Food is hereunto affixed on 11th March 1970.

(L.S.)

Cledwyn Hughes,
Minister of Agriculture, Fisheries and Food.

R. H. S. Crossman,
Secretary of State for Social Services.

12th March 1970.

(a) S.I. 1953/536 (1953 I, p. 665).
(b) S.I. 1953/1889, 1955/1900, 1958/717, 1959/471, 1961/440, 1965/2199, 1967/861, 1864, 1865, 1866, 1867 (1953 I, p. 685; 1955 I, p. 930; 1958 I, p. 1173; 1959 I, p. 1326; 1961 I, p. 1079; 1965 III, p. 6422; 1967 II, p. 2569; III, pp. 5013, 5048, 5056, 5064).

PART I
VITAMINS

Column 1	Column 2
Group and Substance	To be calculated as
1. Vitamin A (Retinol)	Microgrammes of retinol or, until 31st December 1974, international units of vitamin A.
2. Biologically active carotenoids	Microgrammes of retinol equivalents, on the basis that 6 microgrammes of beta-carotene or 12 microgrammes of other biologically active carotenoids equals one microgramme of retinol equivalent, or, until 31st December 1974, international units of vitamin A, on the basis that 0·6 microgrammes of beta-carotene equals one international unit of vitamin A.
3. Vitamin B_1 (Thiamine)	Milligrammes of thiamine hydrochloride.
4. Vitamin B_2 (Riboflavine)	Milligrammes of riboflavine.
5. Nicotinic acid Nicotinamide	Milligrammes of nicotinic acid.
6. Vitamin C (Ascorbic acid) Dehydroascorbic acid	Milligrammes of ascorbic acid.
7. Vitamin D Vitamin D_2 (Ergocalciferol) Vitamin D_3 (Cholecalciferol)	Microgrammes of cholecalciferol or, until 31st December 1974, international units of vitamin D.

1. Each substance specified in column 1 of the above table shall include its biologically active equivalent or derivative.

2. The quantity of any substance specified in column 1 of the above table (as extended by the preceding paragraph) shall be calculated in the manner prescribed in relation thereto in column 2 and may be specified accordingly, so however that such quantity may be specified together with a reference to any of the substances included in the group specified in column 1 in relation to the first mentioned substance.

PART II
MINERALS

Column 1	Column 2
Substance	To be calculated and specified as
Calcium	Milligrammes of calcium
Iodine	Microgrammes of iodine
Iron	Milligrammes of iron

MANNER OF MARKING OR LABELLI

General requirements as to manner of marking or labelling

1. Any designation, name, particulars, list, statement, declaration, indication of origin, word or words referred to in regulation 28(1)—

(*a*) shall be clear and legible;

(*b*) shall be in a conspicuous position on a label marked on, or securely attached to, the container or, as the case may be, on a ticket or notice on or in immediate proximity to the food to which the ticket or notice relates in such a manner that in either case it will be readily discernible and easily read by an intending purchaser or consumer under normal conditions of purchase or use;

(*c*) shall not be interrupted by other written or pictorial matter where such interruption might mislead the purchaser or consumer as to the nature of the food;

(*d*) shall not be in any way hidden or obscured or reduced in conspicuousness by any other matter, whether pictorial or not, appearing on a label.

2. The letters in each word in any designation, name, particulars, list, statement, declaration, indication of origin or words to which paragraph 1 above applies—

(*a*) shall be in characters of uniform colour and size, save that the initial letter of any word may be taller than any other letter in that word and the letters in any preposition, conjunction or participle may be shorter than the letters in any other word;

(*b*) shall appear on a contrasting ground, so however that where there is no ground other than such as is provided by a transparent container and the contents of that container are visible behind the letters, those contents shall be taken to be the ground for the purposes of this paragraph.

3. For the purposes of this Schedule—

(*a*) the height of any lower case letter shall be taken to be the x-height thereof, disregarding any ascender or descender thereof;

(*b*) any requirement that letters or characters shall be of uniform height, colour or size shall be construed as being subject to the saving that any inconsiderable variation in height, colour or size, as the case may be, may be disregarded.

Special requirements as to manner of marking or labelling

4. Any designation, name, particulars, list, statement, declaration, indication of origin, word or words referred to in regulation 28(1) shall respectively comply with such of the following provisions of this Schedule as are appropriate.

Appropriate designation or common or usual name

5.—(1) Any appropriate designation or common or usual name of a food shall appear in characters of such size that the height of the shortest letter in any word, other than a preposition, conjunction or participle, in that designation or name shall be not less than the height specified in the second column of the table in paragraph 13 of this Schedule in relation to that range of dimensions specified in the first column of that table within which the greatest dimension of the container of the food falls.

(2) Subject to the provisions of sub-paragraph (4) of this paragraph, any appropriate designation or common or usual name of a food shall be more prominent than any other matter required by these regulations to be stated, listed, indicated or declared on the same or any other label on the container and shall be so prominent in height, visual emphasis and position on the label as to be conspicuous by comparison with any other matter (whether pictorial or not) on the same or any other label on the container.

(3) Subject to the provisions of sub-paragraph (4) of this paragraph, the height of the letters in any word in that designation or name shall be such as is not calculated by any undue or insufficient prominence to mislead as to the nature, substance or quality of the food to which that designation or name relates; and without prejudice to the generality of the foregoing the height of the letters in any such word describing a minor ingredient shall be such as does not give undue prominence to that ingredient when the designation or name is taken as a whole.

(4) As respects any appropriate designation or common or usual name which appears on a label which was marked on or attached to the container on or before 1st January 1970, or which is identical in design and content to a label which was marked on or attached to a container before that date, the provisions of sub-paragraphs (2) and (3) of this paragraph shall be deemed to be complied with if—

(*a*) the height of the shortest letter in any word, other than a preposition, conjunction or participle, in that designation or name is not less than one-quarter of the height of the tallest letter, other than an initial letter, in any word of more than one letter appearing on any label on the container; and

(*b*) the height of the tallest letter, other than an initial letter, in that designation or name is not more than twice the height of the shortest letter in any word, other than a preposition, conjunction or participle, in that designation or name, so however that the tallest letter in any word in that designation or name describing a minor ingredient shall be such as does not give undue prominence to that ingredient when the designation or name is taken as a whole.

List of appropriate designations of ingredients or constituents and the word or words heading or preceding that list

6.—(1) Any list of appropriate designations of ingredients or constituents required by regulation 5(2) or 24(1)(i)(*ae*) and the word or words heading or preceding it in accordance with regulation 5(5) shall be—

(*a*) in immediate proximity to the appropriate designation or common or usual name of the food to which it relates, or

(*b*) so situated as to be simultaneously visible together with that appropriate designation or common or usual name to an intending purchaser under normal conditions of purchase or use, or

(*c*) within a surrounding line or on a panel which is clearly distinguished in colour from the adjacent parts of the label and no other written or pictorial matter, save such as may be required by virtue of the provisions of any enactment or any order or regulations made thereunder to appear on a label marked on or attached to the container, shall appear within that surrounding line or on that panel, as the case may be.

(2) The height of the shortest letter in any word, other than a preposition, conjunction or participle, in that list, word or words, as the case may be, shall, having regard to the greatest dimension of the container of the food to which it or they relate, be not less than the height specified in the third column of the table in paragraph 13 of this Schedule in relation to the appropriate greatest dimension specified in the first column of that table.

(3) The word or words heading or preceding that list may be taller than the other words in the list.

Bottles containing soft drinks

7. Notwithstanding the provisions of regulation 5(2) and any other provisions of this Schedule, in the case of any soft drink where no words appear on any label on the container save such as appear on a cork, stopper or cap closing that container or are embossed or fired on the container, being words which do not contravene any provisions of these regulations, the appropriate designation or common or usual name of the soft drink, the list of appropriate designations of ingredients or constituents required by regulation 5(2) and the word or words heading or preceding it in accordance with regulation 5(5) may appear not on a label on the container but on the exposed surface of the cork, stopper or cap closing that container and the said list and word or

words need not be in immediate proximity, or simultaneously visible with, the appropriate designation or common or usual name of the soft drink within a surrounding line or on a panel if the height of the shortest letter in any word other than a preposition, conjunction or participle, in the appropriate designation or common or usual name or in the said list, word or words is not less than 1 millimetre.

Exempted pre-packed food for sale by retail

8. The provisions of paragraph 6(2) of this Schedule shall apply to any statement required by regulation 7 as they apply to a list required by regulation 5(2).

Food other than pre-packed, for sale by retail

9. In any statement required by regulation 9(1) the height of the letters in any word in the statement shall be such as is not calculated by any undue or insufficient prominence to mislead as to the nature, substance or quality of the food to which that statement relates; and without prejudice to the generality of the foregoing the height of the letters in any such word describing a minor ingredient shall be such as does not give undue prominence to that ingredient when the statement is taken as a whole.

Acetic acid

10. The words required by regulation 12(*b*) shall appear in red characters not less than 3 millimetres in height on a white ground.

Intoxicating liquor

11.—(1) Any declaration required by regulation 16(4) shall appear within a surrounding line and no matter other than that required by the appropriate sub-paragraph of that paragraph shall appear within that surrounding line.

(2) The height of the shortest letter or numeral in any declaration required or permitted by regulation 16(4) or (5) shall be not less than 3 millimetres, save that in the case of any intoxicating liquor pre-packed in a bottle smaller in size than the normal half bottle the height of any such letter or numeral shall be not less than 2 millimetres.

Tenderised meat

12.—(1) The provisions of paragraph 9 of this Schedule shall apply to any appropriate designation or common or usual name of meat on a ticket to which regulation 18(2) relates as they apply to a statement required by regulation 9(1).

(2) The letters of the word "tenderised" on any ticket to which regulation 18(2) relates shall be of uniform height and not shorter than the tallest letter, other than an initial letter, of any word in any appropriate designation or common or usual name of meat on that ticket.

13. TABLE

Greatest dimension of container	Minimum height of letters referred to in paragraph 5(1)	Minimum height of letters referred to in paragraph 6(2)
Not exceeding 12 centimetres	2 millimetres	1 millimetre
Exceeding 12 centimetres, but not exceeding 30 centimetres	3 millimetres	1·5 millimetres
Exceeding 30 centimetres, but not exceeding 45 centimetres	6 millimetres	3 millimetres
Exceeding 45 centimetres	8 millimetres	4 millimetres

SCHEDULE 6

Column 1	Column 2	Column 3	Column 4
Orders and regulations revoked	References	Extent of revocation	Date of revocation
The Labelling of Food Regulations 1967 ...	S.I. 1967/1864 (1967 III, p. 5013).	The whole regulations.	31st March 1970
The Labelling of Food Order 1953... ...	S.I. 1953/536 (1953 I, p. 665).	The whole order.	1st January 1973
The Labelling of Food (Amendment) Order 1953	S.I. 1953/1889 (1953 I, p. 685).	The whole order.	
The Labelling of Food (Amendment) Regulations 1959	S.I. 1959/471 (1959 I, p. 1326).	The whole regulations.	
The Labelling of Food (Amendment) Regulations 1961	S.I. 1961/440 (1961 I, p. 1079).	The whole regulations.	
The Preservatives in Food Regulations 1962	S.I. 1962/1532 (1962 II, p. 1655).	Regulation 5 and paragraphs 1 to 3 of Schedule 3.	
The Bread and Flour Regulations 1963 ...	S.I. 1963/1435 (1963 II, p. 2464).	Regulation 23.	
The Meat Pie and Sausage Roll Regulations 1967	S.I. 1967/860 (1967 II, p. 2557).	Regulation 10.	
The Canned Meat Product Regulations 1967	S.I. 1967/861 (1967 II, p. 2569).	Regulations 4 and 11.	
The Sausage and Other Meat Product Regulations 1967	S.I. 1967/862 (1967 II, p. 2583).	Regulation 9.	
The Fish and Meat Spreadable Products Regulations 1968	S.I. 1968/430 (1968 I, p. 1130).	Regulation 8.	

EXPLANATORY NOTE

(This Note is not part of the regulations.)

These regulations, which apply to England and Wales only, revoke the Labelling of Food Regulations 1967 on 31st March 1970 (before those regulations would have come into operation for all foods other than those containing cyclamates). They supersede the Labelling of Food Order 1953, as amended, on 1st January 1973.

The principal changes are that the regulations—

(*a*) amend the provisions relating to the labelling of pre-packed food for sale by retail and the list of foods to which they apply (regulations 5 to 7 and 28, Schedules 1, 2 and 5);

(*b*) similarly amend the provisions relating to the labelling of pre-packed food sold otherwise than by retail and the list of foods to which they apply (regulation 8);

(*c*) impose requirements as to the labelling of certain foods for sale by retail which are not pre-packed (regulations 9 and 28, Schedules 3 and 5);

(*d*) impose requirements as to the advertisement of food for sale from vending machines (regulation 10);

(*e*) impose restrictions on the use of the words "milk", "butter" and "cream" on labels or in advertisements (regulation 20);

(*f*) impose restrictions on claims based on the value in, or benefit to be derived from, foods; on their ability to provide energy; on their calorie content; or that they are a source of protein (regulations 21, 27 and 28, Schedule 5);

(*g*) impose restrictions on claims as to the presence of vitamins and minerals in food (regulations 22, 27 and 28, Schedules 4 and 5);

(*h*) impose restrictions on claims that foods are starch-reduced or can aid slimming, and prohibit claims that foods have specific weight reducing properties (regulations 23, 24, 27 and 28, Schedule 5);

(*j*) impose restrictions on claims that foods are suitable, or have been specially prepared, for diabetics (regulations 25 and 28, Schedule 5);

(*k*) impose restrictions on claims that foods have tonic, restorative or medicinal properties (regulations 26 to 28, Schedule 5).

The regulations also contain provisions as to the labelling and advertisement of acetic acid (regulation 12), chocolate confectionery containing intoxicating liquor (regulation 13), dried and dehydrated food (regulation 14), dry mixes (regulation 15), intoxicating and other liquor (regulations 16 and 17), tenderised meat (regulation 18) and processed peas (regulation 19), and as to the labelling and advertisement of food as respects flavours (regulation 11).

The regulations—

(*a*) further amend the Labelling of Food Order 1953, as amended, as respects the designation of a permitted artificial sweetener when forming an ingredient of some other food during the period from 31st March to 31st December 1970 (regulation 33);

(*b*) amend the Fish and Meat Spreadable Products Regulations 1968 as respects the presentation of descriptions during the period from 31st March 1970 to 31st December 1972 (regulations 34 and 35, Schedule 6).

The regulations do not apply—

(a) except insofar as they relate to ad[illegible]ments, to any food intended for export or supplied for consump[illegible]y Her Majesty's forces or a visiting force;

(b) until 3rd January 1983, to any bottles containing a drink which were first used before 4th January 1971 and which bear a fired-on or embossed, but no other, label (regulation 4).

Printed in England by McCorquodale & Co. Ltd., London
and published by
Her Majesty's Stationery Office: 1970
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(27/P36031/1) (D 78) K112 3/70 McC.

SBN 11 000400 0

1971 No. 1410

MEDICINES

The Medicines (Exemption from Licences) (Foods and Cosmetics) Order 1971

Made - - - -	*23rd August* 1971
Laid before Parliament	*3rd September* 1971
Coming into Operation	*6th September* 1971

The Secretaries of State respectively concerned with health in England, in Wales and in Scotland and the Minister of Health and Social Services for Northern Ireland, acting jointly, in exercise of their powers under section 15(1) of the Medicines Act 1968(a) (as having effect subject to the provisions of Article 2(2) of and Schedule 1 to the Transfer of Functions (Wales) Order 1969(b)) and of all other powers enabling them in that behalf, after consulting such organisations as appear to them to be representative of interests likely to be substantially affected by the following order, hereby make the following order:—

Citation, commencement and interpretation

1.—(1) This order may be cited as the Medicines (Exemption from Licences) (Foods and Cosmetics) Order 1971 and shall come into operation on 6th September 1971.

(2) In this order, unless the context otherwise requires—

"the Act" means the Medicines Act 1968;

"cosmetic" means any substance or preparation intended to be applied to the various surfaces of the human body including epidermis, pilary system and hair, nails, lips and external genital organs, or the teeth and buccal mucosa wholly or mainly for the purpose of perfuming them, cleansing them, protecting them, caring for them or keeping them in condition, modifying their appearance (whether for aesthetic purposes or otherwise) or combating body odours or normal body perspiration;

"food" includes beverages, confectionery and articles and substances as ingredients in the preparation of food and includes any manufactured substance to which there has been added any vitamin and which is advertised (within the meaning of Section 92 of the Act) as available and for sale to the general public as a dietary supplement;

(a) 1968 c. 67.

(b) S.I. 1969/388 (1969 I, p. 170).

[H. 172]

"medicinal product" includes articles or substance specified in orders made under section 104 or section 105 of the Act which are [illegible] time being in force and which direct that Part II of the Act shall hav[illegible] [illegible]t in relation to such articles or substances as that Part has effect in relation to medicinal products within the meaning of the Act;

"mineral salts" means salts of any one or more of the following, iron, iodine, calcium, phosphorus, fluorine, copper, potassium, manganese, magnesium or zinc;

"vitamin preparation" means any medicinal product the active ingredients of which consist only of vitamins or vitamins and mineral salts;

"vitamins" means any one or more of the following, vitamins A, B1, B2, B6, C, D and E, biotin, nicotinamide, nicotinic acid, pantothenic acid and its salts, biflavonoids, inositol, choline, para-aminobenzoic acid, cyanocobalamin or folic acid;

and other expressions have the same meaning as in the Act.

(3) Except in so far as the context otherwise requires, any reference in this order to any enactment shall be construed as a reference to that enactment as amended, extended or re-enacted by any other enactment.

(4) The Interpretation Act 1889(a) applies for the purpose of the interpretation of this order as it applies for the purpose of the interpretation of an Act of Parliament.

Exemption from licences for certain foods and cosmetics

2.—(1) Subject to the provisions of paragraph (2) of this Article, the restrictions imposed by sections 7 and 8 of the Act (licences for dealings in and manufacture of medicinal products) shall not apply to anything done in relation to a medicinal product which is wholly or mainly for use by being administered to one or more human beings and which is or is to be for sale either for oral administration as a food or for external use as a cosmetic.

(2) The exemption conferred by the preceding paragraph of this Article does not apply to a medicinal product as aforesaid which—

(*a*) is or is to be sold with, accompanied by or having in relation to it, any particulars in writing specifying that product's curative or remedial function in relation to a disease specified or the use of that product for such curative or remedial purposes, or

(*b*) being a product for oral administration as a food as aforesaid, comes within any of the descriptions contained in the Schedule to this order, or

(*c*) being a product for external use as a cosmetic as aforesaid contains any antibiotic or any hormone in a proportion in excess of 0·4 per cent or resorcinol in a proportion in excess of 1 per cent (the said proportions calculated on the weight of the medicinal product), or

(a) 1889 c. 63.

(d [illegible] a vi[illegible] pre[illegible]ion [illegible] sub[illegible]e co[illegible] with [illegible] desc[illegible] in paragraph 3 of the said Schedule, is or is to be sold with, acc[illegible]ed by or having in relation to it any particulars in writing spec[illegible] the dosage relevant to that product's medicinal purpose.

Signed by authority of the Secretary of State for Social Services.

Paul Dean,
Parliamentary Under Secretary of State,
Department of Health and Social Security.

13th August 1971.

Peter Thomas,
Secretary of State for Wales.

18th August 1971.

Gordon Campbell,
Secretary of State for Scotland.

23rd August 1971.

W. K. Fitzsimmons,
Minister of Health and Social Services
for Northern Ireland.

16th August 1971.

SCHEDULE Article 2(2)(*b*)

Description of certain medicinal product foods which are not exempt from licences

1. Any vitamin preparation in relation to which there are no written particulars or directions as to dosage.

2. Any vitamin preparation in relation to which there are written particulars or directions as to dosage specifying a recommended daily dosage for adults which involves a daily intake in excess of—

(i) the equivalent of 2,500 Units (within the meaning of the 1968 edition of the British Pharmacopoeia) of vitamin A activity, or

(ii) the equivalent of 250 Units (within the meaning of the 1968 edition of the British Pharmacopoeia) of antirachitic activity, or

(iii) 25 micrograms of folic acid, or

(iv) 5 micrograms of cyanocobalamin.

3. Any medicinal product, not being a vitamin preparation, to which one or more of the ingredients vitamin A or D, folic acid or cyanocobalamin has been added and in relation to which product there are written particulars or directions as to recommended use of that substance which involves a daily intake in excess of the quantities and ingredients specified in the preceding paragraph.

EXP[illegible] ATC[illegible] NO[illegible]

(*This Note is not part of the Order.*)

This Order exempts from the restrictions imposed by Part II of [illegible] Medicines Act 1968 as to dealings in and manufacture of medicinal products except in accordance with a licence granted under that Act, certain medicinal products which are for oral administration as foods or which are for external use as cosmetics.

Printed in England by McCorquodale Printers Ltd., London
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Her Majesty's Stationery Office: 1971
5p net

(92/947221/0) (T 77) K40 9/71 McC. SBN 11 011410 8

[DESI 3444 Docket No. FDC-D-518; NDA 3-444]

CERTAIN ORAL PREPARATIONS CONTAINING ERGOCALCIFEROL OR VITAMIN A PALMITATE

Drugs for Human Use; Drug Efficacy Study Implementation

The Food and Drug Administration has evaluated reports received from the National Academy of Sciences-National Research Council, Drug Efficacy Study Group, on the following drugs:

1. Hard gel capsules containing ergocalciferol 50,000 U.S.P. units per capsule; Winthrop Laboratories, Division of Sterling Drug Inc., 90 Park Avenue, New York, N.Y. 10016 (NDA 3-444).

2. Aqua Capsules containing vitamin A palmitate, 50,000 U.S.P. Vitamin A units per capsule; Cole Pharmacal Co., Inc., 3715-31 Laclede Avenue, St. Louis, Mo. 63108 (NDA 3-934).

Such drugs are regarded as new drugs (21 U.S.C. 321(p)). Supplemental new drug applications are required to revise the labeling in and to update previously approved applications providing for such drugs. A new drug application is required from any person marketing such drug without approval.

A. *Effectiveness classification*—1. *Ergocalciferol.* The Food and Drug Administration has considered the Academy's report, as well as other available evidence, and concludes that ergocalciferol:

a. Is effective for the indications described in the labeling conditions which follow [illegible]

[illegible] substantial evidence of effectiveness for use in human subjects.

2. *Vitamin A Palmitate.* The Food and Drug Administration has considered the Academy's report, as well as other available evidence, and concludes that vitamin A palmitate is effective for the indications described in the labeling conditions which follow.

B. *Conditions for approval and marketing.* The Food and Drug Administration is prepared to approve abbreviated new drug applications and abbreviated supplements to previously approved new drug applications under conditions described herein.

1. *Form of drug.* These preparations are in capsule form suitable for oral administration of 50,000 U.S.P. units per capsule.

2. *Labeling conditions.* a. The labels bear the statement "Caution: Federal law prohibits dispensing without prescription."

b. Each drug is labeled to comply with all requirements of the Act and regulations. Its labeling bears adequate information for safe and effective use of the drug and is in accord with the guidelines for drug labeling published in the FEDERAL REGISTER of February 6, 1970. The "Indications" sections are as follows:

(i) *Ergocalciferol.*

INDICATIONS

[illegible] in the treatment of hypoparathyroidism and refractory rickets.

(ii) *Vitamin A Palmitate.*

INDICATIONS

For treatment of severe vitamin A deficiency and associated nyctalopia (night blindness) or hyperkeratosis of the skin.

3. *Marketing status.* Marketing of such drugs may be continued under the conditions governed in the notice entitled "Conditions for Marketing New Drugs Evaluated in Drug Efficacy Study," published in the FEDERAL REGISTER July 14, 1970 (35 F.R. 11273), as follows:

a. For holders of "deemed approved" new drug applications (i.e., an application which became effective on the basis of safety prior to October 10, 1962), the submission of a supplement for revised labeling and an abbreviated supplement for updating information as described in paragraphs (a)(1)(ii) and (iii) of the notice of July 14, 1970.

b. For any person who does not hold an approved or effective new drug application, the submission of an abbreviated new drug application as described in paragraph (a)(3)(i) of that notice.

c. For any distributor of the drug, the use of labeling in accord with this announcement for any such drug shipped within the jurisdiction of the Act as described in paragraph (b) of that notice.

C. *Opportunity for a hearing.* 1. The Commissioner of Food and Drugs proposes to issue an order under the provisions of section 505(e) of the Federal Food, Drug, and Cosmetic Act withdrawing approval of all new drug applications and all amendments and supplements thereto providing for the indications for which substantial evidence of effectiveness is lacking as described in paragraph A 1 b of this announcement. An order withdrawing approval of the applications will not issue if such applications are supplemented, in accord with this notice, to delete such indications. Any related drug for human use, not the subject of an approved new drug application, offered for the indications for which substantial evidence of effectiveness is lacking may be affected by this action.

2. In accordance with the provisions of section 505 of the Act (21 U.S.C. 355) and the regulations promulgated thereunder (21 CFR Part 130), the Commissioner will give the holders of any such applications, and any interested person who would be adversely affected by such an order, an opportunity for a hearing to show why such indications should not be deleted from labeling. A request for a hearing must be filed within 30 days after the date of publication of this notice in the FEDERAL REGISTER.

3. A request for a hearing may not rest upon mere allegations or denials but must set forth specific facts showing that a genuine and substantial issue of fact requires a hearing, together with a well-organized and full-factual analysis of the clinical and other investigational data that the objector is prepared to prove in a hearing. Any data submitted in response to this notice must be previously unsubmitted and include data from adequate and well-controlled clinical investigations (identified for ready review) as described in § 130.12(a)(5) of the regulations published in the FEDERAL REGISTER of May 8, 1970 (35 F.R. 7250). Carefully conducted and documented clinical studies obtained under uncontrolled or partially controlled situations are not acceptable as a sole basis for approval of claims of effectiveness, but such studies may be considered on their merits for corroborative support of efficacy and evidence of safety.

4. If a hearing is requested and is justified by the response to this notice, the issues will be defined, a hearing examiner will be named, and he shall issue a written notice of the time and place at which the hearing will commence.

A copy of the Academy's report has been furnished to each firm referred to above. Communications forwarded in response to this announcement should be identified with the reference number DESI 3444, directed to the attention of the appropriate office listed below, and addressed to the Food and Drug Administration, 5600 Fishers Lane, Rockville, Maryland 20852:

Supplements (identify with NDA number): Office of Scientific Evaluation (BD-100), Bureau of Drugs.

Original abbreviated new drug applications (identify as such): Drug Efficacy Study Implementation Project Office (BD-60), Bureau of Drugs.

Requests for Hearing (identify with Docket Number): Hearing Clerk, Office of General Counsel (GC-1), Room 6-62, Parklawn Building.

Requests for the Academy's report: Drug Efficacy Study Implementation Control Office (BD-67), Bureau of Drugs.

All other communications regarding this announcement: Drug Efficacy Study Implementation Project Office (BD-60), Bureau of Drugs.

This notice is issued pursuant to the provisions of the Federal Food, Drug, and Cosmetic Act (secs. 502, 505, 52 Stat. 1050-53, as amended, 21 U.S.C. 352, 355) and under the authority delegated to the Commissioner of Food and Drugs (21 CFR 2.120).

Dated: July 19, 1971.

SAM D. FINE,
Associate Commissioner for Compliance.

[FR Doc.71-12106 Filed 8-18-71;8:52 am]

[DESI 5554]

CERTAIN PLASMA VOLUME EXPANDERS—POVIDONE OR GELATIN IN SODIUM CHLORIDE INJECTION

Drugs for Human Use; Drug Efficacy Study Implementation

The Food and Drug Administration has evaluated reports received from the National Academy of Sciences-National Research Council, Drug Efficacy Study Group, on the following plasma volume expanding drugs for intravenous administration:

1. Polyvinylpyrrolidone in Normal Saline; containing 3.5 percent povidone in sodium chloride injection; Don Baxter,

NATIONAL ACADEMY OF SCIENCES—NATIONAL RESEARCH COUNCIL
Division of Medical Sciences

0188

DRUG EFFICACY STUDY

DESI 3444

Form A
(To be submitted in duplicate by applicant)

274

1. NDA Number 3444 ECF 2. Date Originally Approved January 11, 1941 3. Rx ☑ OTC ☐

4. Brand Name DRISDOL

5. Applicant's Name Winthrop Laboratories

and Address 90 Park Avenue, New York, New York 10016

6. Quantitative Formula

Established (Non-Proprietary) Name of Active Ingredients (in order shown on label)	Amount (per tablet, per ml., etc.)
Ergocalciferol	1.25 mg. (50,000 U.S. Vitamin D units) per capsule

7. Dosage form (tablets, etc.) Capsules

8. Route of Adm. (Oral, etc. Where a new drug application covers different modes of administration, separate forms should be used.) Oral

9. Therapeutic Claims—Attach 10 labels and 10 package inserts (if used) to original Form A (blue) and 1 copy to duplicate Form A (white).

10. List of literature references most pertinent to an evaluation of the effectiveness of the drug for the purposes for which it is offered in the label, the package insert, or brochure. Approximately 5 to 10 key references are requested, if available. (Attach 10 copies to original Form A (blue) and 1 copy to duplicate Form A (white).)

11. The applicant is invited, if he so desires, to submit any unpublished material that is pertinent to the evaluation of the drug by the Academy—Research Council. This supplementary material should be packaged with Form A (white). A single copy of this material is requested.

In this space, please list and describe briefly the supplementary material that is submitted with Form A (white).

Blue copy is original

The back of this form may be

This drug has been evaluated by the following Panels:

1. Panel on Drugs Used in Metabolic Disorders
2. Panel on Drugs Used in Dermatology

Evaluations follow:

0188

276

DRISDOL
NDA 3444
LOG 722

Panel on Drugs Used in Metabolic Disorders

INDICATIONS

I. "Parathyroprivic" tetany -- acute, chronic, or latent forms. Parathyroid insufficiency.

EVALUATION: Effective, but . . . .

COMMENTS: The use of vitamin D supplemented with calcium is effective in hypoparathyroidism. The Panel advises the change of "parathyroprivic" to "hypoparathyroidism." The initial dose of vitamin D recommended (100,000 to 200,000 U.S.P.) is considered excessive for pediatric use; it would be better to start at a lower dose and one according to serum calcium levels.

DOCUMENTATION:

1. Albright, F., and E. C. Reifenstein, Jr. The Parathyroid Glands and Metabolic Bone Disease. Baltimore: The Williams and Wilkens Co., 1948.

II. Refractory rickets.

EVALUATION: Effective.

COMMENTS: The use of vitamin D supplemented with calcium is effective in refactory rickets.

DOCUMENTATION:

1. Stanbury, S. W., and G. A. Lumb. Metabolic Studies of Renal Osteodystrophy. I. Calcium, phosphorus, and nitrogen metabolism in rickets, osteomalacia and hyperparathyroidism complicating chronic uremia and in the osteomalacia of the adult Fanconi syndrome. Medicine 41:1-34, 1962.
2. Winters, R. W., J. B. Graham, T. F. Williams, V. W. McFalls, and C. H. Burnett. A genetic study of familial hypophosphatemia and vitamin D resistant rickets with a review of the literature. Medicine 37:97-142, 1958.

GENERAL COMMENTS

I. Most important in titrating the dose of vitamin D and calcium are routine serum calcium levels. A urine Sulkowitch offers little help in avoiding the effects of hypercalcemia.

II. Under "Side Effects," the following claim is made: "Intravenous administration of 250 ml. of 2.5 per cent sodium citrate solution at four-hour intervals has been suggested to tide patients over the toxic episode." The Panel feels that more recent work shows that sodium sulfate and sodium chloride are more effective than sodium citrate in the treatment of hypervitaminosis D.

0188

DRISDOI.
NDA 3444
LOG 722

DOCUMENTATION:

1. Chakmakjian, Z. H., and J. E. Bethune. Sodium sulphate treatment of hypercalcemia. New Eng. J. Med. 275:862-869, 1966.

Approved by

Chairman

0188

V76

DRISDOL CAPSULES
NDA 5444
LOG 722

Panel on Drugs Used in Dermatology

INDICATIONS

I. For use in lupus vulgaris.

EVALUATION: Effective, but . . . .

COMMENTS: The Panel feels that this product is now obsolete for the treatment of lupus vulgaris for with the use of this product, there is an associated danger of renal damage.

DOCUMENTATION: None applicable.

Signed Harvey Blank

Harvey Blank, M.D., Chairman,
Dermatology Panel

The Drug Efficacy Study of the National Academy of Sciences - National Research Council has requested that the following qualifying addendum be conveyed with their reports to the ultimate recipients of these reports:

> "Drugs of identical chemical composition (so-called generic drugs) formulated and marketed by numerous individual firms under generic or trademarked names have been evaluated for efficacy as a group without consideration of 'therapeutic equivalence.' In the event that no evidence for pharmacological availability or therapeutic efficacy in man can be presented for any of the indications claimed for the use of any of the drugs in the attached listing, their classifications of effectiveness may need to be modified if regulations of the Food and Drug Administration require such proof."

0190

1972 No. 1510

FOOD AND DRUGS

The Labelling of Food (Amendment) Regulations 1972

Made - - -	10*th October* 1972
Laid before Parliament	18*th October* 1972
Coming into Operation	
Regulation 4(4)	5*th January* 1976
Remainder	8*th November* 1972

The Minister of Agriculture, Fisheries and Food and the Secretary of State for Social Services, acting jointly, in exercise of the powers conferred on them by sections 7 and 123 of the Food and Drugs Act 1955(a), as read with the Secretary of State for Social Services Order 1968(b), and of all other powers enabling them in that behalf, hereby make the following regulations after consultation with such organisations as appear to them to be representative of interests substantially affected by the regulations and after reference to the Food Hygiene Advisory Council under section 82 of the said Act:—

Citation, commencement and interpretation

1.—(1) These regulations may be cited as the Labelling of Food (Amendment) Regulations 1972, and shall come into operation as respects regulation 4(4) on 5th January 1976 and as respects the remainder on 8th November 1972.

(2) In these regulations "the 1970 Regulations" means the Labelling of Food Regulations 1970(c) and "the 1964 Regulations" means the Soft Drinks Regulations 1964(d) as amended (e).

(3) The Interpretation Act 1889(f) shall apply to the interpretation of these regulations as it applies to the interpretation of an Act of Parliament.

Amendment of the 1970 *Regulations*

2.—(1) In this regulation a reference to a numbered regulation or schedule is a reference to the regulation or schedule so numbered in the 1970 Regulations.

(2) Regulation 2(1) shall be amended—

(*a*) by substituting in the definition of flour confectionery for the words "any meat or fish" the words "any meat, or fish or any animal, vegetable or microbial material processed before or during the preparation of the product to resemble the texture of meat or fish";

(a) 4 & 5 Eliz. 2. c. 16.
(b) S.I. 1968/1699 (1968 III, p. 4585).
(c) S.I. 1970/40 (1970 I, p. 1383).
(d) S.I. 1964/760 (1964 II, p. 1605).
(e) S.I. 1969/1318, 1970/1597 (1969 III, p. 5644; 1970 III, p. 5331).
(f) 1889 c. 63.

(*b*) by inserting in paragraph (2)(*a*) thereof after the word "carbohydrate" the words "(expressed as monosaccharide)".

(8) The following regulation shall be substituted for regulation 22:—

"22.—(1) Subject to the provisions of regulation 27, no person shall—

(*a*) give with any food sold by him any label, whether attached to or borne on the container or not, or display with any food offered or exposed by him for sale any ticket or notice, or

(*b*) publish, or be party to the publication of, any advertisement for food,

which bears or includes any words, device or description—

(i) constituting a general claim that vitamins or minerals are present in that food unless that food contains, in the case of a claim as to vitamins, one or more of the substances specified in column 1 of the table in, or in paragraph 1 of, Part I of Schedule 4 or, in the case of a claim as to minerals, one or more of the substances specified in column 1 Part II of that Schedule and the words, device or description, as the case may be, are or is accompanied by a true statement in compliance with this regulation;

(ii) constituting any claim or in any way suggesting that any particular substance or substances specified in column 1 of the table in, or in paragraph 1 of, Part I of Schedule 4 or in column 1 of Part II of that Schedule is or are present in that food unless the words, device or description, as the case may be, are or is accompanied by a true statement in compliance with this regulation:

Provided that the requirement of this sub-paragraph shall not apply in relation to any vitamin or mineral not specified if the only claim or suggestion that the substance is present in a food is contained in a list of the ingredients of that food made on a label on the container thereof in compliance with the provisions of regulation 5;

(iii) constituting any claim or in any way suggesting that any particular vitamin or mineral not specified in Schedule 4 is present in that food:

Provided that the requirements of this sub-paragraph shall not apply in relation to any vitamin or mineral not specified in Schedule 4 if the only claim or suggestion on the label or in the advertisement that the vitamin or mineral is present in a food is a mention of such vitamin or mineral in an appropriate designation of the food in compliance with the provisions of regulation 5, in a list of ingredients in compliance with those provisions, in a true statement in compliance with the provisions of regulation 26 or in a statement of the minimum vitamin or mineral content (being a statement which, when taken as a whole, does not give undue prominence to that vitamin or mineral) as part of a statement of the total nutrient content or chemical analysis of such food.

(2) The true statement referred to in paragraph (1)(i) and (1)(ii) of this regulation shall specify in the manner prescribed in Schedule 4, the minimum quantity of every substance present in the food and referred to in the said paragraph (1)(i) or (1)(ii), as the case may be, contained in each

ounce, fluid ounce, hundred grammes or hundred millilitres of the food, so that where the minimum quantity of the food in the container is stated by weight, the minimum quantity of every such substance shall be specified per ounce or, if the minimum quantity of the food in the container is stated in grammes or in kilogrammes shall be specified per hundred grammes, and where the minimum quantity of the food in the container is stated by volume, the minimum quantity of every such substance shall be specified per fluid ounce or, if the minimum quantity of the food in the container is stated in millilitres or litres, shall be specified per hundred millilitres.".

(9) Regulation 24(2)(*a*) shall be amended by inserting after the words "minerals or vitamins" the words "or both vitamins and minerals".

MR HANSON OR

0190

SCHEDULE 2

VITAMINS

Column 1	Column 2
Substance and Alternative Name or Names	To be calculated as
Group 1	
Biologically active carotenoids	Microgrammes of retinol, on the basis that 6 microgrammes of beta-carotene or 12 microgrammes of other biologically active carotenoids equals 1 microgramme of retinol, or until 31st December 1974, international units of vitamin A, on the basis that 0·6 microgrammes of beta-carotene equals one international unit of vitamin A.
Group 2	
Retinol or Vitamin A	Microgrammes of retinol, or until 31st December 1974, international units of vitamin A.
Group 3	
Thiamin or Vitamin B_1	Milligrammes of thiamin hydrochloride.
Group 4	
Riboflavin or Vitamin B_2	Milligrammes of riboflavin.
Group 5	
Nicotinic acid or Niacin Nicotinamide or Niacinamide	Milligrammes of nicotinic acid.
Group 6	
Ascorbic acid or Vitamin C Dehydroascorbic acid or Vitamin C	Milligrammes of ascorbic acid.
Group 7	
Ergocalciferol or Vitamin D or Vitamin D_2 Cholecalciferol or Vitamin D or Vitamin D_3	Microgrammes of cholecalciferol, or until 31st December 1974, international units of vitamin D.

1. Each substance specified in column 1 of the table shall include its biologically active equivalent or derivative and the Groups specified in column 1 shall be constituted accordingly.

2. The quantity of any substance specified in column 1 (as extended by the preceding paragraph) shall be calculated in the manner prescribed in relation thereto in column 2 and shall either be specified accordingly or in the manner prescribed in paragraphs 3 or 4 of this Part of this Schedule.

3. Where the quantities of any substances included in Groups 1 [illegible] 2 specified in column 1 (as extended by paragraph 1 of this Part of this Sch[illegible] are calculated—

(*a*) as international units of vitamin A, they may, until 31st December 1974, be added together and specified accordingly;

(*b*) as microgrammes of retinol, they may be added together and specified accordingly.

4. The quantity of any substance included in Groups 2, 3, 4, 5, 6 or 7 specified in column 1 (as extended by paragraph 1 of this Part of this Schedule) may be specified—

(*a*) by reference to any one of the names or alternative names, specifically listed in column 1, of the substances in the Group to which the first mentioned substance belongs;

(*b*) where the first mentioned substance is a biologically active equivalent or derivative of a substance specifically listed in column 1, by reference to the name of that first mentioned substance.

SCHEDULE 3

Regulation 4(4)(*d*)

LABELLING OF CONTAINERS AND VENDING MACHINES

1. The provisions of Schedule 5 to the Labelling of Food Regulations 1970 shall apply to every letter and word required to appear on a label on a container by virtue of regulations 7, 8(2), 9, 10 and 11 of these regulations, as they apply to any appropriate designation or common or usual name required by regulation 5(2) of the Labelling of Food Regulations 1970.

2. Every letter and word appearing on a vending machine which is required to appear by virtue of regulation 12 of these regulations shall be printed in dark block type upon a light coloured ground or in a light block type upon a dark coloured ground, shall be of uniform colour and size and—

(*a*) if it is part of the description or descriptive name referred to in that regulation, shall not be less than 12 millimetres in height;

(*b*) if it is part of any expression specified in that regulation, shall not be less than 6 millimetres in height.

3. For the purposes of paragraph 2 any requirement that letters or words shall be of uniform height, colour or size shall be construed as being subject to the saving that any inconsiderable variation in height, colour or size, as the case may be, may be disregarded.

EXPLANATORY NOTE

(This Note is not part of the regulations.)

These regulations, which apply to England and Wales only, amend the Labelling of Food Regulations 1970 ("the 1970 Regulations") which come into operation on 1st January 1973. Transitional arrangements are provided until 1st July 1973 (regulation 3). The regulations—

(*a*) amend the definitions of "flour confectionery", "intoxicating liquor" and "pre-packed" in the 1970 Regulations and add a definition of the word "mineral" (regulation 2(2));

(*b*) amend the provisions relating to appropriate designations in regulation 3 of and Schedule 1 to the 1970 Regulations, as to names of ingredients used adjectivally in such designations, as to the designa-

tio spe of fi id a the f ap iate gnat prescribed or permitted by reg tion 3(2), (5) and (6) (regulation 2(3) and (10) and Schedule 1);

(*c*) amend the exemption provisi n regulation 6 of and Schedule 2 to the 1970 Regulations (regulation 2(4) and (11));

(*d*) substitute a regulation as to the labelling and advertisement of dry mixes in place of regulation 15 of the 1970 Regulations (regulation 2(6));

(*e*) amend the restrictions imposed by regulation 21 of the 1970 Regulations on claims that foods are a source of protein (regulation 2(7));

(*f*) substitute a regulation imposing restrictions on claims as to the presence of vitamins and minerals in place of regulation 22 of the 1970 Regulations and replace Part 1 of Schedule 4 to those regulations (regulation 2(8) and (12) and Schedule 2).

Other amendments are made to regulation 9(2) (Labelling of food, other than pre-packed, for sale by retail), regulation 24(2)(*a*) (Slimming claims) and Schedule 5 (Manner of marking or labelling) (regulations 2(5), (9) and (13)).

The regulations also further amend the Soft Drinks Regulations 1964, so that:—

(*a*) until 5th January 1976 any label on the container of a soft drink which complies with the provisions of the 1970 Regulations as to labelling and manner of marking will not need to comply with regulations 13 and 14 of the Soft Drinks Regulations 1964 (regulation 4(3));

(*b*) from 5th January 1976 (the expiry date of the exemption provided for certain soft drinks by regulation 6(7) of the 1970 Regulations) new provision is made for the labelling and manner of marking of soft drinks (regulation 4(4)(*b*), (*c*) and (*d*) and Schedule 3);

(*c*) from 5th January 1976 the existing provisions of the Soft Drinks Regulations 1964 as to declarations on vending machines containing soft drinks will continue to apply in an amended form (regulation 4(4)(*a*) and (*d*) and Schedule 3).

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New Ideas on Vitamin D

0194

Few efforts in clinical and biochemical research have been so well rewarded in recent years as those devoted to the action of vitamin D. Firstly, several metabolites of vitamin D with greater biological activity than the parent compound have been identified, and some have been administered to patients with bone disease. Secondly, long-term administration of enzyme-inducing agents has been shown to alter the rate of metabolism of vitamin D, and perhaps to cause osteomalacia. Thirdly, a new and plausible hypothesis of the interrelationship between vitamin D and parathyroid hormone has been proposed and in part substantiated.

Vitamin D was the name given by E. V. McCollum and his colleagues in 1922 to the antirachitic factor isolated from cod liver oil.[1] The active vitamin is found in few natural substances, but precursors (or provitamins) are found in both plants and animals. The precursors are sterols. Plant provitamin D (or ergosterol) acquires vitamin D activity when exposed to ultraviolet light. It is then known as vitamin D_2 or calciferol. Animal provitamin D (7-dehydrocholesterol) is found in the skin. It too is converted to an active compound (vitamin D_3 or cholecalciferol) by ultraviolet radiation. In recent years the metabolic fate of vitamin D_3 in man and animals has been studied by using synthetic radioactive-labelled cholecalciferol. Dietary vitamin D (mostly as D_2) is absorbed from the small intestine, and is supplemented by vitamin D_3 produced in the skin. It is now thought that neither of these vitamins is biologically active but that both are hydroxylated in the liver to form 25-hydroxycholecalciferol (25-HCC for short) and 25-hydroxyergocalciferol, which are active. More is known about 25-HCC than the other metabolite. It is produced in the liver, but in response to what stimulus is uncertain.

It has been suggested that barbiturates induce the hepatic enzymes responsible for the production of 25-HCC.[2,3] Since 25-HCC is more active than vitamin D, this hypothesis is difficult to reconcile with the observation that epileptic patients on long-term barbiturate therapy may develop vitamin D deficiency as shown by hypocalcaemia and even osteomalacia.[4] However, inducing agents may perhaps also transform 25-HCC into further (inactive) metabolites. Support for this view comes from the recent observation that the serum concentration of 25-HCC is lower in drug-treated epileptic patients than in controls and, further, serum concentrations of 25-HCC correlate well with those of serum calcium.[5] While these findings suggest that hypocalcaemia results from a drug-induced inactivation of 25-HCC, the effect of anticonvulsant drugs on other aspects of the kinetics of vitamin D, such as its absorption, protein binding, and elimination, must not be discounted.

The fate of 25-HCC has not only in the liver, it has now been clearly shown that it is further hydroxylated in the kidney to form 1, 25-dihydroxycholecalciferol (1, 25-DHCC).[6] There is now considerable evidence that 1, 25-DHCC is the physiological medium of active calcium transport from the gut and also of mobilization of calcium from bone. The formation of 1, 25-DHCC may be under the control of the parathyroid gland. Its production is regulated either by the level of calcium in the serum or by some factor responsive to them. As the level of calcium in the serum falls, more 1, 25-DHCC is produced. This in turn makes more calcium available from intestine and bone. Since parathyroid hormone also responds to the concentration of calcium in the serum, this may regulate the production of 1, 25-DHCC. Support for this idea comes from the observation that animals from which thyroid and parathyroid glands have been removed cannot synthesize 1, 25-DHCC, but their capacity to do so is restored when parathyroid hormone is administered.[7] Thus 1, 25-DHCC can itself be thought of as a hormone, produced by the kidney and responsible for calcium mobilization.

In nephrectomized animals 1, 25-DHCC is more potent than either vitamin D_3 or 25-HCC.[8] It has further been suggested that renal damage may impair the production of 1, 25-DHCC, and this may account for the vitamin-D-deficient-like state that occurs in patients with chronic uraemia.[9] The administration of small doses of 1, 25-DHCC (2-7 μg for 6-10 days) increases serum calcium and phosphate concentrations, and in three patients with chronic renal failure caused an increase in calcium absorption ranging from 30% to 220%. In contrast 1 mg of vitamin D_3 given to the same patients caused none of these changes.

But other workers have shown that vitamin D may cause a clinical improvement of disordered calcium homeostasis in uraemic patients. In fact it may cure the osteomalacia of azotaemia in anephric patients maintained on haemodialysis.[10] This may be due to the action of 25-HCC, whose production is not impaired in uraemia. Whether 1, 25-DHCC will prove a long-term therapeutic benefit in such patients is uncertain, since its effect on serum calcium depends not only on its desirable effect of increasing calcium absorption from the gut but also on its unwanted property of increasing bone resorption. In the future, molecular manipulation may produce an analogue of 1, 25-DHCC with a specific action on the gut. What ever else, the story of vitamin D is not over.

1 McCollum, E. V., Simmonds, N., Becker, J. E., and Shipley, P. G., *Journal of Biological Chemistry*, 1922, 53, 293.
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3 Silver, J., Neale, G., Davies, D., Breckenridge, A., and Thompson, G. R., *Clinical Science*, 1972, 42, 12p.
4 Dent, C. E., Richens, A., Rowe, D. J. F., and Stamp, T. C. B., *British Medical Journal*, 1970, 4, 69.
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10 Salusky, S. W., and Massry, E. E., in *Eighth Symposium on Advances in Medicine*, ed. G. Neale, p. 11. London, Pitman Medical, 1972.

JOURNAL OF NUTRITION

January 1973
Volume 103
Number 1

Nomenclature Policy: Generic Descriptors and Trivial Names for Vitamins and Related Compounds*

INTRODUCTION

0.1 The following nomenclature policy of THE JOURNAL OF NUTRITION is based on the "Tentative Rules for Generic Descriptors and Trivial Names for Vitamins and Related Compounds" recommended by the International Union of Nutrition Sciences Committee on Nomenclature, and the Committee on Nomenclature of the American Institute of Nutrition. The history of development of the "Tentative Rules," and general principles underlying them, were included in their first publication in The Journal (*101:* 133–140, 1971).

0.2 Trivial names and generic descriptors for the vitamins and related compounds are useful and necessary in nutrition literature. In each instance, the generic descriptor should be used to describe the family of compounds having vitamin activity and to modify such terms as "activity," "deficiency," etc. The trivial names should be used only to identify the specific compound. Synonyms for a specific chemical compound are undesirable and lead to unnecessary complications in indexing and in computer retrieval. Therefore only one name is recommended for each vitamin or related compound.

0.3 The summation of the contributions to the dietary intake of different forms of the same vitamin is a practical necessity to nutritionists.

For vitamin A and its provitamins summation should be made in mg (μg) *retinol equivalent*. Provitamins, if their separate summation is required, should be expressed in mg (μg) *β-carotene equivalent*. It is emphasized that discretion must be used in the acceptance of values from food tables and in the interpretation of findings from biological tests. Thus, although retinol is to be used as the summation substance it is greatly inferior, in view of its instability, to all-*trans*-retinyl acetate (9-*trans*,11-*trans*, 13-*trans*-retinyl acetate) as the standard for biological tests.

For vitamin E summations should be made in mg (μg) *α-tocopherol equivalent*. Although α-tocopherol is to be used as the summation substance it is greatly inferior, in view of its instability and variable biological activity, to α-tocopheryl acetate (2D,4′D,8′D-α-tocopheryl acetate from natural sources) as the standard for biological tests. Furthermore, selenium compounds or antioxidants which may show activity more or less similar to some functions of vitamin E should not be included in the term α-tocopherol equivalent.

Other members of vitamin families suggested for purposes of summation, with similar reservations, are:

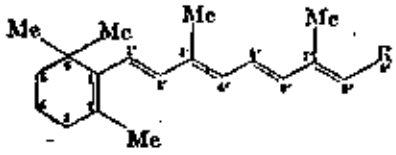
vitamin D	cholecalciferol equivalent
vitamin K	phytylmenaquinone (phylloquinone) equivalent
vitamin B-12	cyanocobalamin equivalent
folacin	folic acid equivalent
niacin (tryptophan)	nicotinamide equivalent

FAT-SOLUBLE VITAMINS AND RELATED COMPOUNDS

V.1 *Vitamin A and Provitamin A Carotenoids*

1.1 The term *vitamin A* should be used as the generic descriptor for all β-ionone derivatives, other than provitamin A carotenoids, exhibiting qualitatively the biological activity of retinol. Thus, phrases such as "vitamin A activity," "vitamin A deficiency" and "vitamin A in the form of . . ." represent preferred usage.

* Comments on nomenclature policy may be directed to the Editor who will bring them to the attention of the AIN Committee on Nomenclature.



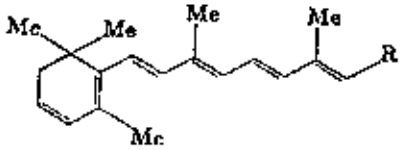
I

1.2 The compound with formula I (R = —CH_2OH), also known as vitamin A, vitamin A alcohol, vitamin A_1, vitamin A_1 alcohol, axerophthol or axerol, should be designated *retinol*.

1.3 Esters of retinol should be designated *retinyl esters*.

1.4 The compound with formula I (R = —CHO), also known as vitamin A (A_1) aldehyde, retinene or retinal, should be designated *retinaldehyde*.

1.5 The compound with formula I (R = —COOH), having partial vitamin A activity and also known as vitamin A acid, should be designated *retinoic acid*.



II

1.6 The compound 3-dehydroretinol, with formula II (R = —CH_2OH), also known as vitamin A_2, should be designated *dehydroretinol*.

1.7 The compound 3-dehydroretinaldehyde, with formula II (R = —CHO), also known as retinene-2, 3-dehydroretinal or dehydroretinal, should be designated *dehydroretinaldehyde*.

1.8 The compound 3-dehydroretinoic acid, with formula II (R = —COOH), should be designated *dehydroretinoic acid*.

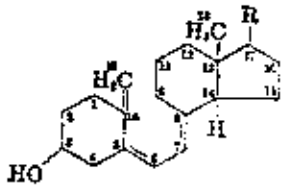
1.9 The term *provitamin A carotenoid* should be used as the generic descriptor for all carotenoids exhibiting qualitatively the biological activity of β-carotene. When referring to the biological activity of the provitamin A carotenoids, the phrase "provitamin A activity" represents preferred usage.

1.10 Trivial names for specific carotenoids are under consideration.

1.11 The term *retinol equivalent* may be used as described in section 0.4 of the Introduction.

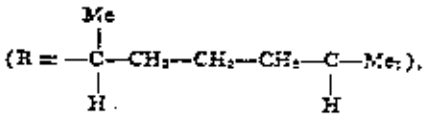
V.2 Vitamin D

2.1 The term *vitamin D* should be used as the generic descriptor for all steroids exhibiting qualitatively the biological activity of cholecalciferol. Thus, phrases such as "vitamin D activity" and "vitamin D deficiency" represent preferred usage.



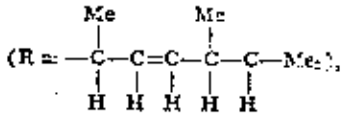
III

2.2 The compound with formula III



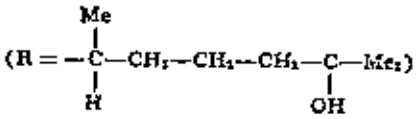
also known as vitamin D_3, should be designated *cholecalciferol*.

2.3 The compound with formula III



also known as vitamin D_2 or calciferol, should be designated *ergocalciferol*.

2.4 The compound with formula III



should be designated *25-hydroxycholecalciferol*.

2.5 Esters of cholecalciferol and ergocalciferol should be designated *cholecalciferyl* esters and *ergocalciferyl* esters, respectively.

V.3 Vitamin E *

3.1 The term *vitamin E* should be used as the generic descriptor for all tocol and tocotrienol derivatives exhibiting qualita-

* Revision of nomenclature of stereoisomers of α-tocopherol is currently under consideration by committees of AIN, IUNS and IUPAC.

tively the biological activity of α-tocopherol. Thus, phrases such as "vitamin E activity," "vitamin E deficiency" and "vitamin E in the form of . . ." represent preferred usage.

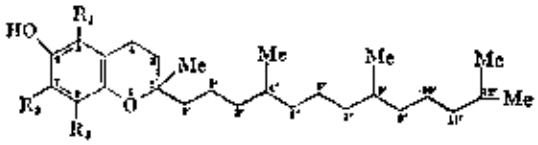
3.2 The term *tocopherols* should be used as the generic descriptor for all methyl tocols. Thus the term "tocopherol" is not synonymous with the term "vitamin E."

3.3 The compound isolated from natural sources, 2D,4'D,8'D-α-tocopherol or 2R, 4'R, 8'R-α-tocopherol with the formula IV ($R_1 = R_2 = R_3 = -Me$), also known as *d*-α-tocopherol, should be designated *α-tocopherol.*

3.4 The mixture of the eight possible stereoisomers of α-tocopherol commonly synthesized from racemic isophytol and also known as 2DL,4'DL,8'DL-α-tocopherol, 2RS,4'RS,8'RS-α-tocopherol or *dl*-α-tocopherol should be designated all-*rac.-α-tocopherol.*

3.5 The mixture of two isomers of α-tocopherol synthesized from natural phytol and also known as 2DL,4'D,8'D-α-tocopherol, 2RS,4'R,8'R-α-tocopherol or 2-*dl*-α-tocopherol should be designated 2*dl*-*α-tocopherol.* The acetate ester of this mixture of two isomers was the former International Standard for vitamin E (J. Nutrition, *90*, 109 (1966)).

3.6 The compound 2L,4'D,8'D-α-tocopherol or 2*S*,4'R,8'R-α-tocopherol, also known as the epimer of *d*-α-tocopherol, 2*l*-α-tocopherol or *l*-α-tocopherol, should be designated 2*l*-*α-tocopherol.*

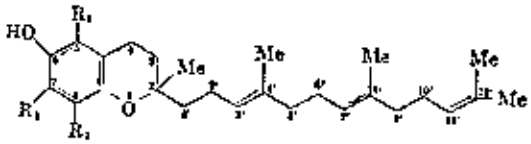


IV

3.7 The compound 5,8-dimethyltocol with formula IV ($R_1 = R_3 = -Me$; $R_2 = -H$), isolated from natural sources, should be designated *β-tocopherol.*

3.8 The compound 7,8-dimethyltocol with formula IV ($R_1 = -H$; $R_2 = R_3 = -Me$), isolated from natural sources, should be designated *γ-tocopherol.*

3.9 The compound 8-methyltocol with formula IV ($R_1 = R_2 = -H$; $R_3 = -Me$), isolated from natural sources, should be designated *δ-tocopherol.*



V

3.10 The compound 5,7,8-trimethyltocotrienol with formula V ($R_1 = R_2 = R_3 = -Me$), isolated from natural sources and also known as ζ_1 or ζ_2-tocopherol or tocochromanol-3, should be designated *α-tocotrienol.*

3.11 The compound 5,8-dimethyltocotrienol with formula V ($R_1 = R_3 = -Me$; $R_2 = -H$), isolated from natural sources and also known as ε-tocopherol, should be designated *β-tocotrienol.*

3.12 The compound 7,8-dimethyltocotrienol with formula V ($R_1 = -H$; $R_2 = R_3 = -Me$), isolated from natural sources and also known as γ-tocopherol or plastochromanol-3, should be designated *γ-tocotrienol.*

3.13 The compound 8-methyltocotrienol with formula V ($R_1 = R_2 = -H$; $R_3 = -Me$), isolated from natural sources, should be designated *δ-tocotrienol.*

3.14 Esters of tocopherols and tocotrienols should be designated *tocopheryl* esters and *tocotrienyl* esters, respectively.

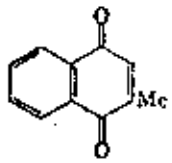
3.15 The term *α-tocopherol equivalent* may be used as described in section 0.4 of the Introduction.

V.4 Vitamin K *

4.1 The term *vitamin K* should be used as the generic descriptor for 2-methyl-1,4-naphthoquinone and all derivatives exhibiting qualitatively the biological activity of phytylmenaquinone (phylloquinone). Thus, phrases such as "vitamin K activity" and "vitamin K deficiency" represent preferred usage.

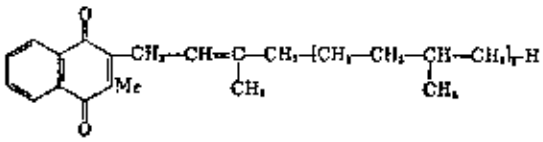
4.2 The compound 2-methyl-1,4-naphthoquinone with formula VI, having partial vitamin K activity and also known as menadione, should be designated *menaquinone.*

* Revision currently under consideration by AIN Committee on Nomenclature to reconcile differences with IUPAC nomenclature.



VI

4.3 The compound 2-methyl-3-phytyl-1,4-naphthoquinone with formula VII, also known as vitamin K_1 or phylloquinone, should preferably be designated *phytylmenaquinone*.

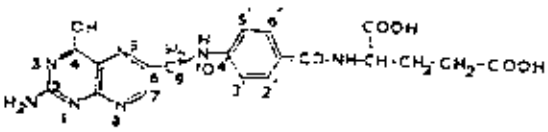


VII

4.4 The compounds known as vitamin K_2 should be designated *multiprenylmenaquinones*. Thus, a vitamin K_2 having a side chain consisting of 6 prenyl units and also known as menaquinone-6 should preferably be designated *prenylmenaquinone-6;* a vitamin K_2 having a side chain consisting of 7 prenyl units and also known as menaquinone-7 should preferably be designated *prenylmenaquinone-7*.

4.5 In general the use of abbreviations for the compounds dealt with in V.4. 3–4 above is not recommended. Should abbreviation be necessary the following forms are permissible: phytylmenaquinone, PMQ; prenylmenaquinone-6, MQ-6; prenylmenaquinone-7, MQ-7.

4.6 The IUPAC–IUB Tentative Rules for Nomenclature of Quinones with Isoprenoid Side Chains (J. Biol. Chem., *241*, 2989–2991 (1966)) may be used with the reservations following from V.4. 2–5 above.



VIII

WATER-SOLUBLE VITAMINS AND RELATED COMPOUNDS

V.5 Folacin

5.1 The term *folacin* should be used as the generic descriptor for folic acid and related compounds exhibiting qualitatively the biological activity of folic acid. Thus, phrases such as "folacin activity" and "folacin deficiency" represent preferred usage.

5.2 The compound monopteroylglutamic acid with formula VIII should be designated *folic acid*.

5.3 Folic acid derivatives in which the glutamic acid residue is combined through a peptide bond with another glutamic acid residue which may or may not be similarly combined with a third glutamic acid residue and so on should be designated *folic acid glutamates* (*N*), where N indicates the number of glutamic acid residues.

5.4 The compound tetrahydropteroylglutamic acid, also known as $PGAH_4$, THFA or tetrahydrofolacin, should be designated *tetrahydrofolic acid*.

5.5 The compound N^5-formyltetrahydropteroylglutamic acid, also known as N^5-F-$PGAH_4$, citrovorum factor, "CF," leucovorin, folinic acid or N^5-formyl THFA, should be designated *5-formyltetrahydrofolic acid*.

5.6 The compound N^{10}-formyltetrahydropteroylglutamic acid, also known as N^{10}-F-$PGAH_4$, heat-labile citrovorum factor, "HLCF" or N^{10}-formyl THFA, should be designated *10-formyltetrahydrofolic acid*.

5.7 The compound N^5-methyltetrahydropteroylglutamic acid, also known as N^5-M-$PGAH_4$, "prefolic A," N^5-methyltetrahydrofolacin or N^5-methyl THFA, should be designated *5-methyltetrahydrofolic acid*.

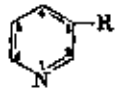
5.8 In general the use of abbreviations for the compounds dealt with in V.5. 4–7 above is not recommended. Should abbreviation be necessary the following forms are permissible: tetrahydrofolic acid, H_4-folic acid; 5-formyltetrahydrofolic acid, 5-CHO-H_4-folic acid; 10-formyltetrahydrofolic acid, 10-CHO-H_4-folic acid; 5-methyltetrahydrofolic acid, 5-CH_3-H_4-folic acid.

5.9 Related compounds with folacin activity should be named and abbreviated in accordance with the IUPAC–IUB Tentative Rules for Nomenclature and Symbols for Folic Acid and Related Compounds (J. Biol. Chem., *241*, 2991–2992 (1966)).

V.6 Niacin

6.1 The term *niacin* should be used as the generic descriptor for pyridine 3-carboxylic acid and derivatives exhibiting qualitatively the biological activity of nicotinamide. Thus, phrases such as "niacin activity" and "niacin deficiency" represent preferred usage.

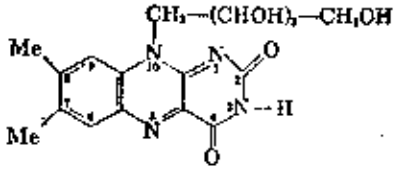
6.2 The compound pyridine 3-carboxylic acid with formula IX (R = —COOH), also known as niacin or vitamin PP, should be designated *nicotinic acid*.



IX

6.3 The compound with formula IX (R = —$CONH_2$), also known as niacinamide or nicotinic acid amide, should be designated *nicotinamide*.

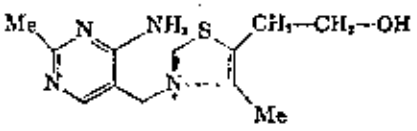
6.4 The term *nicotinamide equivalent* may be used as described in section 0.4 of the Introduction.



X

V.7 *Riboflavin*

7.1 The compound with formula X, also known as vitamin B_2, lactoflavin(e) or riboflavine, should be designated *riboflavin*. Its use in phrases such as "riboflavin activity" and "riboflavin deficiency" represents acceptable usage.



XI

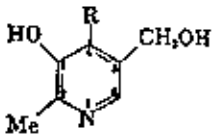
V.8 *Thiamin*

8.1 The cation with formula XI, also known as vitamin B_1, aneurin(e) or thiamine, should be designated *thiamin*. Its use in phrases such as "thiamin activity" and "thiamin deficiency" represents acceptable usage.

V.9 *Vitamin B-6*

9.1 The term *vitamin B-6* should be used as the generic descriptor for all 2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine. Thus, phrases such as "vitamin B-6 activity" and "vitamin B-6 deficiency" represent preferred usage.

9.2 The compound 3-hydroxy-4,5-bis(hydroxymethyl)-2-methylpyridine with formula XII (R = —CH_2OH), also known as vitamin B_6, adermin or pyridoxol, should be designated *pyridoxine*.



XII

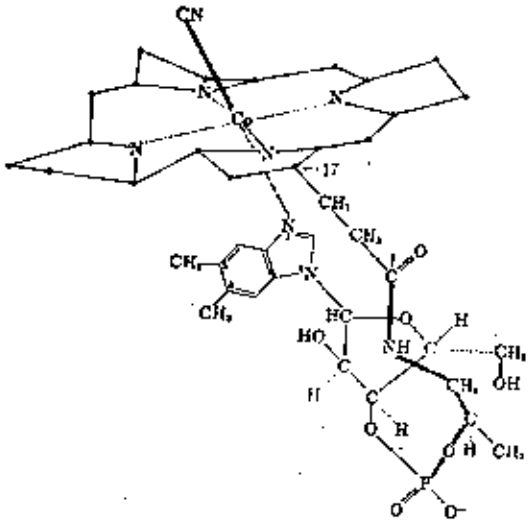
9.3 The compound with formula XII (R = —CHO), also known as pyridoxaldehyde, should be designated *pyridoxal*.

9.4 The compound 3-hydroxy-4-methylamino-5-hydroxymethyl-2-methylpyridine with formula XII (R = —CH_2NH_2) should be designated *pyridoxamine*.

V.10 *Vitamin B-12*

10.1 The term *vitamin B-12* should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. Thus, phrases such as "vitamin B-12 activity" and "vitamin B-12 deficiency" represent preferred usage.

10.2 The term *corrinoids* should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term "corrinoid" is not synonymous with the term "vitamin B-12".



XIII

10.3 The compound α-(5,6-dimethylbenzimidazolyl) cobamide cyanide with formula XIII, also known as vitamin B_{12} or cyanocobalamine, should be designated *cyanocobalamin.*

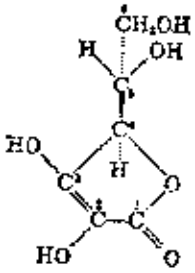
10.4 The compound α-(5,6-dimethylbenzimidazolyl) hydroxocobamide, also known as vitamin B_{12a}, vitamin B_{12b}, aquocobalamin or hydroxocobalamine, should be designated *hydroxocobalamin.*

10.5 The compound α-(5,6-dimethylbenzimidazolyl) cobamide nitrite, also known as vitamin B_{12c} or nitritocobalamine, should be designated *nitritocobalamin.*

10.6 Related compounds with vitamin B-12 activity should be named in accordance with the IUPAC-IUB Tentative Rules for Nomenclature of Corrinoids (J. Biol. Chem., *241*, 2992–2994 (1966)).

V.11 Vitamin C

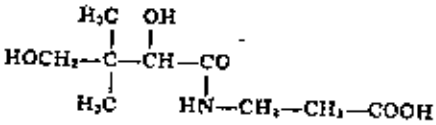
11.1 The term *vitamin C* should be used as the generic descriptor for all compounds exhibiting qualitatively the biological activity of ascorbic acid. Thus, phrases such as "vitamin C activity" and "vitamin C deficiency" represent preferred usage.



XIV

11.2 The compound with formula XIV, also known as vitamin C or L-ascorbic acid, should be designated *ascorbic acid.*

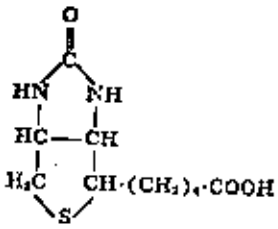
11.3 The compound also known as L-dehydroascorbic acid should be designated *dehydroascorbic acid.*



XV

V.12 Pantothenic acid

12.1 The compound with formula XV, also known as pantoyl-β-alanine, should be designated *pantothenic acid.* Its use in phrases such as "pantothenic acid activity" and "pantothenic acid deficiency" represents acceptable usage.



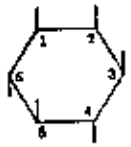
XVI

V.13 Biotin

13.1 The compound with formula XVI, also known as coenzyme R, should be designated *biotin.* Its use in phrases such as "biotin activity" and "biotin deficiency" represents acceptable usage.

V.14 Choline

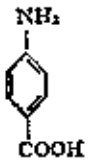
14.1 The compound with formula $(CH_3)_3N^+CH_2CH_2OH$ should be designated *choline.* Its use in phrases such as "choline activity" and "choline deficiency" represents acceptable usage.



XVII

V.15 Myo-inositol

15.1 The compounds with formula XVII, also known as inositol or mesoinositol, should be designated *myo-inositol*. Its use in phrases such as "myo-inositol activity" and "myo-inositol deficiency" represents acceptable usage (see European J. Biochem., 5, 1–12 (1968)).



XVIII

V.16 p-Aminobenzoic acid

16.1 The compound with formula XVIII should be designated *p-aminobenzoic acid*. Its use in phrases such as "*p*-aminobenzoic acid activity" and "*p*-aminobenzoic acid deficiency" represents acceptable usage.

NOMENCLATURE POLICY: ABBREVIATED DESIGNATIONS OF AMINO ACIDS

The following rules for nomenclature of the amino acids are based on the Rules of the IUPAC-IUB Commission on Biochemical Nomenclature for designation of amino acids and peptides (1, 2, 3), and were adopted by recommendation of the AIN Committee on Nomenclature as the nomenclature of the official Journals of the AIN.

1. *General considerations*

1.1 The symbols chosen are derived from the trivial names or chemical names of the amino acids and of chemicals reacting with amino acids and polypeptides. For the sake of clarity, brevity, and listing in tables, the symbols have been, wherever possible, restricted to three letters, usually the first letters of the trivial names.

1.2 The symbols represent not only the names of the compounds but also their structural formulas.

1.3 The amino acid symbols by themselves represent the amino acids. The use of the symbols to represent the free amino acids is *not* recommended in textual material, but such use may occasionally be desirable in tables, diagrams, or figures. Residues of amino acids are represented by addition of hyphens in specific positions.

1.4 Heteroatoms of amino acid residues (e.g. O^{γ} and S^{γ} of serine and cysteine, respectively, N^{ζ} of lysine, N^{α} of glycine, etc.) do not explicitly appear in the symbol; such features are understood to be encompassed by the abbreviation.

1.5 Amino acid symbols denote the L configuration unless otherwise indicated by D or DL appearing before the symbol and separated from it by a hyphen. When it is desired to make the number of amino acid residues appear in a clearer manner, the hyphen between the configurational prefix and the symbol may be omitted (see Ref. 1). (Note: the designation of an amino acid residue as DL is inappropriate for compounds having another amino acid residue with an asymmetrical center.)

1.6 Structural formulas of complicated features may be used along with the abbreviated notation whenever necessary for clarity.

2. *Abbreviations for amino acids*

2.1 *Common Amino Acids*

Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine [1]	Asn [1]	Methionine	Met
Aspartic acid	Asp	Ornithine	Orn
Cysteine	Cys	Phenylalanine	Phe
Cystine	Cys-Cys *	Proline	Pro
Glutamic acid	Glu	Serine	Ser
Glutamine [1]	Gln [1]	Threonine	Thr
Glycine	Gly	Tryptophan	Trp
Histidine	His	Tyrosine	Tyr
Isoleucine	Ile	Valine	Val

[1] Asparagine and glutamine may also be denoted as Asp (NH_2) or Asp, and Glu (NH_2) or Glu, respectively.

* Cystine does not appear in 2.1 (ref. 1). We propose Cys-Cys as consistent with 2.1 but this abbreviation should not be used to denote structure.

2.2 *Less Common Amino Acids*

—Abbreviations for less common amino acids should be defined in each publication in which they appear. The following principles and notations are recommended.

2.2.1 Hydroxyamino Acids

Hydroxylysine	Hyl
3-Hydroxyproline	3Hyp
4-Hydroxyproline	4Hyp

2.2.2 *allo*-Amino Acids

allo-Isoleucine	aIle
allo-Hydroxylysine	aHyl

2.2.3 *"Nor" Amino Acids*—"Nor" (*e.g. in norvaline*) is not used in its accepted sense (denoting a lower homologue) but to change the trivial name of a branched chain compound into that of a straight chain compound (compare with "iso," paragraph 2.1). "Nor" should therefore be treated as part of the trivial name without special emphasis.

Norvaline	Nva
Norleucine	Nle

2.2.4 *Higher Unbranched Amino Acids*

—We suggest the following general rules for guidance in forming abbreviations: the functional prefix "amino" should be included in the symbol as the letter "A," diamino as "D."

The trivial name of the parent acid should be abbreviated to leave no more than two or three letters, as convenient and necessary for clarity. The word "acid" ("*-saure,*" etc.) should be omitted from the symbol as carrying no significant information. Unless otherwise indicated (see Ref. 1), single groups are in the α position, two amino groups in the α,ω (monocarboxylic acids) or α,α' positions (dicarboxylic acids). The location of amino acids in positions other than α and ω is shown by the appropriate Greek letter prefix.

Examples:

ω-Aminobutyric acid	Abu
α-Aminoadipic acid	Aad
ω-Aminopimelic acid	Apm
α,γ-Diaminobutyric acid	Dbu
α,β-Diaminopropionic acid	Dpr
α,α'-Diaminopimelic acid	Dpm
β-Alanine	βAla
ϵ-Aminocaproic acid	ϵAcp
β-Aminoadipic acid	βAad

For further details in using symbols to designate structure, the reader is referred to the following references.

REFERENCES

1. IUPAC-IUB Commission on Biochemical Nomenclature 1966 Abbreviated designation of amino acid derivatives and peptides. Tentative Rules. J. Biol. Chem., 241: 2491–2495.
2. IUPAC-IUB Commission on Biochemical Nomenclature 1967 Rules for naming synthetic modifications of natural peptides. Tentative Rules. J. Biol. Chem., 242: 555–557.
3. IUPAC-IUB Combined Commission on Biochemical Nomenclature 1968 Tentative rules for abbreviated nomenclature of synthetic polypeptides (polymerized amino acids). Biochemistry, 7: 183–185.

[21 CFR Part 125]

USE OF INTERNATIONAL UNITS FOR VITAMINS A AND D

Notice of Proposed Rule Making

Elsewhere in this issue of the FEDERAL REGISTER the Commissioner of Food and Drugs is promulgating final regulations for food for special dietary uses. Exceptions filed to the tentative order in that proceeding pointed out that § 125.5(c)(5) uses "U.S.P. Units" as the units of measurement for vitamins A and D, whereas the tentative order used "International Units" for these vitamins.

The Commissioner has concluded that International Units should be used as the standard units of measurement for vitamins A and D. Accordingly, it is proposed that § 125.5(c)(5) (21 CFR 125.5) be revised to refer to "International Units" rather than "U.S.P. Units" for vitamins A and D.

The Commissioner recognizes that this is a technical change and that there will be no need to require immediate label changes to reflect this revision. The Commissioner therefore proposes that, when adopted, this revision may be reflected in labeling when other labeling changes are made, in order to prevent any unnecessary costs to consumers.

Therefore, pursuant to provisions of the Federal Food, Drug, and Cosmetic Act (secs. 403, 701, 52 Stat. 1047–1048, 1055; 21 U.S.C. 343, 371) and under authority delegated to him (21 CFR 2.120), the Commissioner proposes to amend Part 125 in § 125.5(c)(5) to insert "International Units" in lieu of "U.S.P. Units" as the units of measurement for vitamins A and D.

Interested persons may, on or before September 1, 1974, file with the Hearing Clerk, Food and Drug Administration, Rm. 6–88, 5600 Fishers Lane, Rockville, MD 20852, written comments (preferably in quintuplicate) regarding this proposal. Comments may be accompanied by a memorandum or brief in support thereof. Received comments may be seen in the above office during working hours, Monday through Friday.

Dated: July 25, 1973.

A. M. SCHMIDT,
Commissioner of Food and Drugs.

[FR Doc.73–15690 Filed 8–1–73;8:45 am]

Department of Health and Social Security

Medicines Commission

Report on a General Sale List of Medicinal Products for human use

Acting Chairman:

Professor Andrew Wilson CBE PhD MD FRCP FPS

NOTE

These recommendations of the Medicines Commission are published by the Health Ministers for the purpose of consultation under Section 129(6) of the Medicines Act 1968. Any organisation or individual wishing to submit comments should write, not later than 14 September 1973, to the Secretary, Department of Health and Social Security, Finsbury Square House (Room 321), 33/37a Finsbury Square, London EC2A 1PP.

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THE FIRST GENERAL SALE LIST OF MEDICINAL PRODUCTS FOR HUMAN USE

Recommendations of the Medicines Commission

To: The Secretary of State for Social Services

The Secretary of State for Wales

The Secretary of State for Scotland

The Secretary of State for Northern Ireland

1. INTRODUCTION

1.1 Section 51(1) of the Medicines Act 1968 empowers the Health and Agriculture Ministers by order to specify general sale lists of medicinal products which in their opinion can "with reasonable safety" be sold or supplied at the retail level "otherwise than by, or under the supervision of, a pharmacist".

1.2 On 25 September 1970 the Medicines Commission was asked to give advice on the human and veterinary medicines to be included in such an order. In December 1970 with the approval of the Ministers, it appointed the "General Sale Lists Committee" to study the matter and—

> "To make recommendations (to the Commission) in respect of the medicinal products that should be included in the first General Sale Lists covering human and veterinary medicines".

1.3 The late Lord Rosenheim was Chairman of the Committee from its inception until his sudden death on 2 December 1972. At that time under his wise and tactful leadership the Committee had nearly completed its work and the Commission would like to place on record its appreciation of the invaluable contribution that he made.

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1.4 At present the Committee consists of the following members:

Professor O L Wade MD FRCP (Chairman)
*Mr K J P Barraclough CBE TD JP
Professor A M Barrett BPHARM PHD
*Professor R J Fitzpatrick BSC PHD MRCVS
Dr J B Harman MD FRCP FRCS
Professor J M Rowson PHD MSC FPS FLS FRMS
Mr P M F Sambrook BSC MRCVS

**Member of the Medicines Commission*

1.5 The Committee has examined the composition of approximately 7,000 products currently marketed for human use and has submitted to the Commission recommendations that could form the basis of consultation with the pharmaceutical industry and other interests likely to be affected. The Committee has not yet completed its work; on the human medicines side, it has still to make recommendations in respect of certain additional medicines which contain less familiar substances, generally in the form of dilutions obtained from a mother tincture. Similarly the Committee's recommendations on veterinary products, which are being examined separately, are still awaited.

1.6 The Medicines Commission has examined the General Sale Lists Committee's recommendations, and has made a number of changes in the light of its discussion. Although the Commission is not yet able to make comprehensive recommendations on the contents of the General Sale Lists, it believes the conclusions it has reached so far are sufficiently extensive to be a realistic basis for consultation by the Ministers with the interests likely to be affected. The Commission therefore submits the lists with a recommendation that the Ministers should institute consultations under Section 129(6) of the Medicines Act, and should refer back to the Commission any objections of substance that are brought to their notice in the course of the consultations.

2. CRITERIA FOR INCLUSION IN THE LIST

2.1 Section 51, read with Sections 52 and 53, provides that the products or classes of products to be included in the General Sale List of human medicines should be pre-packed products that can "with reasonable safety" be sold or supplied by retail at shops other than registered pharmacies. In considering what might be covered by "reasonable safety" the Committee examined the more exclusive phraseology used in the 1967 White Paper "Forthcoming Legislation on the Safety, Quality and Description of Drugs and Medicines" (Cmnd 3395) which preceded the introduction of the Medicines Bill into Parliament. The White Paper contemplated that in the field of human medicines the General Sale List would comprise products "where the hazard to health, the risk of misuse, or the need to take special precautions in handling is small and where wider sale would be a convenience to the purchaser". Both the Committee and the Commission accept this as an appropriate elaboration of the expression "with reasonable safety" and have used it in reaching their conclusions and recommendations.

2.2 In the absence of any reference to efficacy or quality in Section 51 these factors were deliberately ignored as criteria justifying inclusion or exclusion of a product from the proposed list. The inclusion of a product in a General Sale List simply means that it is regarded by the Commission as one that can "with reasonable safety" be sold at shops and premises other than registered pharmacies when used for the medicinal purposes for which it is marketed in accordance with the directions given on the container. Inclusion in no way implies that the Commission regards the product as having therapeutic value. Nor does it imply that no harm could ever come from the use, whether in accordance with the directions on the containers or not, of any General Sale List product.

3. SELF-SERVICE

The Council of the Pharmaceutical Society of Great Britain urged that, in the interests of safety, no medicinal products should be offered for sale on a self-service basis, because the cashier at the shop till could not be expected to distinguish customers to whom a sale might involve hazard.

The Committee and the Commission both thought that such a limitation might prove difficult to enforce equitably and that all they could do was to ensure that the products recommended for inclusion in the lists were reasonably safe. The Commission accordingly makes no recommendation about restricting or prohibiting the offering of General Sale List products for sale on a self-service basis.

4. MEDICINAL PRODUCTS THAT RESEMBLE SWEETS

The Committee and Commission were concerned about the fact that some medicines currently being marketed closely resemble

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ons the y a: ar-e ples ous i re es wl he fact that "wider sale would be a convenience to the pu er" cannot be overlooked, but that one could not rely on commercial considerations to limit the size of pack stocked. Notwithstanding consideration (ii) in 5.1 above therefore it considers that it would be prudent to introduce a restriction on pack size. The Commission also recommends that the maximum strength of each tablet or sachet containing these substances should be limited as proposed in paragraph 9.1 and Appendix A of this report.

6. LABELLING OF CERTAIN PRODUCTS ON GENERAL SALE

The Commission makes the following *RECOMMENDATIONS* on the labelling of certain medicinal products which it proposes should be included in the first General Sale List of human medicines:—

(i) **In relation to products containing aloxipirin, aspirin or paracetamol**—that the statement of composition and cautionary particulars on the containers of products which have any of these substances as an active ingredient should be required to refer to the substance by one of these names to the exclusion of synonyms, to state a recommended dose and to include a warning that medical advice should be sought if the symptoms persist.

(ii) **In relation to products containing vitamin D**—that the particulars on the containers of products marketed for use by children and containing vitamin D should be required to indicate that half the dose should be taken when a child is being fed on dried milk.

The second recommendation links up with another recommendation detailed in paragraph 9.1 and Appendix A of this report, namely that in relation to products containing Vitamin D, only those with a recommended maximum daily dose not exceeding 400 iu (10 micrograms cholecalciferol) are recommended as suitable for general sale.

7. THE AUTOMATIC MACHINES SECTION

7.1 Section 51(3) of the Act enables the Ministers to designate in the automatic machines section of a general sale list the medicinal

9. PRODUCTS RECOMMENDED FOR INCLUSION IN THE LIST

9.1 The Commission *RECOMMENDS* that the under-mentioned products should be included in the first General Sale List of human medicines:—

(i) products marketed for either internal or external use (or both) containing the substances listed in Appendix A to this report, whose strength, route of administration or pharmaceutical form is within the limitations indicated, and subject in the case of aloxipirin, aspirin and paracetamol to the quantities per pack indicated;

(ii) products marketed for external use only containing the substances listed in Appendix B to this report, whose strength or pharmaceutical form is within the limitations indicated;

and, in either case, with or without:—

(*a*) any excipient, vehicle, base, colour, flavour, perfume, stabiliser, emulsifier or preservative commonly used as an inactive ingredient in the preparation of medicines, with the exception of liquid paraffin in nasal preparations;

or (*b*) haemoglobin or any of the following parts of animals (except extracts from such parts):—

bone, horn, brain, genitals, prostate and spleen;

or (*c*) any substance of vegetable origin (including fruit, seed, nuts, roots, leaves, oils and juices) commonly used as human foodstuffs or in the preparation of such foodstuffs; and extracts of any such substance.

9.2 Automatic Machines Section

The Commission *RECOMMENDS*:—

(*a*) that medicinal products containing any of the substances annotated with the letters "AMS" in Appendix A or B to this report (subject, where relevant, to the limitation indicated), with or without any excipient, vehicle, base, colour, flavour, perfume, stabiliser, emulsifier or preservative commonly used as an inactive ingredient in the preparation of medicines, should be included in the automatic machines section of the first General Sale List;

(*b*) the imposition of such requirements as may be deemed necessary in relation to the construction and/or the location

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:omatic machines to prevent easy access by children to the medicinal product in question.

10. LIMITATIONS

10.1 As will be seen from Appendices A and B, the Commission describes the medicinal products recommended for inclusion in the first General Sale List in terms of the substances they contain. Presentation in this way *does not mean that the Commission regards these substances as "reasonably safe" in any combination,* but simply that this criterion has been met in the products that it has examined. If a new product containing an unacceptable combination of these substances were promoted in future, it might be necessary to amend the list. An amendment to the list might also be necessary if a new product containing an acceptable combination of substances in the same concentrations as a product covered by the list were promoted with different directions for use.

10.2 A number of the products submitted contained particular substances in concentrations higher than those which the Commission could accept as being "reasonably safe" for general sale to the public. The Comission's proposals effectively exclude these products from the General Sale List by placing a limitation on the substances in question either in the form of a "maximum recommended dose" or a "maximum strength" (both these terms are defined in the notes to Appendix A).

11. LICENSING IMPLICATIONS

In making these recommendations only products submitted to the Committee for inclusion in the General Sale List have been considered; the lists have not been checked against products that are the subject of applications to the licensing authority for licences of right. *The Commission's recommendations do not in any way imply that inclusion in the proposed list shall carry with it any entitlement to a product licence either now or in the future.*

(Signed) A Wilson
Acting Chairman
April 1973 Medicines Commission

SUBSTANCES RECOMMENDED FOR INCLU I IN THE FIRST GENERAL SALE LIST OF MEDICI FOR HUMAN USE WHEN IN PRODUCTS MARKETED FOR INTERNAL OR EXTERNAL USE (OR BOTH)

Notes:—

1. *m.r.d.* = maximum recommended dose not to exceed.....

   "Maximum recommended dose" means the maximum amount recommended on the label of a medicinal product for internal use to be taken at any one time.

2. *m.r.d. (daily)* = maximum recommended daily dose not to exceed.....

   "Maximum recommended daily dose" means the maximum amount recommended on the label of a medicinal product for internal use to be taken within any period of 24 hours.

3. *m.s.* = maximum strength not to exceed.....

   "Maximum strength" means the maximum percentage of the substance contained in a medicinal product calculated in terms of weight in weight, weight in volume or volume in volume as appropriate.

4. "External" means local application to the skin, mouth, throat, nose, ear, eye, vagina, or rectum.

5. [AMS] = substance considered suitable for inclusion in the Automatic Machines Section of the General Sale List.

Abietis Oil
Absinthium
Acetic Acid:—
(i) *m.s. internal* 7·5%
(ii) *m.s. external* 15%
Acetomenaphthone:—
m.r.d. internal 10 *mg*
Adenosine
Aesculus
Agrimony
Albumin Tannate
Alcohol BP
Alginic Acid
Almond Oil
Almond Oil (Essential)
Aloes
Aloin:—
m.r.d. internal 20 *mg*
Aloxiprin:—
(i) *Maximum amount per tablet or sachet* = 400 *mg (adults,)* 100 *mg (children).*
(ii) *When combined with aspirin total aspirin content of the product not to exceed* 325 *mg (adults) or* 90 *mg (children).*
(iii) *Maximum tablets or sachets per pack* = 25.

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Tocopherol
Tocopheryl Acetate
Tocopheryl Acid Succinate
Tolu Balsam
Tormentil
Tormentilla
Tragacanth

Unicorn Root, False
Unicorn Root, True

Valerian
Verbena
Viburnum opulus
Violet
Vitamin A:—
m.r.d. (daily) internal 7,500 *iu* (2,250 *micrograms retinol equivalent*)
Vitamin A Acetate:—
m.r.d. (daily) internal equivalent to 7,500 *iu Vitamin A* (2,250 *micrograms retinol equivalent*)
Vitamin A Palmitate:—
m.r.d. (daily) internal equivalent to 7,500 *iu Vitamin A* (2,250 *micrograms retinol equivalent*)
Vitamin D (Calciferol):—
m.r.d. (daily) internal 400 *iu* (10 *micrograms cholecalciferol*)

Wheat Germ Oil
Whey Powder
White Pond Lily
Wild Carrot
Wild Cherry
Wild Thyme
Willow Black
Willow White
Wintergreen
Wood Betony
Woodsage

Xanthoxylum

Yarrow
Yellow Dock

Zinc:—
m.r.d. (daily) internal 4 *mg*
Zinc Phosphate:—
(i) *m.r.d. (daily) internal equivalent to* 4 *mg elemental Zinc;*
(ii) *m.s. external* 0·15%
Zinc Sulphate:—
(i) *m.r.d. (daily) internal equivalent to* 4 *mg elemental Zinc*
(ii) *m.s. external* 0·25%
Zizyphus vulgaris

Anon.

J. Nutr. 104:144-150, 1974

Nomenclature Policy: Generic Descriptors and Trivial Names for Vitamins and Related Compounds*

INTRODUCTION

0.1 The following nomenclature policy of THE JOURNAL OF NUTRITION is based on the "Tentative Rules for Generic Descriptors and Trivial Names for Vitamins and Related Compounds" recommended by the International Union of Nutrition Sciences Committee on Nomenclature, and the Committee on Nomenclature of the American Institute of Nutrition. With the "Tentative Rules" for vitamin E now under revision, provisional nomenclature on this subject is provided by the Editor as a guide for authors of manuscripts.

0.2 Trivial names and generic descriptors for the vitamins and related compounds are useful and necessary in nutrition literature. In each instance, the generic descriptor should be used to describe the family of compounds having vitamin activity and to modify such terms as "activity," "deficiency," etc. The trivial names should be used only to identify the specific compound. Synonyms for a specific chemical compound are undesirable and lead to unnecessary complications in indexing and in computer retrieval. Therefore only one name is recommended for each vitamin or related compound.

0.3 The summation of the contributions to the dietary intake of different forms of the same vitamin is a practical necessity to nutritionists.

For vitamin A and its provitamins summation should be made in mg (μg) *retinol equivalent*. Provitamins, if their separate summation is required, should be expressed in mg (μg) *β-carotene equivalent*. It is emphasized that discretion must be used in the acceptance of values from food tables and in the interpretation of findings from biological tests. Thus, although retinol is to be used as the summation substance it is greatly inferior, in view of its instability, to all-*trans*-retinyl acetate (9-*trans*,11-*trans*, 13-*trans*-retinyl acetate) as the standard for biological tests.

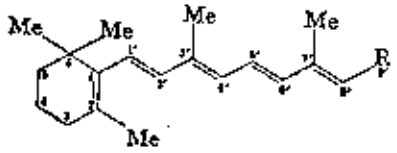
Other members of vitamin families suggested for purposes of summation, with similar reservations, are:

vitamin D	cholecalciferol equivalent
vitamin K	phytylmenaquinone (phylloquinone) equivalent
vitamin B-12	cyanocobalamin equivalent
folacin	folic acid equivalent
niacin (tryptophan)	nicotinamide equivalent

FAT-SOLUBLE VITAMINS AND RELATED COMPOUNDS

V.1 *Vitamin A and Provitamin A Carotenoids*

1.1 The term *vitamin A* should be used as the generic descriptor for all β-ionone derivatives, other than provitamin A carotenoids, exhibiting qualitatively the biological activity of retinol. Thus, phrases such as "vitamin A activity," "vitamin A deficiency" and "vitamin A in the form of . . ." represent preferred usage.



I

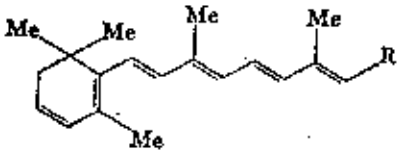
1.2 The compound with formula I (R = $-CH_2OH$), also known as vitamin A, vitamin A alcohol, vitamin A_1, vitamin A_1 alcohol, axerophthol or axerol, should be designated *retinol*.

* Comments on nomenclature policy may be directed to the Editor, who will bring them to the attention of the AIN Committee on Nomenclature.

1.3 Esters of retinol should be designated *retinyl esters.*

1.4 The compound with formula I (R = —CHO), also known as vitamin A (A_1) aldehyde, retinene or retinal, should be designated *retinaldehyde.*

1.5 The compound with formula I (R = —COOH), having partial vitamin A activity and also known as vitamin A acid, should be designated *retinoic acid.*



II

1.6 The compound 3-dehydroretinol, with formula II (R = $-CH_2OH$), also known as vitamin A_2, should be designated *dehydroretinol.*

1.7 The compound 3-dehydroretinaldehyde, with formula II (R = —CHO), also known as retinene-2, 3-dehydroretinal or dehydroretinal, should be designated *dehydroretinaldehyde.*

1.8 The compound 3-dehydroretinoic acid, with formula II (R = —COOH), should be designated *dehydroretinoic acid.*

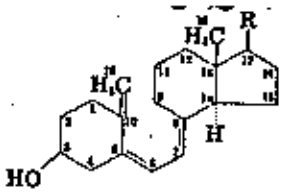
1.9 The term *provitamin A carotenoid* should be used as the generic descriptor for all carotenoids exhibiting qualitatively the biological activity of β-carotene. When referring to the biological activity of the provitamin A carotenoids, the phrase "provitamin A activity" represents preferred usage.

1.10 Trivial names for specific carotenoids are under consideration.

1.11 The term *retinol equivalent* may be used as described in section 0.4 of the Introduction.

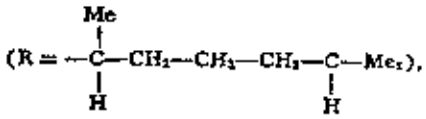
V.2 Vitamin D

2.1 The term *vitamin D* should be used as the generic descriptor for all steroids exhibiting qualitatively the biological activity of cholecalciferol. Thus, phrases such as "vitamin D activity" and "vitamin *D* deficiency" represent preferred usage.



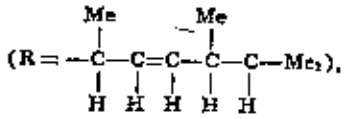
III

2.2 The compound with formula III



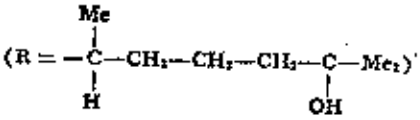
also known as vitamin D_3, should be designated *cholecalciferol.*

2.3 The compound with formula III



also known as vitamin D_2 or calciferol, should be designated *ergocalciferol.*

2.4 The compound with formula III



should be designated *25-hydroxycholecalciferol.*

2.5 Esters of cholecalciferol and ergocalciferol should be designated *cholecalciferyl* esters and *ergocalciferyl* esters, respectively.

V.3 Vitamin E

Nomenclature of vitamin E is under consideration by committees of AIN, IUNS, IUPAC and CBN. Pending their recommendations and adoption of revised nomenclature by AIN, the Journal of Nutrition will accept in submitted manuscripts usage which is consistent with the following provisional nomenclature.

3.1 The term *vitamin E* should be used as the generic descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α-tocopherol.

Thus, phrases such as "vitamin E activity," "vitamin E deficiency" and "vitamin E in the form of . . ." represent preferred usage.

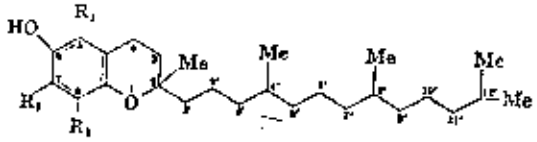
3.2 The term *tocopherols* should be used as the generic descriptor for all methyl tocols. Thus the term "tocopherol" is not synonymous with the term "vitamin E."

3.3. The compound, 2D,4'D,8'D-α-tocopherol or 2R, 4'R, 8'R-α-tocopherol with the formula IV ($R_1 = R_2 = R_3 = -Me$) may also be referred to as *d*-α-tocopherol. This is the naturally occurring *α-tocopherol.*

3.4 The mixture of the eight possible stereoisomers of α-tocopherol commonly synthesized from racemic isophytol, and designated as 2DL,4'DL,8'DL-α-tocopherol or 2RS,4'RS,8'RS-α-tocopherol, may also be referred to as *dl*-α-tocopherol or all-*rac.-α-tocopherol.*

3.5 The mixture of two isomers of α-tocopherol synthesized from natural phytol, and designated 2DL,4'D,8'D-α-tocopherol or 2RS,4'R,8'R-α-tocopherol may be referred to as *2dl-α-tocopherol.* The acetate ester of this mixture was the former International Standard for vitamin E (J. Nutrition, *90*, 109 (1966)).

3.6 The compound designated 2L,4'D,8'D-α-tocopherol or 2S,4'R,8'R-α-tocopherol, may be referred to as *2l*-α-tocopherol or 2-epi-*α-tocopherol.*



IV

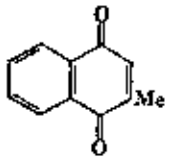
Sections 3.7 through 3.13 are temporarily deleted.

3.14 Esters of tocopherols and tocotrienols should be designated *tocopheryl* esters and *tocotrienyl* esters, respectively.

V.4 Vitamin K [a]

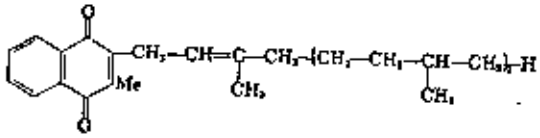
4.1 The term *vitamin K* should be used as the generic descriptor for 2-methyl-1,4-naphthoquinone and all derivatives exhibiting qualitatively the biological activity of phytylmenaquinone (phylloquinone). Thus, phrases such as "vitamin K activity" and "vitamin K deficiency" represent preferred usage.

4.2 The compound 2-methyl-1,4-naphthoquinone with formula VI, having partial vitamin K activity and also known as menadione, should be designated *menaquinone.*



VI

4.3 The compound 2-methyl-3-phytyl-1,4-naphthoquinone with formula VII, also known as vitamin K_1 or phylloquinone, should preferably be designated *phytylmenaquinone.*



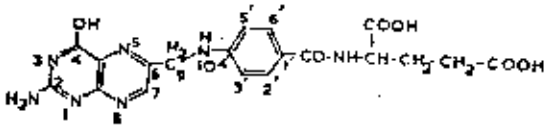
VII

4.4 The compounds known as vitamin K_2 should be designated *multiprenylmenaquinones.* Thus, a vitamin K_2 having a side chain consisting of 6 prenyl units and also known as menaquinone-6 should preferably be designated *prenylmenaquinone-6;* a vitamin K_2 having a side chain consisting of 7 prenyl units and also known as menaquinone-7 should preferably be designated *prenylmenaquinone-7.*

4.5 In general the use of abbreviations for the compounds dealt with in V.4. 3–4 above is not recommended. Should abbreviation be necessary the following forms are permissible: phytylmenaquinone, PMQ; prenylmenaquinone-6, MQ-6; prenylmenaquinone-7, MQ-7.

4.6 The IUPAC–IUB Tentative Rules for Nomenclature of Quinones with Isoprenoid Side Chains (J. Biol. Chem., *241*, 2989–2991 (1966)) may be used with the reservations following from V.4. 2–5 above.

[a] Revision currently under consideration by AIN Committee on Nomenclature to reconcile differences with IUPAC nomenclature.



VIII

WATER-SOLUBLE VITAMINS AND RELATED COMPOUNDS

V.5 *Folacin*

5.1 The term *folacin* should be used as the generic descriptor for folic acid and related compounds exhibiting qualitatively the biological activity of folic acid. Thus, phrases such as "folacin activity" and "folacin deficiency" represent preferred usage.

5.2 The compound monopteroylglutamic acid with formula VIII should be designated *folic acid.*

5.3 Folic acid derivatives in which the glutamic acid residue is combined through a peptide bond with another glutamic acid residue which may or may not be similarly combined with a third glutamic acid residue and so on should be designated *folic acid glutamates* (*N*), where N indicates the number of glutamic acid residues.

5.4 The compound tetrahydropteroylglutamic acid, also known as $PGAH_4$, THFA or tetrahydrofolacin, should be designated *tetrahydrofolic acid.*

5.5 The compound N^5-formyltetrahydropteroylglutamic acid, also known as N^5-F-$PGAH_4$, citrovorum factor, "CF," leucovorin, folinic acid or N^5-formyl THFA, should be designated *5-formyltetrahydrofolic acid.*

5.6 The compound N^{10}-formyltetrahydropteroylglutamic acid, also known as N^{10}-F-$PGAH_4$, heat-labile citrovorum factor, "HLCF" or N^{10}-formyl THFA, should be designated *10-formyltetrahydrofolic acid.*

5.7 The compound N^5-methyltetrahydropteroylglutamic acid, also known as N^5-M-$PGAH_4$, "prefolic A," N^5-methyltetrahydrofolacin or N^5-methyl THFA, should be designated *5-methyltetrahydrofolic acid.*

5.8 In general the use of abbreviations for the compounds dealt with in V.5. 4-7 above is not recommended. Should abbreviation be necessary the following forms are permissible: tetrahydrofolic acid, H_4-folic acid; 5-formyltetrahydrofolic acid, 5-CHO-H_4-folic acid; 10-formyltetrahydrofolic acid, 10-CHO-H_4-folic acid; 5-methyltetrahydrofolic acid, 5-CH_3-H_4-folic acid.

5.9 Related compounds with folacin activity should be named and abbreviated in accordance with the IUPAC–IUB Tentative Rules for Nomenclature and Symbols for Folic Acid and Related Compounds (J. Biol. Chem., *241*, 2991–2992 (1966)).

V.6 *Niacin*

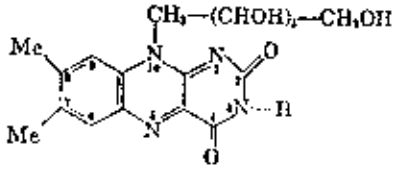
6.1 The term *niacin* should be used as the generic descriptor for pyridine 3-carboxylic acid and derivatives exhibiting qualitatively the biological activity of nicotinamide. Thus, phrases such as "niacin activity" and "niacin deficiency" represent preferred usage.

6.2 The compound pyridine 3-carboxylic acid with formula IX (R = −COOH), also known as niacin or vitamin PP, should be designated *nicotinic acid.*

IX

6.3 The compound with formula IX (R = $-CONH_2$), also known as niacinamide or nicotinic acid amide, should be designated *nicotinamide.*

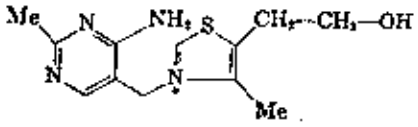
6.4 The term *nicotinamide equivalent* may be used as described in section 0.4 of the Introduction.



X

V.7 *Riboflavin*

7.1 The compound with formula X, also known as vitamin B_2, lactoflavin(e) or riboflavine, should be designated *riboflavin.* Its use in phrases such as "riboflavin activity" and "riboflavin deficiency" represents acceptable usage.



XI

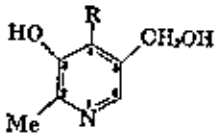
V.8 Thiamin

8.1 The cation with formula XI, also known as vitamin B_1, aneurin(e) or thiamine, should be designated *thiamin*. Its use in phrases such as "thiamin activity" and "thiamin deficiency" represents acceptable usage.

V.9 Vitamin B-6

9.1 The term *vitamin B-6* should be used as the generic descriptor for all 2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine. Thus, phrases such as "vitamin B-6 activity" and "vitamin B-6 deficiency" represent preferred usage.

9.2 The compound 3-hydroxy-4,5-bis(hydroxymethyl)-2-methylpyridine with formula XII ($R = -CH_2OH$), also known as vitamin B_6, adermin or pyridoxol, should be designated *pyridoxine*.



XII

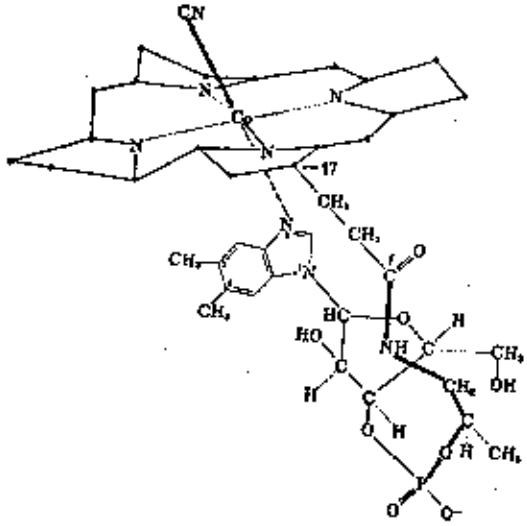
9.3 The compound with formula XII ($R = -CHO$), also known as pyridoxaldehyde, should be designated *pyridoxal*.

9.4 The compound 3-hydroxy-4-methylamino-5-hydroxymethyl-2-methylpyridine with formula XII ($R = -CH_2NH_2$) should be designated *pyridoxamine*.

V.10 Vitamin B-12

10.1 The term *vitamin B-12* should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. Thus, phrases such as "vitamin B-12 activity" and "vitamin B-12 deficiency" represent preferred usage.

10.2 The term *corrinoids* should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term "corrinoid" is not synonymous with the term "vitamin B-12".



XIII

10.3 The compound α-(5,6-dimethylbenzimidazolyl) cobamide cyanide with formula XIII, also known as vitamin B_{12} or cyanocobalamine, should be designated *cyanocobalamin*.

10.4 The compound α-(5,6-dimethylbenzimidazolyl) hydroxocobamide, also known as vitamin B_{12a}, vitamin B_{12b}, aquocobalamin or hydroxocobalamine, should be designated *hydroxocobalamin*.

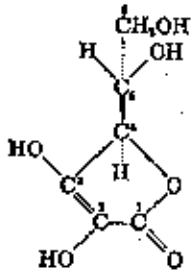
10.5 The compound α-(5,6-dimethylbenzimidazolyl) cobamide nitrite, also known as vitamin B_{12c} or nitritocobalamine, should be designated *nitritocobalamin*.

10.6 Related compounds with vitamin B-12 activity should be named in accordance with the IUPAC-IUB Tentative Rules for Nomenclature of Corrinoids (J. Biol. Chem., *241*, 2992–2994 (1966)).

V.11 Vitamin C

11.1 The term *vitamin C* should be used as the generic descriptor for all compounds exhibiting qualitatively the biological activity of ascorbic acid. Thus, phrases such

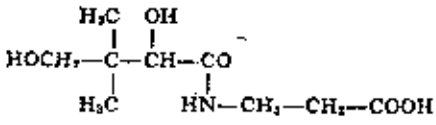
as "vitamin C activity" and "vitamin C deficiency" represent preferred usage.



XIV

11.2 The compound with formula XIV, also known as vitamin C or L-ascorbic acid, should be designated *ascorbic acid.*

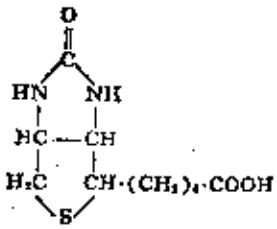
11.3 The compound also known as L-dehydroascorbic acid should be designated *dehydroascorbic acid.*



XV

V.12 *Pantothenic acid*

12.1 The compound with formula XV, also known as pantoyl-β-alanine, should be designated *pantothenic acid.* Its use in phrases such as "pantothenic acid activity" and "pantothenic acid deficiency" represents acceptable usage.



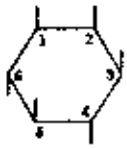
XVI

V.13 *Biotin*

13.1 The compound with formula XVI, also known as coenzyme R, should be designated *biotin.* Its use in phrases such as "biotin activity" and "biotin deficiency" represents acceptable usage.

V.14 *Choline*

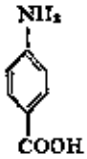
14.1 The compound with formula $(CH_3)_3N^+CH_2CH_2OH$ should be designated *choline.* Its use in phrases such as "choline activity" and "choline deficiency" represents acceptable usage.



XVII

V.15 *Myo-inositol*

15.1 The compounds with formula XVII, also known as inositol or mesoinositol, should be designated *myo-inositol.* Its use in phrases such as "myo-inositol activity" and "myo-inositol deficiency" represents acceptable usage (see European J. Biochem., 5, 1–12 (1968)).



XVIII

V.16 *p-Aminobenzoic acid*

16.1 The compound with formula XVIII should be designated *p-aminobenzoic acid.* Its use in phrases such as "*p*-aminobenzoic acid activity" and "*p*-aminobenzoic acid deficiency" represents acceptable usage.

NOMENCLATURE POLICY: ABBREVIATED DESIGNATIONS OF AMINO ACIDS

The following rules for nomenclature of the amino acids are based on the Rules of the IUPAC-IUB Commission on Biochemical Nomenclature for designation of amino acids and peptides (1, 2, 3), and were adopted by recommendation of the AIN Committee on Nomenclature as the nomenclature of the official Journals of the AIN.

1. *General considerations*

1.1 The symbols chosen are derived from the trivial names or chemical names of the amino acids and of chemicals reacting with amino acids and polypeptides. For the sake of clarity, brevity, and listing in tables, the symbols have been, wherever possible, restricted to three letters, usually the first letters of the trivial names.

1.2 The symbols represent not only the names of the compounds but also their structural formulas.

1.3 The amino acid symbols by themselves represent the amino acids. The use of the symbols to represent the free amino acids is *not* recommended in textual material, but such use may occasionally be desirable in tables, diagrams, or figures. Residues of amino acids are represented by addition of hyphens in specific positions.

1.4 Heteroatoms of amino acid residues (e.g. O^{γ} and S^{γ} of serine and cysteine, respectively, N^{ζ} of lysine, N^{α} of glycine, etc.) do not explicitly appear in the symbol; such features are understood to be encompassed by the abbreviation.

1.5 Amino acid symbols denote the L configuration unless otherwise indicated by D or DL appearing before the symbol and separated from it by a hyphen. When it is desired to make the number of amino acid residues appear in a clearer manner, the hyphen between the configurational prefix and the symbol may be omitted (see Ref. 1). (Note: the designation of an amino acid residue as DL is inappropriate for compounds having another amino acid residue with an asymmetrical center.)

1.6 Structural formulas of complicated features may be used along with the abbreviated notation whenever necessary for clarity.

2. *Abbreviations for amino acids*

2.1 *Common Amino Acids*

Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine [1]	Asn [1]	Methionine	Met
Aspartic acid	Asp	Ornithine	Orn
Cysteine	Cys	Phenylalanine	Phe
Cystine	Cys-Cys *	Proline	Pro
Glutamic acid	Glu	Serine	Ser
Glutamine [1]	Gln [1]	Threonine	Thr
Glycine	Gly	Tryptophan	Trp
Histidine	His	Tyrosine	Tyr
Isoleucine	Ile	Valine	Val

[1] Asparagine and glutamine may also be denoted as Asp (NH_2) or Asp$_{\gamma}$ and Glu (NH_2) or Glu$_{\delta}$, respectively.

$$\begin{array}{c} NH_2 \\ | \end{array} \qquad \begin{array}{c} NH_2 \\ | \end{array}$$

* Cystine does not appear in 2.1 (ref. 1). We propose Cys-Cys as consistent with 2.1 but this abbreviation should not be used to denote structure.

2.2 *Less Common Amino Acids*

—Abbreviations for less common amino acids should be defined in each publication in which they appear. The following principles and notations are recommended.

2.2.1 Hydroxyamino Acids

Hydroxylysine	Hyl
3-Hydroxyproline	3Hyp
4-Hydroxyproline	4Hyp

2.2.2 *allo*-Amino Acids

allo-Isoleucine	*a*Ile
allo-Hydroxylysine	*a*Hyl

2.2.3 *"Nor" Amino Acids*—"Nor" (e.g. in *norvaline*) is not used in its accepted sense (denoting a lower homologue) but to change the trivial name of a branched chain compound into that of a straight chain compound (compare with "iso," paragraph 2.1). "Nor" should therefore be treated as part of the trivial name without special emphasis.

Norvaline	Nva
Norleucine	Nle

2.2.4 *Higher Unbranched Amino Acids*—We suggest the following general rules for guidance in forming abbreviations: the functional prefix "amino" should be included in the symbol as the letter "A," diamino as "A_2."

The trivial name of the parent acid should be abbreviated to leave no more than two or three letters, as convenient and necessary for clarity. The word "acid" ("*-saure*," etc.) should be omitted from the symbol as carrying no significant information. Unless otherwise indicated (see Ref. 1), single groups are in the α position, two amino groups in the α,ω (monocarboxylic acids) or α,α' positions (dicarboxylic acids). The location of amino acids in positions other than α and ω is shown by the appropriate Greek letter prefix.

Examples:

α-Aminobutyric acid	Abu
α-Aminoadipic acid	Aad
α-Aminopimelic acid	Apm
α,γ-Diaminobutyric acid	Adbu
α,β-Diaminopropionic acid	Adpr
α,α'-Diaminopimelic acid	Apm
β-Alanine	βAla
ϵ-Aminocaproic acid	ϵAcp
β-Aminoadipic acid	βAad

For further details in using symbols to designate structure, the reader is referred to the following references.

REFERENCES

1. IUPAC-IUB Commission on Biochemical Nomenclature (1972) Symbols for amino-acid derivatives and peptides. Recommendations (1971). J. Biol. Chem. *247*, 977–983.
2. IUPAC-IUB Commission on Biochemical Nomenclature 1967 Rules for naming synthetic modifications of natural peptides. Tentative Rules, J. Biol. Chem., *243*: 555–557.
3. IUPAC-IUB Commission on Biochemical Nomenclature (1972) Abbreviated nomenclature of synthetic polypeptides (polymerized amino acids). Revised recommendations (1971). Biochemistry *11*, 942–944.

HYPERVITAMINOSIS D

Clinical Nutrition 2nd Ed. pp. 309-311, (1962)

In recent years, patients with rheumatoid arthritis, children with vitamin D resistant rickets, and, in Europe, patients with lupus vulgaris have been treated over periods of several weeks or months, sometimes even years, with daily doses of calciferol equivalent to 500,000 I.U. of vitamin D. Massive doses of vitamin D influence bone resorption more than calcium accretion.[31] Thus, in hypervitaminosis D the common signs are hypercalcemia, hyperphosphatemia, and hypercalciuria. The alkaline phosphatase of the serum remains normal. In addition, marked and generalized resorption of bone develops, together with metastatic calcification in kidneys,[11] blood vessel walls, gastric mucosa, heart, lungs, sclerae, falx, dura mater, thyroid, pancreas and periarticular soft tissues.[32] Band keratitis due to calcium precipitation in the cornea deserves special mention.

Metastatic calcification in the kidneys can often be visualized on the roentgenograms in the form of nephrocalcinosis limited to the medullary pyramids and the renal pelvis. In some cases the calcium deposits in the kidneys remain microscopic in size and do not show up on the x-rays, but the albuminuria and the impairment of the kidney function may be just as serious as when grossly visible nephrocalcinosis is present. Renal stone formation has also been observed. Ultimately, metastatic calcification of the kidneys always leads to uremia.

It is of great importance to recognize hypervitaminosis D as the cause of uremia because, after cessation of the vitamin D intake, calcium and phosphorus both of serum and urine go down to normal levels and the renal function improves. The resorption of small calcium deposits near the interphalangeal joints may take one year. Larger para-articular deposits remain unchanged for a much longer period.

In general, hypervitaminosis D needs to be differentiated from hyperparathyroidism and Boeck's sarcoidosis. In hyperparathyroidism, hypo-

phosphatemia often occurs, while in hypervitaminosis D and in sarcoidosis the serum phosphorus is usually slightly increased. In renal osteodystrophy the serum calcium is nearly always low, or at best normal, the serum phosphorus is high, the urinary calcium and phosphorus excretion are decreased.

Since the dangers of the administration of excessive doses of vitamin D are common knowledge, hypervitaminosis D in adults has become a rarity. However, in infants, at least in the United Kingdom, a new syndrome, "idiopathic hypercalcemia," has now been recognized which may well be due to hypervitaminosis D. A recent report mentions that between 1953 and 1955, 205 such patients, mainly infants between 4 and 8 months of age, have been observed.[7]

In the malignant form of this disease, dwarfism, mental retardation, microcephaly and cerebral signs are present. The benign form of the disease is reversible and is of considerable clinical importance.[32] Children with benign idiopathic hypercalcemia usually suffer from loss of appetite, tenacious vomiting, and constipation. Often, a systolic murmur can be heard, either at the point of maximal impulse or all over the precordium. Hypercalcemia and hypercalciuria are constant findings; the serum phosphorus and alkaline phosphatase usually remain normal. Blood urea nitrogen and cholesterol are increased. Under adequate treatment these abnormal values rapidly return to normal and the systolic murmur disappears.

Nephrocalcinosis has been frequently observed. Linear disposition of abnormally dense bone near the epiphyses of the long bones and osteosclerosis of the skull base and vertebral bodies may be present.

There is strong evidence that hypervitaminosis D played an important role in the etiology of this idiopathic hypercalcemia of infants.[17,19] "National Dried Milk," a popular food for infants in the United Kingdom, contained "not less than" 1,200 I.U. of vitamin D per reconstituted liter, and probably more. The vitamin D content of several other milk preparations used for infant feeding in England was even higher. The "British National Cod Liver Oil Compound" contained not less than 700 to 800 I.U. of vitamin D per teaspoonful, and probably more. Cereals were also fortified and some brands contained 1,500 I.U. per ounce. Infants who received 1.5 pints of reconstituted dried milk, 1 ounce of fortified cereal, and one teaspoon of fortified cod liver oil ingested about 4,000 I.U. of vitamin D per day. This intake was far in excess of the dose of 400 to 700 I.U., which around 1940 was recommended as the daily intake for infants. The daily maximal intake of vitamin D for infants was usually considered to vary around 1,500 I.U.

In this connection, it is of interest that in the United States only a few cases of idiopathic hypercalcemia have been reported. In this country the most popular brand of evaporated milk contains only 420 I.U. of

vitamin D per reconstituted liter. However, the possibility exists that cases of hypervitaminosis D in infants and children may also develop in the United States, since certain halibut liver preparations used in this country contain 6,000 I.U. vitamin D per teaspoonful.

Breast feeding has not become obsolete in England, and it is interesting that until now no case of idiopathic hypercalcemia in a breast-fed infant has been reported. The vitamin content of human milk varies between 50 and 100 I.U. vitamin D per liter, that is, only 25 per cent of the vitamin D content of vitaminized cow's milk.

In most cases of idiopathic hypercalcemia, feeding with low-calcium milk powder (Locasol) and low-calcium cereal (Glaxo) boiled in distilled water brings rapid recovery. In other cases, cortisone, mainly by inhibiting calcium and phosphorus absorption from the intestine, has been helpful.

The British Pediatric Society decided to recommend that no food fortified with vitamin D should be given to infants. Every infant, however, it was stated, should receive cod liver oil of known potency. This, of course, is exactly the same nutritional formula customary half a century ago.

Idiopathic hypercalcemia in older individuals who do not receive excessive amounts of vitamin D with their food[31] may be due to insufficient inactivation or elimination of vitamin D.

1020

Registration
No. Date
SOR/72-149 11 May, 1972

FOOD AND DRUGS ACT

Food and Drug Regulations, amendment Schedule No. 168 and Schedule No. 178

P.C. 1972-976 9 May, 1972

His Excellency the Governor General in Council, on the recommendation of the Minister of National Health and Welfare, pursuant to section 25 of the Food and Drugs Act, is pleased hereby to amend the Food and Drug Regulations made by Order in Council P.C. 1954-1915 of 8th December, 1954[1], as amended[2], in accordance with the Schedules hereto marked Schedule 168 and Schedule 178.

SCHEDULE NO. 168

1. All that portion of section D.01.004 of the French version of the *Food and Drug Regulations* preceding paragraph (*a*) thereof is revoked and the following substituted therefor:

"D.01.004 Lorsque la quantité d'une vitamine dont il est fait mention dans le présent article et qui est contenue dans un aliment n'est pas inférieure à la quantité mentionnée à l'alinéa *b*) de l'article D.01.005 pour cette vitamine, une personne peut, dans la publicité qu'elle fait au sujet de cet aliment ou sur l'étiquette de cet aliment, déclarer,"

2. Subsections (2) and (2*a*) of section D.04.011 of the said Regulations are revoked and the following substituted therefor:

"(2) A person may sell on prescription to the general public,

(*a*) a drug represented as containing a vitamin referred to in paragraph (1)(*a*), or

(*b*) a drug represented as containing vitamin D, if the largest recommended daily dosage shown on the label thereof would, if consumed by a person, result in the daily intake by that person of more than 1000 International Units.

(2*a*) A person may sell a drug referred to in paragraph (2) (*a*) or (*b*) that carries the symbol Pr on the upper left quarter on the main panel of both the inner and outer labels to

(*a*) a drug manufacturer,
(*b*) a practitioner,
(*c*) a wholesale druggist,
(*d*) a registered pharmacist,
(*e*) a hospital certified by the Department of National Health and Welfare,
(*f*) a Department of the Government of Canada or of

[1] SOR/54-161, *Canada Gazette* Part II, Vol. 88, No. 24, December 22, 1954, p. 2080 and *Statutory Orders and Regulations Consolidation 1955*, Vol. 2 p. 1675

[2] SOR/72-140, *Canada Gazette* Part II, Vol. 106, No. 10, May 24, 1972

Enregistrement
N° Date
DORS/72-149 11 mai 1972

LOI DES ALIMENTS ET DROGUES

Règlements des aliments et drogues—Modification Listes de modifications n°s 168 et 178

C.P. 1972-976 9 mai 1972

Sur avis conforme du ministre de la Santé nationale et du Bien-être social et en vertu de l'article 25 de la Loi des aliments et drogues, il plaît à Son Excellence le Gouverneur général en conseil de modifier, conformément aux Listes de modifications n°s 168 et 178 ci-après, les Règlements des aliments et drogues établis par le décret C.P. 1954-1915 du 8 décembre 1954[1], dans leur forme modifiée[2].

LISTE DE MODIFICATIONS N° 168

1. A l'article D.01.004 du texte français des *Règlements des aliments et drogues*, la partie qui précède l'alinéa *a*) est abrogée et remplacée par ce qui suit:

«D.01.004 Lorsque la quantité d'une vitamine dont il est fait mention dans le présent article et qui est contenue dans un aliment n'est pas inférieure à la quantité mentionnée à l'alinéa *b*) de l'article D.01.005 pour cette vitamine, une personne peut, dans la publicité qu'elle fait au sujet de cet aliment ou sur l'étiquette de cet aliment, déclarer,»

2. Les paragraphes (2) et (2*a*) de l'article D.04.011 desdits règlements sont abrogés et remplacés par ce qui suit:

«(2) Une personne peut vendre au public, sur ordonnance,
a) une drogue présentée comme contenant une vitamine mentionnée à l'alinéa (1)*a*), ou
b) une drogue présentée comme contenant la vitamine D, si la plus forte dose quotidienne recommandée telle qu'elle est indiquée sur l'étiquette, apporte à une personne qui la consomme plus de 1000 unités internationales.

(2*a*) Une personne peut vendre une drogue dont il est fait mention à l'alinéa (2)(*a*) ou *b*), lorsque l'angle supérieur gauche de la face principale des étiquettes intérieure et extérieure de la drogue porte le symbole Pr,
a) à un fabricant de drogues,
b) à un praticien,
c) à un pharmacien en gros,
d) à un pharmacien inscrit,
e) à un hôpital reconnu par le ministère de la Santé nationale et du Bien-être social,
f) à un ministère du gouvernement du Canada ou d'une province, dès la réception d'une commande écrite signée

[1] DORS/54-164, *Gazette du Canada* Partie II, Vol. 88, n° 24, 22 décembre 1954, p. 2085 et Décrets, Ordonnances et Règlements Statutaires, Codification de 1955, Vol. 2, p. 1676

[2] DORS/72-140, *Gazette du Canada* Partie II, Vol. 106, n° 10, 24 mai 1972

a province, upon receipt of a written order signed by the Minister thereof or his duly authorized representative, or

(g) any other person, upon receipt of a written order signed by the Director.

(2b) A person may sell

(a) a drug represented as containing a vitamin referred to in paragraphs (b) to (i) and (k) to (l) of subsection (1), or

(b) a drug represented as containing vitamin D, if the largest recommended daily dosage shown on the label thereof would, if consumed by a person, result in the daily intake by that person of more than 400 but not more than 1000 International Units,

if both the inner and outer labels thereof carry a cautionary statement that the drug is for therapeutic use only."

3. Subparagraph (v) of paragraph (b) of section D.05.007 of the French version of the said Regulations is revoked and the following substituted therefor:

"(v) dans le cas de l'iode, au moins 0.05 milligramme,"

par le ministre concerné ou son représentant dûment autorisé, ou

g) à toute autre personne, dès la réception d'une commande écrite signée par le Directeur.

(2b) Une personne peut vendre

a) une drogue présentée comme contenant une vitamine mentionnée dans l'un des alinéas b) à i) et k) à l) du paragraphe (1), ou

b) une drogue présentée comme contenant la vitamine D, si la plus forte dose quotidienne recommandée telle qu'elle est indiquée sur l'étiquette, apporte à une personne qui la consomme plus de 400 unités internationales et au plus 1000 unités internationales,

à la condition que les étiquettes intérieure et extérieure de la drogue portent une mise en garde indiquant que la drogue est réservée à l'usage thérapeutique.»

3. Dans la version française desdits règlements, le sous-alinéa (v) de l'alinéa b) de l'article D.05.007 est abrogé et remplacé par ce qui suit:

«(v) dans le cas de l'iode, au moins 0.05 milligramme,»

SCHEDULE NO. 178

1. (1) Subsection (1) of section C.01.041 of the *Food and Drug Regulations* is revoked and the following substituted therefor:

"(1) Subject to sections C.01.043, C. 01.046 and C.01.601, no person shall sell a substance containing a drug listed or described in Schedule F to the Regulations, (hereinafter referred to as a Schedule F Drug), unless he has received a written or verbal prescription therefor."

(2) Section C.01.041 of the said Regulations is further amended by adding thereto the following subsection:

"(5) Subsection (1) to (4) do not apply to a substance containing

(a) chloral hydrate in preparations for external use, where it constitutes not more than 1% of the substance, or

(b) hexachlorophene and its salts, where it constitutes not more than 0.75% of the substance, calculated as hexachlorophene."

2. (1) Subitem C.3(d) of Part I of Schedule F to the said Regulations is revoked and the following substituted therefor:

"(d) chloral hydrate".

(2) Part I of Schedule F to the said Regulations is further amended by adding thereto, immediately after item H.1 thereof, the following item:

"H.1A hexachlorophene and its salts".

3. Appendix I to the said Regulations is amended by adding thereto the following names:

"Michael J. LeBelle
Clara E. Williams"

4. (1) Part I of Appendix II to the said Regulations is amended by deleting therefrom the following name:

"Richard Allan Brown"

(2) Part I of Appendix II to the said Regulations is further amended by adding thereto the following names:

"Dawn M. Adams	Gaétan Desrosiers
Richard Alan Brown	J. A. Alain Dion
Jean-Marc Charron	Gaétan Soucy"

LISTE DE MODIFICATIONS N° 178

1. (1) Le paragraphe (1) de l'article C.01.041 des *Règlements des aliments et drogues* est abrogé et remplacé par ce qui suit:

«(1) Sous réserve des articles C.01.043, C.01.046 et C.01.601, il est interdit de vendre une substance contenant une drogue mentionnée ou décrite à l'annexe F des présents règlements et désignée ci-après dans lesdits règlements comme drogue de l'annexe F, à moins qu'une ordonnance, écrite ou verbale, n'ait été reçue à cet effet.»

(2) L'article C.01.041 desdits règlements est en outre modifié par l'adjonction du paragraphe suivant:

«(5) Les paragraphes (1) à (4) ne s'appliquent pas à une substance contenant

a) de l'hydrate de chloral, dans des préparations pour usage externe, lorsque celui-ci ne constitue pas plus de 1% de la substance, ou

b) de l'hexachlorophène et ses sels, lorsqu'ils ne constituent pas plus de 0.75% de la substance, calculés en hexachlorophène.»

2. (1) Le sous-poste C.3d) de la partie I de l'annexe F desdits règlements est abrogé et remplacé par ce qui suit:

«d) hydrate de chloral»

(2) La partie I de l'annexe F desdits règlements est en outre modifiée par l'insertion, immédiatement après le poste H.1, de ce qui suit:

«H.1A Hexachlorophène et ses sels»

3. L'appendice I desdits règlements est modifié par l'addition des noms suivants:

«Michael J. LeBelle
Clara E. Williams»

4. (1) La partie I de l'appendice II desdits règlements est modifiée par le retranchement du nom suivant:

«Richard Allan Brown»

(2) La partie I de l'appendice II desdits règlements est en outre modifiée par l'addition des noms suivants:

«Dawn M. Adams	Gaétan Desrosiers
Richard Alan Brown	J. A. Alain Dion
Jean-Marc Charron	Gaétan Soucy»

QUEEN'S PRINTER FOR CANADA, OTTAWA, 1972

IMPRIMEUR DE LA REINE POUR LE CANADA, OTTAWA, 1972

SOR/71-238

FOOD AND DRUGS ACT

Food and Drug Regulations amended—Schedule No. 153

P.C. 1971-1002

25 May, 1971

His Excellency the Governor General in Council, on the recommendation of the Minister of National Health and Welfare, pursuant to section 24 of the Food and Drugs Act, is pleased hereby to amend the Food and Drug Regulations made by Order in Council P.C. 1954-1915 of 8th December, 1954[1], as amended[2], in accordance with the Schedule hereto marked Schedule No. 153.

SCHEDULE NO. 153

1. All that portion of section D.01.004 of the *Food and Drug Regulations* preceding paragraph (*a*) thereof is revoked and the following substituted therefor:

"**D.01.004.** Where the amount of a vitamin referred to in this section that is contained in a food is not less than the amount mentioned in paragraph (*b*) of section D.01.005 in respect of that vitamin, a person may, in advertising that food or on a label of that food, state"

2. Subsection (2) of section D.04.011 of the said Regulations is revoked and the following substituted therefor:

"(2) Where a drug referred to in paragraph (*a*) of subsection (1) carries the symbol Pr on the upper left quarter of the main panel of both the inner and outer labels, a person may sell the drug to

(*a*) the general public, if it is sold on prescription;
(*b*) a drug manufacturer;
(*c*) a practitioner;
(*d*) a wholesale druggist;
(*e*) a registered pharmacist;
(*f*) a hospital certified by the Department of National Health and Welfare;
(*g*) a Department of the Government of Canada or of a province, upon receipt of a written order signed by the Minister thereof or his duly authorized representative; or
(*h*) any other person, upon receipt of a written order signed by the Director.

(2*a*) A person may sell a drug referred to in paragraphs (*b*) to (*f*) of subsection (1) if both the inner and outer labels thereof carry a cautionary statement that the drug is for therapeutic use only."

[1] SOR/54-664, *Canada Gazette* Part II, Vol. 88, No. 24, Dec. 22, 1954, p. 2680 and Statutory Orders and Regulations Consolidation 1955, Vol. 2, p. 1613

[2] SOR/71-164, *Canada Gazette* Part II, Vol. 105, No. 9, May 12, 1971

DORS/71-238

LOI DES ALIMENTS ET DROGUES

Règlements des aliments et drogues—Modifications

C.P. 1971-1002

25 mai 1971

Sur avis conforme du ministre de la Santé nationale et du Bien-être social et en vertu de l'article 24 de la Loi des aliments et drogues, il plaît à Son Excellence le Gouverneur général en conseil, de modifier, conformément à la Liste de modifications n° 153 ci-après, les Règlements des aliments et drogues établis par le décret C.P. 1954-1915 du 8 décembre 1954[1], dans leur forme modifiée[2].

LISTE DE MODIFICATIONS N° 153

1. A l'article D.01.004 des *Règlements des aliments et drogues*, tout ce qui précède l'alinéa *a*) est abrogé et remplacé par ce qui suit:

«**D.01.004.** Lorsque la quantité d'une vitamine dont il est fait mention dans le présent article et qui est contenue dans un aliment n'est pas au moins égale à la quantité mentionnée à l'alinéa *b*) de l'article D.01.005 pour cette vitamine, une personne peut, dans la publicité qu'elle fait au sujet de cet aliment ou sur l'étiquette de cet aliment, déclarer,»

2. Le paragraphe (2) de l'article D.04.011 desdits règlements est abrogé et remplacé par le suivant:

«(2) Lorsqu'une drogue mentionnée à l'alinéa *a*) du paragraphe (1) porte le symbole Pr dans l'angle supérieur gauche de la face principale des étiquettes intérieure et extérieure, une personne peut vendre cette drogue

a) au public, à la condition que ce soit sur ordonnance;
b) à un fabricant de drogues,
c) à un praticien,
d) à un pharmacien en gros,
e) à un pharmacien inscrit,
f) à un hôpital reconnu par le ministère de la Santé nationale et du Bien-être social,
g) à un ministère du gouvernement du Canada ou d'une province, dès la réception d'une commande écrite signée par le Ministre concerné ou son représentant dûment autorisé, ou
h) à toute autre personne, dès la réception d'une commande écrite signée par le Directeur.

(2*a*) Une personne peut vendre une drogue mentionnée dans l'un des alinéas *b*) à *f*) du paragraphe (1) à la condition que les étiquettes intérieure et extérieure de la drogue portent une mise en garde indiquant que la drogue est réservée à l'usage thérapeutique.»

[1] DORS/54-664, *Gazette du Canada* Partie II, Vol. 88, n° 24, 22 décembre 1954, p. 2965 et Décrets, Ordonnances et Règlements Statutaires, Codification de 1955, Vol. 2, p. 1830

[2] DORS/71-164, *Gazette du Canada* Partie II, Vol. 105, n° 9, 12 mai 1971

SOR/71-78

FOOD AND DRUGS ACT

Food and Drug Regulations, amended Schedule No. 90 and Schedule No. 147

P.C. 1971-356

23 February, 1971

His Excellency the Governor General in Council, on the recommendation of the Minister of National Health and Welfare, pursuant to section 24 of the Food and Drugs Act, is pleased hereby to amend the Food and Drug Regulations made by Order in Council P.C. 1954-1915 of 8th December, 1954[1], as amended[2], in accordance with the Schedules hereto marked Schedule No. 90 and Schedule No. 147.

SCHEDULE No. 90

1. Part D of the *Food and Drug Regulations* is revoked and the following substituted therefor:

"PART D

VITAMINS, MINERALS AND AMINO ACIDS

D.01.001. In this Part,

(*a*) "advertise" means to advertise to the general public;

(*b*) "reasonable daily intake" in respect of a food named in an item in Column I of Schedule K means the amount of that food set out in Column II of that item; and

(*c*) "testimonial" with respect to a food or drug that is represented as containing a vitamin, mineral nutrient or mineral means any dramatized or undramatized pictorial, written or oral representation as to the result that is, has been or may be produced by the addition to a person's diet of that vitamin, mineral nutrient or mineral, as the case may be.

DIVISION 1

Vitamins in Foods

D.01.002. (1) In this Division, "vitamin" means any of the following vitamins:

(*a*) vitamin A,

(*b*) thiamine or vitamin B_1,

(*c*) riboflavin or vitamin B_2,

(*d*) niacin or nicotinic acid,

(*e*) niacinamide or nicotinamide,

[1] SOR/54-664, *Canada Gazette* Part II, Vol. 88, No. 24, Dec. 22, 1954, p. 2680 and Statutory Orders and Regulations Consolidation 1955, Vol. 2, p. 1675

[2] SOR/71-64, *Canada Gazette* Part II, Vol. 105, No. 4, Feb. 24, 1971

DORS/71-78

LOI DES ALIMENTS ET DROGUES

Règlements des aliments et drogues—Modifications

C.P. 1971-356

23 février 1971

Sur avis conforme du ministre de la Santé nationale et du Bien-être social et en vertu de l'article 24 de la Loi des aliments et drogues, il plaît à Son Excellence le Gouverneur général en conseil de modifier, conformément aux Listes de modifications n°s 90 et 147 ci-après, les Règlements des aliments et drogues, établis par le décret C.P. 1954-1915 du 8 décembre 1954[1], dans leur forme modifiée[2].

LISTE DE MODIFICATIONS N° 90

1. La partie D des *Règlements des aliments et drogues* est abrogée et remplacée par ce qui suit:

«PARTIE D

VITAMINES, MINÉRAUX ET ACIDES AMINÉS

D.01.001. Dans la présente partie,

a) «faire de la publicité» signifie faire de la publicité auprès du grand public;

b) «ration quotidienne normale», lorsqu'il s'agit d'une substance alimentaire dont le nom figure à l'un des articles de l'annexe K à la colonne I, signifie la quantité de cette substance alimentaire précisée à la colonne II en regard de cet article; et

c) «témoignage», lorsqu'il s'agit d'un aliment ou d'une drogue présentée comme contenant une vitamine, un minéral nutritif ou un minéral, signifie n'importe quelle présentation orale, écrite ou en images, dramatisée ou non, quant à l'effet que produit, a produit ou peut produire l'addition, au régime alimentaire d'une personne, de cette vitamine, de ce minéral nutritif ou de ce minéral, selon le cas.

TITRE 1

Vitamines dans les aliments

D.01.002. (1) Dans le présent titre, «vitamine» désigne l'une des vitamines suivantes:

a) vitamine A,

b) thiamine ou vitamine B_1,

c) riboflavine ou vitamine B_2,

d) niacine ou acide nicotinique,

e) niacinamide ou nicotinamide,

[1] DORS/54-664, *Gazette du Canada* Partie II, Vol. 88, n° 24, 22 décembre 1954, p. 2965 et Décrets, Ordonnances et Règlements Statutaires, Codification de 1955, Vol. 2, p. 1830

[2] DORS/71-64, *Gazette du Canada* Partie II, Vol. 105, n° 4, 24 février 1971

(*f*) pyridoxine or vitamin B_6,
(*g*) d-pantothenic acid,
(*h*) folic acid,
(*i*) biotin,
(*j*) cyanocobalamin or vitamin B_{12},
(*k*) ascorbic acid or vitamin C,
(*l*) vitamin D or vitamin D_2,
(*m*) vitamin D or vitamin D_3,
(*n*) vitamin E,
(*o*) vitamin K, or
(*p*) any salt or derivative of a vitamin, listed in paragraphs (*a*) to (*o*).

(2) This Division applies only to foods that are represented as containing a vitamin for use in human nutrition.

D.01.003. Subject to sections D.01.004 to D.01.008, no person shall, in advertising a food or on a label of a food, make any statement with respect to or any claim based on the vitamin content of that food.

D.01.004. No person may, in advertising a food to which a vitamin has been added or on a label of such a food, if the amount of the vitamin is not less than the amount mentioned in paragraph (*b*) of section D.01.005 in the case of that vitamin, state

(*a*) in the case of ascorbic acid, that it is a factor in the normal development and maintenance of bones, cartilage, teeth and gums;

(*b*) in the case of vitamin D, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood; and

(*c*) in the case of any vitamin listed in paragraph (*b*) of section D.01.005, that it is a factor in the maintenance of good health.

D.01.005. A person may, in advertising a food to which no vitamin has been added or on a label of such food, state

(*a*) that the food is "a good source" or "a good dietary source" of any of the following vitamins, if a reasonable daily intake of that food by a person would result in the daily intake by such person of not less than
(i) in the case of vitamin A, 600 International Units,
(ii) in the case of thiamine, 0.25 milligram,
(iii) in the case of riboflavin, 0.4 milligram,
(iv) in the case of niacin or niacinamide, 2.5 milligrams, and
(v) in the case of ascorbic acid, 7.5 milligrams; or

(*b*) that the food is "an excellent source" or "an excellent dietary source" of any of the following vitamins, if a reasonable daily intake of that food by a person would result in the daily intake by such person of not less than
(i) in the case of vitamin A, 1,200 International Units,
(ii) in the case of thiamine, 0.45 milligram,
(iii) in the case of riboflavin, 0.75 milligram,
(iv) in the case of niacin or niacinamide, 4.5 milligrams,

f) pyridoxine ou vitamine B_6,
g) acide d-pantothénique,
h) acide folique,
i) biotine,
j) cyanocobalamine ou vitamine B_{12},
k) acide ascorbique ou vitamine C,
l) vitamine D ou vitamine D_2,
m) vitamine D ou vitamine D_3,
n) vitamine E,
o) vitamine K, ou
p) tout sel ou dérivé d'une vitamine mentionnée aux alinéas *a*) à *o*).

(2) Ce titre ne s'applique qu'aux aliments présentés comme contenant une vitamine pour usage dans l'alimentation humaine.

D.01.003. Sous réserve des articles D.01.004 à D.01.008, est interdite, dans la publicité faite au sujet d'un aliment ou sur l'étiquette d'un aliment, toute déclaration au sujet de la teneur vitaminique de cet aliment ou toute affirmation fondée sur la teneur vitaminique de cet aliment.

D.01.004. Une personne peut, dans la publicité qu'elle fait au sujet d'un aliment auquel une vitamine a été ajoutée ou sur l'étiquette de cet aliment, si la quantité de vitamine n'est pas inférieure à celle qui est mentionnée à l'alinéa *b*) de l'article D.01.005 pour cette vitamine, déclarer,

a) dans le cas de l'acide ascorbique, que cette vitamine contribue au développement normal et à l'entretien des os, des cartilages, des dents et des gencives;

b) dans le cas de la vitamine D, que cette vitamine contribue au développement normal et à l'entretien des os et des dents, surtout durant l'enfance; et

c) dans le cas de n'importe quelle vitamine mentionnée à l'alinéa *b*) de l'article D.01.005, qu'elle contribue à maintenir une bonne santé.

D.01.005. Une personne peut, dans la publicité qu'elle fait au sujet d'un aliment auquel aucune vitamine n'a été ajoutée ou sur l'étiquette d'un tel aliment, déclarer,

a) que cet aliment est «une bonne source» ou «une bonne source alimentaire» de l'une des vitamines suivantes, si la ration quotidienne normale de cet aliment apporte à une personne qui la consomme, une quantité de cette vitamine au moins égale,
(i) dans le cas de la vitamine A, à 600 unités internationales,
(ii) dans le cas de la thiamine, à 0.25 milligramme,
(iii) dans le cas de la riboflavine, à 0.4 milligramme,
(iv) dans le cas de la niacine ou de la niacinamide, à 2.5 milligrammes, et
(v) dans le cas de l'acide ascorbique, à 7.5 milligrammes; ou

b) que l'aliment est «une excellente source» ou «une excellente source alimentaire» de l'une des vitamines suivantes, si une ration quotidienne normale de cet aliment apporte à une personne qui la consomme,
(i) dans le cas de la vitamine A, au moins 1,200 unités internationales,
(ii) dans le cas de la thiamine, au moins 0.45 milligramme,
(iii) dans le cas de riboflavine, au moins 0.75 milligramme,
(iv) dans le cas de la niacine ou de la niacinamide, au moins 4.5 milligrammes,

(v) in the case of ascorbic acid, 15 milligrams, and
(vi) in the case of vitamin D, 300 International Units.

D.01.006. A person may, in advertising a food to which a vitamin has been added or on a label of such food, state
(*a*) that the food contains the added vitamin; and
(*b*) the amount of the added vitamin that is contained in a specified quantity of the food.

D.01.007. No person shall sell a food to which a vitamin has been added unless the amount of the vitamin present in the food is expressed on the label of the food
(*a*) in the case of vitamin A, vitamin D or vitamin E, in International Units per one hundred grams or millilitres of the food; and
(*b*) in the case of thiamine, riboflavin, niacin, niacinamide, pyridoxine, d-pantothenic acid, folic acid, biotin, vitamin B_{12}, ascorbic acid or vitamin K, in milligrams per one hundred grams or millilitres of the food,
together with the name of the vitamin.

D.01.008. Where a food to which no vitamin has been added is represented as being solely for use in the feeding of children under two years of age, a person may state on the label of the food the amount of any of the following vitamins that are present in the food, if a reasonable daily intake of that food by a child under two years of age would result in the daily intake by the child of not less than
(*a*) in the case of vitamin A, 600 International Units;
(*b*) in the case of thiamine, 0.25 milligram;
(*c*) in the case of riboflavin, 0.4 milligram;
(*d*) in the case of niacin or niacinamide, 2.5 milligrams;
(*e*) in the case of pyridoxine, 0.25 milligram; and
(*f*) in the case of ascorbic acid, 7.5 milligrams.

D.01.009. Subject to section D.01.010, no person shall sell a food to which any of the following vitamins have been added unless a reasonable daily intake of that food by a person would result in the daily intake by such person of not less than,
(*a*) in the case of vitamin A, 1,600 International Units;
(*b*) in the case of thiamine, 0.6 milligram;
(*c*) in the case of riboflavin, 1.0 milligram;
(*d*) in the case of niacin or niacinamide, 6 milligrams;
(*e*) in the case of ascorbic acid, 20 milligrams; and
(*f*) in the case of vitamin D, 300 International Units.

D.01.010. Where a food to which a vitamin has been added is represented as being solely for use in the feeding of children under two years of age, no person shall sell such

(v) dans le cas de l'acide ascorbique, au moins 15 milligrammes, et
(vi) dans le cas de la vitamine D, au moins 300 unités internationales.

D.01.006. Une personne peut, dans la publicité qu'elle fait au sujet d'un aliment auquel une vitamine a été ajoutée ou sur l'étiquette d'un tel aliment, déclarer,
a) que cette vitamine a été ajoutée à l'aliment; et
b) que telle quantité de l'aliment contient telle quantité de la vitamine ajoutée.

D.01.007. Il est interdit de vendre un aliment auquel une vitamine a été ajoutée à moins que ne soit inscrit, sur l'étiquette de l'aliment, le nom de la vitamine ajoutée et la quantité de cette vitamine qui contient l'aliment exprimée
a) dans le cas des vitamines A, D et E, en unités internationales par 100 grammes ou 100 millilitres de cet aliment; et
b) dans le cas de la thiamine, de la riboflavine, de la niacine, de la niacinamide, de la pyridoxine, de l'acide d-pantothénique, de l'acide folique, de la biotine, de la vitamine B_{12}, de l'acide ascorbique ou de la vitamine K, en milligrammes par 100 grammes ou 100 millilitres de cet aliment.

D.01.008. Lorsqu'un aliment auquel aucune vitamine n'a été ajoutée est présenté comme étant destiné exclusivement à l'alimentation des enfants de moins de deux ans, une personne peut déclarer sur l'étiquette de l'aliment la quantité de l'une des vitamines suivantes que contient l'aliment, si une ration quotidienne normale de cet aliment apporte à un enfant de moins de deux ans qui la consomme,
a) dans le cas de la vitamine A, au moins 600 unités internationales,
b) dans le cas de la thiamine, au moins 0.25 milligramme,
c) dans le cas de la riboflavine, au moins 0.4 milligramme,
d) dans le cas de la niacine ou de la niacinamide, au moins 2.5 milligrammes,
e) dans le cas de la pyridoxine, au moins 0.25 milligramme, et
f) dans le cas de l'acide ascorbique, au moins 7.5 milligrammes.

D.01.009. Sous réserve de l'article D.01.010, il est interdit de vendre un aliment auquel l'une des vitamines suivantes a été ajoutée, à moins qu'une ration quotidienne normale de cet aliment n'apporte à une personne qui la consomme,
a) dans le cas de la vitamine A, au moins 1,600 unités internationales,
b) dans le cas de la thiamine, au moins 0.6 milligramme,
c) dans le cas de la riboflavine, au moins 1 milligramme,
d) dans le cas de la niacine ou de la niacinamide, au moins 6 milligrammes,
e) dans le cas de l'acide ascorbique, au moins 20 milligrammes, et
f) dans le cas de la vitamine D, au moins 300 unités internationales.

D.01.010. Lorsqu'un aliment auquel a été ajoutée une vitamine est présenté comme étant destiné exclusivement à l'alimentation des enfants de moins de deux ans, il est

food unless a reasonable daily intake of that food by a child under two years of age would result in the daily intake by the child of not less than,

(*a*) in the case of vitamin A, 1,000 International Units;
(*b*) in the case of thiamine, 0.4 milligram;
(*c*) in the case of riboflavin, 0.6 milligram;
(*d*) in the case of niacin or niacinamide, 4 milligrams;
(*e*) in the case of pyridoxine, 0.6 milligram;
(*f*) in the case of ascorbic acid, 20 milligrams;
(*g*) in the case of vitamin D, 300 International Units; and
(*h*) in the case of vitamin E, 5 International Units.

D.01.011. No person shall sell a food to which any of the following vitamins have been added if a reasonable daily intake of that food by a person would result in the daily intake by such person of more than,

(*a*) in the case of vitamin A, 2,500 International Units;
(*b*) in the case of thiamine, 2 milligrams;
(*c*) in the case of riboflavin, 3 milligrams;
(*d*) in the case of niacin or niacinamide, 20 milligrams;
(*e*) in the case of pyridoxine, 1.5 milligrams;
(*f*) in the case of ascorbic acid, 60 milligrams;
(*g*) in the case of vitamin D, 400 International Units; and
(*h*) in the case of vitamin E, 15 International Units.

D.01.012. No person shall, in advertising a food that is represented as containing a vitamin or on a label of such food,

(*a*) give any assurance or guarantee of any kind with respect to the result that may be, has been or will be obtained by the addition of the vitamin to a person's diet; or
(*b*) refer to, reproduce or quote any testimonial.

DIVISION 2

Mineral Nutrients in Foods

D.02.001. (1) In this Division, "mineral nutrient" means any of the following chemical elements whether alone or in a compound with one or more other chemical elements:

(*a*) calcium;
(*b*) phosphorus;
(*c*) iron;
(*d*) sodium;
(*e*) potassium;
(*f*) iodine;
(*g*) zinc;
(*h*) copper;
(*i*) magnesium; and
(*j*) manganese.

interdit de vendre un tel aliment à moins qu'une ration quotidienne normale de cet aliment n'apporte à un enfant de moins de deux ans qui la consomme,

a) dans le cas de la vitamine A, au moins 1,000 unités internationales,
b) dans le cas de la thiamine, au moins 0.4 milligramme,
c) dans le cas de la riboflavine, au moins 0.6 milligramme,
d) dans le cas de la niacine ou de la niacinamide, au moins 4 milligrammes,
e) dans le cas de la pyridoxine, au moins 0.6 milligramme,
f) dans le cas de l'acide ascorbique, au moins 20 milligrammes,
g) dans le cas de la vitamine D, au moins 300 unités internationales, et
h) dans le cas de la vitamine E, au moins 5 unités internationales.

D.01.011. Il est interdit de vendre un aliment auquel une des vitamines suivantes a été ajoutée si une ration quotidienne normale de cet aliment apporte à une personne qui la consomme,

a) dans le cas de la vitamine A, plus de 2,500 unités internationales,
b) dans le cas de la thiamine, plus de 2 milligrammes,
c) dans le cas de la riboflavine, plus de 3 milligrammes,
d) dans le cas de la niacine ou de la niacinamide, plus de 20 milligrammes,
e) dans le cas de la pyridoxine, plus de 1.5 milligramme,
f) dans le cas de l'acide ascorbique, plus de 60 milligrammes,
g) dans le cas de la vitamine D, plus de 400 unités internationales, et
h) dans le cas de la vitamine E, plus de 15 unités internationales.

D.01.012. Il est interdit, dans la publicité faite au sujet d'un aliment présenté comme contenant une vitamine ou sur l'étiquette d'un tel aliment,

a) de donner quelque garantie que ce soit quant à l'effet que peut produire, qu'a produit ou que produira l'addition de la vitamine au régime d'une personne, ou
b) de mentionner, reproduire ou citer un témoignage quelconque.

TITRE 2

Minéraux nutritifs dans les aliments

D.02.001. (1) Dans le présent titre, «minéral nutritif» désigne n'importe lequel des éléments chimiques suivants, soit seul, soit en combinaison avec un ou plusieurs autres éléments chimiques:

a) calcium,
b) phosphore,
c) fer,
d) sodium,
e) potassium,
f) iode,
g) zinc,
h) cuivre,
i) magnésium, et
j) manganèse.

(2) This Division applies only to foods that are represented as containing a mineral nutrient for use in human nutrition.

D.02.002. Subject to sections D.02.003 to D.02.007, no person shall, in advertising a food or on a label of a food, make any statement with respect to or any claim based on the mineral nutrient content of that food.

D.02.003. Where the amount of any of the following mineral nutrients that are contained in a food is not less than the amount mentioned in paragraph (*b*) of section D.02.004 in respect of that mineral nutrient, a person may, in advertising that food or on a label of that food, state,

(*a*) in the case of calcium or phosphorus, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood;

(*b*) in the case of iron, that it is a factor in the prevention of iron deficiency; and

(*c*) in the case of calcium, phosphorus or iron, that it is a factor in the maintenance of good health.

D.02.004. A person may, in advertising a food to which no mineral nutrient has been added or on a label of such food, state

(*a*) that the food is "a good source" or "a good dietary source" of any of the following mineral nutrients, if a reasonable daily intake of that food by a person would result in the daily intake by such person of not less than,

(i) in the case of calcium, 150 milligrams,

(ii) in the case of phosphorus, 150 milligrams, and

(iii) in the case of iron, 2 milligrams; or

(*b*) that the food is "an excellent source" of any of the following nutrients, if a reasonable daily intake of that food by a person would result in the daily intake by such person of not less than,

(i) in the case of calcium, 300 milligrams,

(ii) in the case of phosphorus, 300 milligrams, and

(iii) in the case of iron, 4 milligrams.

D.02.005. A person may, in advertising a food to which a mineral nutrient has been added or on a label of such food, state

(*a*) that the food contains the added mineral nutrient; and

(*b*) the amount of the added mineral nutrient that is contained in a specified quantity of the food.

D.02.006. No person shall sell any food, other than salt for table or general household use, to which a mineral nutrient has been added unless the amount of the mineral nutrient present in the food is expressed on the label of the food

(*a*) by using the name for that mineral nutrient that is used in section D.02.001, and

(*b*) in milligrams per one hundred grams or millilitres of the food.

D.02.007. Where a food to which no mineral nutrient has been added is represented as being solely for use in the feeding of children under two years of age, a person

(2) Le présent titre ne s'applique qu'aux aliments présentés comme contenant un minéral nutritif pour usage dans l'alimentation humaine.

D.02.002. Sous réserve des articles D.02.003 à D.02.007, est interdite, dans la publicité faite au sujet d'un aliment ou sur l'étiquette d'un aliment, toute déclaration au sujet de la teneur en minéral nutritif de cet aliment ou toute affirmation fondée sur la teneur en minéral nutritif de cet aliment.

D.02.003. Lorsque la quantité d'un des minéraux nutritifs suivants, contenus dans un aliment, n'est pas au moins égale à la quantité mentionnée à l'alinéa *b*) de l'article D.02.004 pour ce minéral nutritif, une personne peut, dans la publicité qu'elle fait au sujet de cet aliment ou sur l'étiquette de cet aliment, déclarer,

a) dans le cas du calcium et du phosphore, que ces minéraux nutritifs contribuent au développement normal et à l'entretien des os et des dents, surtout durant l'enfance;

b) dans le cas du fer, que ce minéral nutritif contribue à prévenir la carence de fer; et

c) dans le cas du calcium, du phosphore et du fer, que ces minéraux nutritifs contribuent à maintenir une bonne santé.

D.02.004. Une personne peut, dans la publicité qu'elle fait au sujet d'un aliment auquel aucun minéral nutritif n'a été ajouté ou sur l'étiquette d'un tel aliment, déclarer,

a) que cet aliment est «une bonne source» ou «une bonne source alimentaire» d'un des minéraux nutritifs suivants, si la ration quotidienne normale de cet aliment apporte à une personne qui la consomme,

(i) dans le cas du calcium, au moins 150 milligrammes,

(ii) dans le cas du phosphore, au moins 150 milligrammes, et

(iii) dans le cas du fer, au moins 2 milligrammes; ou

b) que l'aliment est «une excellente source» ou «une excellente source alimentaire» d'un des minéraux nutritifs suivants, si la ration quotidienne normale de cet aliment apporte à une personne qui la consomme,

(i) dans le cas du calcium, au moins 300 milligrammes,

(ii) dans le cas du phosphore, au moins 300 milligrammes, et

(iii) dans le cas du fer, au moins 4 milligrammes.

D.02.005. Une personne peut, dans la publicité qu'elle fait au sujet d'un aliment auquel aucun minéral nutritif a été ajouté ou sur l'étiquette d'un tel aliment, déclarer,

a) que ce minéral nutritif a été ajouté à l'aliment; et

b) que telle quantité de l'aliment contient telle quantité du minéral nutritif ajouté.

D.02.006. Il est interdit de vendre un aliment, autre qu'un sel de table ou d'usage domestique général, auquel un minéral nutritif a été ajouté à moins que la quantité de ce minéral nutritif que contient l'aliment ne soit exprimée sur l'étiquette de l'aliment

a) en utilisant le nom qui désigne ce minéral nutritif à l'article D.02.001, et

b) en milligrammes par cent grammes ou cent millilitres d'aliment.

D.02.007. Lorsqu'un aliment auquel aucun minéral nutritif n'a été ajouté est présenté comme étant destiné exclusivement à l'alimentation des enfants de moins de deux

may state on the label of the food the amount of any of the following mineral nutrients that are present in the food, if a reasonable daily intake of that food by a child under two years of age would result in the daily intake by the child of not less than,

(*a*) in the case of calcium, 150 milligrams;
(*b*) in the case of phosphorus, 150 milligrams;
(*c*) in the case of iron, 2 milligrams; and
(*d*) in the case of iodine, 0.05 milligram.

D.02.008. No person shall, in advertising a food that is represented as containing a mineral nutrient or on a label of such food,

(*a*) give any assurance or guarantee of any kind with respect to the result that may be, has been or will be obtained by the addition of the mineral nutrient to a person's diet, or
(*b*) refer to, reproduce or quote any testimonial.

D.02.009. No person shall sell a food to which any of the following mineral nutrients have been added unless a reasonable daily intake of that food by a person would result in the daily intake by such person of not less than,

(*a*) in the case of calcium, 300 milligrams;
(*b*) in the case of phosphorus, 300 milligrams;
(*c*) in the case of iron, 4 milligrams; and
(*d*) in the case of iodine, 0.10 milligram.

DIVISION 3

Foods to which Vitamins, Mineral Nutrients or Amino Acids may be Added

D.03.001. In this Division, the expressions "vitamin" and "mineral nutrient" have the same meaning as in Divisions 1 and 2.

D.03.002. Subject to section D.03.003, no person shall sell a food to which a vitamin, mineral nutrient or amino acid has been added unless the food is listed in Column I of the Table to this section and the vitamin, mineral nutrient or amino acid, as the case may be, is listed opposite that food in Column II of the Table.

TABLE

Foods to which a vitamin, mineral nutrient or amino acid may be added

Column I Food	Column II Vitamin, Mineral Nutrient or Amino Acid
1. Breakfast cereals	Thiamine, riboflavin, niacin or niacinamide, iron.
2. Fruit nectars, fruit drinks and bases, concentrates and mixes for fruit drinks and a mixture of vegetable juices	Vitamin C.
3. Infant cereal products	Thiamine, riboflavin, niacin or niacinamide, calcium, phosphorus, iron, iodine.
4. Margarine and other similar substitutes for butter	Vitamin A, vitamin D.
5. Alimentary pastes	Thiamine, riboflavin, niacin or niacinamide, iron.

ans, une personne peut déclarer sur l'étiquette de l'aliment la quantité de l'un des minéraux nutritifs suivants, contenu dans l'aliment, si une ration quotidienne normale de cet aliment apporte à un enfant de moins de deux ans qui la consomme,

a) dans le cas du calcium, au moins 150 milligrammes,
b) dans le cas du phosphore, au moins 150 milligrammes,
c) dans le cas du fer, au moins 2 milligrammes, et
d) dans le cas de l'iode, au moins 0.05 milligramme.

D.02.008. Il est interdit, dans la publicité faite au sujet d'un aliment présenté comme contenant un minéral nutritif ou sur l'étiquette d'un tel aliment,

a) de donner quelque garantie que ce soit quant à l'effet que peut produire, qu'a produit ou que produira l'addition de ce minéral nutritif au régime d'une personne, ou
b) de mentionner, reproduire ou citer un témoignage quelconque.

D.02.009. Il est interdit de vendre un aliment auquel un des minéraux nutritifs suivants a été ajouté à moins qu'une ration quotidienne normale de cet aliment n'apporte à une personne qui la consomme,

a) dans le cas du calcium, au moins 300 milligrammes,
b) dans le cas du phosphore, au moins 300 milligrammes,
c) dans le cas du fer, au moins 4 milligrammes, et
d) dans le cas de l'iode, au moins 0.10 milligramme.

TITRE 3

Aliments auxquels des vitamines, des minéraux nutritifs ou des acides aminés peuvent être ajoutés

D.03.001. Dans le présent titre, les expressions «vitamine» et «minéral nutritif» ont le même sens que dans les titres 1 et 2 respectivement.

D.03.002. Sous réserve de l'article D.03.003, il est interdit de vendre un aliment auquel une vitamine, un minéral nutritif ou un acide aminé a été ajouté à moins que cet aliment ne figure à la colonne I du tableau du présent article et à moins que la vitamine, le minéral nutritif ou l'acide aminé, selon le cas, ne figure dans la colonne II dudit tableau en regard du nom de l'aliment.

TABLEAU

Aliments auxquels une vitamine, un minéral nutritif ou un acide aminé peuvent être ajoutés

Colonne I Aliment	Colonne II Vitamine, minéral nutritif ou acide aminé
1. Céréales à déjeuner	Thiamine, riboflavine, niacine ou niacinamide, fer.
2. Nectar aux fruits, breuvages aux fruits, bases, concentrés et mélanges pour breuvages aux fruits, et mélanges de jus de légumes	Vitamine C.
3. Produits céréaliers pour bébés	Thiamine, riboflavine, niacine ou niacinamide, calcium, phosphore, fer, iode.
4. Margarine et autres succédanés similaires du beurre	Vitamine A, vitamine D.
5. Pâtes alimentaires	Thiamine, riboflavine, niacine ou niacinamide, fer.

Column I Food	Column II Vitamin, Mineral Nutrient or Amino Acid
6. Prepared infant formulas	Vitamin A, thiamine, riboflavin, niacin or niacinamide, pyridoxine, d-pantothenic acid, folic acid, vitamin B12, vitamin C, vitamin D, vitamin E, calcium, phosphorus, iron, sodium, potassium, iodine, zinc, copper, magnesium, manganese, lysine, methionine, tryptophane.
7. Flavoured beverage mixes and bases recommended for addition to milk	Vitamin A, thiamine, niacin or niacinamide, vitamin C, iron.
8. Foods represented as meat substitutes	Lysine, methionine.
9. Ready breakfast, instant breakfast and other similar breakfast replacement foods however described	Vitamin A, thiamine, riboflavin, niacin or niacinamide, vitamin C, iron.
10. Condensed milk, milk, milk powder, sterilized milk	Vitamin D.
11. Modified skim milk, modified partly skimmed milk, any flavoured milk described in section B.08.016, chocolate drink, any flavoured dairy drink described in section B.08.023, skim milk, partly skimmed milk, partly skimmed milk powder, skim milk powder, any flavoured skimmed milk described in section B.08.026	Vitamin A, vitamin D.
12. Evaporated milk	Vitamin C, vitamin D.
13. Evaporated skim milk, concentrated skim milk, evaporated partly skimmed milk, concentrated partly skimmed milk	Vitamin A, vitamin C, vitamin D.
14. Apple juice, reconstituted apple juice, grape juice, reconstituted grape juice, pineapple juice, reconstituted pineapple juice, concentrated fruit juice, apple and any juice described in section B.11.132	Vitamin C.
15. Enriched flour	Thiamine, riboflavin, niacin or niacinamide, calcium, iron.
16. Enriched vitamin B white flour	Thiamine, riboflavin, niacin or niacinamide, iron.
17. Table salt, table salt substitutes	Iodine.

D.03.003. Section D.03.002 does not apply to unstandardized foods recommended for special dietary use that are not advertised.

DIVISION 4

Vitamins in Drugs

D.04.001. In this Division,

(*a*) "proper name" means for a vitamin listed in subparagraphs (i) to (xv) of paragraph (*b*) or for any salt or derivative of that vitamin a name for that vitamin listed in those subparagraphs; and

(*b*) "vitamin" means any of the following vitamins:

(i) vitamin A,

Colonne I Aliment	Colonne II Vitamine, minéral nutritif ou acide aminé
6. Formules préparées pour bébés	Vitamine A, thiamine, riboflavine, niacine ou niacinamide, pyridoxine, acide d-pantothénique, acide folique, vitamine B12, vitamine C, vitamine D, vitamine E, calcium, phosphore, fer, sodium, potassium, iode, zinc, cuivre, magnésium, manganèse, lysine, méthionine, tryptophane.
7. Mélanges et bases aromatisés qu'il est recommandé d'ajouter au lait	Vitamine A, thiamine, niacine ou niacinamide, vitamine C, fer.
8. Aliments présentés comme succédanés de la viande	Lysine, méthionine.
9. Déjeuner tout prêt, déjeuner instantané et autres aliments semblables de remplacement de déjeuner, quelle qu'en soit la description	Vitamine A, thiamine, riboflavine, niacine ou niacinamide, vitamine C, fer.
10. Lait condensé, lait, poudre de lait, lait stérilisé	Vitamine D.
11. Lait écrémé modifié, lait partiellement écrémé modifié, lait aromatisé conforme à l'article B.08.016, breuvage au chocolat, breuvage lacté aromatisé décrit à l'article B.08.023, lait écrémé, lait partiellement écrémé, poudre de lait partiellement écrémé, poudre de lait écrémé, lait écrémé aromatisé décrit à l'article B.08.026	Vitamine A, vitamine D.
12. Lait évaporé	Vitamine C, vitamine D.
13. Lait écrémé évaporé, lait écrémé concentré, lait partiellement écrémé évaporé, lait partiellement écrémé concentré	Vitamine A, vitamine C, vitamine D.
14. Jus de pomme, jus de pomme reconstitué, jus de raisin, jus de raisin reconstitué, jus d'ananas, jus d'ananas reconstitué, jus de fruits concentrés, jus de pomme et d'autres fruits décrits à l'article B.11.132	Vitamine C.
15. Farine enrichie	Thiamine, riboflavine, niacine ou niacinamide, calcium, fer.
16. Farine blanche enrichie en vitamine B.	Thiamine, riboflavine, niacine ou niacinamide, fer.
17. Sel de table, succédanés du sel de table	Iode.

D.03.003. L'article D.03.002 ne s'applique pas aux aliments non normalisés recommandés pour certains régimes spéciaux qui ne font pas l'objet d'une publicité.

TITRE 4

Vitamines dans les drogues

D.04.001. Dans le présent titre,

(*a*) «nom propre» désigne, pour l'une des vitamines, énumérées aux sous-alinéas (i) à (xv) de l'alinéa (*b*) ou pour tout sel ou dérivé de cette vitamine, le nom par lequel est désignée la vitamine audit alinéa; et

(*b*) «vitamine» désigne l'une des vitamines suivantes:

(i) vitamine A,

(ii) thiamine, thiamine hydrochloride or vitamin B_1,
(iii) riboflavin or vitamin B_2,
(iv) niacin or nicotinic acid,
(v) niacinamide or nicotinamide,
(vi) pyridoxine, pyridoxine hydrochloride or vitamin B_6,
(vii) d-pantothenic acid or pantothenic acid,
(viii) folic acid,
(ix) biotin,
(x) cyanocobalamin or vitamin B_{12},
(xi) ascorbic acid or vitamin C,
(xii) vitamin D or vitamin D_3,
(xiii) vitamin D or vitamin D_2,
(xiv) vitamin E,
(xv) vitamin K, or
(xvi) any salt or derivative of a vitamin listed in subparagraphs (i) to (xv).

D.04.002. This Division applies only to drugs for internal or parenteral use in humans.

D.04.003 (1) Subject to subsection (2), the potency of each vitamin present in a drug that is represented as containing a vitamin shall be stated on both the inner and outer labels of the drug by using the proper name for that vitamin in conjunction with a statement of the amount of the vitamin present in the drug expressed,

(*a*) where the drug is in solid or viscous liquid form,
(i) in International Units per gram of the drug in the case of vitamin A, vitamin D or vitamin E,
(ii) in milligrams per gram of the drug in the case of thiamine, riboflavin, niacin or niacinamide, pyridoxine, d-pantothenic acid, folic acid, vitamin C or vitamin K, or
(iii) in micrograms per gram of the drug in the case of biotin or vitamin B_{12}; or

(*b*) where the drug is in other than solid or viscous liquid form,
(i) in International Units per millilitre of the drug in the case of vitamin A, vitamin D or vitamin E,
(ii) in milligrams per millilitre of the drug in the case of thiamine, riboflavin, niacin or niacinamide, pyridoxine, d-pantothenic acid, folic acid, vitamin C or vitamin K, or
(iii) in micrograms per millilitre of the drug in the case of biotin or vitamin B_{12}.

(2) Where a drug is in tablet or capsule form, the potency of each vitamin present in the drug may be expressed per tablet or capsule of the drug.

(3) Where a vitamin present in a drug is a salt or derivative of a vitamin mentioned in subparagraphs (i) to (xv) of paragraph (*b*) of section D.04.001, the common name of the salt or derivative of that vitamin shall be shown on both the inner and outer labels of the drug.

D.04.004. No person shall sell a drug that is represented as containing a vitamin unless both the inner and outer labels of the drug show a month and a year and carry a statement that that month and year indicates the expiration of the period during which the drug will maintain its potency.

(ii) thiamine, chlorhydrate de thiamine ou vitamine B_1,
(iii) riboflavine ou vitamine B_2,
(iv) niacine ou acide nicotinique,
(v) niacinamide ou nicotinamide,
(vi) pyridoxine, chlorhydrate de pyridoxine ou vitamine B_6,
(vii) acide d-pantothénique ou acide pantothénique,
(viii) acide folique,
(ix) biotine,
(x) cyanocobalamine ou vitamine B_{12},
(xi) acide ascorbique ou vitamine C,
(xii) vitamine D ou vitamine D_3,
(xiii) vitamine D ou vitamine D_2,
(xiv) vitamine E,
(xv) vitamine K, ou
(xvi) tout sel ou dérivé d'une vitamine mentionnée aux sous-alinéas (i) à (xv).

D.04.002. Le présent titre ne s'applique qu'aux drogues d'usage interne ou parentéral chez les humains.

D.04.003. (1) Sous réserve du paragraphe (2), les étiquettes intérieure et extérieure d'une drogue présentée comme contenant une vitamine doivent porter, outre le nom propre de cette vitamine, une mention de son activité, soit la quantité de vitamine que contient la drogue, exprimée,

a) s'il s'agit d'une drogue présentée sous forme de solide ou de liquide visqueux,
(i) en unités internationales par gramme de drogue dans le cas des vitamines A, D, ou E,
(ii) en milligrammes par gramme de drogue dans le cas de la thiamine, de la riboflavine, de la niacine ou de la niacinamide, de la pyridoxine, de l'acide d-pantothénique, de l'acide folique, de la vitamine C ou de la vitamine K, ou
(iii) en microgrammes par gramme de drogue dans le cas de la biotine ou de la vitamine B_{12}; ou

b) s'il s'agit d'une drogue présentée sous une autre forme que celle de solide ou de liquide visqueux,
(i) en unités internationales par millilitre de drogue dans le cas des vitamines A, D, ou E,
(ii) en milligrammes par millilitre de drogue dans le cas de la thiamine, de la riboflavine, de la niacine ou de la niacinamide, de la pyridoxine, de l'acide d-pantothénique, de l'acide folique, de la vitamine C ou de la vitamine K, ou
(iii) en microgrammes par millilitre de drogue dans le cas de la biotine ou de la vitamine B_{12}.

(2) Lorsqu'une drogue est présentée sous forme de comprimés ou de capsules, l'activité de chacune des vitamines qu'elle contient peut être exprimée par comprimé ou par capsule.

(3) Lorsqu'une drogue contient un sel ou un dérivé d'une vitamine mentionnée aux sous-alinéas (i) à (xv) de l'alinéa *b*) de l'article D.04.001, le nom usuel de ce sel ou de ce dérivé doit figurer sur les étiquettes intérieure et extérieure de la drogue.

D.04.004. Il est interdit de vendre une drogue, présentée comme contenant une vitamine, à moins que les étiquettes intérieure et extérieure de la drogue ne portent une mention expresse du mois et de l'année qui marquent la fin de la période d'activité de la drogue.

D.04.005. The manufacturer of a drug that is represented as containing a vitamin shall

(*a*) carry out such investigations as are necessary to determine the period during which the drug will maintain the potency specified in respect of that vitamin pursuant to section D.04.003; and

(*b*) whenever requested to do so by the Director, submit to the Director a report, satisfactory to him in form and as to content, of all investigations carried out by the manufacturer pursuant to paragraph (*a*) and of all other investigations carried out by him to determine that the drug as sold maintains the potency of the vitamin specified pursuant to section D.04.003 during the period of time determined pursuant to paragraph (*a*).

D.04.006. No person shall, in advertising a drug or on a label of a drug, make any statement with respect to the action of

(*a*) pyridoxine;
(*b*) d-pantothenic acid;
(*c*) folic acid;
(*d*) biotin;
(*e*) vitamin B_{12};
(*f*) vitamin E;
(*g*) vitamin K; or
(*h*) any salt or derivative of any vitamin listed in paragraphs (*a*) to (*g*).

D.04.007. No person shall sell for oral use any product consisting of or that is represented as containing cod liver oil, an extract of cod liver, an extract of cod liver oil or an emulsion of cod liver oil unless

(*a*) the product contains vitamin A and vitamin D in an amount not less than the amount specified in respect of those vitamins in section D.04.009 or D.04.010, whichever is applicable; and

(*b*) the label of the product shows that it contains vitamin A and vitamin D.

D.04.008. No person shall sell for oral use any product consisting of or that is represented as containing halibut liver oil unless

(*a*) it contains vitamin A in an amount not less than the amount specified in respect of that vitamin in section D.04.009 or D.04.010, as the case may be; and

(*b*) the label therefor shows that it contains vitamin A.

D.04.009. Subject to section D.04.010, no person shall sell a drug that is represented as containing a vitamin unless

(*a*) the smallest recommended daily dosage of the drug is specified on the label, and

(*b*) the smallest recommended daily dosage of the drug specified on the label is an amount that, if consumed by a person, would result in the daily intake by such person of not less than

(i) in the case of vitamin A, 1,600 International Units,
(ii) in the case of thiamine, 0.6 milligram,
(iii) in the case of riboflavin, 1.0 milligram,
(iv) in the case of niacin or niacinamide, 6 milligrams,

D.04.005. Le fabricant d'une drogue présentée comme contenant une vitamine doit

a) faire les recherches nécessaires pour déterminer la période durant laquelle la vitamine que contient la drogue conserve l'activité qui lui est attribuée conformément à l'article D.04.003; et

b) si le Directeur général le lui demande, présenter à ce dernier, un rapport acceptable quant à la forme et au contenu sur toutes les recherches qu'il a effectuées conformément à l'alinéa *a*) et sur toutes les autres recherches effectuées par lui pour s'assurer que la drogue, telle qu'elle est vendue, conservera l'activité attribuée à la vitamine conformément à l'article D.04.003 durant la période déterminée conformément à l'alinéa *a*).

D.04.006. Il est interdit de faire, dans toute publicité au sujet d'une drogue ou sur l'étiquette d'une drogue, une déclaration concernant l'action de

a) la pyridoxine,
b) l'acide d-pantothénique,
c) l'acide folique,
d) la biotine,
e) la vitamine B_{12},
f) la vitamine E,
g) la vitamine K, ou de
h) tout sel ou dérivé d'une substance mentionnée aux alinéas *a*) à *g*).

D.04.007. Il est interdit de vendre, pour usage oral, un produit contenant ou présenté comme contenant de l'huile de foie de morue, un extrait de foie de morue, un extrait d'huile de foie de morue ou une émulsion d'huile de foie de morue, à moins que

a) le produit ne contienne de la vitamine A et de la vitamine D en quantités au moins égales aux quantités mentionnées pour ces vitamines à l'article D.04.009 ou D.04.010, selon le cas; et que

b) l'étiquette du produit n'indique qu'il contient de la vitamine A et de la vitamine D.

D.04.008. Il est interdit de vendre, pour usage oral, un produit contenant ou présenté comme contenant de l'huile de foie de flétan, à moins que

a) cette huile ne contienne de la vitamine A en quantité au moins égale à la quantité mentionnée pour cette vitamine à l'article D.04.009 ou D.04.010, selon le cas; et que

b) l'étiquette du produit n'indique qu'il contient de la vitamine A.

D.04.009. Sous réserve de l'article D.04.010, il est interdit de vendre une drogue présentée comme contenant une vitamine, à moins que

a) la plus petite dose quotidienne recommandée de la drogue ne soit spécifiée sur l'étiquette, et que

b) la plus petite dose quotidienne recommandée de la drogue, telle qu'elle est spécifiée sur l'étiquette, n'apporte à la personne qui la consomme,

(i) dans le cas de la vitamine A, au moins 1,600 unités internationales;
(ii) dans le cas de la thiamine, au moins 0.6 milligramme;
(iii) dans le cas de riboflavine, au moins 1.0 milligramme;
(iv) dans le cas de la niacine ou de la niacinamide, au moins 6 milligrammes;

(v) in the case of pyridoxine, 1 milligram,
(vi) in the case of d-pantothenic acid, 5 milligrams,
(vii) in the case of folic acid, 0.05 milligram,
(viii) in the case of vitamin B_{12}, 2 micrograms,
(ix) in the case of vitamin C, 20 milligrams,
(x) in the case of vitamin D, 200 International Units, and
(xi) in the case of vitamin E, 10 International Units.

(v) dans le cas de la pyridoxine, au moins 1 milligramme;
(vi) dans le cas de l'acide d-pantothénique, au moins 5 milligrammes;
(vii) dans le cas de l'acide folique, au moins 0.05 milligramme;
(viii) dans le cas de la vitamine B_{12}, au moins 2 microgrammes;
(ix) dans le cas de la vitamine C, au moins 20 milligrammes;
(x) dans le cas de la vitamine D, au moins 200 unités internationales; et
(xi) dans le cas de la vitamine E, au moins 10 unités internationales.

D.04.010. Where a drug that is represented as containa vitamin is recommended for use by children under six years of age, the smallest recommended daily dosage of the drug specified on a label for a child under six years of age may be an amount that, if consumed by a child, would result in the daily intake by the child or not less than

(*a*) in the case of vitamin A, 1,000 International Units;
(*b*) in the case of thiamine, 0.4 milligram;
(*c*) in the case of riboflavin, 0.6 milligram;
(*d*) in the case of niacin or niacinamide, 4 milligrams;
(*e*) in the case of pyridoxine, 0.6 milligram;
(*f*) in the case of d-pantothenic acid, 3 milligrams;
(*g*) in the case of folic acid, 0.05 milligram;
(*h*) in the case of vitamin B_{12}, 2 micrograms;
(*i*) in the case of vitamin C, 20 milligrams;
(*j*) n the case of vitamin D, 200 International Units; and
(*k*) in the case of vitamin E, 5 International Units.

D.04.010. Lorsqu'une drogue présentée comme contenant une vitamine est recommandée pour les enfants de moins de 6 ans, la plus petite dose quotidienne recommandée de la drogue, telle qu'elle est spécifiée sur l'étiquette pour un enfant de moins de six ans, peut être la quantité qui apporte à un tel enfant qui la consomme,

a) dans le cas de la vitamine A, au moins 1,000 unités internationales;
b) dans le cas de la thiamine, au moins 0.4 milligramme;
c) dans le cas de la riboflavine, au moins 0.6 milligramme;
d) dans le cas de la niacine ou de la niacinamide, au moins 4 milligrammes;
e) dans le cas de la pyridoxine, au moins 0.6 milligramme;
f) dans le cas de l'acide d-pantothénique, au moins 3 milligrammes;
g) dans le cas de l'acide folique, au moins 0.05 milligramme;
h) dans le cas de la vitamine B_{12}, au moins 2 microgrammes;
i) dans le cas de la vitamine C, au moins 20 milligrammes;
j) dans le cas de la vitamine D, au moins 200 unités internationales; et
k) dans le cas de la vitamine E, au moins 5 unités internationales.

D.04.011. (1) Subject to subsections (2) and (3), no person shall sell or advertise a drug that is represented as containing a vitamin if the largest recommended daily dosage of that drug shown on a label thereof would, if consumed by a person, result in the daily intake by such person of more than

(*a*) in the case of vitamin A, 10,000 International Units;
(*b*) in the case of thiamine, 4.5 milligrams;
(*c*) in the case of riboflavin, 7.5 milligrams;
(*d*) in the case of niacin or niacinamide, 45 milligrams;
(*e*) in the case of pyridoxine, 3 milligrams;
(*f*) in the case of d-pantothenic acid, 15 milligrams;
(*g*) in the case of folic acid, 0.1 milligrams;
(*h*) in the case of vitamin B_{12}, 14 micrograms;
(*i*) in the case of vitamin C, 150 milligrams;

D.04.011. (1) Il est interdit, sous réserve des paragraphes (2) et (3), de vendre une drogue présentée comme contenant une vitamine, ou de faire de la publicité au sujet d'une telle drogue, si la plus forte dose quotidienne recommandée pour cette drogue, telle qu'elle est indiquée sur l'étiquette, apporte à une personne qui la consomme,

a) dans le cas de la vitamine A, plus de 10,000 unités internationales;
b) dans le cas de la thiamine, plus de 4.5 milligrammes;
c) dans le cas de la riboflavine, plus de 7.5 milligrammes;
d) dans le cas de la niacine ou de la niacinamide, plus de 45 milligrammes;
e) dans le cas de la pyridoxine, plus de 3 milligrammes;
f) dans le cas de l'acide d-pantothénique, plus de 15 milligrammes;
g) dans le cas de l'acide folique, plus de 0.1 milligramme;
h) dans le cas de la vitamine B_{12}, plus de 14 microgrammes;
i) dans le cas de la vitamine C, plus de 150 milligrammes;

(j) in the case of vitamin D, 400 International Units;
(k) in the case of vitamin E, 25 International Units; and
(l) in the case of vitamin K, 0.0 milligram.

(2) A person may sell a drug referred to in subsection (1) if both the inner and outer labels thereof carry a cautionary statement that the drug is for therapeutic use only.

(3) Subsection (1) does not apply to a drug
(a) that is prepared for parenteral use only; or
(b) that is required to be sold on prescription by Regulations made under the Act or the *Narcotic Control Act*.

D.04.012. No person shall, in advertising a drug or on a label of a drug, make any statement with respect to the action of any vitamin in the drug other than,
(a) in the case of vitamin C, that it is a factor in the normal development and maintenance of bones, cartilage, teeth and gums;
(b) in the case of vitamin D, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood; and
(c) in the case of any vitamin other than those mentioned in section D.04.006, that it is a factor in the maintenance of good health.

D.04.013. No person shall, in advertising a drug that is represented as containing a vitamin or on a label of such drug,
(a) give any assurance or guarantee of any kind with respect to the result that may be, has been or will be obtained from the use of the drug; or
(b) refer to, reproduce or quote any testimonial.

DIVISION 5

Minerals in Drugs

D.05.001. In this Division, "mineral" means any chemical element whether alone or in a compound with one or more other chemical elements.

D.05.002. This Division applies only to drugs that are represented as being for use in the treatment, mitigation or prevention of a dietary deficiency in humans.

D.05.003. (1) Subject to subsection (2), where a drug contains a mineral, both the inner and outer labels of the drug shall carry the chemical name of the mineral in conjunction with a statement of the amount of the mineral present in the drug expressed,
(a) where the drug is in solid or viscous liquid form, in milligrams per gram of the drug; or
(b) where the drug is in other than solid or viscous liquid form, in milligrams per millilitre of the drug.

(2) Where a drug is in tablet or capsule form, the amount of the mineral present in the drug may be expressed in milligrams per tablet or capsule of the drug.

f) dans le cas de la vitamine D, plus de 400 unités internationales;
k) dans le cas de la vitamine E, plus de 25 unités internationales, et
l) dans le cas de la vitamine K, plus de 0.0 milligramme.

(2) Une personne peut vendre une drogue mentionnée au paragraphe (1) à la condition que les étiquettes intérieure et extérieure de cette drogue portent une mise en garde indiquant que la drogue est réservée à l'usage thérapeutique.

(3) Le paragraphe (1) ne s'applique pas à une drogue
a) qui est préparée exclusivement pour usage parentéral; ou
b) qui, selon les exigences des règlements établis en vertu de la Loi ou de la *Loi sur les stupéfiants*, doit être vendue sur ordonnance.

D.04.012. Il est interdit de faire, dans toute publicité au sujet d'une drogue ou sur l'étiquette d'une drogue, une déclaration sur l'action d'une vitamine que contient la drogue sauf les déclarations suivantes:
a) dans le cas de la vitamine C, qu'elle contribue au développement normal et à l'entretien des os, des cartilages, des dents et des gencives;
b) dans le cas de la vitamine D, qu'elle contribue au développement normal et à l'entretien des os et des dents, surtout durant l'enfance; et
c) dans le cas d'une autre vitamine que celles qui sont mentionnées à l'article D.04.006, qu'elle contribue à maintenir une bonne santé.

D.04.013. Il est interdit, dans toute publicité faite au sujet d'une drogue présentée comme contenant une vitamine ou sur l'étiquette d'une telle drogue,
a) de donner quelque garantie que ce soit quant à l'effet que peut produire, qu'a produit ou que produira l'usage de la drogue; ou
b) de mentionner, reproduire ou citer un témoignage quelconque.

TITRE 5

Minéraux dans les drogues

D.05.001. Dans le présent titre «minéral» signifie tout élément chimique, soit seul, soit en combinaison avec un ou plusieurs autres éléments chimiques.

D.05.002. Le présent titre ne s'applique qu'aux drogues présentées comme étant destinées au traitement, au soulagement ou à la prévention d'une carence alimentaire chez l'homme.

D.05.003. (1) Lorsqu'une drogue contient un minéral, les étiquettes intérieure et extérieure de la drogue doivent porter le nom chimique du minéral ainsi qu'une déclaration de la quantité de minéral que contient la drogue, exprimée,
a) dans le cas d'une drogue présentée sous forme de solide ou de liquide visqueux, en milligrammes par gramme de drogue, et
b) dans le cas d'une drogue présentée sous une autre forme que celle de solide ou de liquide visqueux, en milligrammes par millilitre de drogue.

(2) Nonobstant le paragraphe (1), si une drogue est présentée sous forme de comprimés ou de capsules, la quantité de minéral que contient la drogue peut être exprimée en milligrammes par comprimé ou par capsule.

D.05.004. Subject to section D.05.005, no person shall, in advertising a drug or on a label of a drug, make any statement with respect to the action of any mineral in the drug.

D.05.005. Where a drug contains a mineral mentioned in this section, a person may, in advertising such drug or on a label of such drug, state,

(*a*) in the case of calcium or phosphorus, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood;

(*b*) in the case of iron, that it is a factor in the prevention of iron deficiency; and

(*c*) in the case of calcium, phosphorous or iron, that it is a factor in the maintenance of good health.

D.05.006. (1) Subject to subsection (2), no person shall

(*a*) sell a drug that is represented as containing a mineral other than calcium, phosphorus, iron or magnesium unless both the inner and outer labels of the drug carry a cautionary statement that the drug is for therapeutic use only; or

(*b*) advertise a drug that is represented as containing a mineral other than calcium, phosphorus, iron or magnesium.

(2) Subsection (1) does not apply to a drug

(*a*) that is prepared for parenteral use only; or

(*b*) that is required to be sold on prescription by Regulations made under the Act or the *Narcotic Control Act*.

D.05.007. No person shall sell a drug that contains a mineral mentioned in this section unless

(*a*) the smallest recommended daily dosage of the drug is specified on both the inner and outer labels of the drug; and

(*b*) the smallest recommended daily dosage of the drug specified on both the inner and outer labels is an amount that, if consumed by a person, would result in the daily intake by that person of not less than

(i) in the case of calcium, 125 milligrams,

(ii) in the case of phosphorus, 125 milligrams,

(iii) in the case of iron, 4 milligrams,

(iv) in the case of fluorine, 0.5 milligram,

(v) in the case of iodine, 0.05 milligram,

(vi) in the case of copper, 1.0 milligram, and

(vii) in the case of magnesium, 50 milligrams.

D.05.008. No person shall sell a drug containing fluorine if the largest recommended daily dosage of that drug as shown on the label thereof would, if consumed by a person, result in the daily intake by that person of more than one milligram of fluoride ion.

D.05.009. Where a drug contains fluorine, both the inner and outer labels of the drug shall carry a cautionary statement that, if the drug is used in an area where the drinking water has a natural fluorine content in

D.05.004. Sous réserve de l'article D.05.005, est interdite, dans la publicité faite au sujet d'une drogue ou sur l'étiquette d'une drogue, toute déclaration concernant l'action d'un minéral que contient la drogue.

D.05.005. Lorsqu'une drogue contient un minéral mentionné dans le présent article, une personne peut, dans la publicité qu'elle fait au sujet de cette drogue ou sur l'étiquette de la drogue, déclarer,

a) dans le cas du calcium ou du phosphore, qu'ils contribuent au développement normal et à l'entretien des os et des dents, surtout durant l'enfance;

b) dans le cas du fer, qu'il aide à prévenir la carence de fer; et

c) dans le cas du calcium, du phosphore ou du fer, qu'ils contribuent à maintenir une bonne santé.

D.05.006. (1) Sous réserve du paragraphe (2), il est interdit

a) de vendre une drogue présentée comme contenant un autre minéral que le calcium, le phosphore, le fer ou le magnésium, à moins que les étiquettes intérieure et extérieure de la drogue ne portent une mise en garde indiquant que la drogue est réservée à l'usage thérapeutique; ou

b) de faire de la publicité au sujet d'une drogue présentée comme contenant un minéral autre que le calcium, le phosphore, le fer ou le magnésium.

(2) Le paragraphe (1) ne s'applique pas à une drogue

a) qui est préparée exclusivement pour usage parentéral; ou

b) qui, selon les exigences des règlements établis en vertu de la Loi ou de la *Loi sur les stupéfiants*, doit être vendue sur ordonnance.

D.05.007. Il est interdit de vendre une drogue contenant un minéral mentionné dans le présent article à moins que

a) la plus petite dose quotidienne recommandée de cette drogue ne soit spécifiée sur les étiquettes intérieure et extérieure; et que

b) la plus petite dose quotidienne recommandée et spécifiée sur les étiquettes intérieure et extérieure de cette drogue n'apporte à une personne qui la consomme,

(i) dans le cas du calcium, au moins 125 milligrammes;

(ii) dans le cas du phosphore, au moins 125 milligrammes;

(iii) dans le cas du fer, au moins 4 milligrammes;

(iv) dans le cas du fluor, au moins 0.5 milligramme;

(v) dans le cas de l'iode, au moins 0.5 milligramme,

(vi) dans le cas du cuivre, au moins 1.0 milligramme et

(vii) dans le cas du magnésium, au moins 50 milligrammes.

D.05.008. Il est interdit de vendre une drogue contenant du fluor si la plus forte dose quotidienne recommandée de cette drogue, telle qu'elle est spécifiée sur l'étiquette de la drogue, apporte à une personne qui la consomme plus d'un milligramme d'ion fluor.

D.05.009. Lorsqu'une drogue contient du fluor, les étiquettes intérieure et extérieure de la drogue doivent porter une mise en garde précisant que, si la drogue est utilisée dans une région où l'eau potable naturelle a une teneur en

excess of 0.7 parts of fluoride ion per million parts of water or is artifically fluoridated, mottling of the tooth enamel of a user of the drug may result.

D.05.010. No person shall, in advertising a drug that is represented as containing a mineral or on a label of such a drug

(*a*) give any assurance or guarantee of satisfaction with respect to the result that may be, has been or will be obtained from the use of the drug; or

(*b*) refer to, reproduce or quote any testimonial."

2. The said Regulations are further amended by adding thereto the following Schedule:

fluor supérieure à 0.7 partie d'ion fluor par million de parties d'eau ou est fluorurée par des moyens artificiels, il peut s'ensuivre une tacheture de l'émail des dents chez l'usager de la drogue.

D.05.010. Il est interdit, dans toute publicité faite au sujet d'une drogue présentée comme contenant un minéral ou sur l'étiquette d'une telle drogue,

a) de donner une garantie de satisfaction quant à l'effet que peut produire, qu'a produit ou que produira l'usage de cette drogue; ou

b) de mentionner, reproduire ou citer un témoignage quelconque.»

2. Lesdits règlements sont en outre modifiés par l'addition de l'annexe ci-après:

SCHEDULE K

Reasonable Daily Intake for Various Foods

Column I Name and Description	Column II R.D.I.	
1. Alimentary Pastes, dry	3.0 oz.	(85 gm.)
2. Beverage Bases and Mixes, Flavoured, for Addition to Milk (ready to serve)	16.0 fl. oz.	(454 ml.)
3. Bread, 5 slices	5.3 oz.	(150 gm.)
4. Butter	2.0 oz.	(57 gm.)
5. Buttermilk	8.0 fl. oz.	(227 ml.)
6. Cereals, Breakfast or Infant	1.0 oz.	(28 gm.)
7. Cereals, puffed	0.5 oz.	(14 gm.)
8. Cheese (other than Cottage Cheese)	2.0 oz.	(57 gm.)
9. Cheese, Cottage	3.5 oz.	(100 gm.)
10. Chocolate Drink, Chocolate Milk	30.0 fl. oz.	(852 ml.)
11. Concentrated Milk, Concentrated Skim Milk (reconstituted and ready to serve)	30.0 fl. oz.	(852 ml.)
12. Condensed Milk	15.0 fl. oz.	(426 ml.)
13. Cream, whipping	2.0 oz.	(57 gm.)
14. (Naming the Flavour) Dairy Drink	30.0 fl. oz	(852 ml.)
15. Evaporated Milk, Evaporated Skim Milk	15.0 fl. oz.	(426 ml.)
16. Fish, Shell Fish	3.5 oz.	(100 gm.)
17. Fruits, dried	2.0 oz.	(57 gm.)
18. Fruits (other than banana, lemon, lime, watermelon)	3.5 oz.	(100 gm.)
19. Fruits, Banana	5.3 oz.	(150 gm.)
20. Fruits, Lemon	1.8 oz.	(50 gm.)
21. Fruits, Lime	1.8 oz.	(50 gm.)
22. Fruits, Watermelon	7.0 oz.	(200 gm.)
23. Fruit Drinks, Fruit Nectars (ready to serve)	4.0 fl. oz.	(114 ml.)
24. Fruit Drink Bases, Mixes and Concentrates (ready to serve)	4.0 fl. oz.	(114 ml.)
25. Fruit Juices (other than lemon juice and lime juice)	4.0 fl. oz.	(114 ml.)
26. Fruit Juices, Lemon	1.0 fl. oz.	(28 ml.)
27. Fruit Juices, Lime	1.0 fl. oz.	(28 ml.)
28. Ice Cream, Ice Milk	3.5 oz.	(100 gm.)
29. Infant Formulas, Prepared (ready to serve)	As Directed by Label	
30. Instant Breakfast, Ready Breakfast (ready to serve)	As Directed by Label	
31. Margarine	2.0 oz.	(57 gm.)
32. Meat, Prepared Meat	3.5 oz.	(100 gm.)
33. Meat Substitutes	3.5 oz.	(100 gm.)
34. Milk, whole	30.0 fl. oz.	(852 ml.)
35. Milk Powder (reconstituted and ready to serve)	30.0 fl. oz.	(852 ml.)
36. Modified Skim Milk	30.0 fl. oz.	(852 ml.)
37. Molasses, Table or Blackstrap	1.5 oz.	(43 gm.)
38. (Naming the Flavour) Milk	30.0 fl. oz.	(852 ml.)
39. Nuts	1.0 oz.	(28 gm.)
40. Peanut Butter	1.0 oz.	(28 gm.)

ANNEXE K

Ration quotidienne normale de diverses substances alimentaires

Colonne I Nom et description	Colonne II R.Q.N.	
1. Pâtes alimentaires sèches	3.0 oz.	(85 g)
2. Breuvages, bases et mélanges aromatisés pour ajouter au lait (prêts à servir)	16.0 oz. l.	(454 ml)
3. Pain, 5 tranches	5.3 oz.	(150 g)
4. Beurre	2.0 oz.	(57 g)
5. Babeurre (lait de beurre)	8.0 oz. l.	(227 ml)
6. Céréales à déjeuner pour bébé	1.0 oz.	(28 g)
7. Céréales soufflées	0.5 oz.	(14 g)
8. Fromage (autre que le fromage cottage)	2.0 oz.	(57 g)
9. Fromage cottage	3.5 oz.	(100 g)
10. Breuvages au chocolat, lait au chocolat	30.0 oz. l.	(852 ml)
11. Lait concentré, lait écrémé concentré (reconstitué et prêt à servir)	30.0 oz. l.	(852 ml)
12. Lait condensé	15.0 oz. l.	(426 ml)
13. Crème fouettée	2.0 oz.	(57 g)
14. Breuvages laités à (nom de l'arôme)	30.0 oz. l.	(852 ml)
15. Lait évaporé, lait écrémé évaporé	15.0 oz. l.	(426 ml)
16. Poissons, coquillages	3.5 oz.	(100 g)
17. Fruits secs	2.0 oz.	(57 g)
18. Fruits (sauf les bananes, citrons, limes, pastèques)	3.5 oz.	(100 g)
19. Fruits, banane	5.3 oz.	(150 g)
20. Fruits, citron	1.8 oz.	(50 g)
21. Fruits, lime	1.8 oz.	(50 g)
22. Fruits, pastèque	7.0 oz.	(200 g)
23. Breuvages aux fruits, nectars de fruits (prêts à servir)	4.0 oz. l.	(114 ml)
24. Breuvages aux fruits, bases, mélanges et concentrés (prêts à servir)	4.0 oz. l.	(114 ml)
25. Jus de fruits (autre que le jus de citron et le jus de lime)	4.0 oz. l.	(114 ml)
26. Jus de fruits, citron	1.0 oz. l.	(28 ml)
27. Jus de fruits, lime	1.0 oz. l.	(28 ml)
28. Crème glacée, lait glacé	3.5 oz.	(100 g)
29. Formules préparées pour bébés (prêtes à servir)	Selon l'étiquette	
30. Déjeuner instantané, déjeuner tout prêt (prêt à servir)	Selon l'étiquette	
31. Margarine	2.0 oz.	(57 g)
32. Viande, viande préparée	3.5 oz.	(100 g)
33. Viande, succédanés	3.5 oz.	(100 g)
34. Lait entier	30.0 oz. l.	(852 ml)
35. Lait en poudre (reconstitué et prêt à servir)	30.0 oz. l.	(852 ml)
36. Lait écrémé modifié	30.0 oz. l.	(852 ml)
37. Mélasse de table ou de cuisine	1.5 oz.	(43 g)
38. Lait aromatisé	30.0 oz. l.	(852 ml)
39. Noix	1.0 oz.	(28 g)
40. Beurre d'arachide	1.0 oz.	(28 g)

Column I Name and Description	Column II R.D.I.	
41. Poultry Meat, Prepared Poultry Meat	3.5 oz.	(100 gm.)
42. Skim Milk, Partly Skimmed Milk	30.0 fl. oz.	(852 ml.)
43. (Nonfat the Flavour) Skim Milk	30.0 fl. oz.	(852 ml.)
44. Skim Milk Powder, Partly Skimmed Milk Powder (reconstituted and ready to serve)	30.0 fl. oz.	(852 ml.)
45. Soup (ready to serve)	7.0 fl. oz.	(200 ml.)
46. Sterilized Milk	30.0 fl. oz.	(852 ml.)
47. Vegetable Juices	4.0 fl. oz.	(114 ml.)
48. Vegetables (other than baked beans and cooked potatoes)	3.5 oz.	(100 gm.)
49. Vegetables, baked beans	8.5 oz.	(250 gm.)
50. Vegetables, cooked potatoes	7.0 oz.	(200 gm.)
51. Yeast	0.5 oz.	(14 gm.)
52. Yogurt, plain	5.0 oz.	(150 gm.)

Colonne I Nom et description	Colonne II R.Q.N.	
41. Viande de volaille, viande de volaille préparée	3.5 oz.	(100 g)
42. Lait écrémé, lait partiellement écrémé	30.0 oz. l.	(852 ml)
43. Lait écrémé aromatisé	30.0 oz. l.	(852 ml)
44. Poudre de lait écrémé et poudre de lait partiellement écrémé (reconstitué et prêt à servir)	30.0 oz. l.	(852 ml)
45. Soupe (prête à servir)	7.0 oz. l.	(200 ml)
46. Lait stérilisé	30.0 oz. l.	(852 ml)
47. Jus de légumes	4.0 oz. l.	(114 ml)
48. Légumes (autre que les fèves au four et les pommes de terre cuites)	3.5 oz.	(100 g)
49. Légumes, fèves au four	8.5 oz.	(250 g)
50. Légumes, pommes de terre cuites	7.0 oz.	(200 g)
51. Levure	0.5 oz.	(14 g)
52. Yogourt, nature	5.0 oz.	(150 g)

SCHEDULE NO. 147

1. Part II of Schedule F to the *Food and Drug Regulations* is amended by adding thereto, immediately after item N.2 thereof, the following item:

"N.2A Neomycin and its salts"

LISTE N° 147

1. La partie II de l'annexe F des *Règlements des aliments et drogues* est modifiée par l'adjonction, immédiatement après le poste N. 2 du poste suivant:

«N.2A Néomycine et ses sels»

QUEEN'S PRINTER FOR CANADA, OTTAWA, 1971 IMPRIMEUR DE LA REINE POUR LE CANADA, OTTAWA, 1971

PART D

VITAMINS, MINERALS AND AMINO ACIDS

D.01.001. In this Part,

(*a*) "advertise" means to advertise to the general public;

(*b*) "reasonable daily intake" in respect of a food named in an item in Column I of Schedule K[1] means the amount of that food set out in Column II of that item; and

(*c*) "testimonial" with respect to a food or drug that is represented as containing a vitamin, mineral nutrient or mineral means any dramatized or undramatized pictorial, written or oral representation as to the result that is, has been or may be produced by the addition to a person's diet of that vitamin, mineral nutrient or mineral, as the case may be.

DIVISION 1

Vitamins in Foods

D.01.002. (1) In this Division, "vitamin" means any of the following vitamins:

(*a*) vitamin A,

(*b*) thiamine or vitamin B_1,

(*c*) riboflavin or vitamin B_2,

(*d*) niacin or nicotinic acid,

(*e*) niacinamide or nicotinamide,

(*f*) pyridoxine or vitamin B_6,

(*g*) d-pantothenic acid,

(*h*) folic acid,

(*i*) biotin,

(*j*) cyanocobalamin or vitamin B_{12},

(*k*) ascorbic acid or vitamin C,

(*l*) vitamin D or vitamin D_2,

(*m*) vitamin D or vitamin D_3,

(*n*) vitamin E,

(*o*) vitamin K, or

(*p*) any salt or derivative of a vitamin, listed in paragraphs (*a*) to (*o*).

(2) This Division applies only to foods that are represented as containing a vitamin for use in human nutrition.

D.01.003. Subject to sections D.01.004 to D.01.008, no person shall, in advertising a food or on a label of a food, make any statement with respect to or any claim based on the vitamin content of that food.

25-5-71 **D.01.004.** Where the amount of a vitamin referred to in this section that is contained in a food is not less than the amount mentioned in paragraph (*b*) of section D.01.005 in respect of that vitamin, a person may, in advertising that food or on a label of that food, state

(*a*) in the case of ascorbic acid, that it is a factor in the normal development and maintenance of bones, cartilage, teeth and gums;

(*b*) in the case of vitamin D, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood; and

(*c*) in the case of any vitamin listed in paragraph (*b*) of section D.01.005, that it is a factor in the maintenance of good health.

[1] See page 135D.

127, May 25, 1971.
Replaces page 127, February 23, 1971.

D.01.005. A person may, in advertising a food to which no vitamin has been added or on a label of such food, state

(*a*) that the food is "good source" or "a good dietary source" of any of the following vitamins, if a reasonable daily intake[1] of that food by a person would result in the daily intake by such person of not less than

(i) in the case of vitamin A, 600 International Units,
(ii) in the case of thiamine, 0.25 milligram,
(iii) in the case of riboflavin, 0.4 milligram,
(iv) in the case of niacin or niacinamide, 2.5 milligrams, and
(v) in the case of ascorbic acid, 7.5 milligrams; or

(*b*) that the food is "an excellent source" or "an excellent dietary source" of any of the following vitamins, if a reasonable daily intake[1] of that food by a person would result in the daily intake by such person of not less than

(i) in the case of vitamin A, 1,200 International Units,
(ii) in the case of thiamine, 0.45 milligram,
(iii) in the case of riboflavin, 0.75 milligram,
(iv) in the case of niacin or niacinamide, 4.5 milligrams,
(v) in the case of ascorbic acid, 15 milligrams, and
(vi) in the case of vitamin D, 300 International Units.

D.01.006. A person may, in advertising a food to which a vitamin has been added or on a label of such food, state

(*a*) that the food contains the added vitamin; and

(*b*) the amount of the added vitamin that is contained in a specified quantity of the food.

D.01.007. No person shall sell a food to which a vitamin has been added unless the amount of the vitamin present in the food is expressed on the label of the food

(*a*) in the case of vitamin A, vitamin D or vitamin E, in International Units per one hundred grams or millilitres of the food; and

(*b*) in the case of thiamine, riboflavin, niacin, niacinamide, pyridoxine, d-pantothenic acid, folic acid, biotin, vitamin B_{12}, ascorbic acid or vitamin K, in milligrams per one hundred grams or millilitres of the food,

together with the name of the vitamin.

D.01.008. Where a food to which no vitamin has been added is represented as being solely for use in the feeding of children under two years of age, a person may state on the label of the food the amount of any of the following vitamins that are present in the food, if a reasonable daily intake[1] of that food by a child under two years of age would result in the daily intake by the child of not less than

(*a*) in the case of vitamin A, 600 International Units;

(*b*) in the case of thiamine, 0.25 milligram;

(*c*) in the case of riboflavin, 0.4 milligram;

(*d*) in the case of niacin or niacinamide, 2.5 milligrams;

(*e*) in the case of pyridoxine, 0.25 milligram; and

(*f*) in the case of ascorbic acid, 7.5 milligrams.

D.01.009. Subject to section D.01.010, no person shall sell a food to which any of the following vitamins have been added unless a reasonable daily intake[1] of that food by a person would result in the daily intake by such person of not less than,

(*a*) in the case of vitamin A, 1,600 International Units;

(*b*) in the case of thiamine, 0.6 milligram;

(*c*) in the case of riboflavin, 1.0 milligram;

(*d*) in the case of niacin or niacinamide, 6 milligrams;

(*e*) in the case of ascorbic acid, 20 milligrams; and

(*f*) in the case of vitamin D, 300 International Units.

[1] See D.01.001 (*b*).

128, February 23, 1971.
Replaces page 128, March 25, 1965.

D.01.010. Where a food to which a vitamin has been added is represented as being solely for use in the feeding of children under two years of age, no person shall sell such food unless a reasonable daily intake[1] of that food by a child under two years of age would result in the daily intake by the child of not less than,

(*a*) in the case of vitamin A, 1,000 International Units;

(*b*) in the case of thiamine, 0.4 milligram;

(*c*) in the case of riboflavin, 0.6 milligram;

(*d*) in the case of niacin or niacinamide, 4 milligrams;

(*e*) in the case of pyridoxine, 0.6 milligram;

(*f*) in the case of ascorbic acid, 20 milligrams;

(*g*) in the case of vitamin D, 300 International Units; and

(*h*) in the case of vitamin E, 5 International Units.

D.01.011. No person shall sell a food to which any of the following vitamins have been added if a reasonable daily intake[1] of that food by a person would result in the daily intake by such person of more than,

(*a*) in the case of vitamin A, 2,500 International Units;

(*b*) in the case of thiamine, 2 milligrams;

(*c*) in the case of riboflavin, 3 milligrams;

(*d*) in the case of niacin or niacinamide, 20 milligrams;

(*e*) in the case of pyridoxine, 1.5 milligrams;

(*f*) in the case of ascorbic acid, 60 milligrams;

(*g*) in the case of vitamin D, 400 International Units; and

(*h*) in the case of vitamin E, 15 International Units.

D.01.012. No person shall, in advertising a food that is represented as containing a vitamin or on a label of such food,

(*a*) give any assurance or guarantee of any kind with respect to the result that may be, has been or will be obtained by the addition of the vitamin to a person's diet; or

(*b*) refer to, reproduce or quote any testimonial.[2]

[1] See D.01.001 (*b*).
[2] See D.01.001 (*c*).

129, February 23, 1971.
Replaces page 128A, June 18, 1964.

Mineral Nutrients in Foods

D.02.001. (1) In this Division, "mineral nutrient" means any of the following chemical elements whether alone or in a compound with one or more other chemical elements:

(*a*) calcium;
(*b*) phosphorus;
(*c*) iron;
(*d*) sodium;
(*e*) potassium;
(*f*) iodine;
(*g*) zinc;
(*h*) copper;
(*i*) magnesium; and
(*j*) manganese.

(2) This Division applies only to foods that are represented as containing a mineral nutrient for use in human nutrition.

D.02.002. Subject to sections D.02.003 to D.02.007, no person shall, in advertising a food or on a label of a food, make any statement with respect to or any claim based on the mineral nutrient content of that food.

D.02.003. Where the amount of any of the following mineral nutrients that are contained in a food is not less than the amount mentioned in paragraph (*b*) of section D.02.004 in respect of that mineral nutrient, a person may, in advertising that food or on a label of that food, state,

(*a*) in the case of calcium or phosphorus, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood;
(*b*) in the case of iron, that it is a factor in the prevention of iron deficiency; and
(*c*) in the case of calcium, phosphorus or iron, that it is a factor in the maintenance of good health.

D.02.004. A person may, in advertising a food to which no mineral nutrient has been added or on a label of such food, state

(*a*) that the food is "a good source" or "a good dietary source" of any of the following mineral nutrients, if a reasonable daily intake[1] of that food by a person would result in the daily intake by such person of not less than,
  (i) in the case of calcium, 150 milligrams,
  (ii) in the case of phosphorus, 150 milligrams, and
  (iii) in the case of iron, 2 milligrams; or
(*b*) that the food is "an excellent source" or "an excellent dietary source" of any of the following nutrients, if a reasonable daily intake[1] of that food by a person would result in the daily intake by such person of not less than,
  (i) in the case of calcium, 300 milligrams,
  (ii) in the case of phosphorus, 300 milligrams, and
  (iii) in the case of iron, 4 milligrams.

D.02.005. A person may, in advertising a food to which a mineral nutrient has been added or on a label of such food, state

(*a*) that the food contains the added mineral nutrient; and
(*b*) the amount of the added mineral nutrient that is contained in a specified quantity of the food.

D.02.006. No person shall sell any food, other than salt for table or general household use, to which a mineral nutrient has been added unless the amount of the mineral nutrient present in the food is expressed on the label of the food

(*a*) by using the name for that mineral nutrient that is used in section D.02.001, and
(*b*) in milligrams per one hundred grams or millilitres of the food.

[1] See D.01.001 (*b*).

130, February 23, 1971.
Replaces page 129, May 12, 1962.

D.02.007. Where a food to which no mineral nutrient has been added is represented as being safety for use in the feeding of children under two years of age, a person may state on the label of the food the amount of any of the following mineral nutrients that are present in the food, if a reasonable daily intake[1] of that food by a child under two years of age would result in the daily intake by the child of not less than,

(*a*) in the case of calcium, 150 milligrams;

(*b*) in the case of phosphorus, 150 milligrams;

(*c*) in the case of iron, 2 milligrams; and

(*d*) in the case of iodine, 0.05 milligram.

D.02.008. No person shall, in advertising a food that is represented as containing a mineral nutrient or on a label of such food,

(*a*) give any assurance or guarantee of any kind with respect to the result that may be, has been or will be obtained by the addition of the mineral nutrient to a person's diet, or

(*b*) refer to, reproduce or quote any testimonial.

D.02.009. No person shall sell a food to which any of the following mineral nutrients have been added unless a reasonable daily intake[1] of that food by a person would result in the daily intake by such person of not less than,

(*a*) in the case of calcium, 300 milligrams;

(*b*) in the case of phosphorus, 300 milligrams;

(*c*) in the case of iron, 4 milligrams; and

(*d*) in the case of iodine, 0.10 milligram.

[1] See D.01.001 (*b*).

131, February 23, 1971.
Replaces page 130, February 28, 1964.

DIVISION 3

Foods to which Vitamins, Mineral Nutrients or Amino Acids may be Added

D.03.001. In this Division, the expressions "vitamin" and "mineral nutrient" have the same meaning as in Divisions 1 and 2.

D.03.002. Subject to section D.03.003, no person shall sell a food to which a vitamin, mineral nutrient or amino acid has been added unless the food is listed in Column I of the Table to this section and the vitamin, mineral nutrient or amino acid, as the case may be, is listed opposite that food in Column II of the Table.

TABLE

FOODS TO WHICH A VITAMIN, MINERAL NUTRIENT OR AMINO ACID MAY BE ADDED

COLUMN I Food	COLUMN II Vitamin, Mineral Nutrient or Amino Acid
1. Breakfast cereals	Thiamine, riboflavin, niacin or niacinamide, iron.
12-4-72 2. Fruit nectars, fruit drinks, bases, concentrates and mixes for fruit drinks, bases and mixes for vegetable drinks, and a mixture of vegetable juices	Vitamin C.
3. Infant cereal products	Thiamine, riboflavin, niacin or niacinamide, calcium, phosphorus, iron, iodine.
4. Margarine and other similar substitutes for butter	Vitamin A, vitamin D.
5. Alimentary pastes	Thiamine, riboflavin, niacin or niacinamide, iron.
6. Prepared infant formulas	Vitamin A, thiamine, riboflavin, niacin or niacinamide, pyridoxine, d-pantothenic acid, folic acid, vitamin B_{12}, vitamin C, vitamin D, vitamin E, calcium, phosphorus, iron, sodium, potassium, iodine, zinc, copper, magnesium, manganese, lysine, methionine, tryptophane.
7. Flavoured beverage mixes and bases recommended for addition to milk	Vitamin A, thiamine, niacin or niacinamide, vitamin C, iron.
8. Foods represented as meat substitutes	Lysine, methionine.
9. Ready breakfast, instant breakfast and other similar breakfast replacement foods however described	Vitamin A, thiamine, riboflavin, niacin or niacinamide, vitamin C, iron.
10. Condensed milk, milk, milk powder, sterilized milk	Vitamin D.
11. Modified skim milk, modified partly skimmed milk, any flavoured milk described in section B.08.016, chocolate drink, any flavoured dairy drink described in section B.08.023, skim milk, partly skimmed milk, partly skimmed milk powder, skim milk powder, any flavoured skimmed milk described in section B.08.026	Vitamin A, vitamin D.
12. Evaporated milk	Vitamin C, vitamin D.
13. Evaporated skim milk, concentrated skim milk, evaporated partly skimmed milk, concentrated partly skimmed milk	Vitamin A, vitamin C, vitamin D.

132, April 12, 1972.
Replaces page 132, February 23, 1971.

COLUMN I Food	COLUMN II Vitamin, Mineral Nutrient or Amino Acid
14. Apple juice, reconstituted apple juice, grape juice, reconstituted grape juice, pineapple juice, reconstituted pineapple juice, concentrated fruit juice, apple and any juice described in section B.11.132	Vitamin C.
15. Enriched flour	Thiamine, riboflavin, niacin or niacinamide, calcium, iron.
16. Enriched vitamin B white flour	Thiamine, riboflavin, niacin or niacinamide, iron
17. Table salt, table salt substitutes	Iodine.

D.03.003. Section D.03.002 does not apply to unstandardized foods recommended for special dietary use that are not advertised.

133, February 23, 1971.
Replaces page 132, December 29, 1966.

DIVISION 4

Vitamins in Drugs

D.04.001. In this Division,

(*a*) "proper name" means for a vitamin listed in subparagraphs (i) to (xv) of paragraph (b) or for any salt or derivative of that vitamin a name for that vitamin listed in those subparagraphs; and

(*b*) "vitamin" means any of the following vitamins:

(i) vitamin A,
(ii) thiamine, thiamine hydrochloride or vitamin B_1,
(iii) riboflavin or vitamin B_2,
(iv) niacin or nicotinic acid,
(v) niacinamide or nicotinamide,
(vi) pyridoxine, pyridoxine hydrochloride or vitamin B_6,
(vii) d-pantothenic acid or pantothenic acid,
(viii) folic acid,
(ix) biotin,
(x) cyanocobalamin or vitamin B_{12},
(xi) ascorbic acid or vitamin C,
(xii) vitamin D or vitamin D_2,
(xiii) vitamin D or vitamin D_3,
(xiv) vitamin E,
(xv) vitamin K, or
(xvi) any salt or derivative of a vitamin listed in subparagraphs (i) to (xv).

D.04.002. This Division applies only to drugs for internal or parenteral use in humans.

D.04.003. (1) Subject to subsection (2), the potency of each vitamin present in a drug that is represented as containing a vitamin shall be stated on both the inner and outer labels of the drug by using the proper name for that vitamin in conjunction with a statement of the amount of the vitamin present in the drug expressed,

(*a*) where the drug, is in solid or viscous liquid form,

(i) in International Units per gram of the drug in the case of vitamin A, vitamin D or vitamin E,
(ii) in milligrams per gram of the drug in the case of thiamine, riboflavin, niacin or niacinamide, pyridoxine, d-pantothenic acid, folic acid, vitamin C or vitamin K, or
(iii) in micrograms per gram of the drug in the case of biotin or vitamin B_{12}; or

(*b*) where the drug is in other than solid or viscous liquid form,

(i) in International Units per millilitre of the drug in the case of vitamin A, vitamin D or vitamin E,
(ii) in milligrams per millilitre of the drug in the case of thiamine, riboflavin, niacin or niacinamide, pyridoxine, d-pantothenic acid, folic acid, vitamin C or vitamin K, or
(iii) in micrograms per millilitre of the drug in the case of biotin or vitamin B_{12}.

(2) Where a drug is in tablet or capsule form, the potency of each vitamin present in the drug may be expressed per tablet or capsule of the drug.

(3) Where a vitamin present in a drug is a salt or derivative of a vitamin mentioned in subparagraphs (i) to (xv) of paragraph (b) of section D.04.001, the common name of the salt or derivative of that vitamin shall be shown on both the inner and outer labels of the drug.

D.04.004. No person shall sell a drug that is represented as containing a vitamin unless both the inner and outer labels of the drug show a month and a year and carry a statement that that month and year indicates the expiration of the period during which the drug will maintain its potency.

D.04.005. The manufacturer of a drug that is represented as containing a vitamin shall

(*a*) carry out such investigations as are necessary to determine the period during which the drug will maintain the potency specified in respect of the vitamin pursuant to section D.04.003; and

(*b*) whenever requested to do so by the Director,[1] submit to the Director[1] a report, satisfactory to him in form and as to content, of all investigations carried out by the manufacturer pursuant to paragraph (a) and of all other investigations carried out by him to determine that the drug as sold maintains the potency of the vitamin specified pursuant to section D.04.003 during the period of time determined pursuant to paragraph (a).

[1] See A.01.010 (*d*).

D.04.006. No person shall, in advertising a drug or on a label of a drug, make any statement with respect to the action of

(*a*) pyridoxine;

(*b*) d-pantothenic acid;

(*c*) folic acid;

(*d*) biotin;

(*e*) vitamin B_{12};

(*f*) vitamin E;

(*g*) vitamin K; or

(*h*) any salt or derivative of any vitamin listed in paragraphs (*a*) to (*g*).

D.04.007. No person shall sell for oral use any product consisting of or that is represented as containing cod liver oil, an extract of cod liver, an extract of cod liver oil or an emulsion of cod liver oil unless

(*a*) the product contains vitamin A and vitamin D in an amount not less than the amount specified in respect of those vitamins in section D.04.009 or D.04.010, whichever is applicable; and

(*b*) the label of the product shows that it contains vitamin A and vitamin D.

D.04.008. No person shall sell for oral use any product consisting of or that is represented as containing halibut liver oil unless

(*a*) it contains vitamin A in an amount not less than the amount specified in respect of that vitamin in section D.04.009 or D.04.010, as the case may be; and

(*b*) the label therefor shows that it contains vitamin A.

D.04.009. Subject to section D.04.010, no person shall sell a drug that is represented as containing a vitamin unless

(*a*) the smallest recommended daily dosage of the drug is specified on the label, and

(*b*) the smallest recommended daily dosage of the drug specified on the label is an amount that, if consumed by a person, would result in the daily intake by such person of not less than

(i) in the case of vitamin A, 1,600 International Units,

(ii) in the case of thiamine, 0.6 milligram,

(iii) in the case of riboflavin, 1.0 milligram,

(iv) in the case of niacin or niacinamide, 6 milligrams,

(v) in the case of pyridoxine, 1 milligram,

(vi) in the case of d-pantothenic acid, 5 milligrams,

(vii) in the case of folic acid, 0.05 milligram,

(viii) in the case of vitamin B_{12}, 2 micrograms,

(ix) in the case of vitamin C, 20 milligrams,

(x) in the case of vitamin D, 200 International Units, and

(xi) in the case of vitamin E, 10 International Units.

D.04.010. Where a drug that is represented as containing a vitamin is recommended for use by children under six years of age, the smallest recommended daily dosage of the drug specified on a label for a child under six years of age may be an amount that, if consumed by a child, would result in the daily intake by the child of not less than

(*a*) in the case of vitamin A, 1,000 International Units;

(*b*) in the case of thiamine, 0.4 milligram;

(*c*) in the case of riboflavin, 0.6 milligram;

(*d*) in the case of niacin or niacinamide, 4 milligrams;

(*e*) in the case of pyridoxine, 0.6 milligram;

(*f*) in the case of d-pantothenic acid, 3 milligrams;

(*g*) in the case of folic acid, 0.05 milligram;

(*h*) in the case of vitamin B_{12}, 2 micrograms;

(*i*) in the case of vitamin C, 20 milligrams;

(*j*) in the case of vitamin D, 200 International Units; and

(*k*) in the case of vitamin E, 5 International Units.

135, February 23, 1971.
Replaces page 134, September 8, 1966.

D.04.011. (1) Subject to subsections (2) and (3), no person shall sell or advertise a drug that is represented as containing a vitamin if the largest recommended daily dosage of that drug shown on a label thereof would, if consumed by a person, result in the daily intake by such person of more than

(*a*) in the case of vitamin A, 10,000 International Units;

(*b*) in the case of thiamine, 4.5 milligrams;

(*c*) in the case of riboflavin, 7.5 milligrams;

(*d*) in the case of niacin or niacinamide, 45 milligrams;

(*e*) in the case of pyridoxine, 3 milligrams;

(*f*) in the case of d-pantothenic acid, 15 milligrams;

(*g*) in the case of folic acid, 0.1 milligram;

(*h*) in the case of vitamin B_{12}, 14 micrograms;

(*i*) in the case of vitamin C, 150 milligrams;

(*j*) in the case of vitamin D, 400 International Units;

(*k*) in the case of vitamin E, 25 International Units; and

(*l*) in the case of vitamin K, 0.0 milligram.

(2) A person may sell on prescription to the general public,

(*a*) a drug represented as containing a vitamin referred to in paragraph (1) (*a*), or

(*b*) a drug represented as containing vitamin D, if the largest recommended daily dosage shown on the label thereof would, if consumed by a person, result in the daily intake by that person of more than 1000 International Units.

(2a) A person may sell a drug referred to in paragraph (2) (*a*) or (*b*) that carries the symbol Pr on the upper left quarter on the main panel of both the inner and outer labels to

(*a*) a drug manufacturer,

(*b*) a practitioner,

(*c*) a wholesale druggist,

(*d*) a registered pharmacist,

9-5-72 (*e*) a hospital certified by the Department of National Health and Welfare,

(*f*) a Department of the Government of Canada or of a province, upon receipt of a written order signed by the Minister thereof or his duly authorized representative, or

(*g*) any other person, upon receipt of a written order signed by the Director.

(2b) A person may sell

(*a*) a drug represented as containing a vitamin referred to in paragraphs (*b*) to (*i*) and (*k*) to (*l*) of subsection (1), or

(*b*) a drug represented as containing vitamin D, if the largest recommended daily dosage shown on the label thereof would, if consumed by a person, result in the daily intake by that person of more than 400 but not more than 1000, International Units,

if both the inner and outer labels thereof carry a cautionary statement that the drug is for therapeutic use only.

(3) Subsection (1) does not apply to a drug

(*a*) that is prepared for parenteral use only; or

(*b*) that is required to be sold on prescription by Regulations made under the Act[1] or the *Narcotic Control Act*.

D.04.012. No person shall, in advertising a drug or on a label of a drug, make any statement with respect to the action of any vitamin in the drug other than,

(*a*) in the case of vitamin C, that it is a factor in the normal development and maintenance of bones, cartilage, teeth and gums;

(*b*) in the case of vitamin D, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood; and

(*c*) in the case of any vitamin other than those mentioned in section D.04.006, that it is a factor in the maintenance of good health.

[1] See A.01.010 (*b*).

135A, May 9, 1972.
Replaces page 135A, May 25, 1971.

D.04.013. No person shall, in advertising a drug that is represented as containing a vitamin or on a label of such drug,

(a) give any assurance or guarantee of any kind with respect to the result that may be, has been or will be obtained from the use of the drug; or

(b) refer to, reproduce or quote any testimonial.[1]

[1] See D.01.001 (d).

DIVISION 5

Minerals in Drugs

D.05.001. In this Division, "mineral" means any chemical element whether alone or in a compound with one or more other chemical elements.

D.05.002. This Division applies only to drugs that are represented as being for use in the treatment, mitigation or prevention of a dietary deficiency in humans.

D.05.003. (1) Subject to subsection (2), where a drug contains a mineral, both the inner and outer labels of the drug shall carry the chemical name of the mineral in conjunction with a statement of the amount of the mineral present in the drug expressed,

(*a*) where the drug is in solid or viscous liquid form, in milligrams per gram of the drug; or

(*b*) where the drug is in other than solid or viscous liquid form, in milligrams per millilitre of the drug.

(2) Where a drug is in tablet or capsule form, the amount of the mineral present in the drug may be expressed in milligrams per tablet or capsule of the drug.

D.05.004. Subject to section D.05.005, no person shall, in advertising a drug or on a label of a drug, make any statement with respect to the action of any mineral in the drug.

D.05.005. Where a drug contains a mineral mentioned in this section, a person may, in advertising such drug or on a label of such drug, state,

(*a*) in the case of calcium or phosphorus, that it is a factor in the normal development and maintenance of bones and teeth, especially in infancy and childhood;

(*b*) in the case of iron, that it is a factor in the prevention of iron deficiency; and

(*c*) in the case of calcium, phosphorus or iron, that it is a factor in the maintenance of good health.

D.05.006. (1) Subject to subsection (2), no person shall

(*a*) sell a drug that is represented as containing a mineral other than calcium, phosphorus, iron, fluorine, iodine, copper or magnesium unless both the inner and outer labels of the drug carry a cautionary statement that the drug is for therapeutic use only; or

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(*b*) advertise a drug that is represented as containing a mineral other than calcium, phosphorus, iron, fluorine, iodine, copper or magnesium.

(2) Subsection (1) does not apply to a drug

(*a*) that is prepared for parenteral use only; or

(*b*) that is required to be sold on prescription by Regulations made under the Act[1] or the *Narcotic Control Act*.

D.05.007. No person shall sell a drug that contains a mineral mentioned in this section unless

(*a*) the smallest recommended daily dosage of the drug is specified on both the inner and outer labels of the drug; and

(*b*) the smallest recommended daily dosage of the drug specified on both the inner and outer labels is an amount that, if consumed by a person, would result in the daily intake by that person of not less than

(i) in the case of calcium, 125 milligrams,
(ii) in the case of phosphorus, 125 milligrams,
(iii) in the case of iron, 4 milligrams,
(iv) in the case of fluorine, 0.5 milligram,
(v) in the case of iodine, 0.05 milligram,
(vi) in the case of copper, 1.0 milligram, and
(vii) in the case of magnesium, 50 milligrams.

D.05.008. No person shall sell a drug containing fluorine if the largest recommended daily dosage of that drug as shown on the label thereof would, if consumed by a person, result in the daily intake by that person of more than one milligram of fluoride ion.

[1] See A.01.010 (*b*).

135B, May 25, 1971.
Replaces page 135B, February 23, 1971.

D.05.009. Where a drug contains fluorine, both the inner and outer labels of the drug shall carry a cautionary statement that, if the drug is used in an area where the drinking water has a natural fluorine content in excess of 0.7 parts of fluoride ion per million parts of water or is artificially fluoridated, mottling of the tooth enamel of a user of the drug may result.

D.05.010. No person shall, in advertising a drug that is represented as containing a mineral or on a label of such a drug

(a) give any assurance or guarantee of satisfaction with respect to the result that may be, has been or will be obtained from the use of the drug; or

(b) refer to, reproduce or quote any testimonial.[1]

[1] See D.01.001 (c).

Schedule K

REASONABLE DAILY INTAKE[1] FOR VARIOUS FOODS

Column I Name and Description	Column II R.D.I.	
1. Alimentary Pastes, dry	3.0 oz.	(85 gm.)
2. Beverage Bases and Mixes, Flavoured, for Addition to Milk (ready to serve)	16.0 fl. oz.	(454 ml.)
3. Bread, 5 slices	5.3 oz.	(150 gm.)
4. Butter	2.0 oz.	(57 gm.)
5. Buttermilk	8.0 fl. oz.	(227 ml.)
6. Cereals, Breakfast or Infant	1.0 oz.	(28 gm.)
7. Cereals, puffed	0.5 oz.	(14 gm.)
8. Cheese (other than Cottage Cheese)	2.0 oz.	(57 gm.)
9. Cheese, Cottage	3.5 oz.	(100 gm.)
10. Chocolate Drink, Chocolate Milk	30.0 fl. oz.	(852 ml.)
11. Concentrated Milk, Concentrated Skim Milk (reconstituted and ready to serve)	30.0 fl. oz.	(852 ml.)
12. Condensed Milk	15.0 fl. oz.	(426 ml.)
13. Cream, whipping	2.0 oz.	(57 gm.)
14. (Naming the Flavour) Dairy Drink	30.0 fl. oz.	(852 ml.)
15. Evaporated Milk, Evaporated Skim Milk	15.0 fl. oz.	(426 ml.)
16. Fish, Shell Fish	3.5 oz.	(100 gm.)
17. Fruits, dried	2.0 oz.	(57 gm.)
18. Fruits (other than banana, lemon, lime, watermelon)	3.5 oz.	(100 gm.)
19. Fruits, Banana	5.3 oz.	(150 gm.)
20. Fruits, Lemon	1.8 oz.	(50 gm.)
21. Fruits, Lime	1.8 oz.	(50 gm.)
22. Fruits, Watermelon	7.0 oz.	(200 gm.)
23. Fruit Drinks, Fruit Nectars (ready to serve)	4.0 fl. oz.	(114 ml.)
24. Fruit Drink Bases, Mixes and Concentrates (ready to serve)	4.0 fl. oz.	(114 ml.)
25. Fruit Juices (other than lemon juice and lime juice)	4.0 fl. oz.	(114 ml.)
26. Fruit Juices, Lemon	1.0 fl. oz.	(28 ml.)
27. Fruit Juices, Lime	1.0 fl. oz.	(28 ml.)
28. Ice Cream, Ice Milk	3.5 oz.	(100 gm.)
29. Infant Formulas, Prepared (ready to serve)	As Directed by Label	
30. Instant Breakfast, Ready Breakfast (ready to serve)	As Directed by Label	
31. Margarine	2.0 oz.	(57 gm.)
32. Meat, Prepared Meat	3.5 oz.	(100 gm.)
33. Meat Substitutes	3.5 oz.	(100 gm.)
34. Milk, whole	30.0 fl. oz.	(852 ml.)
35. Milk Powder (reconstituted and ready to serve)	30.0 fl. oz.	(852 ml.)
36. Modified Skim Milk	30.0 fl. oz.	(852 ml.)
37. Molasses, Table or Blackstrap	1.5 oz.	(43 gm.)
38. (Naming the Flavour) Milk	30.0 fl. oz.	(852 ml.)
39. Nuts	1.0 oz.	(28 gm.)
40. Peanut Butter	1.0 oz.	(28 gm.)
41. Poultry Meat, Prepared Poultry Meat	3.5 oz.	(100 gm.)
42. Skim Milk, Partly Skimmed Milk	30.0 fl. oz.	(852 ml.)
43. (Naming the Flavour) Skim Milk	30.0 fl. oz.	(852 ml.)
44. Skim Milk Powder, Partly Skimmed Milk Powder (reconstituted and ready to serve)	30.0 fl. oz.	(852 ml.)
45. Soup (ready to serve)	7.0 fl. oz.	(200 ml.)
46. Sterilized Milk	30.0 fl. oz.	(852 ml.)
47. Vegetable Juices	4.0 fl. oz.	(114 ml.)
48. Vegetables (other than baked beans and cooked potatoes)	3.5 oz.	(100 gm.)
49. Vegetables, baked beans	8.5 oz.	(250 gm.)
50. Vegetables, cooked potatoes	7.0 oz.	(200 gm.)
51. Yeast	0.5 oz.	(14 gm.)
52. Yogurt, plain	5.0 oz.	(150 gm.)

[1] See D.01.001 (4).

SERUM PARATHYROID HORMONE LEVELS IN ACQUIRED VITAMIN D DEFICIENCY OF INFANCY

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ABSTRACT. The later stages of the vitamin D deficiency syndrome during infancy are associated with impaired renal tubular reabsorption of amino acids, phosphate, and other metabolites. Secondary hyperparathyroidism has been implicated as the cause of the renal abnormality. Serum immunoreactive parathyroid hormone levels are elevated in infants with vitamin D deficiency (160 to 238 μ Eq/ml; normal <140 μ Eq/ml). The highest levels were found in the more advanced stages of vitamin D deficiency. *Pediatrics*, 49:837, 1972. VITAMIN D DEFICIENCY, SERUM PARATHYROID HORMONE LEVELS.

VITAMIN D deficiency in man is accompanied by impaired renal tubular function, and there is reduced net reabsorption of phosphate, amino acids, and bicarbonate (literature cited in Reference 1). Investigation of vitamin D deficiency in the human infant by Fraser, Kooh and Scriver[1] revealed three stages of the syndrome. In the first, calcium is low in serum while phosphorus is normal; there is no hyperphosphaturia or hyperaminoaciduria at this time and bone changes are minimal. Hypophosphatemia develops in the second and third stages when serum calcium is normal or low respectively; hyperphosphaturia and hyperaminoaciduria occur in both of the later stages of vitamin D deficiency and typical signs of rickets are found in bone. The impairment of tubular conservation of phosphate and amino acids, in Stage-II and Stage-III vitamin D deficiency, is attributed to secondary hyperparathyroidism, and indirect tests support this hypothesis.[2] Grose and Scriver[3] observed that rats exposed to a low-calcium, vitamin D-free diet developed hypocalcemia and subsequent impairment in the tubular absorption of phosphate and amino acids; the degree of parathyroid gland hypertrophy which was found in these animals correlated directly with the tubular dysfunction.

Until now there have been few direct measurements of circulating parathyroid hormone in the vitamin D deficiency syndrome. This report presents the first observations on the relationship of immunoreactive parathyroid hormone (IPTH) levels in serum, to the stages of vitamin D deficiency in the human infants.

METHODS

Five patients, all French-Canadian male infants, were studied prior to adequate treatment with vitamin D. The criteria used for diagnosis of vitamin D deficiency are those described previously.[1] Briefly, these were: a clinical history compatible with vitamin D deficiency, plus complete rapid healing of bone lesions and any biochemical abnormality on a therapeutic dosage of vitamin D (e.g., 5,000 units/day × 4 weeks) without relapse when the normal intake (400 units/day) was resumed there-

Abbreviations

IPTH: Immunoreactive parathyroid hormone

PTH: Parathyroid hormone

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TABLE I

BIOCHEMICAL AND CLINICAL CORRELATES AND SERUM IPTH IN VITAMIN D DEFICIENCY

Patient	Age (mo)	Presenting Clinical Feature	Serum IPTH (μl Eq/ml)	Serum Calcium (mg/100 ml)	Serum Phosphorus (mg/100 ml)	Amino Aciduria (units 859 μg total N)	X-ray Signs*	Comment†
SB	2	Convulsions	60	8.8	2.2	18	mild	Stage-I deficiency
BJ	18	Weakness & DBI	68	9.7	3.2	38	moderate to severe	Stage-II deficiency; received 5000 u vit. D_2 & Ca++ gluconate 2 wk. before IPTH assay.
EC	6	Convulsions	112	8.0	3.4	26	moderate	Stage-III deficiency. Repeated I.V. infusions of Ca++ gluconate for 2 days before IPTH assay.
SB	9	DBI & Failure to thrive	182	8.7	3.3	30	moderate	Stage-III deficiency
MF	14	Rickets	i) 168 ii) 238	8.0 8.3	1.9 2.3	— 34	moderate to severe	i) Untreated ii) 6 days on vitamin D_2 5000 u/day.

Normal values: Serum calcium 9–11 mg/100 ml; serum phosphorus 4–7 mg/100 ml; urine amino acids 5–15 units per 250 μg total nitrogen; serum IPTH 10–40 μl Eq/ml.

* Designation according to criteria described: mild = calcium demineralized; mild-moderate = minimal cartochondral and long bone changes; moderate = typical rickets changes; moderate-severe = advanced rickets; severe = evidence of hyperparathyroidism in phalanges, clavicles and tibia, and so forth.

† Designation for stage of deficiency assigned according to criteria described in detail.

Stage-I: Low serum calcium, normal serum phosphorus; normal amino aciduria; demineralized calvarium; convulsions are major symptom.

Stage-II: Normal serum calcium; low serum phosphorus; hyperaminoaciduria; rickets in ribs and long bones; convulsions infrequent.

Stage-III: Low serum calcium and phosphorus; hyperaminoaciduria; florid rickets, convulsions common.

after. Early morning sera were analyzed for total calcium and inorganic phosphorus by automated methods on a Technicon Auto Analyzer.* Urine samples were obtained at the same time by spontaneous voiding and the amino acid content was examined by two dimensional partition chromatography. The intensity of excretion in relation to a coefficient of total nitrogen was evaluated by the methods described previously.[3] IPTH was measured in the same sera used for calcium and phosphorus determination; the analyses were performed in Dr. Arnaud's laboratory by the radioimmune method of Arnaud, *et al.*[3] on material kept at −20 °C.

RESULTS

The five patients with proven vitamin D deficiency represented all three stages of the syndrome (Table I). Whereas, the normal IPTH level in human serum is less than 40 μl Eq/ml,[3] serum IPTH is slightly elevated (60 μl Eq/ml) in early vitamin D deficiency and high levels of serum IPTH (112–238 μl Eq/ml) are found in patients with the late stages of vitamin D deficiency, when impaired tubular reabsorption of amino acids and phosphate is apparent. Sufficient treatment with calcium and vitamin D should suppress parathyroid secretion[3] and two patients who had received this regimen (BJ and EC, Table I) had lower serum IPTH than patients at the equivalent stage of deficiency who were untreated.

DISCUSSION

A strong correlation between impaired tubular reabsorption of phosphorus and

* Technicon Instruments Corporation, Tarrytown, New York 10591.

omino acids, and the apparent presence of econdary hyperparathyroidism, is found in nan[1] and the rat[2] made deficient in vitamin D. Infusion of calcium or extirpation of parahyroid tissue rapidly ablates the tubular ransport disorder, despite an unchanged tatus of vitamin D nutrition.[1,2] These obervations imply that circulating parathyroid hormone may play a major role in the athogenesis of the renal tubular lesion in itamin D deficiency. The present investigation confirms that the level of circulating arathyroid hormone is abnormally elevated in the later stages of vitamin D deficiency in man. This finding compliments our recent report[4] that serum IPTH is elevated in untreated hereditary vitamin D dependency, a condition which mimics the physiological correlates of severe vitamin D deficiency.[5] On the other hand, serum IPTH is normal in untreated patients with X-linked hypophosphatemic rickets,[6] a disease in which an inborn error of phosphate ransport in kidney, and perhaps in other issues it is believed to be the primary esion.[7]

The only other studies of serum IPTH in itamin D deficiency known to us, did not relate levels to the stage of deficiency. Lepain[5] described elevated levels in two children with unclassified deficiency rickets; serum IPTH fell in these subjects after treatment with vitamin D. Melick and Martin,[8] and Arnaud, *et al.*[9] reported modest elevation of serum IPTH in adult osteomalacia aused by vitamin D depletion.

The finding of elevated serum PTH in advanced vitamin D deficiency of infancy should not alter the physician's approach to the treatment of stage-II and stage-III deficiency with vitamin D. Therapeutic doses of the vitamin will restore the normal mechanisms for calcium access from intestine, thus allowing subsequent restoration of extracellular calcium with suppression of hyperparathyroidism and healing of bone. The anomalous hypocalcemia without significant hyperparathyroidism in stage-I deficiency may be accompanied by seizures hat is usually responsive to intravenous calcium: repair of this stage of deficiency could be enhanced by the therapeutic use of parathyroid hormone as well as vitamin D in adequate dosage.

The mechanism by which PTH produces its inhibition of net tubular reabsorption in vitamin D deficiency remains unknown. Morris, *et al.*[4] showed that PTH is essential for the pathogenesis of impaired renal tubular function in hereditary fructosemia, a condition where vitamin D depletion in renal cells plays no role in the tubular lesion. Chase, Melson, and Aurbach[10] demonstrated that the phosphaturic response to PTH in normal human kidney is mediated in part by adenosine 3′, 5′-monophosphate. We assume that PTH acts by a similar mechanism in vitamin D deficiency and that its effect is somehow coupled to a PTH-sensitive tubular transport system for phosphate,[7] and the various membrane transport systems which serve amino acid transport, bicarbonate, and other solutes.[7]

Important questions which still require answers are raised by the present findings. Stage-I vitamin D deficiency with hypocalcemia persists for several days before stage-II develops.[3] Why, then, is the feedback response in PTH secretion so minimal and slow, despite the profound hypocalcemic stimulus? Why is serum PTH greatly elevated in stage-II vitamin D deficiency, when serum calcium is normal and capable of suppressing the feedback stimulus for PTH secretion? These observations suggest a special modulation of parathyroid responsiveness to serum calcium by residual vitamin D in the deficiency state, and further investigation of this possibility is indicated.

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Science 173: 845, 1971

Serum Parathyroid Hormone in X-Linked Hypophosphatemia

Abstract. *Serum immunoreactive parathyroid hormone (IPTH) is normal in patients with X-linked hypophosphatemic rickets who are not treated with phosphate salts. Phosphate raises IPTH in these patients. Endogenous IPTH does not influence the existing defect in tubular reabsorption of phosphate in male patients.*

X-linked hypophosphatemic rickets is a dominant disease in which a low concentration of orthophosphate in plasma is the most constant index of the phenotype (*1*). In males, the mutant allele also causes bone disease, while in carrier females it is variably expressed, some showing only hypophosphatemia, while others have bone disease as well. Impairment of renal tubular reabsorption of phosphate has long been recognized as another important phenotypic feature of this trait.

Two opposing views have evolved concerning the pathogenesis of X-linked hypophosphatemia. One opinion favors a primary disorder of intestinal calcium absorption due to impaired endogenous conversion of vitamin D (*2*) to normal biologically active polar derivatives (*3*). This would result in secondary hyperparathyroidism, renal loss of phosphate, hypophosphatemia, and bone disease as an ultimate consequence.

The well-known suppressive effect of intravenous calcium infusion upon the hyperphosphaturia (*4*) appeared to support this hypothesis. The failure of X-linked hypophosphatemia to respond

Table 1. Response of serum IPTH to change in dietary phosphate in patients with X-linked hypophosphatemic rickets and acquired secondary hyperparathyroidism.

Subject	Sex	Dietary regimen: Phosphate (3 g/day); vitamin D_2 (< 25,000 unit/day)			Dietary regimen: Reduced phosphate (1 g/day); vitamin D_2 (100,000 unit/day)		
		Serum values		IPTH	Serum values		IPTH
		P*	Ca*	(μl eq/ml)	P*	Ca*	(μl eq/ml)
M.C.	F	5.4	8.9	290	3.9	10.4	40
I.A.	M	5.0	9.1	100	3.8	11.4	67
I.M.	M	4.4	7.4	750	3.9	10.6	43
	Mean	4.9	8.5	380	3.9	10.8	50

* Values in milligrams per 100 milliliters.

unambiguously to treatment with 25-hydroxycholecalciferol (*5*) is against defective hepatic biosynthesis of vitamin D metabolites; a failure to synthesize the derivative active in the intestine (*3*) has not yet been examined.

The other opinion favors a primary disorder of phosphate transport in the renal tubule (*6*), and perhaps also at the general cellular level (*7*). The ability to heal the bone disease more effectively with phosphate administration alone (*8*), or with phosphate and modest supplements of vitamin D (*9*), than with very large amounts of vitamin D alone in either its precursor or hydroxylated forms (*5*) emphasizes the apparent primacy of the phosphate leak in the pathogenesis of the complete phenotype. If the calcium-transport hypothesis, including its essential codicil about secondary hyperparathyroidism, could be eliminated by direct measurements of circulating parathyroid hormone, investigative efforts could be focused on the phosphate transport hypothesis. The development of a new radioimmunoassay for the measurement of human parathyroid hormone in serum (*10*) provided an opportunity to study this problem.

Serum was obtained from patients with familial hypophosphatemic rickets meeting the criteria for the X-linked trait (*11*). Untreated patients had serum immunoreactive parathyroid hormone concentrations (IPTH) within or close to the normal range (Fig. 1). Patients who had been receiving vitamin D but who still retained their hypophosphatemia, hyperphosphaturia, and active bone disease, also had normal serum IPTH. Only those patients receiving large quantities of therapeutic phosphate salts by mouth had increased serum IPTH. Three patients on the phosphate regimen developed roentgenographic and biochemical signs of hyperparathyroidism; reduction of the phosphate intake and supplementation of their diet with calcium, as calcium gluconate (1 to 3 g/day), and vitamin D_2 (100,000 unit/day) for at least 4 weeks suppressed serum IPTH to normal or near normal in these patients (Table 1).

Tubular reabsorption of phosphate [μmole/100 ml glomerular filtration rate (GFR)] was measured (*12*) on many occasions in four male probands. The results were plotted in relation to the serum IPTH at the same time (Fig. 2). Tubular reabsorption of phosphate was greatly impaired [mean for group = 36.8 μmole/100 ml GFR, normal, > 90 μmole/100 ml GFR (*1*)]. The serum phosphorus concentration was 3.3 ± 1.4 mg/100 ml (mean ± S.D.) for the group at the time the determinations were done and when the patients were taking varying amounts of phosphate by mouth. There was no relationship between tubular reabsorption of phosphate and serum IPTH, indicating that the defect in phosphate transport in male patients is not influenced by endogenous serum IPTH.

We believe that this is the first unambiguous evidence of normal serum IPTH activity in patients with untreated X-linked hypophosphatemia. Serum IPTH is increased only when these patients are treated with phosphate supplements. The mechanism involved in producing this increase in serum IPTH is probably the negative feedback control of parathyroid hormone secretion by the concentration of calcium in serum (*13*). Serum calcium decreased in the patients whose serum phosphorus had been increased by daily oral phosphate administration (Table 1).

A phosphate transport defect is present in male patients with X-linked hypophosphatemia even when their serum IPTH is normal (Fig. 2). The insensitivity of residual phosphate transport to endogenous IPTH suggests to us that the renal tubule of patients with

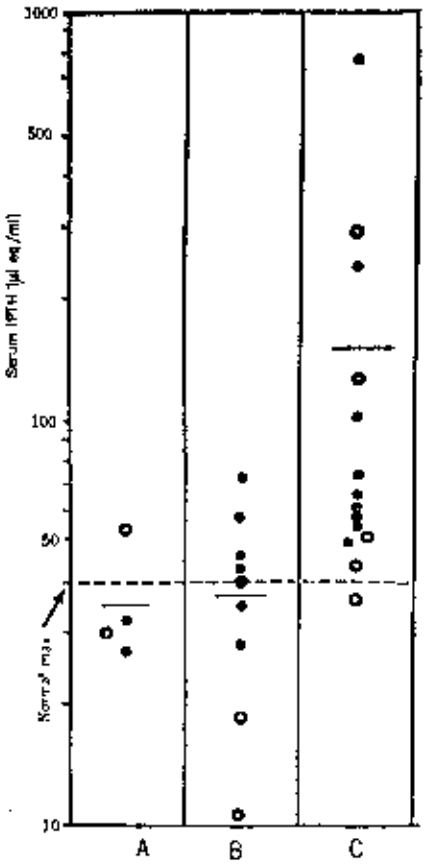

Fig. 1. Serum IPTH in patients with X-linked hypophosphatemic rickets. Column A, no treatment; column B, treatment with large doses of vitamin D alone (about 100,000 unit/day); column C, treatment with a phosphate supplement by mouth (3 g/day, as orthophosphate) and vitamin D (up to 100,000 unit/day). Open circles: female patients; closed circles: male patients. All patients have active disease.

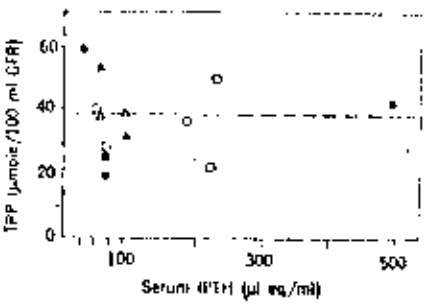

Fig. 2. Relationship of TRP [tubular reabsorption of phosphate, μmole/100 ml glomerular filtration rate (GFR)] to endogenous serum IPTH in four male probands with X-linked hypophosphatemic rickets, at serum phosphate values for group of 3.3 ± 1.4 mg/100 ml (mean ± S.D.). The dotted line indicates the regression of TRP on serum IPTH; the latter does not influence TRP in these patients. Maximum normal serum IPTH, 40 μl eq/ml.

X-linked hypophosphatemia contains a second parathyroid hormone–insensitive phosphate-transport system. We also suggest that this component of phosphate transport in kidney is responsive directly to calcium, perhaps in a manner analogous to that documented in canine kidney by Lavender and Pullman (*14*). This would account for the well-known effect of hypercalcemia on tubular reabsorption of phosphate in X-linked hypophosphatemia (*4*).

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11. The criteria for diagnosis of X-linked hypophosphatemia were: proven hypophosphatemia for age on a morning blood sample after overnight fast; absence of hypercalcemia and rise aminoaciduria in the untreated state; no male-to-male transmission; female clinical phenotype not more severe than male in same degree.
12. Tubular reabsorption of phosphate (TRP) was assessed after an overnight fast. A timed urine collection lasting about 180 minutes was obtained, and venous blood was obtained at the end of the period. Phosphorus and creatinine determinations were made and the TRP value was calculated. TRP was also determined with inulin clearance rates to monitor GFR on several occasions. Inulin and creatinine clearances were comparable in the same subject.
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25 March 1971

PEDIATRICS 46: 871-80, 1970

VITAMIN D DEPENDENCY: AN INHERITED POSTNATAL SYNDROME WITH SECONDARY HYPERPARATHYROIDISM

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ABSTRACT. Three French-Canadian children in a large inbred pedigree each developed hypocalcemic, hypophosphatemic rickets in the latter half of their first year of life; there were also manifestations of generalized renal tubular dysfunction. These abnormalities, which mimic advanced Vitamin D deficiency, disappeared only when Vitamin D_3 or D_2 was given at about 100 times the recommended daily allowance; this indicated the diagnosis of Vitamin D dependency.

Enamel hypoplasia was a prominent clinical finding; only those teeth which calcify postnatally were affected, indicating that the condition found does not affect Vitamin D-dependent nutrition *in utero*.

The level of parathyroid hormone was elevated in serum before treatment; it fell to normal either after treatment with Vitamin D, or during intravenous infusion with a calcium solution sufficient to produce hypercalcemia.

Vitamin D dependency appeared to be inherited as an autosomal recessive trait in this pedigree, but we could observe no phenotypic signs in presumably obligate heterozygotes. One of the three cases in the pedigree arose from inbreeding, suggesting that the mutant allele is probably not particularly rare in the population under our surveillance. *Pediatrics*, 46:871, 1970, VITAMIN D DEPENDENCY, PARATHYROID HORMONE, RICKETS, ENAMEL, HEREDITARY, AUTOSOMAL RECESSIVE.

A FORM of Vitamin D-responsive hypophosphatemic rickets accompanied by hypocalcemia, hyperaminoaciduria, hyperchloremic acidosis, and growth failure has attracted attention recently.[1-3] Because about 100 times the usual daily requirement for Vitamin D* is required to correct this disorder of mineral metabolism, it has been called "Vitamin D dependency" by some,[3] while others have used the term "pseudo-deficiency-rickets"[2] to focus attention on the many features which resemble severe Vitamin D deficiency.[3] The mandatory and high nutritional requirement for the vitamin is a permanent characteristic, and it is evidently inherited as being an autosomal recessive trait.

The following report presents additional information on Vitamin D dependency obtained from the study of a large inbred French-Canadian pedigree containing three members with the trait. We found that parathyroid hormone is elevated in the plasma of the untreated patient; this is the first occasion that such an observation has been made to our knowledge. We also observed the enamel hypoplasia[2] of those teeth which form postnatally and which indicates that the disorder of calcium metabolism which characterizes this hereditary disease develops after birth in the presumed homozygote. The mutant allele is not recognizable, by relatively simple tests, in the presumed heterozygote. Current opinion about this interesting gene-dependent nutritional state relegates the abnormality either to a disorder in the synthesis of the active (25-hydroxy-) form of Vitamin D, or to the binding of this polar derivative to its site of action in cells.

* The recommended daily allowance is 400 units/day.[4]

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TABLE 1

SUMMARY OF INITIAL CLINICAL AND BIOCHEMICAL DATA ON THREE PATIENTS WITH THE VITAMIN D-DEPENDENCY SYNDROME

Case	Age at Diagnosis (mo)	Presenting Signs and Symptoms	Total Serum Calcium (mg/100 ml)	Ionized Calcium (mg/100 ml)	Serum Inorganic Phosphorus (mg/100 ml)	Serum Alkaline Phosphate (K.A.U.)	Radiological Features	Required Vitamin D Intake, I.U. per day*	Outcome
C.J. VI-18	6	bulging fontanelle; irritability; lethargy	10	5.0	2.3	42	florid rickets	45,000 (D_2)	complete healing in 12 weeks
S.J. VI-10	8	convulsions; rachitic rosary; craniotaxes	7.9	3.8	3.8	44	florid rickets	45,000 (D_2)	complete healing in 6 weeks
N.J. VI-6	12	bulging fontanelle; irritability; hypotonia; lethargy; pathological fractures	7.2	—	2.8	127	florid rickets; multiple fractures	25,000 (D_2)	complete healing in 12 weeks

* All patients received 400 I.U. Vitamin D daily from birth.

METHODS AND MATERIALS

Urine

Samples were collected in the fasting state or over timed intervals, measured for volume, and then frozen at −20°C until analyzed. Samples for phosphate determination were acidified before analysis.

Plasma and Serum

Venous blood was collected from the antecubital vein in a heparinized syringe. The whole blood was then centrifuged at 900 × g for 10 minutes and the plasma removed immediately. Serum was obtained from whole blood and allowed to clot at room temperature. All samples for special studies were drawn in the early morning before feeding and stored at −20°C until analysis.

Endogenous Renal Clearance Rates

Endogenous renal clearance rates were determined for amino acids and phosphorus in the manner previously described.[7]

Calcium Infusion

Calcium ion was infused at an estimated rate of 4 mg/kg/hr as described by Fraser, *et al.*[8] and the serum calcium concentration was measured each hour for 12 hours during the infusion.

Amino Acids and Qualitative Analysis

Urine amino acids were identified in aliquots equivalent to 250 μg total nitrogen, by two-dimensional partition chromatography according to the method described by Dent.[7]

Quantitative Analysis

The concentration of amino acids in urine and plasma was measured by elution chromatography on ion exchange resins according to the methods of Spackman, Stein and Moore[6] on a Beckman-Spinco Model 120 amino acid analyzer† modified for simultaneous analysis of "neutral" and "basic" amino acids.[9] Plasma samples were deproteinized for amino acid analysis by the picric acid method.

Creatinine in urine and serum was determined on a Technicon auto analyzer‡ using a modified Folin method.[10]

† Beckman Instruments, Spinco Division, Palo Alto, California.

‡ Technicon Corporation, Chauncey, New York.

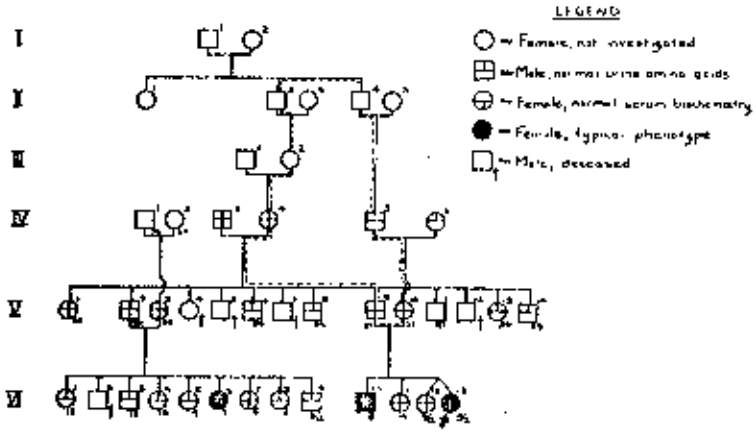

FIG. 1. Pedigree of a French-Canadian family with the Vitamin D dependency syndrome. Consanguinity is indicated by the broken line. Three children (black symbols) manifest the syndrome. Investigations performed on other members in the pedigree are defined by symbols as indicated in the figure.

Calcium in urine and serum was determined by atomic absorption analysis using a modification of the method of Willis.[11] The ultrafilterable calcium fraction was determined by the method of Gonda, *et al.*;[12] the normal range is 45 to 60% of the total calcium.

Inorganic Phosphorus was determined in urine and plasma by a micromodification of the method employed by C. H. Fiske and Y. Subbarow.[13]

Parathyroid Hormone was measured by a radioimmune assay developed by Arnaud and colleagues.[14]

TABLE II

BIOCHEMICAL DETERMINATIONS DONE ON FOUR OBLIGATE HETEROZYGOTES AND FIVE UNCLASSIFIED RELATIVES

Subject (Fig. 1)	Age Year	Total Plasma Calcium* (mg/100 ml)	Ultra Filterable Calcium* (% of total)	Serum Phosphorus* (mg/100 ml)	Serum Alkaline Phosphate (K.A.U.)	Urine Chromatogram
Normal		9-11	45-60%	2.5-3.5	5-30	N = Normal
[illegible]	36	9.7	60%	2.0	6	N
[illegible]	35	9.6	54%	3.5	5	N
[illegible]	20	10.2	-	4.5	8	N
[illegible]	27	10.2	-	4.0	4	N
[illegible]	49	9.2	54%	2.5	7	N
[illegible]	58	9.6	40%	3.3	8	N
[illegible]	37	9.0	56%	2.4	14	N
[illegible]	4	10.1	-	5.8	24	N
[illegible]	10/12	10.4	38%	6.5	38	N

* Patients above dotted line are presumably obligate heterozygotes (See Fig. 1)

* The venous blood samples were drawn in the morning after an overnight fast. Only minimal venous stasis was allowed, to avoid distortion in the serum calcium estimation.[15]

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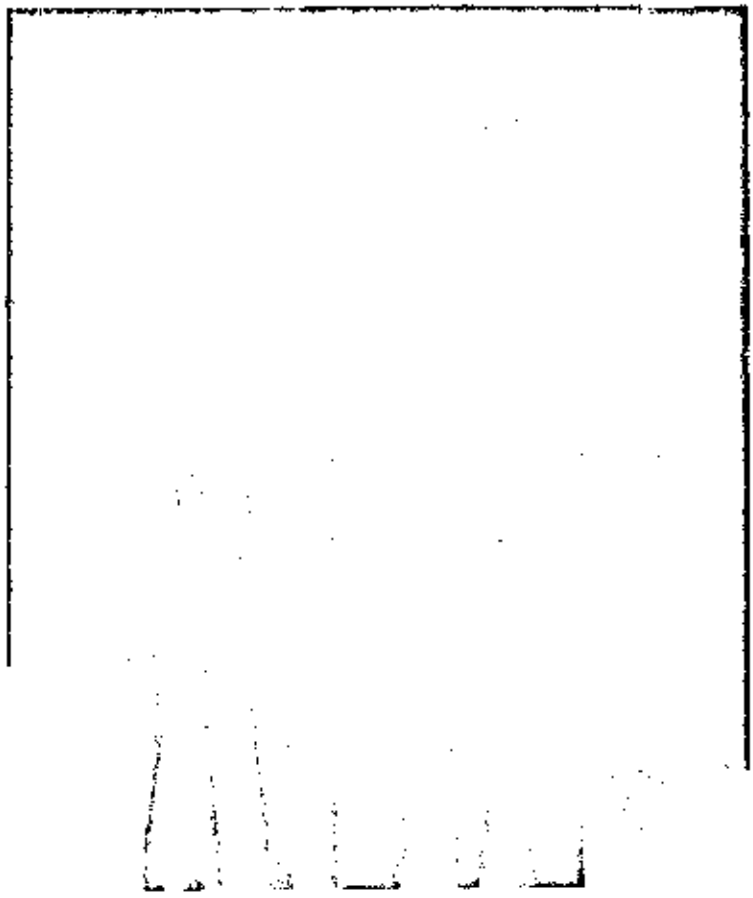

FIG. 2. Radiological examination of the lower teeth in Case S.J. (VI-10). The primary lateral incisors which calcify before birth are normal. The permanent central incisors (the two center teeth in the photograph) and the erupting permanent lateral incisors (the two inferior teeth visible on either side of the photograph) show marked enamel hypoplasia at the tips (dark areas) and enlarged pulp spaces; these teeth calcify after birth and before the eighth month. Vitamin D therapy began at the eighth month of life in this patient.

CASE REPORTS

The important clinical and biochemical data on the three patients reported in this paper are summarized in Table I. Additional information is given below.

Case C.J. (VI-13)

The proband is the fourth child of a consanguineous marriage (Fig. 1). She is one of binovular female twins born after an uneventful birth at 37 weeks' gestation. Her birth weight was 5 lb 12 oz. Her first hospital admission at another hospital was at 6 months of age because of a bulging anterior fontanelle. A pneumoencephalogram and carotid angiogram showed a slight abnormality on the left side and bilateral burr holes were made. The calcium concentration in serum was not recorded on the chart.

Her second hospital admission was to the Montreal Children's Hospital at 10 months of age because of persistent irritability and a bulging fontanelle and burr holes. A careful review of the dietary history revealed that a normal intake of vitamin D had been received during the previous months of the patient's life. Her weight was on the 3rd percentile, and the head circumference was on the 60th percentile for her age. Craniotabes was present. The following determinations on serum

were recorded: total calcium, 10 mg/100 ml; ultrafiltrable calcium, 5.9 mg/100 ml; inorganic phosphorus, 2.7 mg/100 ml; alkaline phosphatase, 35 King-Armstrong Units. A generalized hyperaminoaciduria was observed. There was no evidence of intestinal malabsorption by intestinal radiography and fat absorption studies were normal. X-rays of long bones and the skull showed rickets.

Case S.J.

This boy (VI-10) is the 5-year-old sibling of Case I. His birth history was normal but at age 8 months he was investigated elsewhere for hypophosphatemic rickets. Since that time he has received about 35,000 units of Vitamin D daily in the belief that he had a form of "Vitamin D resistant rickets." When reinvestigated because of the diagnosis in his younger sibling, his mental status was normal and his height and weight were at the 50th percentile. However, many of his permanent teeth were affected by enamel hypoplasia. His long bones show minimal changes of active rickets, while his serum calcium and phosphorus levels were normal at the time.

Case N.J.

This girl (VI-6) is the 7-year-old first cousin of the two previous patients. Her birth history was normal, but a bulging anterior fontanelle was detected at 6 months of age for which repeated bilateral subdural aspirations were performed at a hospital in another city. A diagnosis of Vitamin D dependency was considered by a pediatric consultant when rickets with hyperaminoaciduria, hypocalcemia, and hypophosphatemia was discovered at 1 year of age, despite a normal intake of Vitamin D. The relation of this patient, who lives 250 miles distant from Montreal, to the first two patients became apparent when the pedigree was studied in 1966.

SPECIAL STUDIES

Genetic and Clinical Features

Patients C. J. and S. J. are the offspring of a consanguineous mating between parents who are approximately second cousins (Fig. 1). The father of the third patient (N. J.) is a paternal uncle of the first two patients, while her mother is not related to the members of the J. family.

The appropriate "obligate" heterozygotes were studied, on the assumption that the three affected patients were homozygous for an autosomal recessive mutation. No abnormality of serum calcium, phosphorus, and alkaline phosphatase, or of amino acid excretion in the urine could be detected in these subjects.

Many other members of the pedigree were also examined for one or more parameters (Fig. 1), but again no abnormalities were identified (Table II).

The primary teeth of patient S. J. (VI-10) show normal calcification and enamel formation; these teeth calcify during the prenatal period. Those permanent teeth which normally calcify at about the fourth postnatal month[9] (e.g., central incisors) show marked enamel hypoplasia (Fig. 2). The teeth (e.g., upper lateral incisors) which usually calcify at about the tenth month, are normal. Vitamin D therapy had been instituted at the eighth month of the patient's life.

Biochemical Relationships

Parathyroid hormone was present in abnormally high amounts in the serum of patients C. J. and S. J. after withdrawal of their Vitamin D supplement (Table III). Adequate treatment with Vitamin D, sufficient to produce correction of biochemical abnormalities and complete healing of rickets, restored the level of parathyroid hormone to normal in C. J. After 5 weeks of Vitamin D withdrawal intravenous infusion of calcium into patient S. J. produced hypercalcemic suppression of the abnormal elevation of parathyroid hormone.

Response to Vitamin D

The Vitamin D dependent trait, in its fully expressed form, is accompanied by hypocalcemia, hypophosphatemia, a high renal clearance of phosphorus, hyperaminoaciduria, and a high renal clearance of amino acids.[1,2] The abnormality of renal tubular absorption was documented in one of our patients (Table IV), and we noticed that it resembles that which occurs in stage 3 of the Vitamin D deficiency syndrome.[9] This disorder in tubular transport is reversible[10] and it is believed to reflect endogenous secondary hyperparathyroidism. An appropriate supplement of Vitamin D,

TABLE III

PARATHYROID HORMONE CONCENTRATION IN SERUM OF TWO PATIENTS WITH VITAMIN D DEPENDENCY

Patient	*Date*	*Serum PTH units*	*Serum Calcium (mg/100 ml)*	*Comment*
C.J. (VI-13)	12/9/60	39.9	10.4	45,000 units D_2/day×2 mo
	1/11/69	34	10.5	45,000 units D_2/day×4 mo
	23/12/60	64	9.0	Off Vit D_2 supplement×7 weeks
S.J. (VI-10)	1/11/69	84	10.0	35,000 units D_2/day, mild active rickets
	8/12/60	120	9.8	Off Vitamin D_2 supplement×5 weeks
	9/12/60	24	11.8	10 hours after induction of hypercalcemia

which restores normal calcium homeostasis, should reverse these presumed parathyroid-dependent relationships in Vitamin D dependency. All three patients exhibited this response when approximately 100-fold the recommended daily allowance of Vitamin D was administered for 6 weeks or more (Table I).

The improvement in net tubular reabsorption of phosphorus and amino acids after Vitamin D treatment was specifically documented in one patient (Table IV). This response was associated with a fall in circulating parathyroid hormone to normal levels (Table III).

DISCUSSION

The syndrome of hypocalcemic, hypophosphatemic hyperaminoaciduric rickets has at least three causes. It is most commonly encountered in the third stage of Vitamin D deficiency: patients with deficiency rickets respond within a few weeks to therapeutic doses of Vitamin D_2 or D_3 (e.g., 2,000 IU/day). The syndrome may also occur when there is an intestinal malabsorption syndrome which presumably affects Vitamin D and calcium absorption. Vitamin D responsiveness may not occur until the primary enteropathy is corrected. The third form of the syndrome, "pseudo-deficiency rickets"[2] or "Vitamin D dependency,"[3,18] has now been reported by many authors.[19-24]§ This condition is dramatically responsive to treatment when Vitamin D is given in pharmacologic doses (25,000 to 100,000 I.U./day).

Hamilton and colleagues[25] have shown that intestinal absorption of calcium is specifically impaired in Vitamin D dependency. Consequently the accompanying clinical and biochemical manifestations could reflect a sequence of events whose relationships have been best studied in Vitamin D deficiency.[5,6] Diminished Vitamin D activity in tissues will impair calcium absorption by intestine,[27,28] and the release of calcium from bone;[29] under these conditions extracellular calcium will fall. The parathyroid gland will then respond by increasing the rate of synthesis and release of its hormone[30,31] to stimulate the release of calcium and phosphate from bone and the uptake of calcium from intestine. These peripheral responses to physiological amounts of parathyroid hormone will occur only if sufficient Vitamin D is present to allow a synergistic action between hormone and sterol.[31,32] While restoring calcium levels to normal, the hormone will concomitantly inhibit tubular absorption of phosphate and amino acids,[5,25] an action which does not require Vitamin D.[26,33] When the depletion of Vitamin D is extreme, physiological amounts of parathyroid hormone are unable to enhance uptake of calcium from

§ The patient reported by Soriano, *et al.*[22] is often cited but is atypical and may have a different form of Vitamin D dependency or another disease.

gut and its release from bone; consequently extracellular calcium will fall again.

An argument still prevails as to whether the perturbed Vitamin D activity in tissues does or does not play a direct role in the phosphaturia and aminoaciduria of the deficiency and dependency syndromes; several lines of evidence suggest that it does not. A primary calcium deficiency has been documented in one human infant[5] in whom Vitamin D nutrition was definitely normal; hyperaminoaciduria and increased renal clearance of phosphorus were both present. In the present study, measurement of parathyroid hormone in serum revealed a direct correlation between hyperparathyroidism and the clinical severity of the Vitamin D dependent phenotype: hyperparathyroidism in our patients could be suppressed either by Vitamin D therapy or by calcium infusion. In previous studies[6] Scriver, Kooh and Fraser observed that the impairment of tubular absorption in Vitamin D dependency could be corrected after 9 hours of sustained mild hypercalcemia. These findings suggest that the tubular dysfunction of the hypocalcemic-hypophosphatemic-aminoaciduric rickets syndromes is dependent on secondary hyperparathyroidism.

We can assume that an initial disturbance in calcium homeostasis precipitates the fully expressed phenotype of Vitamin D dependency. The cerebral manifestations of hypocalcemia mentioned in this and other reports on Vitamin D dependency will appear both as early and as late signs as they do in Vitamin D deficiency.[7] The enamel hypoplasia which occurs in this trait reveals that maternal Vitamin D and calcium nutrition protect the fetus; only when

TABLE IV

URINARY EXCRETION AND NET TUBULAR ABSORPTION OF AMINO ACIDS AND PHOSPHORUS BEFORE AND AFTER VITAMIN D TREATMENT IN A PATIENT WITH VITAMIN D DEPENDENCY

Data	Before Treatment			After Treatment		
	Urinary Excretion μmoles/min/1.73m²	Clearance ml/min/1.73m²	Absorption %	Urinary Excretion μmoles/min/1.73m²	Clearance ml/min/1.73m²	Absorption %
Taurine	0.576	9.0	86	0.248	0.8	97.8
Hydroxyproline	0.017	0.6	98.0	0	0	100
Aspartic Acid	0.002	0.1	99.0	0.037	<0.1	93.0
Threonine	0.443	5.5	91.8	0.055	5.0	98.0
Serine	1.55	9.3	86.2	0.290	1.4	96.8
Asp + Glu.	2.89	5.9	91.0	0.452	0.9	98.7
Proline	Trace	Trace	<100.0	0	0	0
Glutamic Acid	0.021	0.1	99.8	0.020	0.4	99.3
Glycine	4.15	17.5	73.5	0.930	5.6	92.2
Alanine	1.65	3.5	94.7	0.198	0.9	98.6
Valine	0.061	0.4	99.3	0.030	0.8	99.5
Methionine	0.035	0.0	96.0	0.015	1.8	98.2
Isoleucine	0.063	1.4	97.0	0.039	1.5	97.0
Leucine	0.051	0.8	98.0	0.010	0.2	99.0
Tyrosine	0.460	8.0	87.3	0.051	1.4	98.0
Phenylalanine	0.176	2.7	95.8	0.030	0.9	98.8
Ornithine	0.025	0.4	99.3	0.010	0.7	99.3
Lysine	0.485	4.1	93.7	0.095	0.1	99.8
Histidine	1.83	31	48.8	0.35	8.0	87.5
Arginine	Trace			Trace		
Phosphorus *	0.02 mg/min	26	61.3	1.00	16	80.0
Creatinine		66			72	

the homozygous infant is dependent on calcium absorption from his own intestine in postnatal life does the phenotype, including enamel hypoplasia, appear in all its aspects.

There are sufficient indications that the cellular mechanisms for calcium transport in the intestine[16,37] are intact in the Vitamin D dependent homozygote, and that it is only the coupling of Vitamin D to the transport process which is defective; this has been demonstrated most explicitly by the Toronto group.[20] Therefore an abnormality in the metabolism and mode of biological action of Vitamin D itself should be considered. DeLuca and his colleagues[37] and Stanbury[38] have identified an important polar metabolite of Vitamin D which they believe to be the important biological form of the vitamin. This 25-hydroxy derivative stimulates synthesis of the calcium transport protein in gut[6,7] and augments calcium mobilization from bone.[9,38] Consequently, a deficiency in the synthesis of the 25-hydroxy derivative or an altered relationship in the binding of this metabolite to the membrane at its cellular site of action could provoke the Vitamin D dependent phenotype.

Several workers[19,28,39] have noted that the serum Vitamin D activity as determined by the rat bioassay is not depressed in patients with Vitamin D dependency. Moreover this type of assay cannot exclude a deficiency of the polar derivatives in serum,[38] since rat liver will convert the precursor forms of Vitamin D in the test serum to the active form,[7] and will thus yield a "normal" assay. Direct chemical measurement of polar Vitamin D metabolites in serum is required to resolve this point.

There are certain clinical indications which favor the alternate hypothesis for aberrant membrane binding of Vitamin D metabolites. The absolute range for the therapeutic response to Vitamin D in the dependent patient is peculiarly narrow, usually being within a few thousand units of the average requirement (which is about 50,000 units). This dose response is not compatible with the Michaelis-Menten kinetics anticipated for a "leaky" mutant which impairs first-order conversion of precursor Vitamin D to polar derivatives. The therapeutic dose-response kinetics of Vitamin D dependency resemble the more complex coenzyme binding kinetics described by Mudd.[40] This hypothesis can be tested when the dose-response to the polar metabolite 25-hydroxycholecalciferol has been determined and compared with the response to cholecalciferol itself in Vitamin D dependent patients.

The present work and that of others[22,29,39] clearly indicates the hereditary basis for human Vitamin D dependency. Whether all patients have the same autosomal recessive trait is still unknown. The tremendous genetic heterogeneity which characterizes so many of the inborn errors of metabolism begs the discovery of several "Vitamin D dependent" alleles. It is already apparent that the trait is not that rare; one case occurred with outbreeding in the present inbred pedigree, and the rising frequency with which the condition is being reported indicates that there may be a substantial reservoir of the allele in the general population. The probability that this trait also occurs as an autosomal recessive in swine[41,42] should assist those who wish to study the pathophysiology of the condition in depth.

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Further Studies on the Interrelationship between Parathyroid Hormone and Vitamin D *

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Calcium homeostasis is achieved by the interplay of a number of humoral agents (11). Of central importance are the activities of parathyroid hormone (PTH) and of vitamin D. This was fully appreciated 35 years ago (2), but in spite of considerable effort, the nature of the relationship remains controversial. Recent opinion has varied from that of a complete dependence of hormone action upon the presence of vitamin D (3) to that of a complete independence of action (4, 5). In our initial studies of this problem, we concluded that in the absence of vitamin D a physiologic concentration of parathyroid hormone exerted its usual effect upon the renal excretion of phosphate but was unable to mobilize calcium from bone (6).

Since these studies were published, two groups of investigators have challenged our conclusions (7, 4). Harrison and Harrison reaffirmed their previous observations (7) and concluded, as they had before, that all of the actions of parathyroid hormone depended upon vitamin D. At the opposite extreme, Ney and associates concluded that all of the effects of parathyroid hormone are exerted in the D-deficient organism. These differences are difficult to explain. However, both studies differed from ours in several respects. In the study of Harrison and Harrison (7), no attempt was made to define or control the level of endogenous parathyroid hormone, although we found this to be of prime importance (6). In the work done by Ney and co-workers a different species was employed, and the degree of D deficiency was assessed by different criteria (4). Nevertheless, both studies raised points that seemed worthy of further examination.

The development of a technique for study of the response of the conscious rat to the long-term perfusion of hormones (8) has allowed us to examine several aspects of this problem more directly. The experimental design permitted the exclusion of altered endogenous secretion of parathyroid hormone or thyrocalcitonin as variables. The results reaffirm our previous conclusion and explain, in large part, the previous disparity of opinion regarding the nature of the parathyroid hormone vitamin D interrelationship.

Methods

Vitamin D deficiency was produced in young growing Holtzman rats as previously described (9). Control animals received the same purified diet but were given 75 U of vitamin D_3 in cottonseed oil by oral intubation daily for the week before study. Urinary calcium and phosphate were measured simultaneously with a Technicon Autoanalyzer (6). Plasma calcium and phosphate were measured by the same means except for a manifold modification that allowed for a microdetermination on 50 to 100 µl of plasma. Magnesium in the urine was measured by absorption flame spectrophotometry with a Perkin-Elmer instrument (10). Urinary sodium and potassium were measured by emission spectrophotometry with a Zeiss instrument. Urinary inulin was measured by an automated procedure with a Technicon Autoanalyzer (11).

The technique of long-term perfusion of the rat was developed as a modification of that described by Cotlove (12). With rats under ether anesthesia, a no. 240 polyethylene catheter[1] was placed in the urinary bladder, a no. 50 polyethylene catheter[1] was placed in the external jugular vein, and the parathyroid or both thyroid and parathyroid glands were removed by surgical means. In some instances parathyroidectomy was per-

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TABLE I

Electrolyte balance during period of control infusion

	No. of experiments	Na	K	Mg	Ca
		μmoles/hr	μmoles/hr	μmoles/hr	μmoles/hr
PTX*					
+D	6	+38 ± 4†	−11 ± 4	+5 ± 0.5	+10 ± 2
−D	5	+26 ± 4	−22 ± 4	+1 ± 0.25	+12 ± 3
High Ca					
−D	4				+8 ± 1
Sham					
+D	4	+28 ± 5	+16 ± 4	+2 ± 0.5	+8 ± 2
−D	4	+15 ± 3	−9 ± 2	+4 ± 0.5	+13 ± 3

* PTX = parathyroidectomized rats; high Ca = rats fed a high calcium vitamin D-deficient diet; sham = sham-operated control rats.
† Mean ± standard deviation.

formed with an electric cautery. After surgery, the animal was placed in a cage similar to that designed by Cotlove (12) with the jugular vein catheter exiting through the top and the bladder catheter and hind legs through the rectangular opening in the bottom. The jugular vein catheter was connected to a peristaltic pump that delivered 3.6 to 4.0 ml per hour. The animals were perfused at this rate with a solution containing 4% dextrose, 5 mM calcium chloride, 5 mM magnesium chloride, 20 mM sodium chloride, 2.5 mM potassium chloride, and 20 mg per L inulin. Urine was collected at half-hour or 2-hour intervals. The animals were perfused for 16 hours after surgery before measurements of urinary volume and determinations of electrolyte content were begun. Collections were then made during a control period of at least 10 hours before parathyroid hormone (5 μg per hour) was added to the perfusate. Hormone infusion was usually continued for 20 hours, and then a period without hormone was recorded. We obtained blood samples by cardiac puncture before surgery in order to measure plasma calcium and phosphate. This was done to be certain of the degree of D deficiency or D repletion. Rats were considered D deficient only if they had stopped gaining weight after 3 weeks of vitamin D free diet and if the concentration of calcium in the plasma (plasma calcium) was below 6.5 mg per 100 ml. The mean plasma calcium value of the D-fed controls was 10.8 ± 0.4 mg per 100 ml before surgery. One group of animals was raised on a high calcium vitamin D-free diet, and their mean preoperative plasma calcium was 9.6 ± 0.3 mg per 100 ml. These animals did not stop gaining weight but were considered vitamin D deficient because littermates raised on a normal calcium D-free diet in adjacent individual cages exhibited classic disturbances in growth and plasma calcium levels. The number of animals studied in each group is indicated in the figure legends; standard statistical methods were used to obtain values for standard error of the mean and significance of the differences between means (15).

The purified parathyroid hormone used was prepared by the method of Hawker, Glass, and Rasmussen (14).

Results

Initially, the reproducibility of the perfusion technique was established. Two problems of immediate concern were the stability of the base line rates of electrolyte excretion and the balance of fluid and electrolytes.

We found that 10 to 15 hours of perfusion with the control solution was necessary before the rate of excretion of the various electrolytes became stable. Therefore, in all experiments to be reported, the animals were perfused for at least 16 hours before urine collections were begun. Collections over the ensuing 10 hours constituted the control period for each experiment.

After the poststurgical equilibration period, the rates of urine flow were from 0.05 to 0.1 ml per hour less than the measured rates of perfusate infusion in all the animals. However, there appeared to be no significant fluid retention. The animals weighed 3 to 4 g less after a 50-hour experiment than before surgery. Postmortem examination revealed no evidence of abnormal fluid

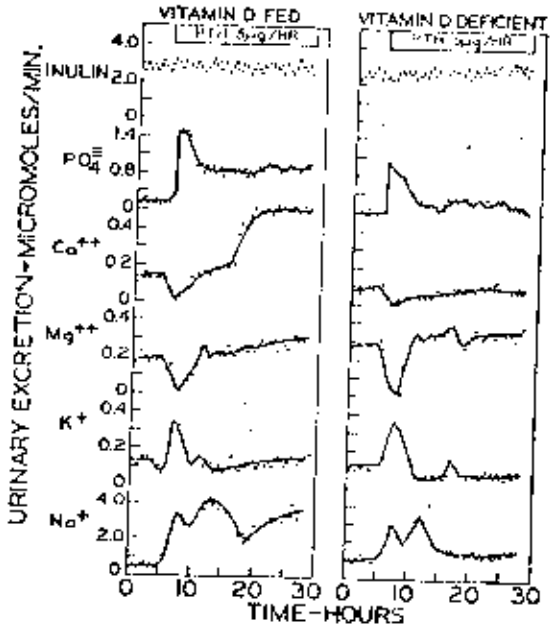

FIG. 1. RATES OF EXCRETION OF INULIN, PHOSPHATE, CALCIUM, MAGNESIUM, POTASSIUM AND SODIUM BEFORE AND DURING THE INFUSION OF PARATHYROID HORMONE (PTH). Five μg per hour PTH was infused into a D-fed (left) and a D-deficient (right) parathyroidectomized rat maintained on a constant infusion of glucose and electrolytes.

retention and the rates of infusion were less than the maximal rates the rats could tolerate. Rates of urine flow up to 5.0 to 6.0 ml per hour could be produced by increasing the rate of infusion. Because of these facts, it was concluded that the slight discrepancies between rates of infusion and rates of urine flow were accounted for by insensible water loss and possibly by slight evaporation from the urine samples when they were in the fraction collector. It should be noted, however, that there was usually sodium retention and always potassium loss during the control period (Table I), possibly as a result of adrenal hyperactivity secondary to the stress of surgery and experimentation. In all instances in which sodium retention occurred, it was followed by sodium diuresis during hormone infusion (Figures 1 and 2). Magnesium excretion balanced intake, but there was always calcium retention that was more marked in the D deficient than in the D-fed animals (Table I).

Our initial studies were carried out in parathyroidectomized animals. Two typical experiments are shown in Figure 1. In the rat fed vitamin D, the infusion of parathyroid hormone at a rate of 5 μg per hour led to a prompt increase in the rate of excretion of phosphate, potassium, and sodium, and an initial fall in magnesium and calcium excretion (Figure 1). The changes in rates of magnesium and potassium excretion were not sustained but returned to baseline or slightly greater values after 4 to 6 hours of perfusion. In contrast, the initial phosphaturia was followed by a sustained phosphate excretion throughout the course of the perfusion. Calcium excretion, after decreasing initially, returned to the control rate and then rose, after 15 to 22 hours of constant hormone perfusion, to a rate considerably higher than that observed initially. Changes in the rate of sodium excretion were more variable but, in general parallel to those of phosphate.

In contrast, the infusion of hormone at the same rate into a D deficient parathyroidectomized rat led to the initial changes in electrolyte excretion that were qualitatively similar to those seen in the D-fed animal, i.e., an increased rate of sodium, potassium, and phosphate excretion, and a fall in excretion of calcium and magnesium. However, a different pattern of response emerged during the later hours of perfusion (Figure 1). The rate of

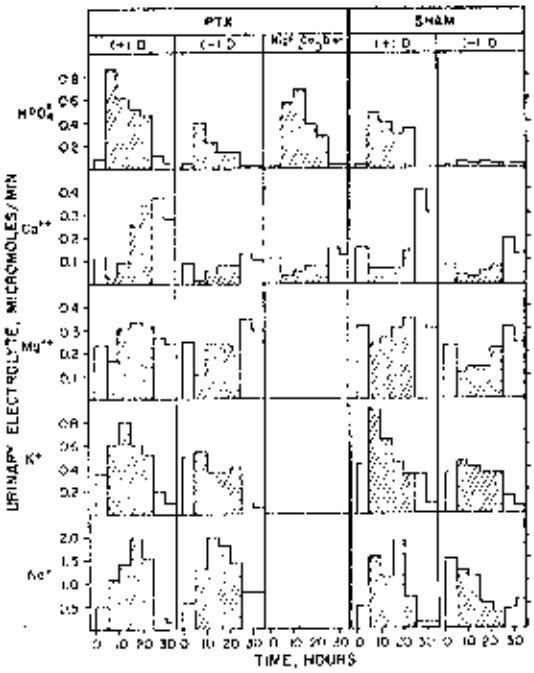

FIG. 2. PATTERNS OF URINARY ELECTROLYTE EXCRETION BEFORE, DURING, AND AFTER THE INFUSION OF PARATHYROID HORMONE, 5 μg PER HOUR, IN PARATHYROIDECTOMIZED (PTX) AND SHAM OPERATED D-DEFICIENT AND D-FED RATS. All animals were maintained on a normal calcium and phosphate diet, except the third group of D-deficient animals, which were maintained on a high calcium normal phosphate diet. The shaded areas represent the values during hormone infusion. Number of animals in each group: +D, 6; −D, 5; −D high calcium diet, 4; +D sham, 4; −D sham, 4.

phosphate excretion fell, after the initial increase, to rates only slightly greater than the control rates; the initial decrease in rate of calcium excretion was followed by a gradual return to a rate nearly equal to, but never in excess of, the control. Also, sodium diuresis was not sustained as in the D fed animal.

The rates of inulin excretion were also measured in these animals. There was no significant change in the excretion of constantly infused inulin during hormone infusion in either animal (Figure 1). Rates of inulin excretion were measured in six additional animals, and in all instances the infusion of parathyroid hormone, at a rate of 5 μg per hour, had no effect upon this rate.

The same procedure was followed in six other D-fed and five other D-deficient animals. The results are recorded as the means of the values

TABLE II

Comparison of responses to parathyroid hormone infusion in parathyroidectomized vitamin D-fed and D-deficient rats

Animals*		Urinary electrolyte excretion in 5 hours				
		HPO_4^{--}	Ca^{++}	Mg	K	Na
		μmoles	μmoles	μmoles	μmoles	μmoles
+D	C†	24.0 ± 4.2	36.0 ± 4.4	69.0 ± 5.2	108 ± 10	150 ± 13
−D		7.5 ± 3.0‡	27.0 ± 4.1	75.0 ± 5.8	150 ± 11‡	180 ± 17
−D(Ca)		6.1 ± 2.6‡	34.0 ± 1.7			
+D	5	258.0 ± 20	7.5 ± 2.3	18.0 ± 5.8	180 ± 11	340 ± 12
−D		120.0 ± 9‡	3.6 ± 0.9‡	33.0 ± 4.9§	165 ± 11	300 ± 14‡
−D(Ca)		201.0 ± 17§	8.0 ± 2.5			
+D	10	188.0 ± 15	28.0 ± 1.3	81.6 ± 6.3	236 ± 15	414 ± 16
−D		65.0 ± 8‡	17.0 ± 5.2‡	73.0 ± 6.1	124 ± 12‡	580 ± 23‡
−D(Ca)		225.0 ± 17	12.0 ± 3.2‡			
+D	15	151.0 ± 17	90.3 ± 8.8	89.4 ± 7.0	179 ± 8	571 ± 29
−D		14.0 ± 9‡	22.0 ± 4.0‡	73.0 ± 5.8	124 ± 12‡	521 ± 20§
−D(Ca)		143.0 ± 16	23.0 ± 5.1‡			
+D	20	138.0 ± 16	107.7 ± 9.0	83.2 ± 5.8	155 ± 13	487 ± 26
−D		44.0 ± 11‡	22.0 ± 4.4‡	69.0 ± 7.1§	132 ± 14§	530 ± 12§
−D(Ca)		99.0 ± 17§	23.0 ± 6.6‡			
+D	PH	30.0 ± 4.2	127.6 ± 11.0	78.2 ± 6.0	55 ± 7	77 ± 11
−D		5.0 ± 2.2‡	42.6 ± 7.1‡	91.3 ± 8.3	40 ± 8	208 ± 15‡
−D(Ca)		4.8 ± 2.1‡	54.7 ± 9.4‡			

* +D = 6 vitamin D-fed controls; −D = 5 vitamin D deficient rats on normal calcium intake; −D(Ca) = 4 vitamin D-deficient rats on high calcium intake.

† C = excretion during 5-hour control period; 5, 10, 15, 20 = excretion during first 5, 10, 15, 20 hours of hormone infusion; and PH = excretion during 5 hours after hormone infusion.

‡ $p < 0.001$ when values (mean ± standard error) between +D and −D animals are compared.

§ $p < 0.025$ when values (mean ± standard error) between +D and −D animals are compared.

for 5-hour periods before, during, and after hormone perfusion in Figure 2 (columns 1 and 2). Also shown are the responses of four sham-operated animals (Figure 2, columns 4 and 5), and the changes in urinary calcium and phosphate excretion on a high calcium, normal phosphate diet (Figure 2, column 3). Table II summarizes the electrolyte data and gives the results of the statistical evaluation of the differences between the mean values for the various 5 hour periods between D-fed and D-deficient animals. Table III shows the mean plasma calcium and phosphate values of animals in each group before surgery, during the period of control perfusion, and after 12 hours of hormone infusion. All three measurements could not be made in all animals in each group. Because of the small number of values in some groups, the mean and range in each group are recorded rather than the standard error.

The plasma values and the changes in electrolyte excretion were highly reproducible from one animal to the next within a given group but varied considerably from group to group. Early in the course of this project, a single bloodletting frequently led to a significant decrease in the urinary output during the next 0.5 hour period, but the urinary output returned to the previous level thereafter. After further experience, it was possible to obtain three blood samples from a single animal within a 24-hour period without greatly altering the rate of urine flow. The number of measurements within each group was too few to obtain precise statements of significance, but as seen in Table III, the results were clear. There was no significant rise in plasma calcium in the D-deficient animals of any group after hormone infusion, although in each instance there was a tendency for the value to rise slightly. On the other hand, there was a rise in plasma calcium in the D-fed animals (Table III). The plasma phosphate decreased in all animals except in the infant D-deficient ones (Table III).

The slight rise in plasma calcium seen in all D-deficient animals was associated with definite

TABLE III

Plasma calcium and phosphate values in rats before parathyroidectomy or thyroparathyroidectomy, during the control infusion, and after 12 hours of hormone infusion

Status	Vitamin D	No. rats	Plasma calcium			Plasma PO_4		
			Pre-op.*	Control inf.†	PTH‡	Pre-op.*	Control inf.†	PTH‡
			mg/100 ml			*mg/100 ml*		
PTX	−	4	11.1 ± 0.4	8.4 ± 0.4	13.1 ± 0.6	11.3 ± 0.3	10.2 ± 0.5	7.4 ± 0.4
PTX	−	4	6.0 ± 0.2	7.0 ± 0.4	8.1 ± 0.3	11.4 ± 0.3	10.8 ± 0.3	8.4 ± 0.3
PTX§	− High Ca	4	9.6 ± 0.3	8.3 ± 0.3	8.9 ± 0.4	9.3 ± 0.4	8.9 ± 0.3	6.2 ± 0.5
Intact	+	3	11.4 ± 0.3	10.7 ± 0.3	12.2 ± 0.3	11.3 ± 0.4	10.7 ± 0.4	8.6 ± 0.1
Intact	−	2	6.1 ± 0.3	7.1 ± 0.2	7.2 ± 0.2	11.8 ± 0.3	10.8 ± 0.4	10.4 ± 0.3
TXPTX‖	+	1	10.8 ± 0.3	8.8 ± 0.3	18.2 ± 1.4	11.3 ± 0.4	11.0 ± 0.2	7.2 ± 0.4
TXPTX¶	−	1	6.1 ± 0.2	9.2 ± 0.4	9.8 ± 0.4	10.8 ± 0.4	10.6 ± 0.3	7.3 ± 0.5

* Values before surgery.
† Values after surgery and at least 10 to 12 hours of perfusion with control solution.
‡ Values after 12 hours of parathyroid hormone (PTH) infusion.
§ Maintained on a high calcium vitamin D-deficient diet.
‖ Thyroparathyroidectomized.
¶ These D-deficient animals were perfused with a solution containing 25 M $CaCl_2$ rather than the usual 5 mM concentration.

renal retention of calcium during hormone infusion and a slight calcium diuresis after the hormone infusion stopped (Figure 2, columns 2 and 3). This pattern was also seen in the intact D-fed animals, but in the D-fed parathyroidectomized animals the initial retention of calcium was followed by a marked diuresis.

It is noteworthy that the magnitude of the initial (first 5 hours) phosphaturia seen in the D-deficient animals (Figure 2, column 2) was approximately 50% ($p < 0.001$) and the potassium diuresis 20% of that seen in the D-fed animals (Figure 2, column 1). Conversely, the initial magnesium retention was twice as great in the D-deficient animals as in the D-fed ($p < 0.025$) (Figure 2, columns 1 and 2). Also, the initial magnesium retention in the D-fed animals was followed by a diuresis of magnesium during the later hours of hormone infusion, whereas this did not occur in the D-deficient animals although the latter had a magnesium diuresis after the cessation of hormone infusion.

If D-deficient animals were maintained on a high calcium diet during the entire period before study, their initial phosphaturia during the first 10 hours of hormone perfusion was considerably greater ($p < 0.01$) than that seen in D-deficient animals raised on a normal calcium diet (Figure 2, columns 2 and 3), and was approximately 80% of that seen in the D-fed animals (Figure 2, columns 1 and 3). The results in Figure 2, column 3 represent the mean values of four animals.

Animals subjected to a sham operation rather than parathyroidectomy responded differently than did the parathyroidectomized animals. Those fed vitamin D (Figure 2, column 4) had qualitatively similar initial changes (compared to D-fed parathyroidectomized animals) in rates of electrolyte excretion. However, the magnitude of the phosphaturia was significantly less ($p < 0.01$) than that seen in parathyroidectomized animals, and the marked secondary rise in rate of calcium excretion, normally seen in the parathyroidectomized rats, was not seen in the sham-operated rats. However, after the cessation of hormone infusion in these latter animals, a striking calcium diuresis was always observed. In contrast, D-deficient sham-operated animals had no significant change in potassium or phosphate excretion during hormone infusion although the rates of excretion of sodium, calcium, and magnesium all fell significantly ($p < 0.01$) and rose again only upon cessation of hormone infusion.

The lack of both a rise in plasma calcium (Table III) and a secondary calcium diuresis (Figures 1 and 2, columns 2 and 3) in the D-deficient parathyroidectomized animals was interpreted to mean that the parathyroid hormone was

TABLE IV

Phosphate balances in D-deficient rats given parathyroid hormone[1]

Rats	No.	Diet	Total decrease ECF HPO_4[2]	Excess urinary HPO_4[3]
			mmoles	
PTX	4	Normal Ca	24	150‡
UTX	5	High Ca	27	340‡
UNPTX	4	Normal Ca	17	128‡

[1] Calculated from the data in Figure 2, columns 2 and 3; Figure 3, column 2; and Tables II and III.
[2] ECF = extracellular fluid.
‡ Significantly greater ($p < 0.001$) than the total decrease in ECF phosphate.

unable to mobilize calcium from bone. If this was the case, it could be argued that it would also be unable to mobilize phosphate from bone. The question then arises as to the source of the urinary phosphate seen in the D-deficient animals (Figure 2, columns 2 and 3). From the data in Figure 2 (columns 2 and 3) and Table III, it was possible to calculate the excess phosphate appearing in the urine of the D-deficient animals during the first 10 hours of hormone infusion. This was done by subtracting the rates during the control period from those seen in the first two 5 hour periods of hormone infusion. The difference was taken as a measure of phosphate loss and recorded in Table IV as excess urinary HPO_4[3]. The total loss of phosphate from plasma and extracellular fluids was calculated from the differences in the plasma phosphate concentrations seen during the control infusion and after 12 hours of hormone infusion (Table I), assuming the plasma and the extracellular fluid volume to be 20% of the body weight. These values are recorded in Table IV as total decrease in extracellular fluid (ECF) HPO_4. In both instances, the phosphate appearing in the urine was considerably in excess of that which disappeared from the extracellular fluids ($p < 0.001$). This was true even if the total ECF volume was as great as 30% of the body weight.

Because of the recent evidence that thyrocalcitonin suppresses the action of parathyroid hormone upon bone resorption (15), it was considered important to determine whether thyrocalcitonin was involved in the altered responses of the D-deficient animals to parathyroid hormone infusion. For this purpose, four D-deficient and four D-fed animals were thyroparathyroidectomized and parafused. The changes in plasma calcium and phosphate and in the rates of urinary excretion of calcium and phosphate are shown in Figure 3 and Table III. In all the D-fed animals, the initial phosphaturia was equal to that seen in the parathyroidectomized animals (compare Figure 3, column 1, with Figure 2, column 1). However, there was no initial calcium retention as seen in the parathyroidectomized animals. Calcium excretion rose slightly during the first 6 hours and then rapidly over the next 6. This was associated with a striking rise in plasma calcium from 8.5 to 18.2 as compared to 8.4 to 13.1 in the para-

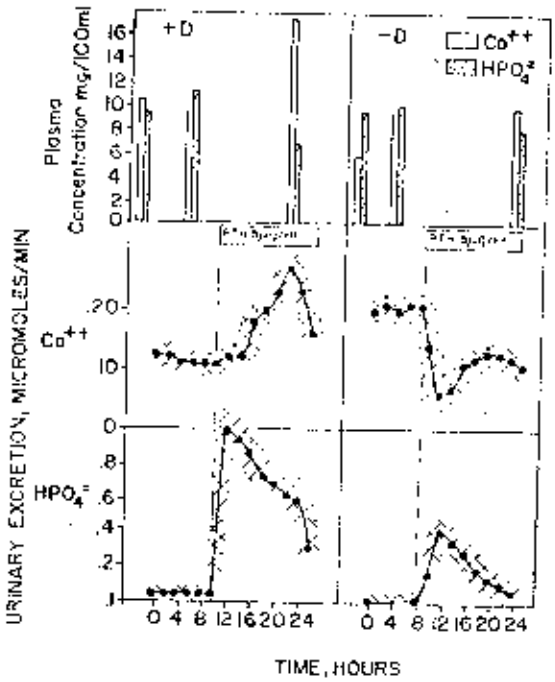

FIG. 3. CONCENTRATIONS OF CALCIUM AND PHOSPHATE IN PLASMA AND RATES OF EXCRETION OF CALCIUM AND PHOSPHATE IN THE URINE BEFORE AND DURING THE INFUSION OF PTH INTO THYROPARATHYROIDECTOMIZED D-FED (LEFT) AND D-DEFICIENT (RIGHT) RATS. There were four animals in each group. The shaded areas along the lower curves represent the range of values, and the heavy line represents the mean. The first pair of plasma values in each case represents the values obtained before the animals were operated on. The latter two pairs represent values taken during the control period and during the period of hormone infusion, respectively. Note that the D-deficient animals were infused with a more concentrated solution of calcium chloride so that the concentration of calcium in their plasma during the period of control infusion was nearly identical to that seen in the D-fed controls.

thyroidectomized animal (Table III). The rates of excretion of both calcium and phosphate fell during the last 4 hours of perfusion, but after 16 to 20 hours of hormone infusion these animals developed oliguria and died. The decline in calcium and phosphate excretion coincided with the onset of oliguria. The apparent cause of death was gross nephrocalcinosis. Because of this, perfusion could not be continued as long in these animals.

In contrast to the D-fed animals, the responses of thyroparathyroidectomized D-deficient animals were not very different from those seen in parathyroidectomized D-deficient animals (compare Figure 3, column 2, with Figure 2, column 2). In an attempt to make the experimental situation between D-fed and D-deficient animals as comparable as possible, we infused the thyroparathyroidectomized D-deficient animals with a solution containing 25 rather than 5 mM $CaCl_2$. This was sufficient to raise their plasma calcium concentrations to values slightly greater than those of the D-fed animals (Table III and Figure 3). This led to a much higher rate of calcium excretion and to the complete disappearance of phosphate from the urine during the control period (Figure 3). In spite of the high rate of urinary calcium excretion, the animals were in significant positive calcium balance, approximately 60 μmoles per hour. Parathyroid hormone infusion led to a prompt and striking fall in urinary calcium, which remained below control values throughout the period of hormone infusion. Phosphaturia was also observed and was approximately 40% of that seen initially in the D-fed controls (Figure 3). The total excess phosphate in the urine was significantly greater ($p < 0.001$) than that which disappeared from the ECF (Table IV). The plasma calcium rose only slightly, 9.2 to 9.8 mg per 100 ml, as compared to the striking rise, 8.8 to 18.2, seen in the D-fed animals. In spite of the high initial rates of calcium infusion and excretion, these animals did not develop nephrocalcinosis.

Discussion

The present results extend our previous observations (6) and those of Harrison and Harrison (7). In D-deficient rats with intact parathyroid glands, the injection (6, 7) or infusion of parathyroid hormone (Figure 2, column 3, and Table II) led to no change in plasma calcium or phosphate and no change in the urinary excretion of phosphate. However (Figure 2, column 5), a significant ($p < 0.025$) decrease in magnesium and sodium and a slight decrease in calcium excretion were observed. Removal of the parathyroid or both thyroid and parathyroid glands of these animals altered this pattern of response. Plasma phosphate fell after hormone infusion (Table III), and phosphaturia as well as magnesium and calcium retention occurred (Figure 2, columns 2 and 3; and Figure 3, column 2). Plasma calcium rose slightly, but in no instance was this significant ($p < 0.2$). This slight rise in plasma calcium was thought to be a consequence of renal retention of calcium. Furthermore, the responses of the thyroparathyroidectomized D-deficient animals (Figure 3, column 2) indicate that the lack of calcium mobilization during hormone infusion was not due to the presence of excess thyrocalcitonin. This is in sharp contrast to the results of hormone perfusion in D-fed animals. The presence of the thyroid gland altered the pattern of response of this group (compare Figure 2, column 1, and Figure 3, column 1). In the absence of endogenous thyrocalcitonin, parathyroid hormone led to a more rapid mobilization of calcium from bone. During the same period, the infusion of hormone into D-deficient animals maintained on a much higher rate of calcium infusion led to no significant hypercalcemia, but to a striking decrease in the rate of urinary calcium excretion (Figure 3).

The major differences in the responses of the parathyroidectomized D-deficient and D-fed rats to the long-term infusion of parathyroid hormone are the less marked initial phosphate and potassium diuresis, the lack of delayed hypercalciuria, and the poorly sustained phosphaturia in the D-deficient group (Figures 1 and 2). The latter two findings probably reflect the failure of the hormone to induce the mobilization of calcium and phosphate from bone in the absence of vitamin D. If this conclusion is accepted, then the data shown in Table IV indicate that parathyroid hormone mobilizes phosphate from a source other than the bone and extracellular fluids, presumably the intracellular fluids. This in turn implies that parathyroid hormone alters intracellular electrolyte concentrations as well as those in extracellular fluids, a point that has been discussed previously

in relation to the effects of this hormone on isolated mitochondria (1).

The less marked initial phosphaturia seen in the D-deficient animals (Figure 2, column 2 vs. column 1; and Figure 3) is difficult to explain. There are several possibilities. It is conceivable that the rats employed in these studies were not adequately depleted of vitamin D and that this vitamin is necessary for the action of parathyroid hormone on the renal tubule as well as on bone. However, if one considers the total response of the tubule to hormone infusion, then it is apparent that the quantitative changes in excretion of sodium and calcium were similar in D-fed and D-deficient parathyroidectomized animals; that the changes in magnesium excretion were greater ($p < 0.025$) in the D-deficient animals (Figure 2, columns 1 and 2); and that even in D-deficient sham-operated animals (Figure 2, column 5) a change in magnesium and calcium excretion was observed. Thus, by some criteria, the kidney of the D-deficient animal responds to parathyroid hormone as well as or better than that of the D-fed animal, but by others it responds less well.

One possibility that may account for the differences in phosphate excretion in the different groups of animals is that they had varying degrees of secondary hyperparathyroidism before these experiments were undertaken; it is possible that this altered the concentrations of intracellular electrolytes and consequently renal responses. There is increasing evidence (6, 16, 17) that significant hyperparathyroidism exists in vitamin D-deficient rats maintained on a normal calcium intake. This appears to be a consequence of the hypocalcemia that results from the vitamin deficiency (6, 17) and can be prevented in large part by putting these animals on high calcium intakes (6, 17). In the present context, perhaps the most important consequence of chronic secondary hyperparathyroidism is possible depletion of intracellular phosphate stores. It is important to point out that, although our animals were studied after their parathyroid glands had been removed, they received no phosphate after this surgery and hence would not have replaced tissue stores of this substance. The most obvious difference between D-deficient animals on a normal and on a high calcium intake (Figure 2, columns 2 and 3; and Table III) is that the former have low levels of plasma calcium and the latter have high levels. Thus, the latter would not have secondary hyperparathyroidism [see also (17)], and the response of these animals measured in terms of phosphate excretion (Figure 2, column 3; and Table II) and plasma phosphate (Table III) was similar to that seen in D-fed controls (Figure 2, column 1). On the other hand, the D-deficient animals with hypocalcemia undoubtedly had secondary hyperparathyroidism with resultant changes in phosphate metabolism (6), and their response measured as phosphate excretion (Figure 2, column 2) was significantly less than that of the D-fed controls. This difference would account for the fact that parathyroid hormone mobilized less total phosphate in the D-deficient animals on a normal calcium intake than it did in the D-deficient animals on a high calcium intake (Table IV). The acceptance of this proposal must await the demonstration that parathyroid hormone alters intracellular phosphate concentrations. These studies are in progress.

The results with the group of D-deficient animals fed a high calcium diet (Figure 2, column 3; and Table III) were of importance for another reason. These animals had the same plasma calcium values as the D-fed animals (Table III) during the period of control infusion (before hormone infusion) and the same degree of calcium retention during this period (Table I). Nevertheless, the infusion of hormone into these D-deficient animals did not lead to significant rise in plasma calcium (Table III) or delayed hypercalciuria as seen in the D-fed animals (Figure 2, columns 3 and 1). Also the fall in plasma phosphate concentration was as great in this group of animals as that seen in the D-fed ones (2.8 vs. 2.7, Table III). Thus, in terms of initial changes in phosphate metabolism, the two groups were nearly identical; in spite of this, parathyroid hormone did not mobilize calcium from bone. These data constitute the strongest argument in support of our previous thesis (6) that parathyroid hormone is unable to mobilize calcium from bone in D-deficient rats but is still able to exert its usual effects on renal function.

This thesis is also supported by the recent studies of the effect of inhibitors of RNA and protein synthesis upon the response of rats to parathyroid hormone and vitamin D (8, 18, 19). The infu-

sion of parathyroid hormone into an actinomycin-treated rat leads to the same pattern of urinary electrolyte changes (8) as seen in the D-deficient rat (Figures 2 and 3), that is, the early renal response is normal, but the secondary changes due to mobilization of calcium and phosphate from bone are absent. Similarly, Zull, Czarnowska-Misztal, and DeLuca (18) and Norman (19) have shown that prior treatment with actinomycin D blocks the normal response of vitamin D-deficient rats to the administration of vitamin D.

Thus, evidence gathered by several different means leads to the conclusion that vitamin D is necessary for the expression of some, but not all, effects of parathyroid hormone. The problem that develops is that of accounting for the divergent views of others (4, 5, 7, 20). There is no basic disagreement between our data and those of Harrison and Harrison (7). The important point is that, in a D deficient rat, exogenous parathyroid hormone has little detectable influence on phosphate metabolism because of high levels of endogenous hormone, which are already exerting a nearly maximal effect. Only by removing the parathyroid glands is it possible to demonstrate a need for, or a response to, exogenous hormone (Figure 2). Other studies in rodents (5, 20), which have led to results different from ours and those of Harrison and Harrison, can be questioned on their lack of sufficient evidence that the animals involved were completely D deficient. The most serious challenge to our thesis comes from the work of Ney and associates (4). These authors concluded, on the basis of the response of D-deficient puppies to parathyroidectomy and to parathyroid extract, that parathyroid hormone exerts its usual effects on bone even in D deficiency. One possible explanation is that the nature of the hormone-vitamin relationship described in the present report is unique to the rat. A more likely explanation is that the dogs studied by Ney and co-workers did not have a severe degree of D deficiency as that developed in the rats. One of the dogs with the most marked hypocalcemia had no change in plasma calcium after parathyroidectomy, and several others had minimal changes both after operation and after the subsequent injection of parathyroid extract. In fact, there was direct correlation between the initial level of plasma calcium and the fall observed after parathyroidectomy (omitting animal T782, which was most unusual), and all animals were initially hypocalcemic. These results could be accounted for if the animals studied had varying degrees of D deficiency, and they are similar to our previous observations in the rat (6).

Unfortunately, there is as yet no simple method by which the degree of D deficiency can be assessed in experimental animals; hence, it is not an easy matter to resolve all controversy. Nevertheless, in the species in which the degree of deficiency can be best controlled, the rat, it seems clear that vitamin D is necessary for physiologic concentrations of parathyroid hormone to mobilize calcium and phosphate from bone. On the other hand, there appears to be no vitamin D requirement for the expression of parathyroid hormone action at the level of the renal tubule.

Summary

The response of the D-deficient parathyroidectomized rat to the infusion of parathyroid hormone is different from that of a D-fed control. Both exhibited qualitatively similar initial changes in plasma phosphate and in the urinary excretion of calcium, phosphate, magnesium, sodium, and potassium, but in the D-deficient animals sustained phosphaturia and secondary hypercalciuria and hypercalcemia did not develop as they did in the D fed animal.

Initial phosphate excretion was less and initial magnesium retention greater in D-deficient animals raised on a normal calcium intake. However, D deficient animals raised on a high calcium intake had nearly the same initial values of plasma calcium and changes in plasma and urinary phosphate after hormone infusion as D-fed animals raised on a normal calcium intake. In spite of this, the D-deficient animals had no rise in either plasma or urinary calcium.

Removal of the thyroid gland did not greatly alter the response of the D deficient rat to parathyroid hormone, but a more immediate and marked calciuria and hypercalcemia were observed in the D-fed animal.

We concluded that vitamin D is necessary for physiologic concentrations of parathyroid hormone to mobilize calcium and phosphate from bone but not for the hormone to act on the renal tubule.

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Intestinal Calcium Absorption: Nature of Defect in Chronic Renal Disease

Abstract. *When compared to that of normal animals, calcium-binding protein activity of duodenal mucosa obtained from uremic rats was decreased. There was no change in this activity after vitamin D_3 therapy. In contrast, prior treatment with 25-hydroxycholecalciferol resulted in increased transport of calcium-45 and calcium-binding protein activity in the intestine.*

The intestinal absorption of calcium is characteristically depressed in patients with chronic renal insufficiency (*1*) and in rats with experimentally induced chronic uremia (*2*). We have presented evidence for an abnormal metabolism of vitamin D_3 in subjects with chronic uremia and a decrease in concentration of a biologically active vitamin D_3 metabolite in the plasma (*3*). This metabolite, which is normally more potent than vitamin D_3 in effecting the intestinal transport of calcium, has been subsequently identified by De Luca and co-workers as 25-hydroxycholecalciferol (*4*). In our earlier studies we suggested that the derangements of vitamin D_3 metabolism noted in uremic subjects could account, at least in part, for the intestinal malabsorption of calcium of chronic renal disease and the reported "resistance" of renal osteodystrophy to vitamin D therapy (*3*). Since there is mounting evidence that some of the effects of vitamin D or metabolites thereof on intestinal calcium transport may be mediated by the induction of a specific calcium-binding protein (Ca BP) (*5*), the possibility existed that, as a consequence of abnormal vitamin D metabolism, the activity of Ca BP was depressed in the chronic uremic state.

We now report that the concentration of Ca BP is decreased in the intestinal mucosa of rats with experimentally induced chronic uremia and defective intestinal calcium transport. Whereas concomitant increments in calcium transport and amounts of intestinal duodenal Ca BP were observed in normal rats after vitamin D_3 therapy, vitamin D_3 was ineffectual in reversing both the abnormal calcium transport and low concentrations of Ca BP in the mucosa of uremic rats. In contrast, 25-hydroxycholecalciferol was extremely effective in reversing the abnormalities in calcium transport and Ca BP activity.

The chronic uremic state was experimentally induced in male Sprague-Dawley rats (100 g) by unilateral (right) nephrectomy and ligation of most of the primary and secondary divisions of the main left renal artery. Sixty days after nephrectomy and segmental infarction of the contralateral kidney, when urea nitrogen in the blood was 70 mg per 100 ml or greater, the animals were placed on a low calcium diet for 4 days and then killed by decapitation. At death, the uremic animals were randomly distributed into one of two groups. In one group a

3- to 4-cm segment of proximal duodenum was excised from each animal and everted, and ^{45}Ca transport was measured in vitro by the everted gut sac techniques (6). In the second group, the duodenum was immediately excised, cooled to 4°C, slit open, rinsed with cold 0.12*M* NaCl and blotted dry. Mucosal tissue was scraped from the underlying muscle layer with a glass slide, and the pooled harvest (from 14 uremic animals), was homogenized in tris buffer (20 percent, weight/volume) with a Potter-Elvehjem homogenizer and a Teflon pestle. The crude homogenate was spun at 38,000*g* for 20 minutes. The supernatant was removed and heated to 60°C for 10 minutes, cooled, and centrifuged again at 38,000*g* for 20 minutes. The calcium-binding activity of a portion of the heat-treated supernatant was determined by the competitive Chelex-100 resin binding assay (7). The protein content of this portion was measured by the Lowry method (8). The remainder of the heat-treated crude mucosal supernatant was concentrated on a Diaflo membrane filter and chromatographed on Sephadex G-100 at 5°C with tris buffer as the eluting agent (9); 200 fractions containing 2.5 ml were collected. Alternate Sephadex fractions were analyzed for protein and calcium-binding activity. Pair-fed sham-operated control animals with ages similar to the uremic group were also placed on a diet low in calcium for 4 days and then killed, and both ^{45}Ca transport and Ca BP activity of crude and Sephadex-fractionated supernatants from duodenal homogenate were measured as outlined above.

The effect of orally administered vitamin D_3 and 25-hydroxycholecalciferol on the ability of the isolated gut sacs to transport ^{45}Ca and the concentration of Ca BP in the mucosa were also tested. Uremic and control animals each received 500 units of either vitamin D_3 or 25-hydroxycholecalciferol in cottonseed oil orally 12 hours before they were killed. At that time ^{45}Ca transport and Ca BP studies were performed as outlined for the untreated normal control and uremic animals.

In the uremic animals ^{45}Ca transport of isolated duodenal gut sacs was depressed (Table 1). The average ratio of the ^{45}Ca concentration in the serosal side to that in the mucosal side in rats with experimentally induced chronic uremia was significantly lower than in the controls. These findings are consistent with the observations of Kessner and Epstein, who reported that chronic renal insufficiency in rats decreases the abiltiy of isolated duodenal sacs to transport calcium against a chemical gradient (2). Whereas a significant increase ($P < .01$) in ^{45}Ca transport was noted in normal animals after vitamin D_3 therapy, vitamin D_3 failed to reverse the duodenal ^{45}Ca transport of uremic animals. In contrast, prior treatment with 25-hydroxycholecalciferol resulted in a twofold increment in ^{45}Ca transport of everted duodenal sacs obtained from rats with chronic uremia.

When compared to that of normal control animals, mucosa from uremic rats (Table 2) showed a significant ($P < .001$) decrease in Ca BP activity. Moreover, no change in calcium-binding protein was observed in the uremic animals treated with vitamin D_3, whereas the binding activity of the supernatants from the duodenum homogenates of uremic rats treated with 25-hydroxycholecalciferol was 2.9 times greater than that of the nontreated

Table 1. Calcium transport in everted intestinal sacs of normal and uremic rats: effects of vitamin D_3 and 25-hydroxycholecalciferol. Normal and uremic rats were given orally either 500 I.U. of vitamin D_3 or 500 I.U. of 25-hydroxycholecalciferol in 0.1 ml of cottonseed oil 12 hours before they were killed. The controls received the oil alone. Calcium transport, expressed as a ratio of ^{45}Ca on the serosal side to that on the mucosal side, was measured as described (7). The values are expressed as mean ± S.E.

Group	Rats (No.)	Dosage (unit)	^{45}Ca(S/M)	*P*
Normal control	10		2.5 ± 0.3	
Normal + vitamin D_3	10	500	3.8 ± 0.2	< .01*
Normal + 25-OH-cholecalciferol	9	500	4.8 ± 0.1	< .01*
Uremic control	10		1.2 ± 0.3	
Uremic + vitamin D_3	11	500	1.5 ± 0.2	> .10†
Uremic + 25-OH-cholecalciferol	10	500	2.5 ± 0.2	< .01†

* As compared to normal control. † As compared to uremic control.

Table 2. Effect of vitamin D_3 and 25-hydroxycholecalciferol on the calcium-binding activity in duodenum of normal and uremic rats. Normal and uremic rats were given orally either 500 I.U. of vitamin D_3 or 500 I.U. of 25-hydroxycholecalciferol in 0.1 ml cottonseed oil; the controls received the oil vehicle alone. Mucosal scrapings of 14 rats were harvested for each determination. The results represent the mean ± standard error of the mean for each group.

Group	Tests (No.)	^{45}Ca in supernatant (%/ml)	Protein (mg/ml)	Specific activity (%^{45}Ca/mg protein)
Normal control	9	10.6 ± 0.4	1.58 ± 0.06	6.7 ± 0.2
Normal + vitamin D_3	7	11.9 ± 0.5	1.48 ± 0.07	7.9 ± 0.2
Normal + 25-OH-cholecalciferol	8	9.6 ± 0.2	0.99 ± 0.02	9.6 ± 0.3
Uremic control	7	3.9 ± 0.6	1.37 ± 0.03	3.0 ± 0.3
Uremic + vitamin D_3	7	4.1 ± 0.3	1.40 ± 0.03	3.0 ± 0.3
Uremic + 25-OH-cholecalciferol	9	11.0 ± 0.4	1.29 ± 0.06	8.6 ± 0.3

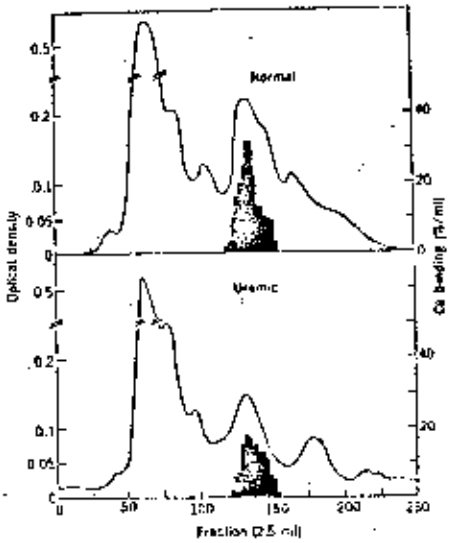

Fig. 1. Distribution of proteins and calcium-binding activity in supernatant fractions of heat-treated supernatants from homogenized mucosa of normal (upper panel) and uremic (lower panel) rats, after Diaflo and dextran-gel filtration (Sephadex G-100). Elution was performed with *p*H 7.4 tris buffer at 5°C, and 2.5-ml fractions were collected. Protein was measured densitometrically in each tube. Portions of alternate fractions were also assayed for protein (8) and calcium-binding (black histogram) activity (7).

uremic control group. Further purification of the Ca BP with Sephadex G-100 chromatography revealed that the Ca BP activity in both normal and uremic rats was associated with a specific protein fraction and therefore was not a general property of all the proteins contained in the crude heat-treated supernatant (Fig. 1). This isolation procedure resulted in a protein fraction (tubes 140 to 155) which contained all the Ca BP activity in the mucosa homogenates of untreated animals as well as from those treated with vitamin D_3 and 25-hydroxycholecalciferol.

Like that of the crude heat-treated supernatant from mucosa homogenates of uremic animals (Table 2), the binding activity of material isolated by Sephadex chromatography rose minimally after vitamin D_3 treatment but more than twofold after administration of the 25-hydroxycholecalciferol vitamin D_3 metabolite. Because 10 to 16 hours may be required before the defective intestinal calcium absorption is measurably restored to normal by oral vitamin D_3 therapy (*10*), calcium-binding activity of duodenal mucosa was also measured in uremic rats 24 and 48 hours after 500 units of vitamin were administered. In each instance, no significant increment in Ca BP was demonstrable when compared to the Ca BP activity of uremic rat mucosa obtained 12 hours after vitamin D_3 administration.

So far, vitamin D dependent Ca BP has been detected in the intestinal mucosa of the rat, dog, and monkey (*11*). The nature of the interaction of vitamin D with the mucosal cell that leads to the formation of the calcium-binding protein is still in doubt. Studies by Zull, Czarnowska-Misztal, and De Luca (*12*) and Norman (*13*) suggest that this interaction represents either a direct effect of 25-hydroxycholecalciferol on the DNA–messenger RNA complex of the mucosal cell or an indirect alteration of the permeability characteristics of the nuclear membrane. Although the product of this nuclear or membrane interaction has been proposed to be a translocase or transport enzyme (*12*), the possibility that the substance formed is identical to the vitamin D dependent Ca BP isolated from the chick mucosa by Wasserman and co-workers (*5, 7, 9, 11*) must be entertained. Should this interpretation prove correct, alterations in vitamin D_3 metabolism in uremia (*3*) resulting in decreased intestinal 25-hydroxycholecalciferol concentrations could lead sequentially to decreased synthesis and concentration of the intestinal Ca BP and contribute to the defective calcium absorption characteristic of the chronic uremic state.

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Histo of the Development of Homogenize and Vitamin D Milks in the United States

*F. Bruce Baldwin, Jr.**

HOMOGENIZATION

The story of the invention and development of the homogenizer, its application to natural emulsions such as milk, the introduction of homogenized milk, and the research on homogenized milk which followed, furnish one of the most interesting episodes in the development of the modern dairy industry.

F. Bruce Baldwin, Jr.

The history of homogenization appears to date back to early 1592, when Paul Marix, a Frenchman, was granted two patents for the purpose of manufacturing margarine. Later in the same year, Julien was granted a patent for making artificial butter from oil, milk, margarine, et cetera; the principle adopted being the same as that of Marix's first patent, except that the mixture was forced through many holes in the mechanism instead of through only one.

Marix's and Julien's patents were concerned with rendering homogeneous a heterogeneous mixture of the constituents of artificial butter. No thought was given to making a stable emulsion out of a natural unstable emulsion, such as milk. This was to come later. With the advent of industrial sterilization and the accompanying transportation of milk over long distances, creaming and churning in transit became a problem. It was therefore necessary to "fix" the composition of milk.

Auguste Gaulin, mechanic, inventor, business entrepreneur, and Chevalier de la Legion d' Honneur, owned and operated the Gaulin Dairy Machinery Equipment plant in Paris. It was he who had seen the unsightly fat globules in the milk and turned thoughtfully to his drawing board and workbench for a solution. It was he who conceived the notion of pumping whole milk under high pressure through a small hole—the engineers called it "a restricted orifice." The results were superb. The large fat globules were broken up, and a smooth homogeneous emulsion emerged. It was a product for the gods. It had taste, it had texture, and was described with exclamation: "Ah, it is the homogenized milk." On December 26, 1899, Gaulin was granted a patent on a "process and apparatus for fixing the composition of liquids," aiming specifically at milk. Whereas Marix and Julien were interested in emulsifying fats mixed with a watery liquid, Gaulin was concerned with fixing the emulsion that occurred naturally.

Milk treated by Gaulin's process and fixed in its composition appeared for the first time at the World's Fair in Paris in 1900, where it stirred up profound curiosity. Because of the stability of the milk's constituents, made homogeneous, the name "lait homogeneise" was given to it. Thus, a new verb, defining anical action unknown up to that time, was added to the dictionary. Milks .. processed were variously referred to as "Gaulin's Milk," "fixed milk," or "homogenized milk."

Following the World's Fair showing of homogenized milk in 1900, much interest was manifested in the development of machinery to render homogeneous liquid mixtures. Speculation also was noted on the use of such machines for pharmaceutic preparations.

Meanwhile, Gaulin, realizing that attainment of perfect homogenization by the principle involved in his first machine seemed impossible, began a scientific study of milk under the microscope to perfect his method. In 1902, he improved his invention by the development of the so called high-pressure machine. In this machine, the milk was forced by a powerful piston pump between two plane surfaces fitting closely together and held in close apposition by a powerful spring. Machines based on this principle have been widely used in the dairy industry ever since. In ice cream mix, they blend the ingredients intimately together, and in evaporated milk they permit the development of a uniform stable suspension that can be stored in cases for many years. Machines of this type utilize high pressures of the magnitude of 2,000 to 3,000 pounds.

Mechanical homogenization can be obtained by other types of machines which have been developed since the inventions of Marix, Julien, and Gaulin. Within more recent years, major attention has been centered upon improving the sanitization of the homogenizing equipment.

The most noticeable effect of homogenization is the breaking up of fat globules into smaller ones. Normal milk contains fat globules which average 4 to 8 microns in diameter. After homogenization the globules average less than 1 micron in diameter. This reduction in size increases the number of globules 200 or more times, and increases the surface area of the globules approximately 6 or more times. An almost permanent dispersion of the fat throughout the milk serum is the result.

Originally intended as a sterilized milk for transportation over long distances and for extended storage, homogenized milk today is a fresh pasteurized milk having many of the qualities of regular pasteurized milk, but possessing some additional characteristics of its own. Its value centers around three factors: (1) the finer subdivision of the butterfat, (2) the softer curd (both of these factors have beneficial effects from a nutritional and digestive standpoint), (3) and the improved flavor. This richer-tasting milk with cream in every drop means greater consumption.

The author will not discuss in this paper the physical and chemical changes resulting from homogenization. These aspects together with others, such as nutritional advantages and the effect of homogenization in rendering milk more easily digestible, will be elaborated in fuller detail in other papers in this symposium.

It appears that Allen first described homogenized milk to American readers in the *Scientific American* in April, 1904. Homogenized milk found recognition in America at La Colle, Quebec, Canada, during the years 1910 to 1912. This new product was unsuccessful there, probably because homogenization of milk was ahead of researches in medicine, bacteriology, engineering, and the dairy science which have since contributed much to modern homogenization technics. Extrava-

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gant cl[illegible] ot supported by scientific evidence, also seem to have been ma-
Not unt[illegible] 7, again in Canada, was homogenized milk successfully introduced. The Laurentian Dairy, Ottawa, Ontario, sold its first bottle of homogenized milk on April 8, 1927. Shortly thereafter the product was introduced in Toronto and other Canadian cities. By 1932 milk plants in many of the large cities and towns of Ontario offered homogenized milk for sale.

As to the introduction of homogenized milk in the United States, data are somewhat limited. Hudson states that some homogenized milk was distributed apparently experimentally on the University of Illinois creamery routes in 1921 and later in 1922, by the Illinois Dairy, Springfield, Illinois, but the product did not become popular until the early thirties. A sales chart (Hudson) shows that in April, 1934, less than 11,000 quarts of homogenized milk were sold. Tracy stated that it was approximately 1932 when the University of Illinois introduced homogenized milk on their milk routes. Doan stated: "In Pennsylvania there is little doubt but that homogenized milk was introduced into some of the state institutions before it was offered to the public. Mont Alto Sanitorium dispensed homogenized milk to the patients first in January, 1930, and this was followed by its appearance in other institutions within a year. This antedated any dealer distributing homogenized milk by about two years."

McDonald introduced homogenized milk successfully in Flint, Michigan, in 1932. By experiments and demonstrations involving regurgitation studies, attention of the public was drawn to homogenized milk sales by the McDonald Dairy Company in the midst of the economic depression. This stimulated much interest not only in Michigan, but throughout the United States. Correspondence with the Anthony Price Milk Company of Nashville, Tennessee, reveals that firm as having first introduced homogenized milk in their area of distribution in 1934.

Perhaps the greatest single impetus in the interest and adoption of homogenized milk in the United States stems directly from studies commenced in Philadelphia in early 1928 (and continuing since) by Doctors Leslie A. Chambers, Joseph Stokes, Jr., Irving J. Wolman, and Bernhard Spur of the Children's Hospital of Philadelphia and the University of Pennsylvania.

Doctors Chambers and Stokes were originally interested in the purely scientific observation that high frequency sound waves had the property of shattering bacteria exposed to these waves in a liquid medium. It was hoped that sonic impact applied to milk might prove a substitute for pasteurization. However, after a number of years of investigation, it was found that the process was not practical. An important incidental observation was that sonic vibration resulted in the homogenization of the fat globules in the milk. This led to the development of the so-called "sonic homogenizer," which was marketed for some years by the Submarine Signal Company of Boston. Though useful in small plants, the device could not handle the large volume of the average-sized dairy.

In 1938, Dr. Irving J. Wolman was applying the classic artificial coagulation approach, developed by Dr. Joseph Brennemann of Chicago, to the milk formulas being used in infant feeding, with special reference to curd properties of sonic-homogenized and pressure-homogenized milks. The preliminary observations indicated that in physical characteristics the curds from these [illegible]genized milks seemed of the same small, soft nature as the curds rendered by boiled milk and similar preparations which were successful in artificial infant-feeding.

After consultations with representatives of the milk dealers of the Philadelphia area, a large-scale investigation was drawn up and subsidized as a collaborative effort between the Children's Hospital of Philadelphia and the Philadelphia Milk Exchange. Assistance was given by the Inter-State Milk Producers' Association, Mr. Henry Woolman, Sr., of the Supplee-Wills-Jones Milk Company; Dr. J. A. Webb of Abbotts Dairies, Inc.; Dr. Thomas Kelly of the Scott-Powell Dairies; Mr. William Fusselbaugh of Harbison Dairies, and the author. Mr. Thomas Waddell of the Philadelphia Milk Exchange was among those particularly active in this project.

The plan was designed to investigate the various technics that might be used for homogenizing milk and to evaluate the results of the different processes on human nutrition and digestion. In one of the Philadelphia dairies, four homogenizers were set up for a large scale study—a sonic apparatus, a low-pressure homogenizer, and two different types of high-pressure homogenizers. Dr. Bernhard Spur took over the responsibility of laboratory control and supervision and developed a special method for measuring the size of the curds in coagulated homogenized milk. These studies resulted in a definition of soft curd milk. Doctor Sydney Borowsky and Rae Nicholas assisted Dr. Wolman in careful supervision of over 800 babies who were fed for the greater part of their first year of life with these various processed milks, using boiled cream-line milk as a control. The results showed that "in digestibility and safety the homogenized milks proved as satisfactory as the control boiled milk." Recommendations were made to the dairy industry for preferred technics of processing with special emphasis upon temperatures and pressures.

This large-scale study was followed with great interest by the dairy industry at large. There were a great many visitors to the Milk Research Laboratory of the Children's Hospital of Philadelphia from all parts of the United States, from Canada, and from foreign countries to follow the procedures of the study. Even before the project was completed, the results were so favorable that milk plants in various parts of the country began to market homogenized milk on a large scale. In Philadelphia, the product was not introduced until March, 1940, after the findings of the study were completely analyzed and thoroughly evaluated.

Commencing in 1940, demand for the product throughout the United States began to increase at a truly astounding rate. In 1946 Bixby reported that 50 per cent of the volume of milk sold in this country was homogenized. Spencer, in 1949, found that approximately 70 per cent of the fluid milk sold in New York City was homogenized. Just what percentage of milk plants are homogenizing is not known. However, it appears safe to state that virtually all plants having a considerable volume of business offer homogenized milk as one of their regular items. The percentage of homogenized milk in the individual plants ranges from 10 to 100 per cent.*

* In writing this history of homogenized milks, the author has drawn freely from the archives of the Manton Gaulin Manufacturing Company, Inc., and from the recently published text *Homogenized Milk* by Dr. G. Malcolm Trout, Professor of Dairy Manufacture, Michigan State College. Scientific investigators, who have contributed much to our present knowledge of this subject, include the names of Hill, Tracy, Doan, Weckel, and many others.

There a[illegible]ably no conditions which contribute more to physical inferiority in man than defects in skeletal development. Most important among these is the result of a syndrome designated as rickets. Rickets leads to deformity of the bones from abnormal enlargement of their ends and from bending, owing to lack of resistance of the bones to the body weight, to muscular tension, and to atmospheric pressure. The latter factor is especially important in changing the form of the thorax. Bowlegs, knock knees, enlarged joints, flat or deformed chest, and abnormal conformation of the skull, all may result from failure of the bones to develop in a normal manner. Rickets is essentially a disease of infancy and early childhood. Vitamin D is the major dietary factor which can prevent and cure most cases of rickets.

With reference to the history and geographic distribution of rickets, the author would like to quote from the article, "The Disease of Poverty and Darkness," which appeared in *Today's Health*, May, 1952, and was presented by Anna May Wilson: "If your ancestors were handsome dark-skinned people who let their children play naked under palm trees, while they ate oranges and drank goat's milk—follow their example. They had something.

"Or, if your ancestors were bright eyed Eskimos who taught their children early how to dip their first two fingers in the family pot of fish oil and slurp it off with their lips—follow your family tradition and at least your children will never get rickets.

"If, however, your forbears came from a crowded city of central Europe—think twice before you follow their lead. They were skilled at weaving, metalwork and other industries. They built exquisite cathedrals and were wonderful in their production of religious art, but at raising babies they did a sorry job. This shows up dramatically in the oil paintings they have passed down to us. Religious subjects often called for children as models. You may be sure the artists chose children they considered the finest in town to represent Christ, but many show signs of rickets.

"Look for instance at 'Virgin and Child' by Hans Burkmair of Nuremberg (1500) and you will see a baby with rachitic head and bowlegs. Study the infant in the 'Adoration of the Christ by Mary,' painted in Cologne about 1460, for an example of the grooved chest and enlarged abdomen caused by rickets. Or observe in 'The Nativity,' the five little cherubim, all with square heads of rickets, carefully painted in Augsberg ten years later."

In his text *The Newer Knowledge of Nutrition*, Dr. E. V. McCollum of Johns Hopkins University stated that in the National Museum at Washington there are several hundreds of skulls of Indians who lived between 200 and 300 years ago. Their sources extend from the Aleutian Islands down the Pacific coast through Honduras and Yucatan into Peru. In the entire collection only a single tooth showed dental caries. Among these primitive peoples, the diet was excellent from the standpoint of chemical completeness, and bone defects and bad teeth were unknown or nearly so.

In his second edition of the above text, published in 1922, Dr. McCollum stated as follows: "From what has been said of recent developments in the study of rickets, there can no longer be any doubt that it is a disease which is essentially due to nutritional disturbances, and that dietetic errors play the don[illegible] role in its etiology. It is also clear that at least three dietary factors, calc[illegible], phosphorus and an organic substance which is more abundant in cod liver oil than in any other substance known, are especially important in that rickets will develop unless proper amounts of these be furnished to the growing animal. Other factors may favor or interfere with the development of the disease."

In 1918 to 1919, Mellanby of England reported the first convincing experiments showing that rickets is a deficiency disease cured by diet. However, he entertained the erroneous idea that the antirachitic factor was vitamin A. Not until 1922 did McCollum and co-workers show that cod liver oil contains two fat soluble vitamins and that the antirachitic factor is distinct from vitamin A. Further experimentation by the same group showed that vitamin A was unstable when boiled for a certain period in the presence of air, whereas the other vitamin which demonstrated definite antirachitic properties was stable under such treatment. This relatively stable factor was later termed vitamin D.

In the meanwhile, definite information was obtained concerning the beneficial effect of ultraviolet light as well as sunshine in the healing of rickets in children and in experimental animals. In 1924 and 1925, a number of papers appeared in which were described the remarkable effect of ultraviolet irradiation in endowing foods, otherwise ineffective, with antirachitic potency. Attention was called to this phenomenon at approximately the same time by Hess and Weinstock and by Steenbock and his students. It was observed by Steenbock and Nelson that a ration which ordinarily induced rickets in rats could be made definitely antirachitic by the simple expedient of exposing it to ultraviolet light. Hess reported that, by means of irradiation, vegetable oils, green vegetables, dry milk, and other substances acquired antirachitic properties. Two discoveries followed, namely, the identification of ergosterol as the parent substance of vitamin D (in 1927) and the isolation of calciferol, the active component of irradiated ergosterol (in 1931–1933). Studies on the irradiation of cholesterol and other sterols followed.

Doctors Alfred F. Hess and J. M. Lewis, in their paper, "An Appraisal of Antirachitics, in Terms of Rat and Clinical Units" (J. A. M. A., July 15, 1933) stated as follows: "There are, in all, five reliable and well established antirachitics, each one of which represents a distinct and different pharmacologic point of view. The first to be established was cod liver oil, a natural product; the second to be introduced was direct irradiation of the body by means of ultraviolet energy; the third was irradiated food, particularly milk, activated by exposure to ultraviolet radiations; the fourth, an irradiated basic substance, ergosterol, extracted from yeast; the fifth, representing still another method, was 'yeast milk,' a biologic product endowed with antirachitic potency as the result of feeding irradiated yeast to the cow. In addition to these five established agents there are numerous others which consist of various modifications or combinations; for example, the addition of a concentrate of cod liver oil to milk, or of viosterol to cod liver oil. Whether one or the other of these several antirachitics should be adopted in the campaign to eradicate rickets from the community will depend not so much on their efficacy in preventing rickets as on their availability, which involves their cost to the con-

sumer. [illegible] readiness with which they are accepted by the public, their palatab[illegible] and o[illegible] nmedical considerations."

As early as 1932 the *Journal of the American Medical Association* carried the following editorial comment: "Obviously, it ought to be of advantage if antirachitic (vitamin D) properties could be imparted without attending deterioration, in a suitable degree to a few foods that enjoy widespread use, particularly in the dietary of childhood. Milk is of particular interest because of its unrivaled content of calcium and phosphorus. . . ."

From 1932 to early 1937, vitamin D milks produced by the addition of concentrates, by direct irradiation, or by the feeding of irradiated yeast to cows began to be marketed throughout the country, including the state of Pennsylvania. In those days, the most extensive supervision of the vitamin D milk project was exercised by what is now called the Council on Foods and Nutrition of the American Medical Association. On January 16, 1937, the Council published a report entitled, "The Present Status of Vitamin D in Milk." It may assumed that publication of this report was intended by the Council to serve as a guide for the medical profession in addition to serving as a statement of the Council's policy in "acceptance" of Vitamin milks. In their report, the Council stated that before they would consider acceptance of any vitamin D fortified food "a general need for the contemplated addition must be evidenced." They then made the following statement: "There is, of course, abundant evidence that infants and very young children need extra vitamin D. Though an increased proportion of infants receive additional vitamin D in some form, many still do not. Many physicians prefer to prescribe pharmaceutical preparations of vitamin D because of the freedom thus afforded to adjust the dosage to the individual patient. The Council on Foods respects this attitude, but at the same time recognizes *that a considerable portion of the population unfortunately does not consult a physician, except for illness.* Consequently it seems to the Council to be in the interests of the public as a whole to recognize and accept the fortification of food with vitamin D."

With specific regard to milk, the Council made this additional statement: "Of all the common foods available, milk is most suitable as a carrier of added vitamin D. Vitamin D is concerned with the utilization of calcium and phosphorus, of which milk is an excellent source."

Other organizations interested in the public health adopted a similar attitude. As a result of this favorable reaction and of stimulation from the National Dairy Council, the sale and distribution of vitamin D milk increased by leaps and bounds.

In the fourth edition of *Infant Nutrition*, published in 1947, about 14 years after the introduction of vitamin D milk, Dr. P. C. Jeans wrote: "Rickets still is found with some frequency in some of our large cities, especially among the dark-skinned races, but it has almost disappeared in the country as a whole."

Today a high proportion of bottled milk and practically all evaporated milk is fortified to the recommended level of 400 U.S.P. units of vitamin D to the quart, which is believed sufficient to prevent rickets and satisfy the growth needs.

An integral part of the program is the protection of the public by regular checking of the milk. This is customarily done by State Health Departments or by special

independent laboratories approved by the respective States. [illegible] importance of this safeguard is not, as yet, universally appreciated. Parent[illegible] ally, one of the first of these testing laboratories to be established in this country is in Philadelphia at the Children's Hospital.

Early fortification of milk with vitamin D, whether by irradiation or by the addition of vitamin concentrate, was applied for the most part to cream line milk. However, with the advent of homogenization, dairies were quick to recognize the advantages of a milk of softened curd and one in which the added vitamin, associated with the fat, would be distributed evenly throughout the body of the milk. The discovery of this method for combating rickets, dental caries, and similar bone disorders has been beneficial to mankind.

While topic discussed here was specifically about homogenized and vitamin D milk, it might also be mentioned that in the cities of some 13 states there are now being distributed market milk containing added multiple vitamins and minerals (iron and iodine). Mention should be made, also, of the recent introduction of nonfat (fat-free) milk fortified with vitamins A and D, and with or without added nonfat milk solids; also nonfat milk with multiple vitamins and added minerals and with or without added nonfat milk solids. Nonfat milks should appeal to those who cannot tolerate fat, as well as those on a reducing diet.

TABLE I

Combined Sales of Homogenized Milks in Philadelphia, Pa. with and without Vitamin D Included—Expressed as Per Cent of the Total Class I "In Area" Sales, for the Month of July 1942, 1948, and 1953

1942	21.3%
1948	45.5%
1953	61.8%

TABLE II

Increase in Sales of Homogenized Milks without Added Vitamin D

	Grade "A"	Grade "B"
July 1948	528,119 lb. (245,637 qt.)	2,826,539 lb. (1,314,669 qt.)
July 1953	346,392 lb. (161,112 qt.)	10,677,092 lb. (4,966,089 qt.)

DISCUSSION

Dr. Rice (Evap. Milk Assn.): Dr. Baldwin's paper is most excellent, and contains information of the history of homogenization and vitamin D fortification the

A review of the literature will reveal the names of many who have contributed generously to our present knowledge of the history of vitamin D and its application to food fortification. The omission of any one or more investigators from this necessarily brief history is purely unintentional.

like of which I haven't heard any pla[illegible] I would just like to add a sentence to that history. The evaporated milk [illegible]ry, back about 1905, or 1907, or 1909, depending on which company's word you take as authoritative, began the homogenization of milk going into cans, so that by 1910 or 1912 this process was quite general in the industry. There was not the thought of soft curd in those days or of greater digestibility of the fat. That came later. There was the very serious problem of fat separation because evaporated milk may stand in one place, in one position, for six months or more. And I believe that we probably were the first ones to use one of the Gaulin homogenizers. I have tried to find out what company first practiced homogenization, but the records are incomplete.

Mr. Calldwell (?): Dr. Baldwin, in your table for Philadelphia sales, you show a decline in Grade A milk from 1948 to 1953, and a great increase in homogenized Grade B without vitamin D added. What is the reason for that trend, in your opinion?

Dr. Baldwin: The decrease in Grade A is undoubtedly due to the increase in golden Guernsey sales, where there has been a switch from Grade A to golden Guernsey in the market.

In standard milk, the increase in sales of plain homogenized milk, and a corresponding decrease in Vitamin D homogenized milk is due to the fact that there is a differential in price between homogenized milk and Vitamin D homogenized milk. As I understand it, several firms have converted all of their standard milk to homogenized milk. The figures do not show the full story.

The point I wanted to make was that vitamin D milk, historically, is very important. It seems foolish, I do believe, that we are not adding more Vitamin D to milk. We should add it and sell homogenized Vitamin D milk instead of just homogenized milk. The Vitamin D is of great importance to the population. These figures show a trend which is alarming because many consumers think they are purchasing homogenized milk containing Vitamin D when they are not, due to the fact they have associated vitamin D with homogenized milk for so long.

Voice: Sounds like a good reason for this symposium.

Sm 2 Dis LA 20 45 12 3 7

CONTROL WITH FORTY UNITS (STEENBOCK) OF COD LIVER OIL VITAMIN D CONCENTRATE IN MILK

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Standardization of potency of antirachitic substances by rat assay has, with the introduction of antirachitic agents other than cod liver oil or its concentrates, lost much of its value through the discovery that the relative prophylactic amounts of these different substances for rats, chickens and infants are not comparable unit for unit. No such dissimilarity was suspected during the time that irradiated products were first being developed until it was found that viosterol, when used for rats, chickens and infants, fell entirely out of line with what was known concerning the preventive dose of cod liver oil for the three species[1] noted. Irradiated milk now seems to be demanding a still further revision of ideas concerning vitamin D effectiveness[2] as measured in rat units. While viosterol measured in rat units when compared to cod liver oil seems relatively impotent,[3] for infants irradiated milk seems to be just the reverse, its relative effectiveness being claimed to be higher, according to rat assay of potency,[3] than that of cod liver oil.

The amount of viosterol, expressed in rat units, required by the infant has during the past few years received considerable attention, while few studies in which present assay methods are used have been made of the minimum requirements of cod liver oil. An investigation reported in 1929,[3a] in which 3 teaspoonfuls of cod liver oil were used for prophylaxis, included rat assays of the oil, but the dosage was based on the observations of Eliot,[4] who found by trial and observation that this dose was most satisfactory, no rat assay being made of the cod liver oil that she used. Accepted standards for cod liver oil are still based on clinical opinion[5] rather than on controlled observational material. Until more definite clinical standards are available for cod liver oil, the relative values of cod liver oil, viosterol and irradiated milk cannot be adequately expressed. The most recent statement[5] gives the following as equivalent dosage:

	Units
Irradiated milk	40
Viosterol	600
Cod liver oil	240

Such a comparison, expressed in rat units, seems premised on the old standard of 3 teaspoonfuls of cod liver oil a day as a proper prophylactic dose for a baby—an amount determined empirically at a time when that amount of cod liver oil contained about 150 rat units of vitamin D, instead of 240 units, as do the good oils of today.

The following report contains data bearing on the aforementioned question, obtained with vitamin D milk made by incorporating a vitamin D concentrate of cod liver oil in milk and indicates that such conclusions are based on a fallacy. The study was begun before the introduction of irradiated milk in purchasable form and the publication of recent data on it, and consequently my observations were made only on the cod liver oil concentrate in milk. It would be highly desirable to have a comparison of results when different types of vitamin D milk are used by infants under the same conditions and at the same time.

Eliot and others have indicated the great need of a reasonably priced antirachitic agent easily available to all classes of population. She showed that salmon oil,[6] although at present not commercially available in sufficient quantities, has a high antirachitic potency, and that it might, with some revision of industrial practice, be made available at a low cost. From the point of view of public health, this entire line of thought is of the utmost importance. With the possibility of a low price and certain advantages in distribution, it was felt that a vitamin D milk, with the added advantage of its palatability and nearly automatic administration for infants, was well worth investigating.

During the winter of 1932 and 1933 further observations were made on the amount of vitamin D necessary for prophylaxis of rickets. My

One Steenbock unit is equal to approximately 2.7 U. S. P. X (1934) revised) units of vitamin D.

1. Steenbock, H.; Kletzien, S. W. F., and Halpin, J. G.: The Reaction of Chickens to Irradiated Ergosterol and Irradiated Yeast as Contrasted with the Natural Vitamin D of Fish Liver Oils, J. Biol. Chem. 97:249 (July) 1932.

2. Hess, A. F., and Lewis, J. M.: An Appraisal of Antirachitics in Terms of Rat and Clinical Units, J. A. M. A. 101:181 (July 15) 1933.

3. (a) Barnes, D. J.; Brady, M. J., and Jones, E. M.: The Comparative Value of Irradiated Ergosterol and Cod Liver Oil as a Prophylactic Antirachitic Agent when Given in Equivalent Dosage According to Rat Units of Vitamin D, Am. J. Dis. Child. 39:15 (Jan.) 1930. (b) DeSanctis, A. G., and Craig, J. D.: Comparative Value of Viosterol and Cod Liver Oil as Prophylactic Antirachitic Agents, J. A. M. A. 94:1285 (April 20) 1930.

4. Eliot, M. M.: The Control of Rickets, J. A. M. A. 85:653 (Aug. 29) 1925.

5. Council on Pharmacy and Chemistry of the American Medical Association: Average Optimal Dosage of Cod Liver Oil, J. A. M. A. 98:316 (Jan. 23) 1932.

6. Eliot, M. M.; Nelson, E. M.; Souther, S. P., and Cary, M. E.: Value of Salmon Oil in Treatment of Infantile Rickets, J. A. M. A. 99:1075 (Sept. 24) 1932.

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preliminary studies[7] with a commercial cod liver oil concentrate[8] homogenized in milk indicated that 150 units, or even 75, was more than necessary for prophylaxis in the average infant. In the study described here, I therefore aimed to approach the minimal prophylactic dose of cod liver oil vitamin by ascertaining the effect of 50 units of the concentrate when given in the medium of milk.

The group consisted of thirty-eight infants from the Child Welfare Clinics of the Detroit Health Department who were observed through the winter. A daily dose of 50 units of vitamin D was given each baby, and this was maintained in spite of individual variations in the amount of milk used in formula feeding by the addition of the proper amount of cod liver oil concentrate to 1 pint (473 cc.) of milk. Each infant received a pint of the special milk every day and, if more milk was needed, plain pasteurized milk was added in sufficient quantity to make the formula. Therefore, irrespective of the total quantity of milk in the day's feeding, the amount of concentrate remained the same. The special milk was prepared under careful supervision[9] by the addition of a specified number of rat units of vitamin D concentrate to a small amount of milk by means of a small homogenizing apparatus, which thoroughly incorporated the vitamin D materials in the milk.[10] This special mixture was then mixed completely with the remainder of the milk in such quantity that each pint contained the requisite amount, namely, 50 Steenbock units of vitamin D.[11] The intake of milk and the methods of making the formulas were carefully checked in the field several times during each two week period between clinic visits by a nurse who devoted her full time to the study and who consequently was able to evaluate the degree of cooperation obtained through her intimate home association. Only cooperating families were included, the rest being dropped from the experiment.

In the group studied, nineteen infants were under 6 months of age, eleven under 4 months and the remaining nineteen between 6 and 12

7. Barnes, D. J.: Antirachitic Concentrates, J. Michigan M. Soc. 32:242 (April) 1933.

8. Zucker, T. F.: Further Observations on the Chemistry of Cod Liver Oil, Proc. Soc. Exper. Biol. & Med. 20:135, 1922-1923. Addition of Vitamin D Concentrate to Milk, Am. J. Pub. Health 23:16 (Jan.) 1933.

9. The patents for the cod liver oil concentrate are under the control of Columbia University.

10. Committee on Foods of the American Medical Association: Vitamin D Fortified Pasteurized Milk (150 Vitamin D Units per Quart) Advertising of W. J. Kennedy Dairy Company, J. A. M. A. 101:34 (July 1) 1933.

11. The potency of the concentrate received an additional check through biologic assays conducted on market samples of vitamin D milk. The concentrate used in my special milk was the same as that used in the commercial product. The biologic assays were conducted by C. A. Hoeppert, Ph.D., of the department of physiological chemistry at Michigan State College.

months. The average age at the start of the observation was 3.64 months, eight of the infants entering the study in November and thirty in December. None had had antirachitic therapy before the special milk containing cod liver oil vitamin D concentrate was given other than some casual exposure to sunlight during the summer. Twenty-five of the babies were white, and thirteen were colored. Roentgen examination[12] of the long bones (wrist and knee) was made approximately every two weeks to determine any rachitic state, present or developing. At the start, since work commenced late in the fall, six babies showed slight rickets. This disappeared, as roentgen examination revealed, during the next few weeks, and no new cases developed during the study.

TABLE 1.—*Summary*

Number of infants in experimental group		38
Race: White	25	
Colored	13	
Average age: At beginning of study	3.64 months	
At end of study	10.63 months	
Roentgenographic findings: Normal throughout	32	
Rachitic on first examination	6	
Healed at end of study	6	
Number of infants in control group		25
Race: White	13	
Colored	12	
Average age: At end of study, i.e., at time of roentgen examination	10.23 months	
Roentgenographic findings: Rachitic	14 (56%)	
Normal-like	11 (44%)	

The gains in weight were in general satisfactory, the gain averaging approximately 1 pound (0.5 Kg.) a month. Detailed data are given in the tables. Intercurrent infections occurred among the group, as would be expected, but no unusual problems in nutrition developed, though the infants were from poverty-stricken homes.

In April the collection of a group of control cases was started. A total of twenty-five babies, taken without selection as they appeared at the clinic, were examined by roentgen rays. They had had no antirachitic treatment during the winter, as they had not come under clinical supervision, and therefore afforded a suitable standard for comparison with the study group. Thirteen were white, and twelve were colored. Their average age was 6.23 months; they did not differ in age materially from the experimental group at the end of the period of study. Of the controls, fourteen (56 per cent) were rachitic, and eleven

12. The roentgenograms were made at the Children's Hospital of Michigan and interpreted by Dr. D. B. Witwer.

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—Prevention (Normal Infants Receiving 50 Units [Steenbock] of Vitamin D per Day in the Form of Vitamin D Milk)

Name, Race, Date of Birth	Date of Examination	Weight Lbs.	Weight Oz.	Roentgen Findings
L. J.	11/28/32	12		Negative
White	12/12/32	12	6	Negative
9/?/32	12/26/32	12	15	Negative
	1/10/33	13	4	Negative
	1/?/33	14	8	Negative
	?/?/33	13	14	Negative
	?/?/33	15	7	Negative
	3/25/33	15	15	Negative
	4/?/33	15	10	Negative
	4/?/33	16		Negative
M. L.	1/7/33	14	5	Negative
White	1/31/33	15	9	Negative
8/21/32	2/?/33	16		Negative
	2/18/33	?	8	Negative
	3/?/33	?	15	Negative
	3/?/33	17	5	Negative
	?/?/33	?	2	Negative
	4/?/33	?	4	Negative
E. D. M.	1/?/33	9	?	Negative
White	?/16/33	10	2	Negative
11/?/32	?/?/33	11	1	Negative
	?/10/33	12	2	Negative
	?/?/33	12		Negative
	3/15/33	13	12	Negative
	4/?/33	13	5	Negative
	4/20/33	14	12	Negative
D. M.	1/?/33	14	10	Negative
Colored	1/?/33	15	7	Negative
7/?/32	2/?/33	?	1	Negative
	2/1?/33	15	9	Negative
	2/?/33	16		Negative
	3/?/33	16	5	Negative
	3/12/33	11	11	Negative
	?/?/33	?	?	Negative
	4/?/33	17	12	Negative
	4/27/33	17	15	Negative
D. M.	1/5/33	21	13	Negative
White	1/?/33	22	6	Negative
2/10/32	2/?/33	22	13	Negative
	2/?/33	23	2	Negative
	3/2/33	23	10	Negative
	3/?/33	24	2	Negative
	3/?/33	21	2	Negative
	4/?/33	21	5	Negative
	4/27/33	22		Negative
L. S.	1/?/33	25	7	Negative
Colored	1/14/33	23	5	Negative
1/?/32	1/28/33	23	13	Negative
	2/11/33	23	11	Negative
	2/25/33	24	5	Negative
	3/11/33	25		Negative
	3/?/33	25		Negative
	4/?/33	25	12	Negative
J. W.	12/?/32	15	11	Negative
White	1/?/33	16	12	Negative
8/19/32	1/?/33	17	12	Negative
	3/18/33	19	?	Negative
	4/22/33	21	3	Negative
S. S.	12/29/32	18	11	Negative
White	1/12/33	18	7	Negative
2/1/32	2/4/33	20	5	Negative
	2/18/33	20	11	Negative
	3/4/33	21	12	Negative
	3/?/33	22	1	Negative
	4/19/33	22	5	Negative
	4/?/33	22	10	Negative
A. C.	12/?/32	11	2	Negative
White	1/?/33	11	7	Negative
8/1/32	2/?/33	11	12	Negative
	2/?/33	12		Negative
	3/?/33	12		Negative
	4/?/33	12	8	Negative
	4/?/33	*		Negative

Name, Race, Date of Birth	Date of Examination	Weight Lbs.	Weight Oz.	Roentgen Findings
M. R.	12/?/32	17	15	Negative
White	1/?/33	18	6	Negative
?/?/32	1/?/33	18	9	Negative
	2/?/33	19	4	Negative
	2/?/33	19	7	Negative
	3/?/33	20	6	Negative
	4/?/33	20	11	Negative
	4/?/33	21	11	Negative
F. M.	12/?/32	11	4	Negative
White	1/11/33	15	2	Negative
8/?/32	2/?/33	19	9	Negative
	?/?/33	19	12	Negative
	3/19/33	?	?	Negative
	?/?/33	22		Negative
	?/?/33	21		Negative
P. C.	12/?/32	17	12	Negative
White	12/?/32	18	8	Negative
2/?/32	12/27/32	18	14	Negative
	1/?/33	18	1	Negative
	?/?/33	19	1	Negative
	2/7/33	19	6	Negative
	2/21/33	20	7	Negative
	3/7/33	20	8	Negative
	3/?/33	20	1	Negative
	4/?/33	20	12	Negative
	4/25/33	21	3	Negative
F. E. W.	11/?/32	?		Negative
White	12/12/32	20	6	Negative
2/?/32	12/?/32	?		Negative
	1/10/33	22	10	Negative
	1/26/33	22	14	Negative
	2/?/33	?	15	Negative
	?/?/33	22	1	Negative
	?/4/33	22	8	Negative
	3/?/33	21		Negative
	4/?/33	22	5	Negative
	4/15/33	22	14	Negative
R. M. W.	11/29/32	16	4	Negative
White	12/21/32	17	3	Negative
?/?/32	1/4/33	17	3	Negative
	1/?/33	17	7	Negative
	2/?/33	18	11	Negative
	3/?/33	19	10	Negative
	3/?/33	20		Negative
	4/?/33	20	9	Negative
	4/?/33	21		Negative
J. Z.	11/30/32	9	7	Negative
White	12/?/32	10	5	Negative
10/15/32	1/?/33	10	9	Negative
	1/17/33	10	?	Negative
	?/?/33	11	2	Negative
	2/?/33	?	4	Negative
	3/?/33	?	5	Negative
	3/?/33	?	9	Negative
	4/?/33	13	15	Negative
	4/?/33	14	7	Negative
D. P.	12/1/32	16	13	Negative
Colored	12/17/32	16	2	Negative
6/2/32	12/31/32	16	4	Negative
	1/14/33	17		Negative
	1/28/33	17	13	Negative
	2/11/33	18	6	Negative
	2/25/33	18	14	Negative
	3/?/33	19		Negative
	3/?/33	20	1	Negative
	4/?/33	20	3	Negative
	4/?/33	20	4	Negative
P. C.	12/?/32	16	7	Negative
White	12/?/32	16	12	Negative
5/?/32	1/?/33	17	4	Negative
	1/?/33	17	8	Negative
	2/?/33	17	9	Negative
	2/?/33	17	9	Negative
	3/?/33	17	13	Negative
	3/18/33	18	2	Negative
	4/?/33	18	14	Negative
	4/?/33	19		Negative

* Not taken.

Table 2.—Prevention (Normal Infants Receiving 50 Units [Steenbock] of Vitamin D per Day in the Form of Vitamin D Milk)—Continued

Name, Race, Date of Birth	Date of Examination	Weight Lbs.	Weight Oz.	Roentgen Findings
J. M. C.	12/1/32	19	2	Negative
Colored	12/17/32	19	13	Negative
4/1/32	1/?/33	20	6	Negative
	1/31/33	?		Negative
	2/7/33	21	8	Negative
	2/?/33	21	13	Negative
	3/?/33	?	9	Negative
	3/?/33	?	6	Negative
	4/9/33	?	12	Negative
	4/22/33	22	8	Negative
C. L.	12/2/32	9	12	Negative
Colored	12/?/32	10	?	Negative
9/1/32	1/?/33	11	4	Negative
	1/?/33	11	14	Negative
	2/11/33	12	6	Negative
	2/?/33	14		Negative
	3/?/33	14	11	Negative
	4/7/33	15	8	Negative
	4/2?/33	16	1	Negative
A. L.	12/2/32	13	9	Negative
Colored	12/?/32	14	13	Negative
8/2/32	1/7/33	16	4	Negative
	1/21/33	17	6	Negative
	2/4/33	17	6	Negative
	2/18/33	17	6	Negative
	3/?/33	17	9	Negative
	3/18/33	18	14	Negative
	3/29/33	20	4	Negative
	4/?/33	20	1	Negative
	4/?/33	20	4	Negative
R. R.	12/?/32	8	14	Negative
White	12/31/32	9	8	Negative
8/6/32	1/14/33	10	14	Negative
	2/?/33	11	0	Negative
	2/?/33	12	4	Negative
	3/?/33	14		Negative
	3/?/33	14	13	Negative
	4/18/33	15	8	Negative
	4/?/33	16	12	Negative
M. S.	12/31/32	19		Negative
White	1/14/33	20	6	Negative
(Twin)	1/?/33	21	8	Negative
8/?/32	2/11/33	21	7	Negative
	2/?/33	21	8	Negative
	3/11/33	21	14	Negative
	4/?/33	22	2	Negative
	4/?/33	22	15	Negative
J. Z.	12/21/32	22		Negative
White	1/?/33	22	1	Negative
(Twin)	1/?/33	24		Negative
2/21/32	1/?/33	24	7	Negative
	2/11/33	24	5	Negative
	2/?/33	24	7	Negative
	3/11/33	24	14	Negative
	4/?/33	25		Negative
	4/22/33	25	8	Negative
D. H.	12/17/32	10	8	Negative
White	12/31/32	11	6	Negative
9/?/32	1/17/33	12	7	Negative
	1/31/33	13	3	Negative
	2/14/33	13	9	Negative
	3/?/33	14		Negative
	3/?/33	14	12	Negative
	4/11/33	15	6	Negative
	4/25/33	15	2	Negative
R. S.	12/17/32	18	8	Negative
Colored	1/?/33	19	12	Negative
3/?/32	1/21/33	20		Negative
	2/18/33	21		Negative
	3/4/33	21	2	Negative
	3/18/33	21	9	Negative
	?/?/33	22	3	Negative
	4/?/33	22	7	Negative
	4/?/33	22	11	Negative

Name, Race, Date of Birth	Date of Examination	Weight Lbs.	Weight Oz.	Roentgen Findings
L. S.	12/13/32	14		Negative
White	12/27/32	14	8	Negative
8/?/32	1/?/33	15	2	Negative
	?/?/33	15	7	Negative
	3/7/33	18	10	Negative
	3/?/33	19	7	Negative
	4/?/33	19	1	Negative
	4/20/33	19	8	Negative
J. T.	12/12/32	16	8	Negative
White	1/?/33	17	2	Negative
8/27/32	2/?/33	17	0	Negative
	2/?/33	18	2	Negative
	3/?/33	20		Negative
	4/?/33	?	?	Negative
F. W.	12/?/32	22	14	Negative
Colored	12/?/32	22	4	Negative
2/20/32	1/?/33	22	15	Negative
	1/19/33	21	1	Negative
	2/2/33	24	6	Negative
	2/?/33	24		Negative
	3/?/33	25	8	Negative
	3/?/33	25	1	Negative
	4/19/33	25	4	Negative
D. T.	12/6/32	12	2	Negative
Colored	12/22/32	12	5	Negative
8/30/32	1/?/33	12	11	Negative
	1/?/33	13	3	Negative
	2/?/33	14	4	Negative
	2/?/33	14	8	Negative
	3/?/33	15	3	Negative
	3/?/33	15	7	Negative
	4/1/33	16	5	Negative
	4/15/33	16	15	Negative
	4/?/33	17	11	Negative
C. C.	12/?/32	14		Negative
White	12/20/32	13	14	Negative
8/28/32	1/3/33	14	6	Negative
	1/17/33	15	3	Negative
	1/31/33	16		Negative
	2/14/33	16	3	Negative
	2/?/33	16	8	Negative
	3/?/33	17		Negative
	3/?/33	17	4	Negative
	4/18/33	17	4	Negative
	4/27/33	17	8	Negative
J. W.	12/5/32	12	6	Negative
Colored	12/?/32	12	7	Negative
7/7/32	1/?/33	11	0	Negative
	1/?/33	12	10	Negative
	2/16/33	13	1	Negative
	3/?/33	12	15	Negative
	3/10/33	11	12	Negative
	3/?/33	12	6	Negative
	4/11/33	12	14	Negative
	4/?/33	13	3	Negative
D. H.	12/?/32	19	8	Negative
Colored	12/?/32	19	13	Negative
12/?/31	1/7/33	20	0	Negative
	1/?/33	20	4	Negative
	2/4/33	20	6	Negative
	2/18/33	20	13	Negative
	3/4/33	20	7	Negative
	3/18/33	20	13	Negative
	?/?/33	20	12	Negative
	?/?/33	21	11	Negative
	4/27/33	21	4	Negative

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apparently the structure of not one of them has been worked out.[14] To judge by physiologic action, however, it is perfectly definite that one form of artificial vitamin D, namely, "viosterol," has properties quite different from the factor originally designated as vitamin D. The properties of the antirachitic factor of irradiated milk, on the other hand, seem to resemble those of natural vitamin D more closely.

In clarifying the situation one must not only give more attention to the determination of the minimum dose but also consider the form in which vitamin D is given. Its degree of dispersion and its physical environment may play a large role. Janes and others[15] have indicated that the degree of dispersion of fat soluble physiologically active substances is of importance, and Hess also alluded to this in discussing the action of vitamin D in irradiated milk. Further, a standardized clinical technic for gathering data is as essential as a proper laboratory procedure if satisfactory comparisons are to be made.

CONCLUSIONS

1. Thirty-two normal infants were completely protected against rickets from November or December to April on 50 units of vitamin D given as a concentrate in milk.

2. Six infants entering the study with slight roentgenographic signs of rickets showed progressive improvement on 50 units of vitamin D in milk a day. Since the rachitic lesions were slight, the healing processes were not striking, but they were nevertheless definite when traced throughout the series of roentgenograms. In no case did the patient grow worse during the study.

3. A control group of twenty-five infants without antirachitic treatment during the winter were examined by roentgen rays in April. Fourteen, or 56 per cent of these, showed active rickets, the remainder being normal.

4. It is shown that for comparison of the various forms of vitamin D the ratio 40:240 for the vitamin D of irradiated milk and the vitamin D of cod liver oil does not hold. When both are given in milk the required units are approximately equal. Considering the relatively small

14. (a) Askew, F. A.; Bruce, H. M.; Callow, R. K.; Philpot, J. St. L. and Webster, T. A.: Nature 128:758, 1931. (b) Windaus, A. H., and Leitringhaus, A.: Ueber das kristallisierte Ergosterin, Deutsche med. Wchnschr. 58:1609 (Oct. 21) 1931. (c) Bills, C. E. and McDonald, F. G.: Crystalline Vitamin D, J. Biol. Chem. 93:189 (April) 1932.

15. Janes, E. R.; Grover, H. F., and Quinn, E. J.: Method of Enhancing the Vitamin A Value of Cod Liver Oil, Proc. Soc. Exper. Biol. & Med. 30:516 (Jan.) 1933. Bills and McDonald.[14c]

number of infants, the error of biologic assay[16] and the difficulty of quantitative clinical evaluation, it is not safe to say that one differs from the other.

5. A standardized clinical procedure is necessary in order to make satisfactory quantitative comparisons of various antirachitic substances.

The Child Welfare Division of the Detroit Health Department, the Detroit Department of Public Welfare and the Staff of the Children's Hospital of Michigan cooperated and assisted with this study.

16. Although rat assays present their difficulties, the large numbers done in various laboratories during the last four years have led to a sufficient knowledge so that when a large enough number of animals is used an acceptable accuracy can be attained. The usual commercial standardization is ±15 per cent; while the use of many more animals would give better results, such practice is infrequent.

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NUTRITION

INHIBITION OF VITAMIN D METABOLISM BY ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE. Lee Ann Baxter*, Hector F. DeLuca, J.-P. Bonjour* and Herbert A. Fleisch*. Dept. of Biochem., Univ. of Wis.-Madison, Madison, WI 53706.

Administration of high doses of disodium-ethane-1-hydroxy-1,1-diphosphonate (EHDP) (20 mg/kg body weight/day for two weeks) to chicks results in an inhibition of intestinal calcium absorption, a decrease in accumulation of bone ash, and a hypercalcemia. Simultaneous administration of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (125 pmole/day) restored intestinal calcium absorption to control levels, while an equal level of 25-hydroxyvitamin D_3 (25-OH-D_3), the immediate precursor of 1,25-$(OH)_2D_3$, was ineffective. The 1,25-$(OH)_2D_3$, however, did not restore the accumulation of bone ash to normal. *In vitro* incubations of kidney mitochondria prepared from EHDP-treated chicks showed a marked decrease in the conversion of ^{3}H-25-OH-D_3 to 1,25-$(OH)_2D_3$ and a marked increase in the conversion to 24,25-dihydroxyvitamin D_3 as compared to controls. These findings were confirmed by *in vivo* experiments. EHDP administration prevents accumulation of 1,25-$(OH)_2D_3$ in intestinal mucosa and kidney following injection of ^{3}H-25-OH-D_3. These results suggest that high doses of EHDP result in inhibition of intestinal calcium absorption via inhibition of the 1-hydroxylation of 25-OH-D_3. (Supported by grants from the NIH, No. AM-14881 and AM-15512.)

On the Abnormal Calcium Absorption in Sarcoidosis*

Evidence for Increased Sensitivity to Vitamin D

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WITH THE TECHNICAL ASSISTANCE OF ESTHER DILLER AND HAROLD SMITH
Bethesda, Maryland

HYPERCALCIURIA and hypercalcemia, reported to occur in from 10 to 40 per cent of patients with sarcoidosis [3 5], may lead to nephrolithiasis, nephrocalcinosis and renal failure [6 8]. In previous studies, it has been demonstrated that the defect in calcium metabolism is one of excessive intestinal absorption of calcium, which can be corrected with cortisone and other carbohydrate-active steroids. It has thus been proposed that the defect may result from either an abnormal sensitivity to vitamin D [9] or an increase in circulating vitamin D [10].

It is the purpose of the present study to evaluate the role of vitamin D in the pathogenesis of the defect in calcium absorption.

METHODS

Four patients between twenty and forty-three years of age, in whom sarcoidosis had been confirmed by biopsy, were maintained on an air-conditioned metabolic ward. Diet and fluid intake were kept constant throughout each study. Stools were collected in four- or six-day periods, and urine in one-, two-, or three-day pools. Stools, diets and urine were analyzed for calcium, phosphorus and nitrogen content by methods previously reported [11]. Blood was drawn at intervals for the determination of calcium, phosphorus and alkaline phosphatase levels. Alkaline phosphatase activity was determined by the method of either Bessey and Lowry [12] (normal adult range 0.8 to 2.9 Bessey-Lowry units) or King and Armstrong [14] (normal adult range 4 to 13 King-Armstrong units).

The effects of prednisone (30 or 50 mg. per day given orally in four divided doses for twelve days (three patients) and 50 mg. a day for twenty days (patient J. R. T.), vitamin D_2 (10,000 units per day given intramuscularly for twelve days) and of prednisone and vitamin D given together were determined in each patient. In two (J. R. T. and E. J. S.), calcium intake was kept constant; in the other two (M. E. and M. C.), calcium intake was increased with each treatment as well as in a control period when no treatment was given.

The effects of vitamin D_2 (10,000 units per day given intramuscularly for twelve days) were also measured in two normal women, aged twenty-two and twenty-six, in whom calcium intake was kept constant.

In two patients (J. R. T. and E. J. S.) the effect of prednisone on the absorption of calcium was determined by the oral administration of $Ca^{47}Cl_2$, 1 μc. per dose [14]. Radioactivity was measured in all subsequent stool specimens until background readings were obtained. Results were expressed as per cent of administered dose excreted (and, by difference, absorbed). In one patient (E. J. S.) and in the two normal subjects, the effect of vitamin D_2 on Ca^{47} absorption was also determined. In this patient (E. J. S.), 10 mg. of prednisone was given each day throughout the study and 10 units of ACTH for part of the study for therapeutic reasons; despite this, the effects of vitamin D and of large doses of prednisone with vitamin D were readily demonstrated.

The methods and rachitogenic diet employed were those described in the A.O.A.C. Methods of Analysis [15], for the bioassay of vitamin D in serum [16] with the standard line test as modified by Shine, Friedman and Tolle [17]. A strain of Sprague-Dawley rats produced for this purpose was used. They were fed 1 to 5 cc. of serum by stomach tube; at least four rats were used for each assay. The effects on rachitic cartilage were compared to healing produced by doses of 4 or 10 units of vitamin D (U.S.P. Reference

* From the Clinical Endocrinology Branch, National Heart Institute, National Institutes of Health, Bethesda, Maryland. This study was presented in part at the International Congress on Sarcoidosis, Washington, D. C., June 1, 1960 [1] and at the Annual Meeting of the Endocrine Society, New York City, New York, June 24, 1961 [2]. Manuscript received April 18, 1963.

† Present address: Northwestern University School of Medicine, Chicago, Illinois.

36: 500-13, (1964)

Standard). This method gives a reproducibility of ±25 per cent. The normal range is 65 to 400 units per 100 ml.

RESULTS

Effects of Prednisone and Vitamin D. (Fig. 1 through 6.) In one patient (J. R. T.), 50 mg. of prednisone per day produced an increase in mean fecal calcium from 270 to 457 mg. per day (Fig. 1, days 9 through 20), a decrease in serum calcium from a range of 9.8 to 11.6 mg. per 100 ml. (days 1 through 8) to as low as 8.2 mg. per 100 ml. (day 25), and a decrease in mean urinary calcium from abnormal (271 mg. per day) to normal (128 mg. per day) values. After the administration of prednisone was stopped (days 21 through 36), serum calcium level rose to 9.8 mg. per 100 ml. (day 33), but the other changes persisted.

The serum phosphorus level fell from 2.9 to 2.1 mg. per 100 ml., and serum alkaline phosphatase activity decreased from 20.4 to 5.0

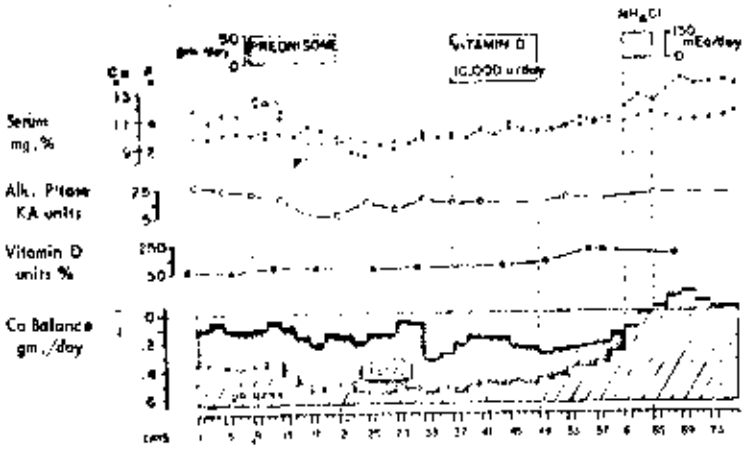

Fig. 1. Patient J. R. T. Effects of prednisone, vitamin D and ammonium chloride (NH_4Cl) on serum calcium, phosphorus, alkaline phosphatase and antirachitic activity and calcium balance.*

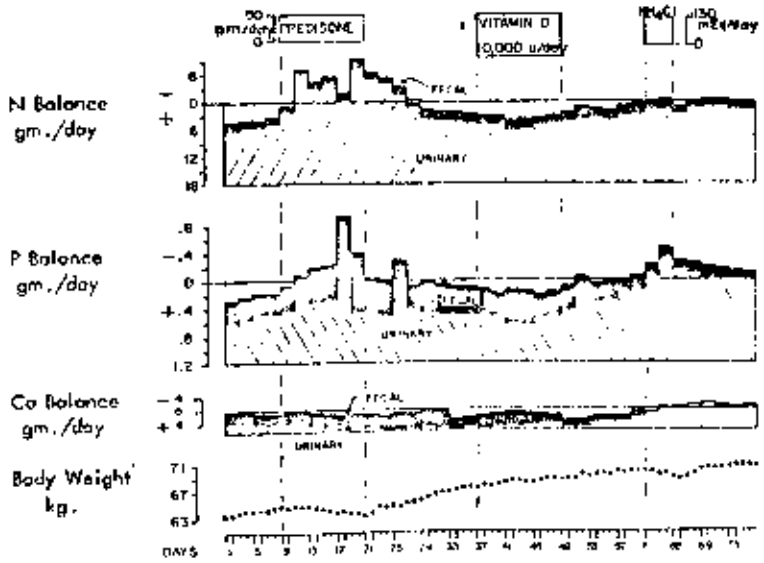

Fig. 2. Patient J. R. T. The effects of prednisone, vitamin D and NH_4Cl on nitrogen, phosphorus and calcium balances and body weight.

* In these and in subsequent charts, balance data are plotted as follows: intake is plotted from the zero line to the bottom of the column, and urinary and fecal values are plotted upwards from the intake line. Thus a positive balance is shown as a clear area below the zero line, a negative one by fecal or urinary values above the line.

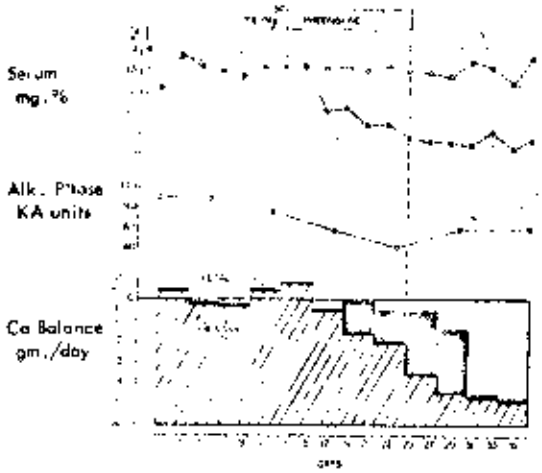

FIG. 3. Patient J. R. T. The effects of prednisone on serum calcium, phosphorus and alkaline phosphatase and calcium balance.

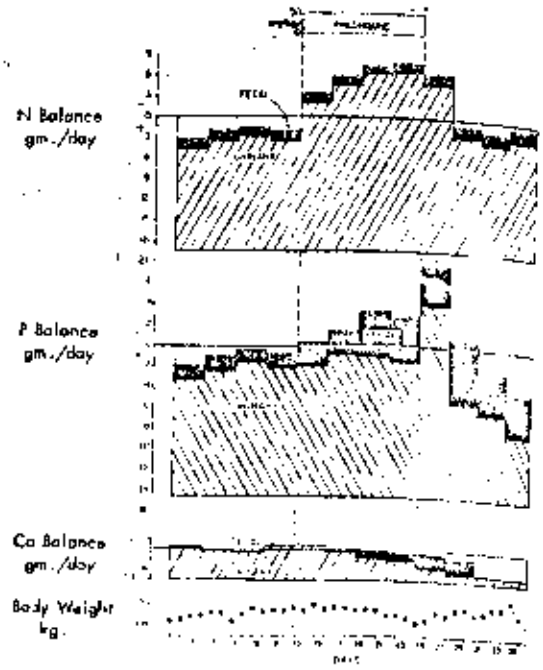

FIG. 4. Patient J. R. T. The effects of prednisone on nitrogen, phosphorus and calcium balances and body weight.

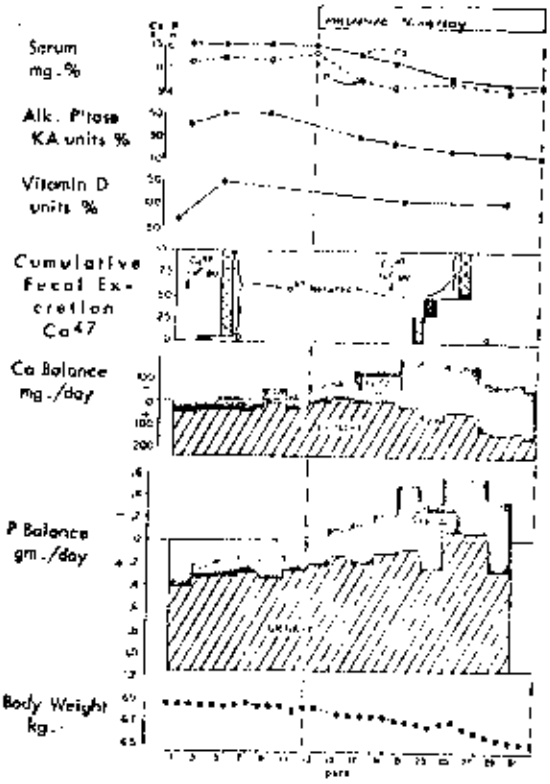

FIG. 5. Patient J. R. T. The effects of prednisone on serum calcium, phosphorus, alkaline phosphatase and antirachitic activity and on calcium and phosphorus balances, Ca^{45} absorption and body weight.

King-Armstrong units per 100 ml. during prednisone therapy. Serum antirachitic activity was 42 and 57 units per 100 ml. without treatment, and 72 and 80 units per 100 ml. when prednisone was given.

With the administration of 10,000 units of vitamin D per day, fecal calcium decreased from an average of 381 (Fig. 1, days 21 through 36) to an average of 303 mg. per day (days 37 through 48). After vitamin D therapy was stopped, fecal calcium continued to decrease until it reached 20 mg. per day; serum calcium increased to 13.6 mg. per 100 ml. (day 69); and urinary calcium increased to a high of 769 mg. per day (days 69 and 70).

During this period serum phosphorus increased to 3.8 mg. per 100 ml. (day 65); serum alkaline phosphatase activity did not change and serum antirachitic activity increased to 194 units per 100 ml.

Excretion of nitrogen and phosphorus increased during prednisone therapy (Fig. 2, days 9 through 20). After treatment with vitamin D, fecal phosphorus decreased and urinary phosphorus showed an increase parallel to that of serum phosphorus. The administration of ammonium chloride, 130 mEq. per day, (days 61

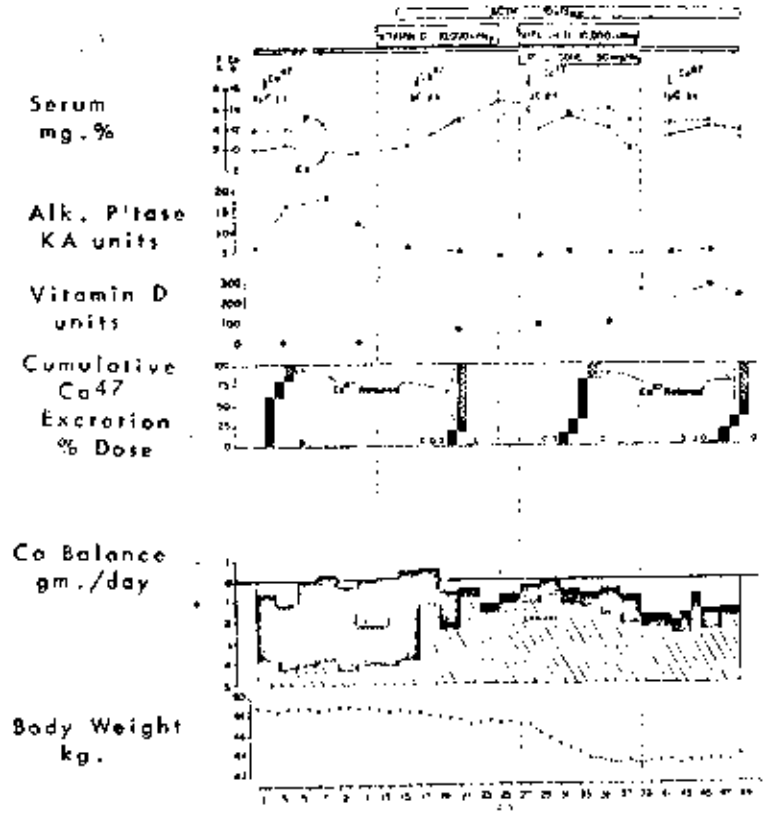

FIG. 6. Patient E. J. S. The effects of vitamin D and prednisone on serum calcium, phosphorus, alkaline phosphatase and antirachitic activity and on calcium balance and Ca^{47} absorption.

through 63) further increased the urinary excretion of phosphorus.

Two months later, the effect of vitamin D was still present (Fig. 3, days 1 through 12). Fecal calcium averaged 14 mg. per day, and urinary calcium 604 mg. per day. Serum calcium ranged from 12.2 to 13.8 mg. per 100 ml. The administration of prednisone, 50 mg. per day, increased mean fecal calcium to 313 mg. per day (days 25 through 29) and decreased serum and urinary calcium to normal (10.0 mg. per 100 ml. and 109 mg. per day, respectively). Fecal calcium decreased to an average of 22 mg. per day during the last four days of the study, after prednisone therapy had been stopped.

Whereas serum phosphorus levels did not change during prednisone therapy, the alkaline phosphatase level fell from 75 to 42 units per 100 ml. The administration of prednisone again caused an increase in urinary nitrogen and phosphorus and an increase in fecal phosphorus (Fig. 4, days 13 through 24).

Two years later, when hypercalcemia and hypercalciuria were again present (Fig. 5), the administration of 50 mg. of prednisone a day produced an increase in mean fecal calcium from 60 to 181 mg. per day (Fig. 5, days 13 through 32), a decrease in serum calcium from a range of 12.3 to 13.2 mg. per 100 ml. (days 1 through 12) to as low as 9.6 mg. per 100 ml. (days 29 and 33) and a decrease in average urinary calcium from 212 to 188 mg. per day.

Serum phosphorus decreased from 5.7 to 4.0 mg. per 100 ml. when prednisone was given and the serum alkaline phosphatase level fell from 51 to 13 King-Armstrong units per 100 ml. Serum antirachitic activity was 70 and 150 units per 100 ml. before treatment and 110 and 105 units per 100 ml. after prednisone was given. Urinary and fecal phosphorus also increased when prednisone was given (Fig. 5, days 13 through 32).

Effects of Prednisone and Vitamin D on Ca^{47} Absorption. (Fig. 5 and 6.) In one patient (J. R. T.), 50 mg. of prednisone a day (Fig. 5, days 13 through 32), produced an increase in the fecal recovery of Ca^{47} from 5.6 (days 2 through 5) to 56.6 per cent (days 20 through 30).

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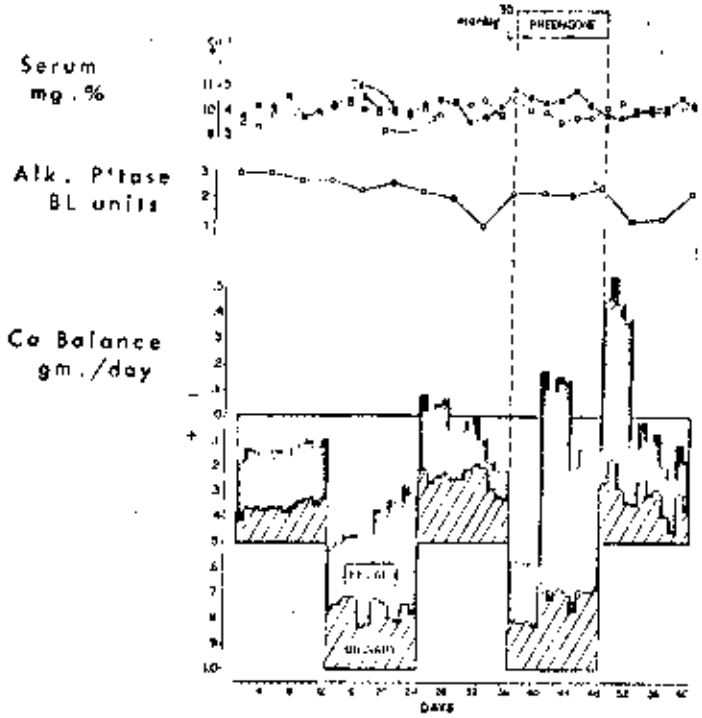

FIG. 7. Patient M. E. The effects of prednisone and of increases in calcium intake on serum calcium, phosphorus and alkaline phosphatase and on calcium balance.

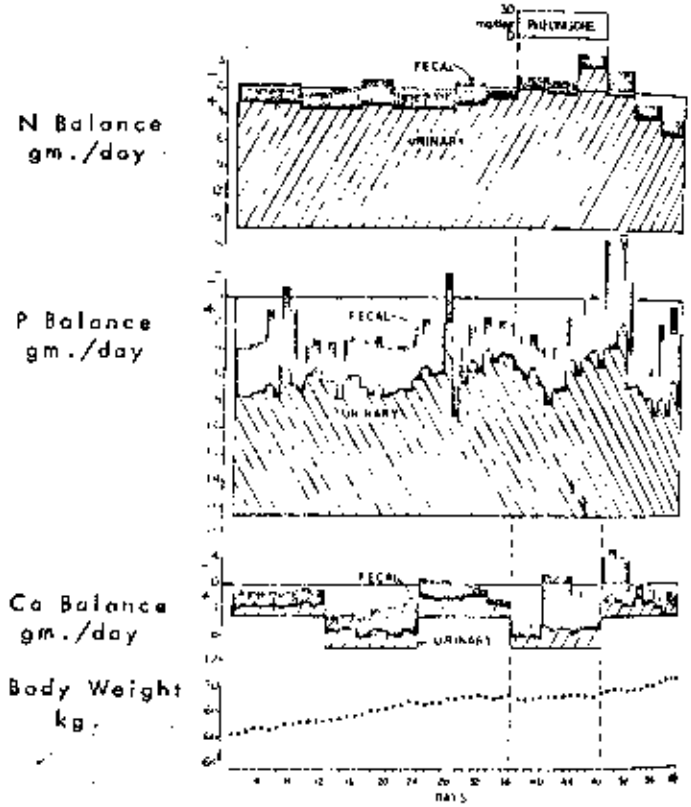

FIG. 8. Patient M. E. The effects of prednisone and of increases in calcium intake on nitrogen, phosphorus and calcium balances and on body weight.

In another patient (E. J. S.), 10 mg. of prednisone a day caused 87 per cent of the dose of Ca^{47} to be excreted in the stools (Fig. 6, days 3 through 6). When vitamin D was given, only 30 per cent was recovered in the stools (days 17 through 21). When 50 mg. of prednisone per day was given together with vitamin D, 85 per cent of a dose of Ca^{47} was excreted (days 28 through 34). Again under "control" conditions (10 mg. of prednisone per day, and 10 units of ACTH per day), 53 per cent was recovered (days 42 through 49).

Effects of An Increase in Calcium Intake. (Fig. 7 through 9.) When the intake of calcium was increased from 506 to 1,006 mg. per day in one patient (M. E.) (Fig. 7, days 13 through 24), fecal calcium increased from a mean value of 346 to a high of 725 mg. per day (days 23 and 24), and urinary calcium increased from normal (mean 138 mg. per day, days 1 through 12) to abnormal values (mean 235 mg. per day, days 13 through 24). Despite reduction of the intake (days 25 through 36), urinary calcium levels remained elevated (mean 249 mg. per day). Fecal calcium averaged 201 mg. per day, and was less than that in the control period. The serum calcium changed little, if at all, with the change in intake, and there was no change in serum, urinary or fecal phosphorus or in urinary or fecal nitrogen (Fig. 8, days 13 through 24).

In another patient (M. C.), an increase in calcium intake from 500 to 1,000 mg. per day produced a mean increase in fecal calcium of only 121 mg. per day from an average of 111 to an average of 232 mg. per day (Fig. 9, periods 5 through 8), but increased serum calcium to abnormal values (above 10.8 mg. per 100 ml.) and urinary calcium from a mean of 558 to 676 mg. per day.

Serum alkaline phosphatase and serum phosphorus levels were not altered by the change in calcium intake. Serum antirachitic activity, which fluctuated between 170 and 336 units per 100 ml., was not affected.

Effects of Prednisone and An Increase in Calcium Intake. (Fig. 7 through 9.) In one patient (M. E.) (Fig. 7) with the same increase in calcium intake, 30 mg. of prednisone per day produced a more marked increase in fecal calcium (mean 544 mg. per day, days 37 through 48), than that observed with an increase in calcium intake alone (mean 356 mg. per day, days 13 through 26). Urinary calcium levels remained elevated (mean 261 mg. per day) until calcium intake was reduced, when it averaged 176 mg. per day (days 49 through 60).

When prednisone was given, serum calcium, phosphorus and alkaline phosphatase levels were normal and did not change (Fig. 7), whereas the urinary and fecal excretion of nitrogen and phosphorus increased (Fig. 8, days 37 through 48).

In another patient (M. C.) with the same increase in calcium intake, 50 mg. of prednisone per day produced an increase in mean fecal calcium to 379 mg. per day (Fig. 9, periods 13 through 16), in contrast to the mean value of 232 mg. per day observed with an increase in calcium intake alone (periods 5 through 8). Prednisone prevented a rise in serum calcium, and decreased mean urinary calcium from 676 to 407 mg. per day. When the added calcium was discontinued, urinary calcium excretion fell to normal levels (average 188 mg. per day, periods 17 through 20).

Effects of Vitamin D and An Increase in Calcium Intake. (Fig. 10 through 13.) In one patient (M. E.), 10,000 units of vitamin D per day and an increase in calcium intake from 320 to 820 mg. per day (Fig. 10, days 13 through 24), mean fecal calcium increased from 85 to 202 mg. per day, and average urinary calcium increased from 238 to 253 mg. per day. When vitamin D was given, fecal calcium level tended to fall and was less than that seen with an increase in calcium intake without vitamin D when it tended to rise (mean fecal calcium 354 mg. per day, Fig. 7, days 13 through 24).

Whereas serum calcium, phosphorus and alkaline phosphatase levels were normal and not altered by vitamin D, serum antirachitic activity increased from 117 to 223 units per 100 ml. (day 35).

When vitamin D was given, nitrogen excretion did not change, but urinary phosphorus decreased (Fig. 11, periods 7 through 12). Fecal phosphorus decreased together with fecal calcium after treatment was stopped (periods 13 through 18).

In another patient (M. C.), 10,000 units of vitamin D per day and an increase in calcium intake from 478 to 978 mg. per day (Fig. 12, days 37 through 48) caused a decrease in fecal calcium from a mean value of 558 (days 25 through 36) to a mean of 430 mg. per day. Mean fecal calcium was only 144 mg. per day in the subsequent twelve-day period (days 49

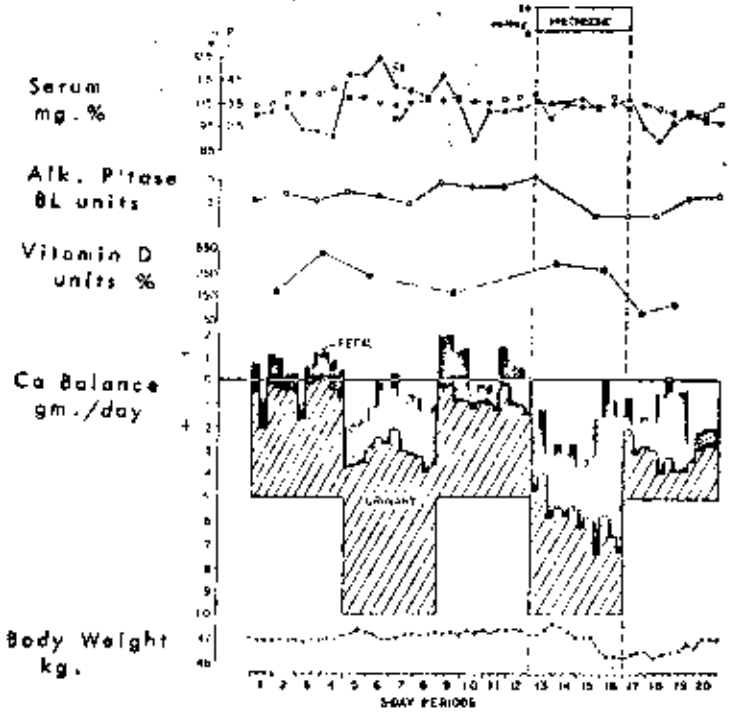

FIG. 9. Patient M. C. The effects of prednisone and of increase calcium intake on serum calci phosphorus, alkaline phos tase and antirachitic activity on calcium balance and body weight.

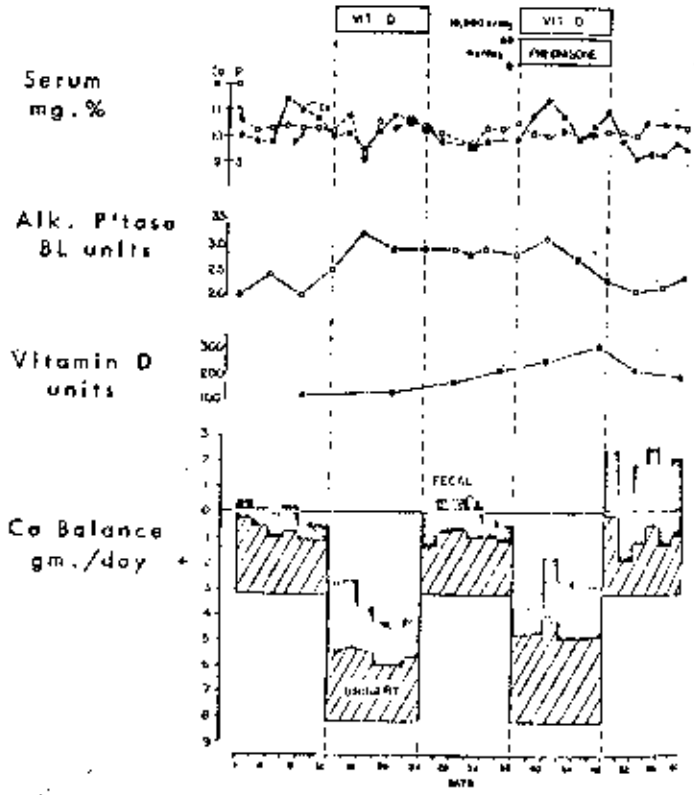

FIG. 10. Patient M. L. The effects of vitamin D and prednisone together with increases in calcium intake on serum calcium, phosphorus, alkaline phosphatase and antirachitic activity and on calcium balance.

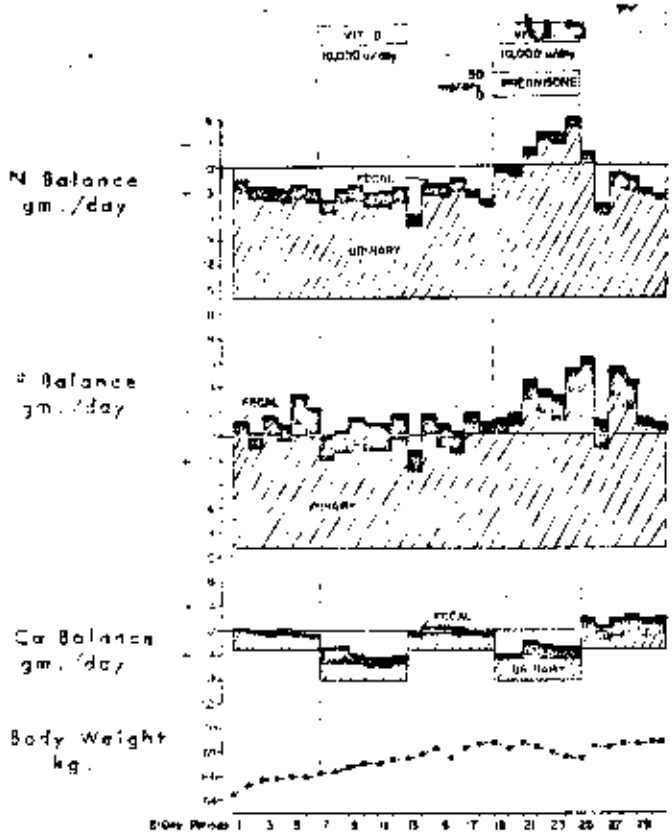

FIG. 11. The effects of vitamin D and prednisone together with increases in calcium intake on nitrogen, phosphorus and calcium balances and on body weight in M. E.

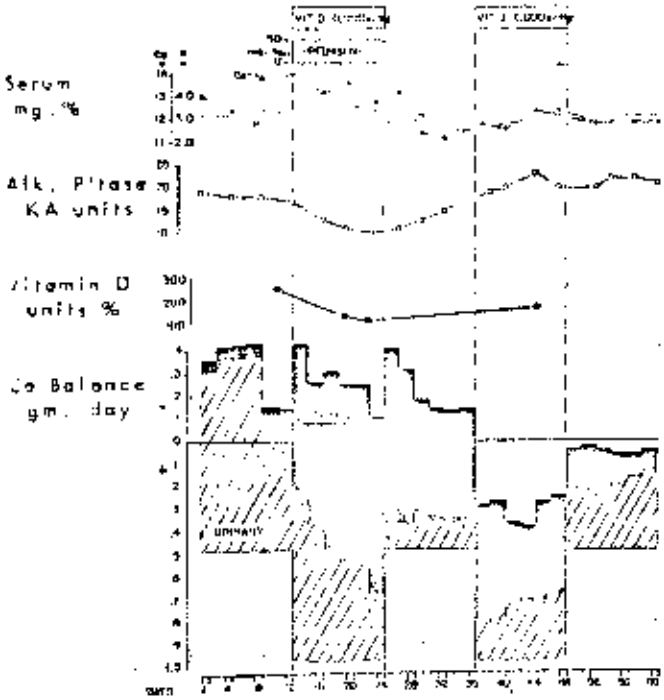

FIG. 12. The effects of vitamin D and prednisone together with increases in calcium intake on serum calcium, phosphorus, alkaline phosphatase and antirachitic activity and on calcium balance in M. C.

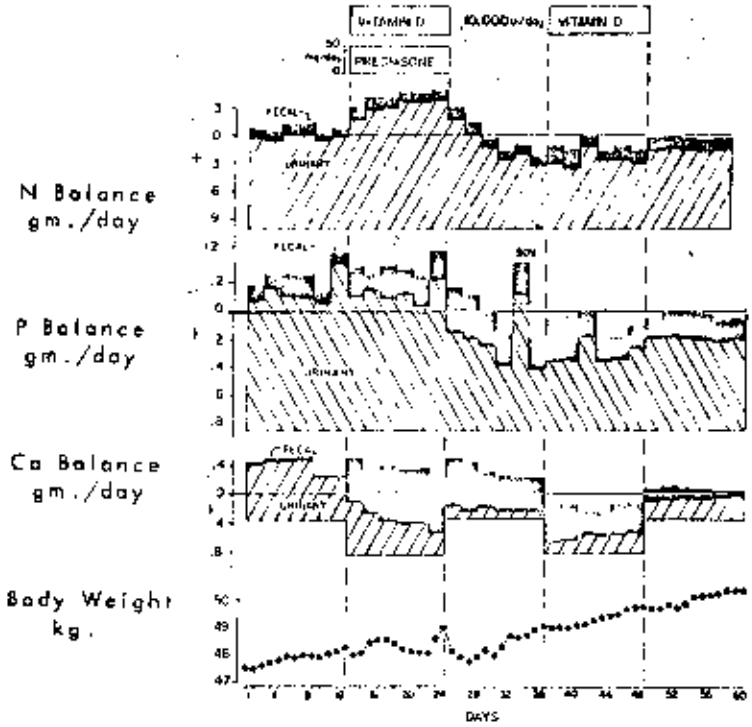

FIG. 13. The effects of vitamin D and prednisone together with increases in calcium intake on nitrogen, phosphorus and calcium balances and on body weight in M. C. Urinary phosphorus (days 33 and 34) could not be confirmed and is undoubtedly an error.

through 60). Urinary calcium increased from normal (mean 143 mg. per day, days 25 through 36) to abnormal values (mean 293 mg. per day, days 49 through 60).

Serum calcium (which was elevated), serum phosphorus and alkaline phosphatase levels did not change when vitamin D was given. A single value for serum antirachitic activity was 168 units per 100 ml. (day 45). Vitamin D had little, if any, effect on nitrogen excretion (Fig. 13, days 37 through 48), but appeared to lower fecal phosphorus.

Effects of Prednisone, Vitamin D and An Increase in Calcium Intake. (Fig. 10 through 13.) In one patient (M. E.), 50 mg. of prednisone per day, 10,000 units of vitamin D per day and an increase in calcium intake (Fig. 10, days 37 through 48) caused an increase in fecal calcium from an average of 111 (days 25 through 36) to 257 mg. per day (days 49 through 60). Serum calcium fluctuated between 9.9 and 11.4 mg. per 100 ml.; urinary calcium, which increased during treatment, returned to control values in the subsequent twelve-day period. The increase in fecal calcium was not as great as that produced by a smaller dose of prednisone given without vitamin D (Fig. 7, days 37 through 48).

Whereas serum phosphorus and alkaline phosphatase levels did not change when vitamin D and prednisone were given, serum antirachitic activity increased progressively to 305 units per 100 ml. (Fig. 10, day 48), and urinary nitrogen, urinary phosphorus and fecal phosphorus increased (Fig. 11, days 37 through 48).

In another patient (M. C.), 50 mg. of prednisone per day, 10,000 units of vitamin D per day and an increase in calcium intake of 500 mg. per day (Fig. 12, days 13 through 24), caused an increase in fecal calcium from a mean of 47 to a mean of 750 mg. per day. After added calcium was withdrawn, mean fecal calcium was 558 mg. per day (days 25 through 36). The serum calcium level fell from 14.0 (day 12) to 11.2 mg. per 100 ml. (day 32) and the urinary calcium level from an average of 751 (days 1 through 12) to an average of 143 mg. per day (days 25 through 36).

Serum phosphorus levels fell from a range of 3.2 to 3.7 mg. per 100 ml. to a range of 2.5 to 2.9 mg. per 100 ml., and serum alkaline phosphatase from 18 to 10 King-Armstrong units per 100 ml. Serum antirachitic activity was 251 units per 100 ml. before treatment and 112 to 132 units per 100 ml. after prednisone and vita-

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min D were given. Urinary nitrogen and phosphorus levels were high, with negative balances, before treatment and rose with the combined treatment, then decreased after treatment was stopped (Fig. 13, days 13 through 24). Fecal phosphorus however, increased together with fecal calcium.

Effects of Vitamin D on Calcium Absorption in Normal Subjects. (Fig. 14.) In a twenty-six year old normal woman (L. B.), 10,000 units of vitamin D per day produced little change in fecal calcium (mean 341 mg. per day) (Fig. 14A, days 15 through 26) and urinary calcium (mean 201 mg. per day) when compared to control values for fecal calcium (mean 352 mg. per day, days 1 through 14) and urinary calcium (mean 227 mg. per day). After the administration of vitamin D was stopped, fecal and urinary calcium levels averaged 352 and 159 mg. per day, respectively, (days 27 through 38) and did not change.

When vitamin D was given serum calcium and phosphorus values were normal and serum antirachitic activity was 135 and 210 units per 100 ml. and did not increase from control values of 215 and 380 units per 100 ml. Fecal and urinary phosphorus levels did not change. (Fig. 14A.)

In a twenty-two year old normal woman (L. H.), 10,000 units of vitamin D per day did not produce a decrease in fecal calcium (Fig. 14B, days 15 through 26) (mean 500 mg. per day) or an increase in urinary calcium (mean 115 mg. a day) after a control period when fecal calcium averaged 382 mg. per day and urinary calcium 141 mg. per day (days 1 through 14). After treatment, mean fecal calcium was 425 mg. per day and mean urinary calcium 113 mg. per day; these values did not change (days 27 through 38).

When vitamin D was given, serum calcium nd phosphorus levels remained normal and rum antirachitic activity increased to 231 nits per 100 ml. from control values of 115 and 155 units per 100 ml. Fecal and urinary phosphorus did not change. (Fig. 14B.)

Effects of Vitamin D on Ca^{47} Absorption in Normal Subjects. (Fig. 14.) In one patient (L. B.), 57 per cent of a dose of Ca^{47} was excreted in the stools (Fig. 14A, days 7 through 10). When 10,000 units of vitamin D per day was given 45 per cent was excreted (days 21 through 28); after treatment, 43 per cent was excreted (days 33 through 36).

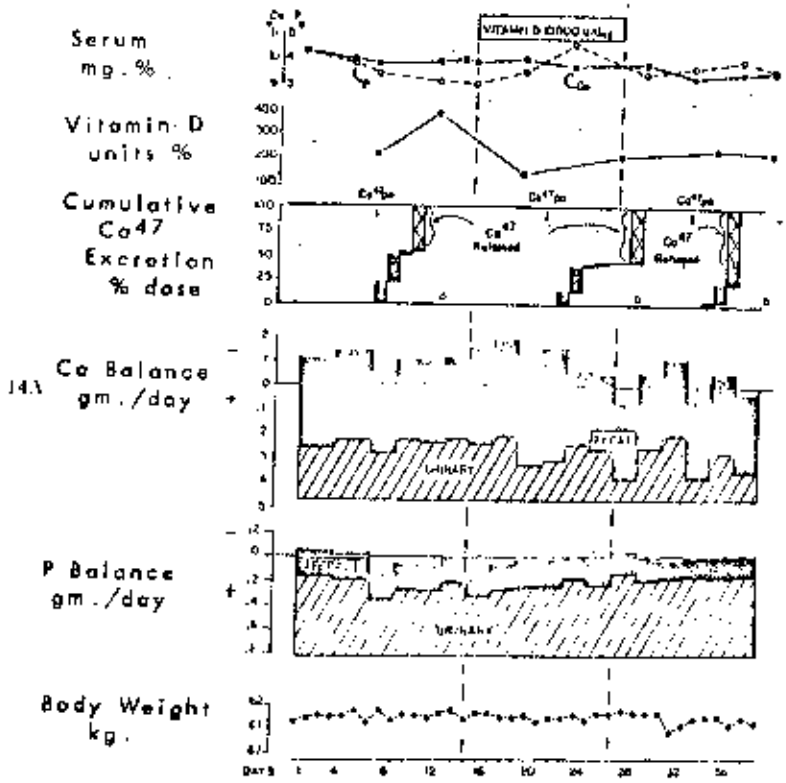
In the other subject (L. H.), 63 per cent of an oral dose of Ca^{47} was excreted in the stools (Fig. 14B, days 7 through 9). When 10,000 units of vitamin D per day was given, 70 per cent was excreted (days 21 through 25); after treatment, 51 per cent was excreted (days 33 through 36).

COMMENTS

In each of the three patients studied without treatment (J. R. T., M. L. and M. C.), fecal calcium was low, urinary calcium increased and serum vitamin D activity low or normal. Further, in all the patients, but not in the normal subjects, an increase could be produced in calcium absorption, with hypercalciuria, by administering small doses of vitamin D without an abnormal increase in antirachitic activity of the serum; in three patients (J. R. T., E. J. S. and M. C.), hypercalcemia was produced. In each of the patients, the administration of prednisone diminished the excessive absorption of calcium which occurred spontaneously or was induced with vitamin D therapy. Thus the balance studies in four patients and the studies with Ca^{47} in two of them indicate that low fecal calcium and increased urinary calcium (with or without hypercalcemia) are characteristic of the defect in calcium metabolism in sarcoidosis, and that this defect can be corrected with prednisone therapy. They also indicate that calcium absorption may be increased further by the administration of small doses of vitamin D without abnormally increasing serum antirachitic activity, and that this effect of vitamin D can be prevented with prednisone.

The incidence of hypercalcemia in patients with sarcoidosis is greater during the summer months when presumably the production of vitamin D_3 from 7-dehydrocholesterol in the skin by ultraviolet radiation is enhanced [14]. Conversely, the hyperabsorption of calcium in such patients can be diminished by feeding them a diet deficient in vitamin D [18]. These results, together with the present studies, support the hypothesis of Anderson and associates [9] that the defect in calcium metabolism is a result of intestinal hyperabsorption of calcium caused by an increased sensitivity to vitamin D, and that prednisone and other carbohydrate-active steroids act by antagonizing this action of vitamin D. They do not support the concept that the syndrome results from an endogenously-produced hypervitaminosis D [10].

Calcium absorption in patients with sar-



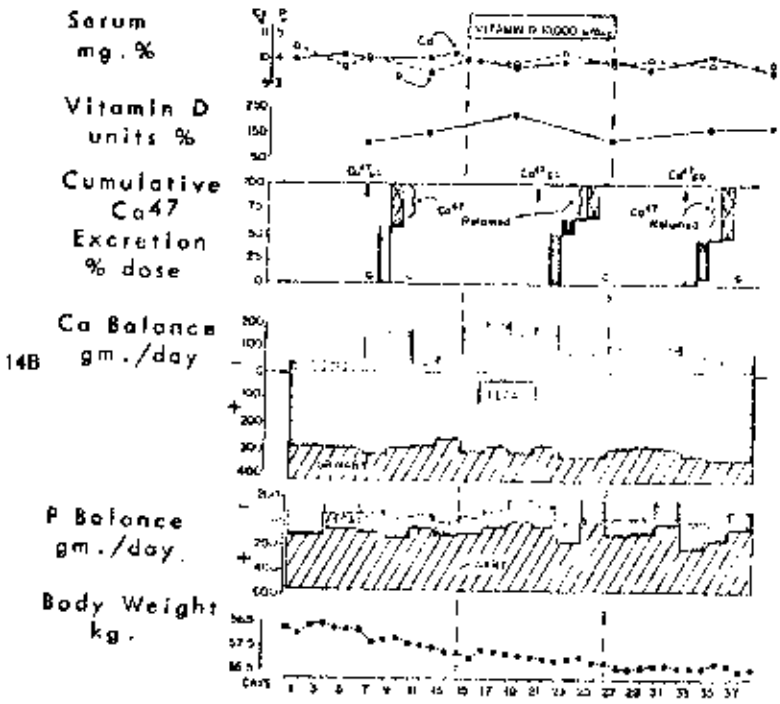


FIG. 14. The effects of vitamin D on serum calcium, phosphorus and antirachitic activity and on calcium and phosphorus balances, Ca^{47} absorption and body weight in normal subjects L. A. B. (A) and L. A. H. (B).

coidosis can be shown to be partially dependent on calcium intake. In two patients (M. E. and M. C.), an increase in calcium intake of 500 mg. a day increased calcium absorption and urinary calcium, and induced a positive calcium balance. In one (M. C.), it led to hypercalcemia as well. Similar results have been reported in previous studies [*1,5,9,19*].

Other investigators have demonstrated that cortisone [*4,9,10,24,37*], hydrocortisone [*5*] and prednisone [*5*] reduce the hyperabsorption of calcium and may produce a decrease in urinary and serum calcium in sarcoidosis. In contrast, such carbohydrate-active steroids may actually increase urinary calcium in normal subjects [*11,22*] and in patients with idiopathic hypercalciuria [*22*] and hyperparathyroidism [*22*].

Effects of calciferol given in doses similar to those used in the present study have been reported in two patients with sarcoidosis and hypercalciuria [*4,9*]. Whereas little or no change in fecal and urinary calcium was produced in one patient [*9*], an increase in the intestinal absorption occurred in the second and led to hypercalcemia and hypercalciuria [*4*]. In both, cortisone (100 to 150 mg. a day) when given with calciferol decreased calcium absorption and produced an increase in fecal calcium and a decrease in urinary calcium, results comparable to those reported here.

In three patients (J. R. T., M. E. and E. J. S.), serum antirachitic activity increased progressively when vitamin D was administered parenterally, and reached a peak during treatment, or shortly after treatment was stopped. These results suggest that bioassay in rachitic rats of serum from these patients provides, in fact, a measure of vitamin D content. Serum antirachitic activity has also been found to be normal in other patients with sarcoidosis and hypercalcemia by other investigators [*23*].

In two patients (J. R. T. and E. J. S.) antirachitic activity of the serum was abnormally low; in one of these (E. J. S.), no activity was demonstrable before vitamin D was administered. Both had nitrogen retention and impaired renal function as a result of long-standing hypercalciuria and recurrent nephrolithiasis. Similar low levels of antirachitic activity have been found in the serum of patients with renal insufficiency and osteitis fibrosa cystica [*24*]. These findings suggest that renal insufficiency may be associated with an impairment in intestinal absorption of vitamin D, and thus could explain the defect in calcium absorption in such patients [*24–28,29*]. Alternatively, the serum of patients with renal failure might contain substances which inhibit the action of vitamin D both in the patients and in the assay animal. Studies are in progress to explore these hypotheses.

Patients with hypercalcemia of infancy have been reported to have hyperabsorption of calcium, reversible with cortisone therapy [*30,31*], and have been said to be hypersensitive to vitamin D [*32,33*]. However, abnormally high levels of serum vitamin D activity, which persisted despite elimination of vitamin D from the diet, have been reported in three infants with this syndrome [*33*]. This has suggested an endogenous production of a substance with vitamin D-like activity [*33*].

In studies with the isolated small intestine of the rat, it has been shown that Ca^{45} is transported against both a chemical [*34,35*] and an electrical gradient [*36*]. Further, calcium transport is too great to be accounted for by passive diffusion alone [*37*]. This transport is most marked in the proximal small intestine and is dependent on vitamin D and on oxidative phosphorylation [*34,35*]. This effect of vitamin D can be antagonized with hydrocortisone [*38*].

In the present studies, fecal phosphorus decreased after treatment with vitamin D, as has been shown in normal subjects [*39*], and increased during treatment with prednisone in three patients (J. R. T., M. E. and M. C.). Although these changes in phosphorus absorption could be secondary to changes in calcium absorption, *in vitro* studies with rat intestine suggest that the effect of vitamin D on absorption of phosphorus is independent of that on absorption of calcium [*40,41*].

It has been suggested that the negative balances of calcium and phosphorus found in patients with sarcoidosis result from an effect of vitamin D on bone [*10*]. Negative balances of calcium and phosphorus were found occurring spontaneously in one patient (M. C.) (Fig. 12) and were induced with vitamin D in another (J. R. T.) (Fig. 1 and 3.) With Ca^{47}, it has recently been found that in sarcoidosis there are abnormalities in rates of bone formation and bone resorption similar to those induced with vitamin D in normal subjects and in patients with hypoparathyroidism [*42*]. These findings suggest that not only the gastrointestinal tract but also the bones of patients with sarcoidosis are abnormally sensitive to vitamin D.

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SUMMARY AND CONCLUSIONS

Calcium, phosphorus and nitrogen balance studies were conducted in four patients with sarcoidosis and hypercalciuria. The effects of prednisone (30 or 50 mg. per day for twelve days), of vitamin D (10,000 units per day for twelve days) and of both drugs given together were studied. The effects of the same dose of vitamin D on calcium and phosphorus balance were also examined in two normal subjects.

In three patients studied without treatment, the fecal calcium level was low, the urinary calcium level elevated and the serum antirachitic activity low or normal.

In all the patients, fecal calcium levels could be decreased further, without an abnormal increase in serum antirachitic activity, by administering vitamin D. Vitamin D had no effect on either fecal calcium or Ca^{47} absorption in the normal subjects.

Prednisone therapy diminished both the spontaneous absorption of calcium and that produced with vitamin D; in two patients, the results of the balance study were confirmed with Ca^{47}.

The results, together with previous findings, support the hypothesis that abnormal calcium metabolism in sarcoidosis is produced not by hypervitaminosis D but by an abnormally increased sensitivity to vitamin D.

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Studies on the Metabolites of Vitamin D

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That vitamin D is metabolised by the rat to a number of more polar breakdown products was first demonstrated by KODICEK [1960]. With the increased availability in recent years of radioactively labelled vitamins D, a number of studies of the distribution of metabolites in tissues have been undertaken, but as yet little progress has been made on their identification. This is mainly a result of the problems involved in the purification of the trace amounts of metabolites present in tissue extracts.

These distribution studies have led to the generally accepted conclusions that an oral dose of vitamin D is absorbed from the intestine into lymph [SCHACHTER, FINKELSTEIN and KOWARSKI, 1964; FRASER and KODICEK, 1968] and is eventually excreted in the faeces, presumably via the bile. Excretion via the urine does not appear to be a significant pathway in the rat; KODICEK [1960] found only 2.1% of the radioactivity of an oral dose of [U-^{14}C] ergocalciferol in the urine of rachitic rats, compared to 79.3% in the faeces and intestinal contents. Similar results have been reported by CALLOW, KODICEK and THOMPSON [1966].

Separation of Biliary Metabolites

Our main interest has lain in the characterisation of the metabolites of vitamin D produced by the rat; attention has therefore been concentrated on those sources yielding metabolites in the largest amounts, namely faeces and bile. Liver has also been shown to contain a considerable proportion of the radioactivity from a dose of vitamin D, but much of this is in the form of

unchanged vitamin. Thus CALLOW *et al.* [1966] found 5.7% of the radioactivity from an oral dose of [1α-^{3}H] cholecalciferol in the livers of rachitic rats 24 h after dosing, but only 1% was present as metabolites more polar than cholecalciferol itself. It therefore appears probable that the metabolites are not stored in the liver but are rapidly excreted in bile.

Faecal extracts, although containing considerable amounts of metabolites, have proved difficult to examine as they are contaminated with large quantities of pigments which interfere with the estimation of radioactivity by scintillation counting. Furthermore, any metabolites excreted in the bile will have been subjected to the action of the intestinal bacteria before being voided in the faeces, possibly giving rise to secondary products as is the case with the bile acids.

To circumvent these difficulties, the metabolism of [1α-^{3}H] cholecalciferol and of [U-^{14}C] ergocalciferol has been studied in rats with cannulated bile ducts. The animals used were young rats which had been fed from weaning on the rachitogenic diet 2965 of STEENBOCK and BLACK [1925]. As it was doubtful whether animals maintained on this diet alone would withstand the extensive surgical procedure involved, the diet was supplemented with vitamin D. In a representative experiment, 0.34 mg of [1α-^{3}H] cholecalciferol of specific activity 141 mC/m-mole, which had been bound onto bovine serum albumin to make it water-dispersible, was slowly infused into a rat by means of a jugular vein cannula. The vitamin was infused at the rate

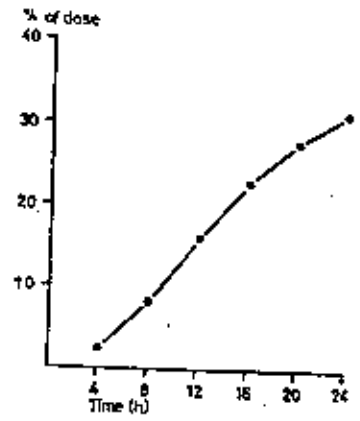

Fig. 1. Cumulative excretion of radioactivity into the bile of a young rat following intravenous infusion of 0.34 mg [1α-^{3}H] cholecalciferol.

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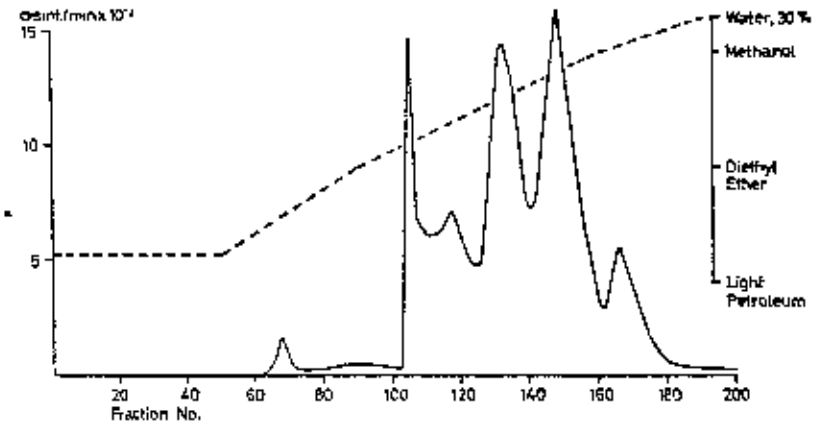

Fig. 2. Separation of metabolites of [1α-^{3}H] cholecalciferol in rat bile by column chromatography on silicic acid. The composition of the eluting solvent is shown by the broken line.

of 16% in the first 2 h, and the remainder at a constant rate over a further 16 h. This simulated the rate of absorption of a similar oral dose into lymph [FRASER and KODICEK, 1968]. The vitamin could not be administered orally owing to the requirement for bile salts in the process of absorption from the intestine. Bile was collected 4-hourly from the commencement of the infusion, each collection being extracted with acetone-ethanol (1:1, v/v). Radioactivity in these extracts was estimated by liquid scintillation counting. The excretion of radioactivity into bile followed a smooth time-curve, as shown in figure 1. Maximum excretion occurred between 8 and 12 h after starting the infusion, and by 24 h 31% of the radioactivity in the administered [1α-^{3}H] cholecalciferol had been excreted in the bile.

Samples of the combined 24 h bile extract were examined by silicic acid column chromatography, eluting successively with light petroleum-diethyl ether (3:1, v/v), a linear gradient to diethyl ether, a linear gradient to methanol, and then to 30% aqueous methanol, with the results shown in figure 2. The extract contained five major components, all more polar than cholecalciferol itself, which in this system is eluted between fractions 20 and 40. We have denoted these peaks as metabolites A, B, C, D and E, in order of increasing polarity. An identical pattern of metabolites was also found in the bile of young rats which had received 0.54 mg of [U-^{14}C] ergocalciferol, also albumin-bound, by intravenous infusion. The absence of additional peaks not observed in the bile of rats given the tritiated vitamin confirms that no

metabolites have passed undetected, as might be the case if metabolites were formed by reactions which caused loss of the tritium label. This experiment also confirms that the products obtained using [1α-^{3}H] cholecalciferol are derived from the vitamin and are not substances labelled by exchange. By repeated gradient- and stepwise-elution column chromatography we have been able to separate and purify the metabolites derived from [1α-^{3}H] cholecalciferol, and have studied their properties using a combination of classical chemical techniques and thin-layer chromatography.

Characterisation of Biliary Metabolites

The least polar component, metabolite A, was examined by thin-layer chromatography on silica gel before and after treatment with a number of reagent likely to affects its chromatographic mobility by modifying functional groups. The component was a neutral substance, unreactive towards alkaline hydrolysis, diazomethane, ketonic reagents such as Girard's reagent and 2,4-dinitrophenylhydrazine, and maleic anhydride. It reacted readily and completely with acetic anhydride in pyridine, leading to the conclusion that it is a hydroxylated derivative of the vitamin. It is believed that this metabolite corresponds to that found in a number of tissues by DELUCA *et al.* [NORMAN, LUND and DELUCA, 1964; LUND and DELUCA, 1966] which has been claimed to be fully biologically active, being as effective as vitamin D itself in curing rickets, stimulating calcium transport by everted intestinal sacs, and in elevating serum calcium concentration [MORII, LUND, NEVILLE and DELUCA, 1967]. Although the reactions discussed were found to be quantitative, indications have been found that this component is not homogenous. In particular, the spread of radioactivity after thin-layer chromatography was such as to allow the possibility that several closely spaced radioactive bands might be present.

That such a situation may occur is shown by the fact that on aeration, cholecalciferol in aqueous emulsion is converted to products which can be eluted from a silicic acid column as a single peak in a position corresponding to that of metabolite A, but which can be resolved by thin-layer chromatography on silica gel in chloroform-methanol (9:1, v/v) into three components, having R_f 0.32, 0.45 and 0.50. Under the same conditions of thin-layer chromatography metabolite A exhibits an 'envelope' of radioactivity extending from R_f 0.27 to 0.73. The most polar component showed ultraviolet absorption maxima at 243, 251 and 260 mμ, characteristic of deriva-

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tives of vitamin D in which the *trans*-5,7-diene structure is intact [SCHUBERT, 1954]. The structure of these oxidation products is at present under investigation. It is not suggested that these substances are necessarily related to the *in vivo* metabolites: the necessity of distinguishing between true metabolites and autoxidation products is however a problem which must be considered.

The third component in order of increasing polarity, metabolite C, has also been examined by similar techniques. From measurements of its distribution coefficient between organic solvents and water or dilute acids, and from its retention on chromatography on anion-exchange resin, it is concluded that metabolite C is ionic. Thus the distribution coefficient for chloroform:water was 0.3, and for chloroform:0.35N hydrochloric acid it was 0.8. These observations were confirmed by a study of the effect of a number of reagents on its chromatographic mobility; in particular the mobility was increased by treatment with diazomethane, or with acetic anhydride in pyridine. It therefore appears that this metabolite contains an acidic function, together with at least one hydroxyl group.

Metabolite D, accounting for 30–40% of the radioactivity in bile, was quantitatively the most important component, and is the one which has been studied in greatest detail [BELL and KOUCEK, 1967]. The metabolite was highly polar, being retained by anion-exchange resins, and having distribution coefficients favouring the aqueous phase. The thin-layer chromatographic mobility of this metabolite was increased by prior treatment with hydrochloric acid, diazomethane, acetic anhydride, periodate or bismuthate. In view of the polarity of the metabolite, a conjugate was the most likely structure, and of the common conjugating agents glucuronic acid is the only one to have properties concordant with the above reactions, i.e. a carboxyl function and an α-glycol. Incubation of the metabolite with β-glucuronidase confirmed this hypothesis, with the results shown in table I. There was a clear increase in lipid-soluble radioactivity with the active enzyme over the value for controls and this increase was largely prevented by the presence of saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase.

It has been reported that β-glucuronidase treatment of bile containing metabolites of vitamin D liberates free vitamin D [DELUCA, 1967]. Accordingly, cholecalciferyl β-D-glucuronide was synthesized from the vitamin and the methyl triacetyl bromo-ester of glucuronic acid by the method used by BUCKER [1965] for the synthesis of the glucuronides of isomers of androsterone, involving condensation of the compounds in refluxing benzene in the presence of anhydrous silver carbonate. The protected glucuronide so formed was demethylated and deacetylated with alcoholic sodium hydroxide to give

Table I. Chloroform-extractable radioactivity from metabolite D after incubation with β-glucuronidase

Enzyme[1]	Incubation time h	Chloroform-extractable radioactivity, %
Boiled	8	13
Active	2	44
Active	8	62
Active	24	72
Inhibited	8	24

[1] Metabolite and bovine liver β-glucuronidase, 400 units, incubated in pH 5.05 acetate buffer at 37°. The inhibitor was 0.01M saccharo-1,4-lactone.

the sodium salt of cholecalciferyl β-D-glucuronide. Both the free glucuronide and its methyl triacetyl ester have been fully characterised; in particular the nuclear magnetic resonance spectrum of the protected glucuronide clearly showed the spectrum of cholecalciferol with that for the glucuronic acid residue superimposed. The free glucuronide had maximum ultraviolet absorption at 265 mμ, indicating that the calciferol moiety was intact, and was hydrolysed by β-glucuronidase, confirming the presence of a β-linked glucuronide residue. Further confirmation was obtained from the infra-red spectra of the compounds, which compared well with the spectra obtained for cholesteryl β-D-glucuronide and its methyl triacetyl ester by NAGAYAMA, SAITO and IDLER [1966].

The authentic glucuronide and a sample of the radioactive metabolite were together converted to their pyridinium salts by the method of MCKENNA and NORYMBERSKI [1960], to eliminate effects due to cationic differences, and were then examined by chromatography on silicic acid using gradient elution. The results, shown in figure 3, show that the two substances are not identical, the metabolite being slightly more polar than cholecalciferyl glucuronide. Additional proof of non-identity was obtained by co-chromatography of the two substances after methylation and acetylation. The products of β-glucuronidase hydrolysis of the metabolite have also been investigated by chromatography. Two products were obtained, one of which corresponded in chromatographic behaviour to the metabolite A already described, the other product being intermediate in polarity between cholecalciferol and metabolite A. No material behaving like cholecalciferol itself could be

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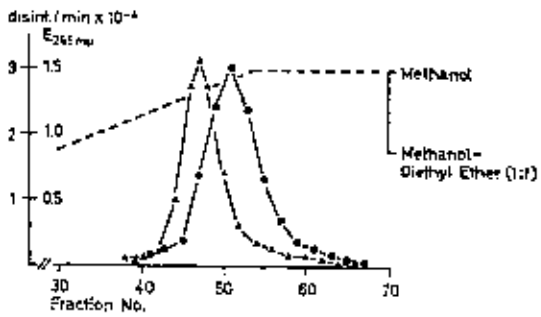

Fig. 3. Silicic acid column chromatography of metabolite D (●–●) and cholecalciferyl glucuronide (▲–▲). The composition of the eluting solvent is shown by the broken line.

obtained under any circumstances. The same products could also be obtained by perchloric acid – catalysed solvolysis of metabolite D in tetrahydrofuran according to the procedure of JACOBSOHN and LIEBERMAN [1962], although in this case some non-polar material was also found, presumably arising by dehydration of the initially formed products.

Interrelations between these metabolites have been studied in experiments in which the principal biliary metabolites were bound onto bovine serum albumin and reinjected into young rats with cannulated bile ducts. When metabolite A was so injected, over 90% of the radioactivity was recovered in the bile within 24 h, of which 86% was in the form of more polar metabolites, 33% being present as metabolite C. 90% of a similar intravenous dose of metabolite C was similarly recovered in bile, but in this case it was substantially unchanged. These results suggest that the acidic metabolite C is the terminal product of a reaction sequence originating from metabolite A.

The only other reports of the separation of biliary metabolites of vitamin D have come from BELL and BRYAN [1965] who reported that [4-^{14}C]-cholecalciferol was metabolised in a manner comparable to that of cholesterol, and that biliary radioactivity in rats given [4-^{14}C] cholecalciferol was principally associated with taurocholic acid, with smaller amounts of radioactivity associated with taurochenodeoxycholic acid and glycocholic acid. After hydrolysis the radioactivity was present as two large peaks, one more polar than cholic acid, and one more polar than lithocholic acid. It was concluded that cholecalciferol is excreted as bile acids conjugated with glycine and taurine which are similar to, but not identical with, cholic and lithocholic acids.

The Metabolic Pathways

The excretion of foreign aromatic compounds in the rat has been extensively studied by WILLIAMS and co-workers [ABOU-EL-MAKAREM, MILLBURN, SMITH and WILLIAMS, 1967; MILLBURN, SMITH and WILLIAMS, 1967], who concluded that the extent of biliary excretion of foreign compounds in rats depends on their molecular weight and on their possessing a strongly polar anionic group. The minimum molecular weight below which little biliary excretion occurs is about 325 ± 50. Both requirements can be met by metabolism, for example to form glucuronides. They suggest that the mechanism of the biliary excretion of foreign compounds may be similar to that of the conjugated bile acids. The more polar metabolites of vitamin D in bile all meet the criteria of possessing an anionic group, and probably also meet the requirement for molecular weight, and may therefore possibly be considered 'foreign compounds' in this sense.

These similarities between the metabolism and excretion of vitamin D and of other groups of compounds suggest a series of degradative transformations that are non-specific. A number of studies with drugs as substrates have shown that a series of reactions such as oxidation, hydroxylation, dealkylation, etc., occur in liver, and are catalyzed by liver microsomal enzymes which are remarkably non-specific [BRODIE, 1956, 1960]. Liver microsomal enzymes are also known to effect the hydroxylation of cholesterol [DANIELSSON and EINARSSON, 1966; MITTON and BOYD, 1965, 1967], and these and other hydroxylations have been shown to require NADPH and molecular oxygen [BRODIE *et al.*, 1955], which is the source of the hydroxyl oxygen. It is therefore quite possible that vitamin D is handled by the liver as any other pharmacologically active 'foreign compound' might be dealt with. It is however clear that the significance of the formation of these metabolites in relation to the physiological role of the vitamin can only be a matter for conjecture so long as their structures remain unknown.

Discussion

(Chairman: Prof. Dr. C. DEN HARTOG)

B. MAWER (Manchester): I should like to ask Dr. BELL two technical questions and then make a short comment. Firstly has he considered replenishing bile in bile fistula rats by introducing bile from a second animal so as to avoid increasing the turnover of vitamin D by interfering with the enterohepatic circulation? Secondly, was it neces-

sary to take any precautions against autoxidation during the period of infusion of the labelled vitamin D?

P. A. BELL: With regard to your first question we have not considered it necessary in this present work to replenish the bile since we are primarily concerned with the chemical nature of the metabolites. Should we wish to make any quantitative measurements we would certainly have to consider this modification to our experimental design. As regards autoxidation of the infused vitamin D, in our experience the protein-bound form of the [1-^{3}H] vitamin D is quite stable compared to the free vitamin. No special precautions were taken during the period of infusion.

B. MAWER: I would like to mention some work of our own which would appear to confirm Dr. BELL's observations. Samples of human serum, obtained shortly after introducing the radioactive vitamin D into patients, were extracted in the manner of DELUCA and showed small amounts of 'Peak IV' radioactivity. On storage of such extracts and in the presence of air and at 4 C the proportion of the radioactivity moving as 'Peak IV' was markedly increased showing that this fraction can easily be augmented artifactually and may well not be homogenous.

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The Effect of Vitamin D on Urinary Citrate in Relation to Calcium, Phosphorus and Urinary pH[1]

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Introduction

With the observation that the increase in urinary citrate induced by the administration of vitamin D can be correlated with an increase in blood citrate (1), it became increasingly important to determine what dietary relations affect this increase. Admittedly, this is hard to establish because, as judged by the increments in citrate excretion following the ingestion of base (2–7), and the great capacity of the animal to catabolize orally ingested citrate (8–12), the range within which an equilibrium can be established must be very wide.

So far, we know that in rachitic rats, while the citrate response to vitamin D is accompanied by an increase in urinary pH, it apparently is not dependent on this increase because it is elicited even when the urines are already strongly alkaline as the result of the inclusion of generous amounts of $NaHCO_3$ in the ration. It remains to be ascertained if vitamin D will have the same effect in the absence of a demonstrable increase in urinary pH.

In addition, we have sought to determine the effect of Ca, P, and other minerals. Our interest in Ca stemmed from the fact that citratemia has been associated with hypercalcemia (11) and that Ca is well known as forming soluble non-ionized complexes with citrate. These complexes might be excreted readily during a hypercalcemia induced by vitamin D (13). Phosphorus has already been shown (7) to affect the intensity of citraturia in that citrate excretion was observed to be at

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. We are indebted to the Wisconsin Alumni Research Foundation for the funds which made this research possible.

the highest level on low-P rations but it was increased the most, by vitamin D, with P-containing rations.

In some of our presently reported experiments the Ca and P contents of the rations were reduced to very low levels (approximately 0.02%). In others, all mineral additions to the organic basal ration, except NaCl, were dispensed with. $NaHCO_3$ was added in one series to increase the urinary pH.

The above experiments were undertaken in full realization of the limitations in the value of urinary excretion as an index of citrate metabolism. Not only could increased synthesis in one tissue or organ be counterbalanced by increased catabolism in another, but changes in blood citrate might not be reflected by comparable changes in excretion (6). Nevertheless, we felt that a demonstration of the generality of a vitamin D effect might emphasize the desirability of more definitive research with tissues and organs.

Experimental

Methods

Young rats varying from 60 to 100 g. in weight in different series, but within a range of 10 g. for individual experiments, were fed a basal synthetic ration of glucose, cooked egg white, roughage, cottonseed oil, salts, and vitamins as previously described (1, 7). The salts in the basal ration were limited to a Ca-free, P-free mixture; the vitamins to a vitamin D-free mixture. The various omissions from, and supplements to, the basal constituents are presented, with the results, in the various tables. When required by the experimental plan, Ca was added as $CaCO_3$, unless otherwise indicated; P was added as a neutral equimolar mixture of KH_2PO_4 and K_2HPO_4, and vitamin D as a cottonseed oil solution of calciferol. The latter supplement, equivalent to 75 I.U., was given orally to each rat every 3 days. Records were kept of food consumed and, in some cases, as noted, consumption was equalized between D and non-D groups. The urines, preserved with toluene, were collected in 3-day periods. They were analyzed for citrate essentially by the use of the Pucher, Sherman, and Vickery technique (14), and for Ca and P, respectively, by $KMnO_4$ titration of the oxalate, and by the Fiske-SubbaRow colorimetric method (15) after perchloric–nitric acid digestion. The pH of the urine was determined at the end of each 3-day period with a glass electrode on fresh samples of urine collected in a few hours in clean, dry metabolism cages.

Results

A gross inspection of the results in Table I reveals significantly that modifications of the low-P, low-Ca basal ration by addition of either P or Ca, singly or in multiple, did not have as much effect on citrate excretion as an increase in base intake in the form of $NaHCO_3$ alone, or re-

TABLE I

The Effect of Vitamin D on Urinary Citrate in Relation to Calcium and Phosphorus[a]

Series	Ration		Vitamin D	Food intake	Weight of rats		Urinary excretion (daily/100 g. rat)			
					Initial	Final	Ca	P	Citric acid	
		%		g.	g.	g.	mg.	mg.	mg.	% increase
III	Low Ca	0.02	0	56	80	107	3.57	0.12	4.25	
	Low P	0.02	+	95	86	107	6.45	0.12	8.63	103
IV	Low Ca	0.02	0	89	87	102	3.44	0.13	21.5	
	Low P	0.02	+	96	87	110	6.28	0.24	33.4	56
	$NaHCO_3$	10.00								
42,S_4	Low Ca	0.01	0	103	103	124	2.98	0.11	2.36	
	Low P	0.03	+	116	106	141	8.10	0.13	4.38	
	NaCl	0.40								85
	No other minerals									
42,S_3	Normal Ca	0.40	0	90	102	130	0.48	6.86	2.22	
	Normal P	0.32	+	125	110	118	0.68	6.19	8.99	300
	NaCl	0.40								
	No other minerals									
V	Low Ca	0.02	0	42	75	68	0.54	24.7	1.70	
	High P	0.62	+	102	100	127	0.40	37.7	7.80	359
II(*a*)	Medium Ca	0.20	0	69	59	70	0.57	10.2	2.30	
	Normal P	0.30	+	98	59	98	0.61	8.56	12.50	445
II(*b*)	Medium Ca[b]	0.21	0	60	90	98	0.69	—	0.91	
	Normal P	0.33	+	105	92	135	0.58	—	2.54	179
I	High Ca	1.26	0	61	62	68	11.6	0.20	21.3	
	Low P	0.02	+	64	60	73	18.2	0.17	36.6	72
	$NaHCO_3$	6.60								

[a] All groups contained six rats except series II(*b*) and 42,S_3 in which there were three rats. All rats were males except those in series 42 which were females. All data are for the last four collections for 3 days each and therefore represent averages for the collections from the 9th to the 21st day except for series II(*a*); in this way they represent the averages for the 6th to the 18th day. The food consumed and body weights, initial and final, are likewise for these 12-day periods. To each rat was given 75 I.U. of vitamin D every 3 days when indicated. A basal mineral mixture "free" from Ca and P was included in all rations except those fed in series 42,S_4 and 42,S_3.

[b] Ca was given as the sulfate; in all other series it was given as the carbonate.

played, in part, by an equivalent amount of $CaCO_3$. Furthermore, even when citrate excretion was raised to a high level, supplementation with vitamin D elicited a further increase which, expressed in milligrams, was as large as that obtained with any of the other rations. Obviously, even

the lowest levels of Ca and P did not prevent the excretion of citrate or the vitamin D effect. Citrate was always excreted in the urine and vitamin D always increased its excretion. This response, though limited to the area covered by our experiments, is expected to be very general, except possibly under more acidic conditions than those studied by us.

The role of an increase in urinary pH or systemic pH is not so easily assessed. We have reported (12) that vitamin D will increase urinary pH when given to rats on rachitogenic rations, that is, on rations low or physiologically low, in assimilable P, but relatively high in Ca. On other rations, e.g., those nonrachitogenic in character, this effect was not obtained (Table II), yet citrate excretion was always increased even in those instances in which a reduction in urinary pH occurred. With urines from the bicarbonate series we were unable to obtain measurement of pH to a sufficient degree of accuracy; they were too heavily charged with CO_2.

If it is accepted that an increase in urinary citrate is not necessarily accompanied by an increase in urinary pH, it does not necessarily follow that the vitamin D effect and pH are not interrelated; an increase in hydroxyl ions may nevertheless be induced at the loci of citrate formation. Folis (16) has reported his inability to demonstrate any deviation in pH from the normal physiological range in bone and cartilage by the

TABLE II

Vitamin D in Relation to Urinary Citrate and pH

Expt.	No.	Ration		Citrate mg./100 g. rat/day		pH	
		No.	Characteristic	Before D[a]	After D[b]	Before D[a]	After D[b]
9	4	11*A*	0.20% calcium as carbonate 0.30% phosphorus	5.30 (4.75–5.81)	16.09 (0.30–20.70)	7.27 (5.53–7.75)	5.51 (5.14–6.04)
20	12	11*C*	Wesson salts[d] 4.0%	3.57 (0.99–4.60)	10.80 (3.66–24.60)	6.29 (5.49–7.61)	6.08 (5.39–7.70)
S_1	6	11*D*	No calcium 0.30% phosphorus	1.31 (0.83–2.30)	6.18 (3.54–9.21)	7.32 (6.25–8.04)	6.18 (6.32–7.23)
				Without D[a]	With D[c]	Without D[c]	With D[c]
42	6	S_4	No salts except 0.40% NaCl	3.51	6.37	7.29	7.29
42	3	S_3	No salts except 0.40% NaCl, 1.0% $CaCO_3$, and 0.32% P	6.70	11.56	7.60	7.60

[a] Collection after 6th–8th day on ration.

[b] Collection after 8th–12th day on ration; 525 I.U. vitamin D was given over the 3-day period.

[c] Average values obtained in 21 days from rats started on rations with and without vitamin D when received from the breeders. The vitamin D supplemented group received 75 I.U. each day, every 3 days.

[d] Wesson, L. G., *Science* 75, 339 (1932).

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application of a series of indicators. It remains to be seen if such a test is adequate. The skeleton has been considered of minor importance in citrate production (17).

Our data do not provide the answer as to whether the increase in citrate elimination was due to an increase in citrate synthesis or to a decrease in its destruction. Tentatively we favor the former because the very high level of citrate excretion induced by 5% $NaHCO_3$, as previously reported (1), was increased only 8% by raising the dietary bicarbonate from the 5% to the 10% level, while vitamin D increased it 34%. In accordance with the neutralization theory of Östberg (3), the excessive intake of bicarbonate should have elicited the excretion of much more citrate if it had been possible for the rats to synthesize more in the absence of vitamin D.

While P is a potent factor in determining citrate equilibrium, our efforts to duplicate the effect of vitamin D by the addition of P were unsuccessful. In fact, in harmony with the low level of citrate excretion obtained with rations which contained P but no vitamin D, supplementing a rachitogenic ration with neutral phosphates reduced the citrate output. Later, when vitamin D was given, a rise in urinary citrate followed promptly. The urinary pH was not increased (Table III).

Further evidence of a possible P effect was obtained with rats on

TABLE III

Effect of Phosphate on Urinary Citrate

The basal ration was 23A, a low-P (0.02%), medium-Ca (0.21% Ca as $CaCO_3$) ration. The P supplement was given gradually in a few days to avoid tetany. In series S-1, 136 mg. P was given per rat as a solution of K_2HPO_4 and KH_2PO_4. In series W-1, P was incorporated in the ration, equivalent to 0.30% P. The vitamin D dose was 625 I.U. given every 3 days.

Collection days	Before P		With P			After D	
	4–6	7–9	10–12	13–15	16–18	19–21	22–24
Mg. citrate, 100 g. rat, daily							
Series W-1 (8 rats)	15.8	10.8	8.1	6.6	1.1	4.7	15.8
Series S-1 (12 rats)	21.3	18.1	9.6	10.0	5.2		
Urinary pH							
	7.0	7.2	8.0	7.2	7.0		

Ration 2065 (18). As is well known, vitamin D increases the availability of P from this cereal ration. With four rats made rachitic to a degree comparable to that produced on our synthetic rations and then given vitamin D, citrate excretion was reduced to a level 42% below that found in a comparable 6-day period immediately preceding the administration of the vitamin. It was not until the lapse of an additional 3 days that the excretion of urinary citrate rose to a level of 35% above the pre-vitamin D period. This effect is quite at variance with the increases of 35–37% obtained with our low P (0.02%) synthetic rations in the first 3 days.

We were unable to find any correlation between the excretions of citrate and Ca or P.

Summary

Vitamin D increased the amount of urinary citrate excreted by young rats kept on rations varying widely in mineral content. These rations ranged in composition from those providing an adequate mineral intake to those made grossly deficient in Ca, P, and other minerals. The biggest percentage increase in citrate was obtained with P-containing rations, but supplements of P, given in the form of neutral solutions of K phosphates to a low-P ration, reduced citrate elimination. An increase in urinary pH induced by vitamin D with rats on low-P rachitogenic rations was not obtained with rations adequate in P, yet an increase in citrate elimination resulted in all instances. Inasmuch as a supplement of vitamin D increased urinary citrate far more than did an additional large intake of $NaHCO_3$, it appears that the effect of vitamin D is due to an increase in citrate synthesis rather than to a decrease in its destruction.

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Effect of Vitamin D Deficiency on Developing Bone*

I. High Calcium-Phosphorus Ratio Diet

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The purpose of this investigation was to determine the influence of rachitogenic diets on the developing membrane bones of the newborn rats. Histochemical and micro-radiographic observations demonstrated a decrease in the deposition of minerals in osteoid. Alterations in the osteoblastic layer and the osteoid secreted by them, were also observed. These findings were similar to those noted in the shaft of long bones during rickets.

AMONG THE essential nutrients involved in the development and maintainence of the calcified tissues, the role of vitamin D in calcification of bone has been generally recognized. Since the primary influence of vitamin D appears to be based on the absorption of calcium from the intestine with a secondary effect on the absorption of phosphate,[1] the deficiency of this vitamin could cause an alteration in the calcium and phosphorus metabolism of the body. If bone is developing when such changes are taking place, they will be reflected as alterations in its composition and structure.

Our knowledge of bone calcification has been derived primarily from studies of rickets, whether, occurring naturally or produced experimentally. Rickets is the developmental disease of the calcified tissues resulting from vitamin D deficiency, from calcium or phosphorus deficiency, or from combined deficiencies of these nutrients. It is characterized by a decrease in, or a complete absence of, the deposition of inorganic materials in bone matrix. The various mechanisms which may lead to rickets have been dealt with in detail by Albright and Reifenstein,[2] and Follis.[3]

The morphological criteria of rickets are found in the bony shaft and in the area of the epiphyseal cartilage. The osteoblastic activity is not affected except when some other disease intervenes. Osteoid, the organic matrix of bone is deposited upon preexisting trabeculae in a normal fashion. The deposition of inorganic salts is either retarded or completely lacking during rickets, while the deposition of mineral salts occurs simultaneously with the deposition of osteoid in normal animals. In the normal growing rat, osteoid is rarely detectable. The amount of osteoid varies depending upon the age of the species from which the specimen is derived. These alterations are similar whether rickets are produced by vitamin D deficiency, by calcium-phosphorus deficiency, or by imbalanced calcium-phosphorus ratio in the diet. The only exception is seen in the rat where hypertrophic uncalcified cartilage and osteoid appear more prominent in diets with high calcium-phosphorus ratio than in those with low calcium-phosphorus ratio. It was suggested[4] that the rat requires very little vitamin D for normal growth, since this animal does not develop rickets following simple deprivation of this vitamin. It has since been demonstrated,[5] that vitamin D deficiency without abnormalities of the calcium and phosphorus content of the diet, produces biochemical abnormalities in the rat which can be corrected by administration of vitamin D. Harrison and Harrison[5] observed that weanling rats placed on a diet of normal mineral composition but lacking in vitamin D, showed no evidence of rickets but their growth rate was reduced. The bone weight was less than that of the normal controls, indicating a decreased total bone

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‡ Instructor, Department of Anatomy.

calcium. This of course would be expected, since absorption of dietary calcium is reduced in the vitamin D deficient animals. A vitamin D deficiency state can, therefore, exist in a rat without the rat having true rickets. In order to produce rickets in the rat, a distortion of the calcium-phosphorus ratio of the diet is necessary in addition to the vitamin D deficiency.

The prenatal influence of vitamin D deficiency was studied by Warkany.[6] He observed skeletal deformities in the offsprings of rats fed a diet with a high calcium-phosphorus content without vitamin D. The histological picture was not characteristic of rickets, but when vitamin D supplements were provided in the maternal diet, skeletal deformities were prevented. When the same diet (no supplement) was given to the weanling rats, typical rachitic changes were noted. The fetal tissues reacted somewhat differently to the deficiency. Wolfe[7] observed skeletal changes in infants born of mothers suffering from osteomalacia. It is obvious that although maternal nutritional deficiency can result in calcification disturbances in the offspring, the alterations may or may not be identical to those observed due to post-natal nutritional deficiencies.

This investigation was, therefore, designed to determine the influence of vitamin D deficiency on the developing bone by histological and microradiographic techniques. Newborn rats whose mothers had been fed a rachitogenic diet were used in this study.

Material and Methods

In order to have an adequate number of rats available for this experiment, breeding cages were organized with the adult female albino rats (Long Evans Strain) from the stock colony. These animals were divided into two groups on the basis of diets received during the experiment.

Group A Normal animals: 12 female rats fed a nutritionally adequate stock diet.

Group B The Steenbock animals: 19 female rats were placed on the modified Steenbock rachitogenic diet (Yellow ground corn, 76 per cent; Wheat Gluten, 20 per cent; Calcium carbonate, 3 per cent; Sodium chloride, 1 per cent; for three to four weeks prior to mating in order to deplete their reserves of vitamin D. This diet has a calcium-phosphorus ratio of 4.5:1 and without vitamin D produces rickets.

Mating of rats in both groups was started. The Steenbock group of females were mated with males fed a diet normal in calcium and phosphorus, but deficient in vitamin D, to prevent contamination with this vitamin. Upon determination of pregnancy, the females were removed to individual maternity cages. As the litters were born, the results of the maternal deficiency diet became apparent. Two litters died at birth, and the number of animals in each surviving litter was small (five to six). Although normal at birth, they did not grow and gain weight like normal animals. The animals were weighed twice weekly for the duration of the experiment.

The experimental and control animals were sacrificed by decapitation at three day intervals beginning from birth to 21 days post-natal. Their heads were cut into two halves.

All specimens were fixed in 10 per cent alcoholic formalin for 24 hours. The left half of the head was decalcified by Morse's technique, dehydrated in alcohol, cleared in chloroform, and embedded in paraffin. The right half was embedded in paraffin without decalcification. Serial sections were cut from both halves (decalcified and undecalcified) at six microns thickness and stained with Hematoxylin and Eosin, and Masson Trichrome stains. The undecalcified sections were stained by Von Kossa's Silver stain[8]- Gomori's modification, and Mowry's procedure[9] for acid and neutral mucopolysaccharides. Some of these sections were used for contact micro-radiography, under vacuum, in Philips CMR-5 unit at voltages between 2.5 MA and 3.5 KV. Corresponding variations in exposure time (15-20 minutes)

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were used in order to obtain maximum image detail and contrast.

Observations

In order to assess the effect of the nutritional deficiency, microscopic observations were made on the developing bone of the cranium and maxilla. Serial sections from experimental and control animals of corresponding age were examined and compared.

In a general consideration of the observations, it should be mentioned that the newborn animals from Steenbock group failed to grow and gain weight. They tended to be sluggish and weak. From equal weights at birth, the animals on normal diet weighed approximately twice as much at 21 days (45 gm. versus 22 gm.). The microscopic observations on developing membrane bones of rat cranium in the experimental group showed abnormalities in osteogenesis. The effect of the deficiency diet appeared to be progressive in nature. To facilitate a comparison and interpretation of the structural changes, the following photographs from control and experimental animals will demonstrate the representative observations of this study.

At birth, the deposition of bone matrix in the control animals (*Figure 1*) appeared normal and the trabeculae enclosing marrow

Fig. 1

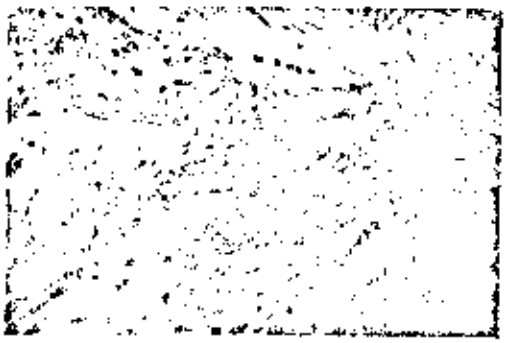

Fig. 2

Fig. 1. Decalcified section of cranium of rat at birth, stained with Masson Trichrome to demonstrate normal bone formation.

Fig. 2. Section of rat skull at birth, mother fed the Steenbock rachitogenic diet. Note homogeneous masses on surface of spicules. Stain: Masson trichrome.

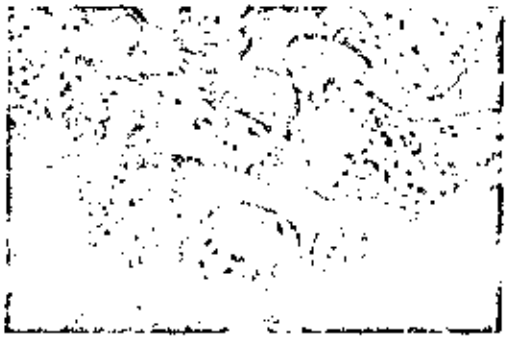

Fig. 3

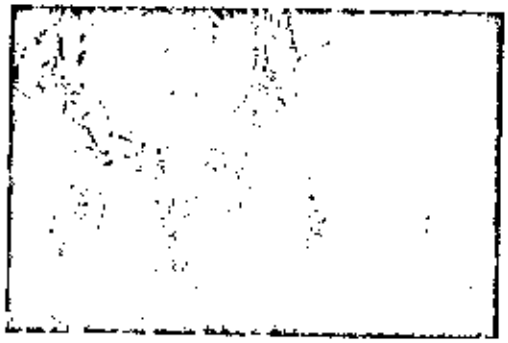

Fig. 4

Fig. 3. Decalcified section from normal rat 18 days old, to demonstrate pattern of bone remodelling. Note the osteoblastic and osteoclastic activity.

Fig. 4. Same age as *figure 2*, rat born of a rickettic mother. Notice large patches of uncalcified osteoid in the spicules, and marrow spaces filled with blood and fibrous tissue.

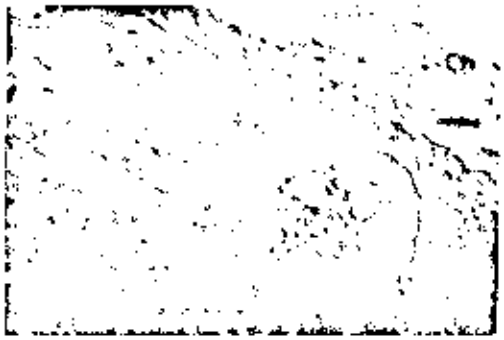

FIG. 5

FIG. 6

FIG. 5. Undecalcified section, ten day old normal animal stained with Von Kossa's silver nitrate method to demonstrate sites of calcification

FIG. 6. Undecalcified section, nine day old rickettic animal, stained with Von Kossa showing sparse sites of calcification in the uncalcified osteoid.

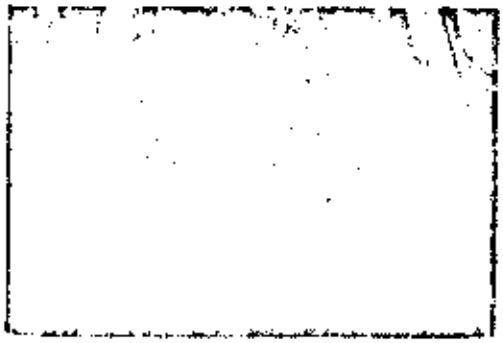

FIG. 7

FIG. 8

FIG. 7. Undecalcified section, normal animal six days-skull, stained with Mowry's method for demonstration of mucopolysaccharides. Note the light color of bone spicules and the darker color in the ground substance

FIG. 8. Section from six day old rickettic animal showing a decreased intensity of the mucopolysaccharides staining material.

spaces were well organized. The osteoblasts were arranged on the surface of growing bone. In animals whose mothers were fed the Steenbock diet (*Figure 2*), the newly formed bone lacked organization and the growth appeared retarded. The osteoblasts were fewer than in normal bone, and capillary invasion of this layer was observed in some areas. Along the surface of some spicules, homogeneous orange-red masses containing nuclei were sometimes noted. Whether this represented uncalcified osteoid with embedded osteocytes or osteoclastic activity was not clear.

As the animals got older (18 days) the membrane bones of the skull in the control animals (*Figure 3*) showed a normal pattern of remodelling. This was indicated by the simultaneous activity of the osteoblasts and the osteoclasts. The experimental group of animals (*Figure 1*) on the other hand, demonstrated large areas of uncalcified eosinophilic material in the trabeculae, the typical appearance of rickettic osteoid. There was an excessive accumulation of osteoid which had failed to undergo resorption. Few osteoclasts were present, but no lacunar resorption was seen. A distortion in the growth pattern of bone was also noted. The osteoblasts appeared to have atrophied and were less numerous than in normal bone. The marrow spaces were filled with blood and

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FIG. 9

FIG. 10

FIGS. 9 & 10. Micro-radiographs of skull of normal animals three days old. Note the uniform density of mineral deposition in developing bone indicated by the opaque areas.

FIG. 11

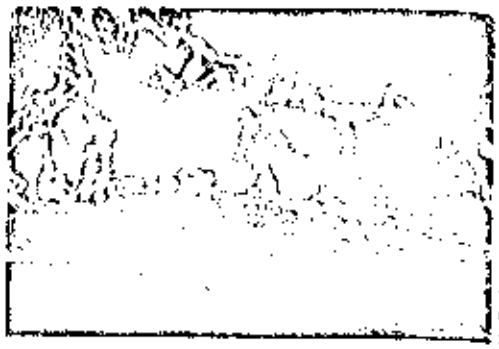

FIG. 12

FIGS. 11 & 12. X-ray micrograph of six day old animal, whose mother received the Steenbock diet. Compare this with *figure 9 and 10* and notice the lack of radio-opaque areas in developing bone indicating defective calcification. The cellular components are visible.

fibrous tissue, and the fibro-osteoid appeared to be functionally inadequate.

Observations on undecalcified specimen from these animals, stained with the Von Kossa silver nitrate method (*Figures 5, 6*) demonstrated the sparse sites of calcification in the uncalcified osteoid of ricketic animals. (The mechanism of staining with silver nitrate is not clear, although it is supposed to demonstrate the presence of phosphates and amorphous carbonates rather than calcium. Theoretically during the preparation of the specimen, the soluble phosphates are washed out and the insoluble calcium phosphate is thereby demonstrated.) This was indicated by traces of silver precipitate scattered in the bone matrix. In normal animals, the calcified spicules of bone showed a dark staining precipitate of silver suggesting normal calcification.

In undecalcified sections stained (*Figures 7, 8*) for the demonstration of mucopolysaccharides, it was apparent that the uncalcified osteoid and connective tissue in the experimental animals showed a decreased intensity of magenta color (Schiff reaction) and the prussian blue reaction (colloidal iron binding) as compared to normal bone matrix.

Micro-radiographs of the tissues revealed obvious differences in the amount of mineral in the control and experimental animals (*Figures 9 12*). The mineral deposit in the bone of normal animals appeared quite uniform in density and the cellular details of the osteoblasts and marrow cells were visible. Parts of the calcified regions, especially close

to the calcification front, seemed granular suggesting initial mineralization. In the offspring of ricketic animals the osteoid showed little or no mineral deposition. The uncalcified bone matrix revealed the structural organization and cellular structure.

Discussion

Histological and micro-radiographic observations on the development of membrane bones of newborn rats whose mothers had been fed a rachitogenic diet, demonstrated a decrease in the deposition of inorganic material in osteoid. Alteration in the osteoblastic layer of cells, was sometimes seen. It is obvious that these findings on membrane bone are similar to the changes during rickets in the shaft of long bones and in the area of epiphyseal cartilage noted by various other investigators.[10, 11, 12]

The exact pathogenesis, that is, the chain of events leading to this defect is not clear. Currently it is believed that naturally occuring or experimentally produced rickets is dependent on an abnormality in the humoral concentrations[13, 14] of calcium and phosphorus in the blood stream, to a degree that they deposit insufficiently or not at all in the bone or cartilage matrix. Follis,[15] on the other hand stresses the decrease in the extracellular concentrations of calcium and phosphorus, which lead to alterations in the cells themselves, which influence the lack of deposition of inorganic materials.

Although the action of vitamin D at the cellular level is still not clear, a number of observations have offered new possibilities for further investigations. The action of vitamin D in increasing the serum and bone citrate concentrations[16] in the vitamin D deficient rats and infants have been reported. They suggest that the action of vitamin D depends on the distribution of citrate between cells and extracellular fluid rather than upon intercellular accumulation of citrate. It is possible therefore, that vitamin D influences the rate of citrate accumulation in cells. Gutman[16] and Dixon and Perkins[17] have demonstrated a number of enzyme systems concerned with the glycogenolytic cycle and the citric acid cycle. The presence of citrogenase in bone was demonstrated by Dixon and Perkins, and the isocitric dehydrogenase activity was reported by Van Reen and Lowe.[18] No evidence of interaction of vitamin D in specific enzyme system has been established. It is possible that certain effects ascribed to vitamin D, might be indirect since citrate utilization *in vitro* is also affected by the concentration of calcium and inorganic phosphate in the medium.

It is suggested that the above factors may cause alterations in the metabolic activity of the matrix producing cells. Change in the osteoid secreted by them may make the impregnation of mineral salts difficult. This area needs further investigation.

Summary

1. This investigation was designed to determine the influence of a rachitogenic diet on the developing membrane bones of the newborn rats (birth-21 days). The Steenbuck rachitogenic diet has a Calcium-Phosphorus ratio of 4.5:1 and, without vitamin D, produces rickets.

2. Serial sections of the cranium were cut and stained with H & E, Masson trichrome for connective tissue, Von Kossa's silver stain for calcium, Mowry's procedure for acid and neutral mucopolysaccharides. Contact micro-radiographs were made with Philips CMR-5 unit.

3. Almost all animals in the experimental group showed abnormalities in osteogenesis and failed to grow and gain weight.

4. The Steenbock animals demonstrated large areas of uncalcified eosinophilic material in the bone spicules. There was an excessive osteoid which failed to undergo resorption.

5. The presence of uncalcified osteoid was confirmed by lack of Von Kossa reaction and the appearance of large radiluscent areas in contact micro-radiographs.

6. The PAS reaction and Mowry's procedure demonstrated a weak reaction for polysaccharides.

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7. Whether the calcification disturbances in developing bone during rickets are due to a lack of mineral deposition alone, or are secondary to alteration in the osteoid production, needs further investigation

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REVIEW

Idiopathic Hypercalcaemia and Vitamin D

J. A. Black

In 1929 Putschar suggested that irradiated ergosterol (vigantol) might have been responsible for the death of a 5½ months old infant in whom nephrocalcinosis was the only significant abnormality found at postmortem (42). Shortly after this Thatcher (49, 50) made a similar suggestion based on a study of two infants who had failed to thrive and subsequently died, and in whom the autopsy findings were similar to those in Putschar's case. All three children had received vitamin D in various forms but none had been given more than an average dose. As no estimations of the serum calcium were done it is impossible to say whether hypercalcaemia was present or not and it is equally probable that they were suffering from infantile renal rickets, in which nephrocalcinosis is found without tissue calcification elsewhere (5).

In 1932 Lightwood described the first case which can properly be considered, in retrospect, to have been an example of idiopathic hypercalcaemia, even though the only estimation of the serum calcium performed was normal (35). The child, aged 2 years and 4 months, was dwarfed and had increased calcification at the base of the skull and at the ends of the long bones; the brachial and femoral arteries were [illegible] in the radiographs. She died shortly after admission to hospital and was found to have marked nephrocalcinosis and widespread calcification of the [illegible] arteries. There was no history of an excessive intake of vitamin D.

[illegible] 1932 that idiopathic hypercalcaemia [illegible] as a definite entity [illegible] and Payne (44) described the [illegible] features of a group of cases [illegible] in the same year in a joint publication [illegible] described two children [illegible] with abnormal dural features [illegible] recognised as characteristic [illegible] of idiopathic hypercalcaemia, in [illegible] cases which formed [illegible] and Payne's group. It is [illegible] that [illegible] idiopathic hypercalcaemia [illegible] of the [illegible] of these two [illegible] and intermediate cases are [illegible] which they have in common are as follows:

1. The onset is usually between 3 and 6 months of age with vomiting, constipation and failure to gain weight.
2. The serum calcium is raised, usually between 11 mg.% and 15 mg.%.
3. The level of the serum cholesterol is usually high in the severe cases, but is often normal in the mild cases.
4. There is evidence of renal damage, which may be temporary or permanent, as shown by a raised blood urea in the absence of dehydration.
5. Nephrocalcinosis can often be detected radiologically, and at post-mortem extensive calcification is found in both renal cortex and medulla.
6. Other autopsy findings may include calcification in the walls of the arteries, in the myocardium, the lungs and the gastric mucosa.

In the severe type there are the following additional features:

1. A curious "elfin" facial appearance which is easily recognisable (see Fig. 1).

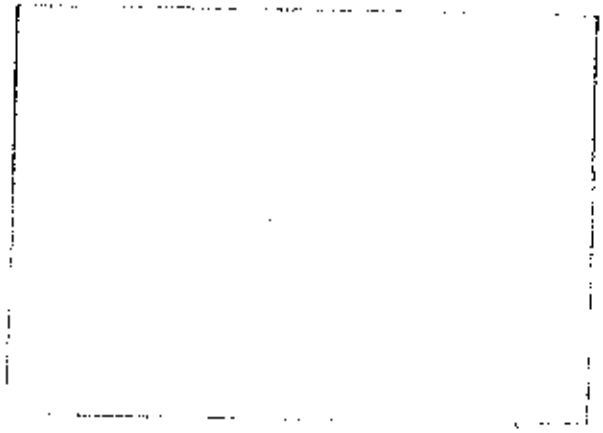

Fig. 1. Two cases of the severe type of idiopathic hypercalcaemia at the ages of six years (left and [illegible] right), showing the typical facial appearances [illegible] (see [44]). Reproduced by kind permission of the [illegible] and the Editor of the British Medical Journal.

2. A precordial systolic murmur, usually with a raised blood pressure.
3. Marked increase in calcification at the base of the skull at the ends of the long bones, in the vertebral bodies and often round the epiphyseal centres. In contrast the mild cases may either show no radiological bone changes, or bands of increased density at the metaphyses.
4. Mental deficiency, the I.Q. being usually between 40% and 70%.

Fig. 9

Fig. 8

Fig. 11

Fig. 8—10. Extensive changes in the mother (29 years older), resembling Paget's disease.

Fig. 11—12. Characteristic changes in the younger brother.

For the article by Weizel (pp. 285—287)

Progressive Diaphyseal Hyperostosis

Fig. 12

Fig. 10

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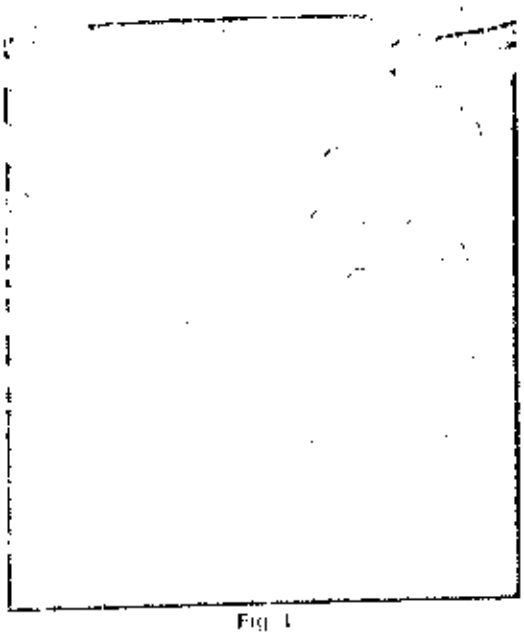

Fig. 1

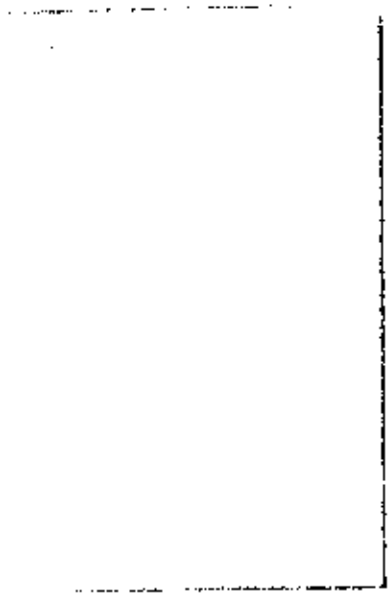

Fig. 2

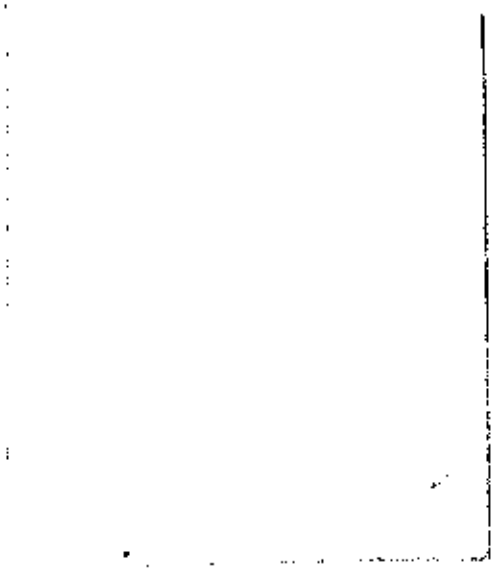

Fig. 3

For the article by Sperling, Thomas (pp. 287–289)

Agenesis of the Lung with Spastic Bronchitis

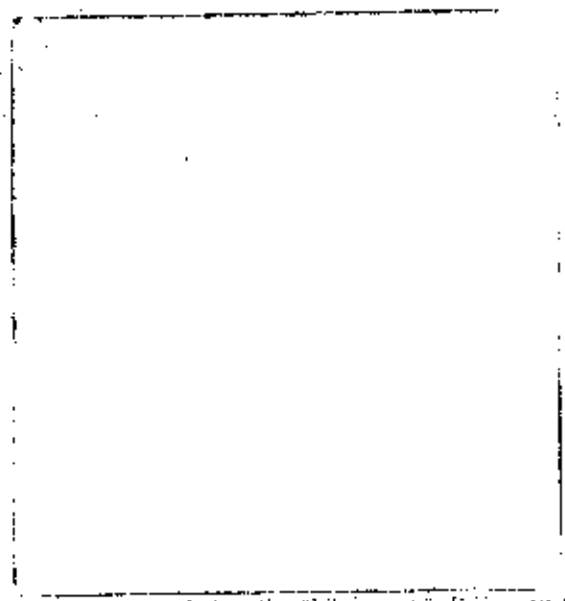

Fig. 4. Tomogram below the "bifurcation". VK = vertebral bodies, A = thoracic aorta, Oe = oesophagus, [illegible] = left border of anterior mediastinal hernia, [illegible] = left border of small posteroinferior mediastinal hernia

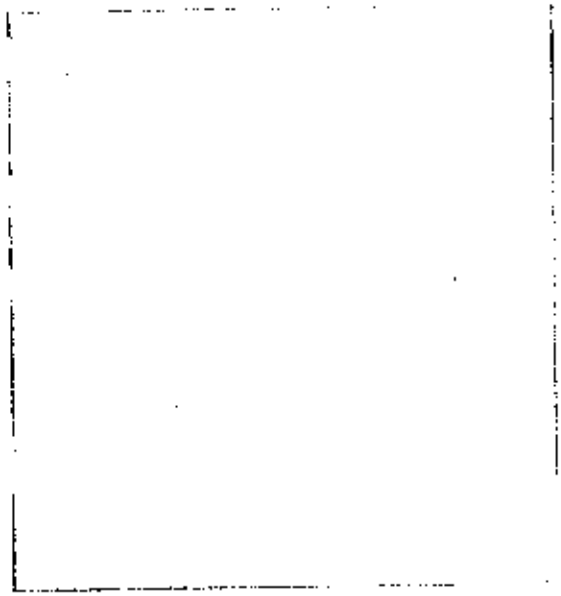

Fig. 5. Angiocardiogram (Dos. Dr. Porstmann). [illegible] right pulmonary artery. The left pulmonary artery is absent. The heart is much displaced and rotated to the left.

Fig. 1. PA radiograph. Oe = oesophagus. [illegible] = edge of large anterior mediastinal hernia. The heart and left cupola of the diaphragm merge into one [illegible]

Fig. 2. PA tomogram at the level of the "bifurcation" of the trachea. T = trachea. OLB = right upper lobe bronchus, ZB = lower lobe bronchus. The bifurcation is absent and there is no left main bronchus.

Fig. 3. PA tomogram of the right hilum. T = trachea. USA = pulmonary arterial branch to upper lobe. The right hilum is twisted clockwise to such a [illegible] that it assumes the form normally seen in a [illegible]

5. A [illegible] and complete facial [illegible]

6. Mi[illegible] and [illegible] have been [illegible]

7. Autop[illegible] described above, the [illegible] valve, may be thick[illegible] there may be a con[illegible] tissue involv[illegible] (15).

There has been considerable discussion whether the [illegible] constitute separate [illegible], or whether there is a continuous gradi[illegible] mild to severe. There is no doubt that [illegible] cases with most or all [illegible] features of the severe type, whether considered from the point of view of renal damage and hyperpyrexia (6) or in terms of the permanent mental [illegible] which [illegible] the survivors. In contr[illegible] appear to recover, with normal [illegible] intelligence (53).

Any [illegible] of idiopathic hypercalcemia [illegible] for both types of hypercalcemia, whatever [illegible] variants of [illegible] the following facts [illegible] (1) the [illegible] evidence for a genetic basis for the condition, (2) the [illegible] in the first year of life and (3) the [illegible] of hypercalcemia which [illegible] after the age of [illegible] years.

Since [illegible] the cause of idiopathic hypercalcemia [illegible], but some [illegible] for all the [illegible]

[illegible] Not Due to Vitamin D

1. Excessive [illegible] of [illegible] (7).
2. [illegible] of [illegible] (9).
3. Reduction [illegible] (13, 19).

It is not [illegible] to discuss these theories as they have been [illegible], they deal with in two recent reviews (20, 25).

[illegible] Involving Vitamin D Indirectly

1. D[illegible] of [illegible] fatty acids (40). Sinclair suggested [illegible] children with idiopathic hypercalcemia have been fed on dried cow's milk, they may [illegible] deficiency of unsaturated fatty acids, [illegible] fed cholesterol and vitamin D. However, [illegible] and co-workers [illegible] of British dried milk and [illegible] of unsaturated fatty acids in the blood of children with idiopathic hypercalcemia failed to substantiate [illegible] hypothesis.

2. Hypercalcemia [illegible] cholesterol (19). Fodor and [illegible] cholesterol and the [illegible] that the highest levels of [illegible] hypercalcemia. It was suggested that there is a disorder of the adrenal [illegible] in an excess of a substance with a biological activity similar to that of vitamin D. If this were [illegible] respect to functional [illegible] of vitamin D actively in the course of patients with idiopathic hypercalcemia.

3. Adrenal insufficiency (3). Anderson [illegible] the adrenal response of two cases of idiopathic hypercalcemia. In one [illegible] there was a normal rise in the urinary total 17-ketosteroids after a 10 day course of A.C.T.H. but in a second case there was no response [illegible] 10 days of A.C.T.H. (6 units a day). Hill and Pinder (15) studied a severe case aged 3 months and found [illegible] rise in 17-ketosteroids and 11-oxysteroids in the urine after clinical [illegible] with A.C.T.H.; they also found a [illegible] return to normal in the blood sugar after injection. In infants the response of the adrenal cortex to A.C.T.H. is more reliably shown by a rise in 17-hydroxycorticosteroids than by increasing the 17-ketosteroids alone (1). It is difficult therefore to come to any definite conclusion from the available data, however, in [illegible] of idiopathic hypercalcemia we have observed [illegible] of response to a standard A.C.T.H. test, as measured by the 17-hydroxycorticoids. We have also seen a [illegible] of response to A.C.T.H. In a case of hypercalcemia [illegible], and a possible explanation for this [illegible] cells of the adrenal cortex might be causing [illegible] calcinosis, whatever the cause. It is also [illegible] adrenal cortical atrophy has not been [illegible] of the fatal cases of idiopathic hypercalcemia.

The situation is somewhat complicated by the fact that hypercalcemia can occur in Addison's disease (4, 5). There is also the evidence that in idiopathic hypercalcemia relatively small doses of cortisone and related drugs will bring down the serum calcium to normal, a response which is also seen in overdosage with vitamin D, and in sarcoidosis. This has been attributed to a local effect of cortisone blocking the vitamin D at the level of the intestinal wall and preventing further absorption of calcium; there is considerable clinical (8) and experimental (27) evidence to support such a mechanism, but the fact that patients will benefit cases of idiopathic hypercalcemia does not necessarily indicate that there is always hypofunction of the adrenal cortex in this disease.

4. Hypothyroidism. Many of the early cases of the severe type of hypercalcemia were considered to be cretins and were treated with thyroid extract, though without benefit. Superficially there are many resemblances to cretinism, such as the peculiar facial appearance, the slow mental and physical development and the constipation; the retarded skeletal development, and the high blood cholesterol may also be misleading. Investigations of thyroid function, such as the protein bound iodine (26, 40) and the uptake of I^{131} (12), have all been normal.

Against this is the beneficial effect of treatment with thyroxine in a severe case of idiopathic hypercalcemia reported by Ibsoff and Vermeulen (34). Though they were careful to point out that they did not regard this as a [illegible] calling hypothyroidism but rather an effect of thyroxine in increasing the excretion of calcium. There is never [illegible] evidence that hypercalcemia sometimes occurs in hypothyroid children (13a, 26) and also that nephrocalcinosis and bone changes at the base of the skull and elsewhere very similar to those seen in idiopathic hypercalcemia may be found in hypothyroidism in childhood (41, 52). As an explanation for these observations Chomaginitsa and Hlandovsky (21) showed that thyroidectomised rats were hypersensitive to vitamin D and Royer and co-workers (43a) demonstrated hypersensitivity to vitamin D in one of their cases of hypothyroidism and hypercalcemia. Though this possibility should always be considered in hypercalcemia in childhood the weight of evidence against hypothyroidism as a factor in the aetiology of idiopathic hypercalcemia

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Theories Involving Vitamin D Directly

There are four possibilities to be considered: there might be a high intake of vitamin D up to toxic levels, there might be a failure of metabolic disposal or inactivation of vitamin D, an endogenously produced substance with activity similar to vitamin D might be present, or there might be a lowered threshold of response (hypersensitivity) to normal amounts of vitamin D. With an excessive intake, or a metabolic error one would expect to find a high level of vitamin D activity in the serum, but in "hypersensitivity" one would expect a normal level.

It is necessary therefore to examine the available evidence to see how closely a known excess of vitamin D (i.e., a toxic amount by ordinary standards) can reproduce the features of the mild and severe types of the disease.

Comparison between Idiopathic Hypercalcaemia and Hypervitaminosis D

1. **Biochemical features.** There is no doubt that the essential abnormality, the hypercalcaemia, is common to both conditions and that the renal damage seen is due to the hypercalcaemia. Hypercholesterolaemia is usual in the severe type of idiopathic hypercalcaemia and is less common or less marked in the mild cases. Forfar and Tompsett (20) have pointed out that though the serum cholesterol is undoubtedly raised in overdosage with vitamin D, in the age group affected by idiopathic hypercalcaemia a raised serum cholesterol is rarely seen. However, the cases described by Lang and Eiardt (31), Koranyi (30), Amann (1) which are considered in more detail later, show that hypercholesterolaemia can occur in chronic overdosage with vitamin D in infancy. The most marked [illegible] features between the two conditions [illegible] the rapidity with which the hypercalcaemia in vitamin D overdosage returns to normal when the vitamin is omitted from the diet, compared to the much slower improvement seen in idiopathic hypercalcaemia, whether of the mild or severe type.

2. **Clinical and radiological features.** The general symptoms due to hypercalcaemia are the same whatever the cause. Debré (13) has given an excellent description of vitamin D poisoning in the young child which could equally have applied to idiopathic hypercalcaemia. Comparison becomes more difficult when one considers some of the characteristic features of the severe type of idiopathic hypercalcaemia. A review of the literature shows that there are only the three cases already mentioned in which undoubted overdosage with vitamin D produced a clinical picture similar to that of severe hypercalcaemia (Table I). Lang and Eiardt's (31) case was aged two years, and had been given a total of approximately 2,000,000 units of vitamin D in addition to ultra-violet irradiation. The face of this boy was somewhat similar to that seen in the severe form of hypercalcaemia; he was mentally retarded, the bones showed increased calcification and the serum cholesterol was raised (310 mg.%), but there was no systolic murmur. There seems little reason to doubt that this was an example of chronic overdosage with vitamin D.

The second case, described by Koranyi (30) was also given very large doses of vitamin D. This child was mentally retarded, with the typical facies of hypercalcaemia; the serum cholesterol was normal (200–216 mg.%) and the bone changes were not marked. In the third case (1) a total of 6,600,000 units of D_3 was given over a period of 6 months; the facies was typical of hypercalcaemia, a squint was noted but there was no cardiac murmur; the serum cholesterol has 485 mg.% and there was a considerable increase in calcification at the ends of the long bones and at the base of the skull. In each case the symp-

Table I. Cases of hypercalcaemia resulting from large doses of vitamin D.

Case	1	2	3
Typical symptoms	+	+	+
Facies	?	+	+
Squint	0	+	0
Mental retardation	+	?	?
Osteosclerosis	+	0	−
Systolic murmur	0	0	0
B.M.	175/120	?	145/95
Hypercalcaemia	+	+	+
Raised blood urea	+	0	+
Serum cholesterol	310 mg.%	200–216 mg.%	485 mg.%
Symptoms after giving vitamin D	Yes	Yes	Yes
Total dose of vitamin D	2,000,000 I.U. "D"	40 mg. D_2 (1,600,000 I.U.)	6,600,000 I.U. D_3
Period of treatment with vitamin D (age in weeks or months)	6/12–20/12	6/52–11/12	4/12–10/12

* Case 1, Lang and Eiardt (31); Case 2, Koranyi (30); Case 3, Amann (1).

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toms developed after vitamin D had been given. In a slightly older child (14) in whom 50,000 units of vitamin D were given daily starting at the age of three years and continuing for two years the bone changes were identical to those seen in the severe type of hypercalcaemia. In view of these well documented cases there seems little doubt that chronic overdosage with vitamin D can produce the clinical and radiological features of the severe type of idiopathic hypercalcaemia.

Pathological features. It is generally agreed that the renal changes seen at autopsy or biopsy in idiopathic hypercalcaemia and hypervitaminosis D are identical. A systolic murmur is commonly present in the severe cases of hypercalcaemia, and on the basis of the evidence already quoted it is accepted that changes in the cardiac valves, usually the aortic valve, are responsible for the murmurs. There is also good evidence that high dosage with vitamin D can produce calcification of the heart valves in infants, as was shown by the Finnish experience (23) where large doses of vitamin D ("stosstherapie") had been given. Hjelt and co-workers reported two cases with hypercalcaemia and a blowing systolic murmur during life, in which there was calcification at the base of the aortic and mitral valves, which were considerably thickened throughout.

Intake of Vitamin D in Idiopathic Hypercalcaemia

With the exception of the three cases already referred to, infants suffering from idiopathic hypercalcaemia have not received doses of vitamin D which would normally be considered sufficient to produce hypercalcaemia. It is clear that there is a great deal of variation between individuals in their response to the same dose of vitamin D. Jeans and Stearns (28) showed that the optimal intake for most infants is in the region of 400 units daily, and that in a few infants on an intake of 1,800–4,600 units daily from birth, growth began to slow down after the age of 5 or 6 months and accelerated again when the intake was reduced to normal. Crenny and Neill (9) found that 51% of infants in Great Britain were receiving between 1000–2000 units of vitamin D daily and 4% were receiving more than 2000 units daily. This is of course far less than the amount which is usually considered to be frankly toxic though any estimate of the toxic dose must take into consideration both the age of the recipient and the duration of treatment.

Investigations have shown that in most cases of idiopathic hypercalcaemia the intake of vitamin D has been between 1000 and 2000 units daily (25), but in two of the three severe cases reported from Japan (39) there was no vitamin D in the diet other than the small amount derived from unfortified cow's milk in one case, and from human milk in the other. Thorough investigation of the maternal diet during pregnancy has never revealed an excessive intake of vitamin D.

Geographical Factors

It is probably significant that more cases of idiopathic hypercalcaemia have been seen in Great Britain than in any other country. In a period of 2½ years between 1952 and 1955, 204 cases were recognised in Great Britain (4) and in the two years ending October 1955 in Dundee, in Scotland, 4.6% (15 cases in all) of all medical admissions to hospital between the ages of 6 months and one year were suffering from hypercalcaemia (38). This high incidence in Great Britain involves both the mild and severe cases, though the mild cases are much commoner than the severe ones; of all the severe cases reported up till 1961, 16 were in Great Britain and 13 were from the United States (23) where the mild form has only rarely been reported. Any accurate assessment of the frequency of idiopathic hypercalcaemia in any particular country is of course impossible, as diagnosis depends upon awareness of the disease and facilities for adequate diagnosis.

Vitamin D Levels in the Serum

Whatever may be the cause of idiopathic hypercalcaemia a study of the level of vitamin D activity in the serum is clearly of great importance in the understanding of the condition. Unfortunately, not a great deal of work has yet been done on this problem, but normal levels of between one and three units per ml have been established by a number of workers (17, 51, 53). Cuthbertson (1963) has found similar levels in Great Britain.

The results in hypercalcaemia are conflicting (Table II); Lang and Fiardi (31) found a slightly raised level (four units per ml) two months after stopping vitamin D, but in view of the enormous doses given, their case should properly be regarded as one of vitamin D poisoning, and the finding of a level of four units per ml two months after stopping treatment is what would be expected in such a case. The findings of Fellers (16) and of Smith and co-workers (47) are much more difficult to explain. Their cases appear to have been typical examples of the severe type of hypercalcaemia, and in no instance was an unusually high intake observed (Table II). The striking feature about these cases is the length of time during which a high level of vitamin D activity was maintained after all vitamin D had been stopped. It seemed unlikely that an unsuspected source of large doses of vitamin D could have remained undetected for so long. In the three cases therefore it is difficult to escape the conclusion that the excessive vitamin D activity in the blood was

Table II. Cases of idiopathic hypercalcaemia with vitamin D assays on serum.

Authors	Vitamin D intake	Clinical type	Vitamin D assay results I.U. per ml.	Period without vitamin D
Lang & Bierdt (31)	Excessive	Severe type* hypercalcaemia	4 2.5	2 months 8 months
Fellers (16)	Normal	Severe type hypercalcaemia	10 19	0 18 months
Fellers (16)	Normal	Severe type hypercalcaemia	20 20 < 2	0 16 months 26 months
Smith, Blizard and Harrison (47)	1500 units daily, then 4,400 units daily for 1 month before admission	Severe type hypercalcaemia	23 5 2	0 6 months 21 months
Schmid, Just & Stalder (45)	300,000 i.u. 2 months before admission & 300,000 i.u. just before admission	Mild type hypercalcaemia	1.75	Not stated
Hewitt & Stephan (25)	Not stated	Intermediate hypercalcaemia	3.6	Not stated
Thomas, Morgan, Cormier, Haddock, Fells & Howard (51)	Not stated	Mild** (2 cases)	"Normal"	Not stated
Cuthbertson (11)	1350 2000 1000	Mild Mild Mild	2.9 3.0 1.5	0 3 days 6 days
Cuthbertson (11)	Not stated 2300 2000 – 3000 1500	Mild Mild Mild Severe	2.0 2.1 1.4 2.0 2.0	7 days 3½ weeks 0 12 days
	1600 3000 1700	Severe Severe Severe	1.7 2.0 1.0	2 weeks 3½ weeks 6½ months

* Probably a case of vitamin D overdosage.
** Serum obtained from 2 cases in Britain (Mitchell — personal communication).

due to some metabolic error involving the disposal of exogenous vitamin D or even that there might have been some substance of similar activity produced endogenously, though this seems unlikely as the levels did in fact decline when vitamin D was omitted, and reached normal levels in one of Fellers' cases and in Bierdt's case and by Smith and co-workers.

In all the other cases in which vitamin D activity in the serum has been assayed it has been found to be normal or only slightly raised. Schmid, Just and Stalder (45) found a level of 1.75 per ml. units in a mild case and Thomas and co-workers (51) found normal levels in the serum of two cases from Dundee in Scotland which were of the mild type (Mitchell, personal communication). Hewitt and Stephan (25) reported a level of 3.6 units per ml., a slightly raised level in an intermediate case. In the largest series so far reported Cuthbertson (11) was unable to assay the vitamin D content of the serum from cases from various parts of Great Britain and found normal levels in every instance. This series included four examples of the severe type of idiopathic hypercalcaemia. There seems no way of reconciling these results with those of Fellers and of Smith and co-workers, except by assuming that we are dealing with a condition in which there may be more than one cause for the hypercalcaemic state.

It seems worth considering the point made by Thomas et al. (51) that, although dihydrotachysterol has the same activity in man weight for weight as has vitamin D, its activity as measured by the rat line test is only 1:400 of the activity of vitamin D. Thus it should not be assumed that the currently used line test is able to detect every agent in the serum which is capable of raising the serum calcium level in the human infant.

Conclusion

Both types of idiopathic hypercalcaemia are easily recognisable clinically and biochemically; there is no obvious endocrine disorder underlying the condition, but it is clear that overdosage with vitamin D can produce a state which is similar if not identical—clinically, biochemically and radiologically—to idiopathic hypercalcaemia. The number of cases in which this has occurred is however extremely small. There appears to be a group of cases, of which three examples have been reported from the United States.

where the vitamin D levels are very high and have remained high for many months after vitamin D has been stopped; in these cases the intake of vitamin D has not been excessive. In all other cases investigated the vitamin D levels have been normal or only slightly raised. It would appear therefore that in the majority of cases of idiopathic hypercalcaemia there is either an increased sensitivity to normal levels of vitamin D in the serum, or that another active agent is present in the serum which remains undetected by our present methods of assay.

It is necessary to consider these facts in relation to national policies for the fortification of milks with vitamin D and also to current use and abuse of vitamin D by the medical profession and by the general public. Whether or not vitamin D or one of its metabolic products is finally found to be the cause of idiopathic hypercalcaemia, it seems obvious that a general level of intake of vitamin D which is three times the optimal requirement is likely to have an additive effect upon any other factors with a vitamin D-like action. Similar consideration apply equally to "stosstherapie" which appears justified only in terms of expediency. The medical profession and the public should realize that high-potency vitamin preparations are extremely toxic and should not be used as a tonic.

The British Paediatric Association has for some years been conducting a survey of the incidence of idiopathic hypercalcaemia in Great Britain with the hope of throwing some further light upon the relationship between the intake of vitamin D and the incidence of idiopathic hypercalcaemia. The report has just been published (Brit. med. J. 1964/I, 1659).

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The Synthesis of 25-Hydroxycholecalciferol. A Biologically Active Metabolite of Vitamin D_3*

0630

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ABSTRACT: Cholesta-5,7-diene-3β,25-diol has been synthesized by two methods and then converted into 25-hydroxycholecalciferol, which was identical in all respects with the biologically active metabolite of vitamin D_3 previously isolated from porcine plasma.

Recently in this laboratory a metabolite of vitamin D_3 was isolated from the plasma of hogs maintained on high daily levels of the vitamin for 26 days. It was identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). This metabolite was shown to be more efficient than vitamin D_3 in curing rickets in rats, and was able to stimulate calcium transport in isolated rat intestinal sacs earlier than a similar amount of vitamin D_3. Also, bone resorption in rats was shown to occur after a dose of 25-hydroxycholecalciferol earlier than after a similar dose of vitamin D_3 (Blunt *et al.*, 1968c). As a result of these experiments, and others cited earlier (DeLuca, 1967), the suggestion was made that 25-hydroxycholecalciferol was possibly the metabolically active form of the vitamin. In order to continue studies to verify this suggestion, and to investigate other possible uses of the metabolite, it became necessary to synthesize 25-hydroxycholecalciferol in larger amounts. Accordingly, cholesta-5,7-diene-3β,25-diol has been synthesized by two methods, and irradiation of this with ultraviolet light produced 25-hydroxycholecalciferol, identical in all respects, including biological activity, with the material isolated previously from porcine plasma.

Experimental Procedure

Ultraviolet spectra were determined in ethanol in a Beckman DB-G spectrophotometer, and nuclear magnetic resonance spectra were obtained from a Varian Associates Model HA 100 spectrometer coupled to a time-averaging computer, using deuteriochloroform solutions with tetramethylsilane as internal standard. Gas–liquid partition chromatography was performed in an F & M Model 402 gas chromatograph, using 4 ft × 0.25 in. glass columns packed with 3% W-98 on 80–100 mesh Diatoport S. High-resolution mass spectra were recorded with an A.E.I. MS-9 spectrometer coupled to a scientific Data Systems Sigma-7 computer.

Synthesis of Cholesta-5,7-diene-3β,25-diol. METHOD A (Figure 1). 25-Hydroxycholesteryl acetate (1 g) (Steraloids, Inc., New York, N. Y.) was refluxed in 50 ml of acetic anhydride with a few drops of pyridine for 1 hr. The cooled solution was poured into 250 ml of water and after 3 hr the white solid was filtered. Recrystallization from methanol gave 850 mg of the diacetate, mp 119–120° (Dauben and Bradlow, 1950). The diacetate (100 mg) was dissolved in 1.5 ml of dry benzene and 1.5 ml of dry Skelly B (a petroleum fraction which boils at 67–69°). This solution was added to 32 mg of finely powdered *N,N'*-dibromodimethylhydantoin in a test tube, which was then immersed in a water bath at 72–74°. This bromination procedure was first described by Hunziker and Müller (1958) and in our hands represents the only successful allylic brominating agent for these compounds. When dissolution of the dibromantin was complete (6–8 min) the solution was cooled in ice and the crystalline precipitate of dimethylhydantoin was removed by filtration. The precipitate was rinsed twice with 1-ml rinses of ice-cold Skelly B. The filtrate was evaporated at less than 40° *in vacuo*, and taken up in 0.4 ml of dry xylene. This was added, with two 0.1-ml rinses, dropwise over 2 min to a solution of 0.1 ml of trimethyl phosphite in 0.3 ml of xylene maintained at 130–135°. The mixture was kept at this temperature for 90 min followed by evaporation of the solvent *in vacuo* at 65°. The residue was applied to a column of 25 g of neutral alumina (grade I) in Skelly B. Elution with diethyl ether–Skelly (1:3) followed by ether–Skelly B (1:1) and collecting 12-ml fractions yielded the diacetate of cholesta-5,7-diene-3β,25-diol, as indicated by its ultraviolet spectrum with maxima at 272, 282, and 294 mμ. Earlier fractions containing this material were contaminated with the 4,6-diene, as indicated by ultraviolet absorption at 239 mμ. Those fractions containing the 5,7-diene free of the 4,6-diene were combined, giving 23 mg by weight and by ultraviolet spectrum (ϵ_{294} 11,000). This material was dissolved in 3 ml of dry ether and treated with a small portion of $LiAlH_4$ for 5 min to remove the acetyl group. Extraction of the product gave 18 mg of cholesta-5,7-diene-3β,25-diol: mp 169–171 (aqueous MeOH); ultraviolet max at 272, 282, and 294 mμ (ϵ_{294} 11,000); nuclear magnetic resonance: C_{18} H_3, δ 0.65; C_{19}-H_3, 0.98; C_{21} H_3, 0.89 (J = 6.5 cps); $C_{26,27}$ H_6, 1.20; $C_{3\alpha}$ H, 5.4 (multiplet).

METHOD B (Figure 2). 3β-Acetoxy-26-norcholest-5 en-25-one (142 mg) in 2.2 ml of dry benzene and 2.2 ml of

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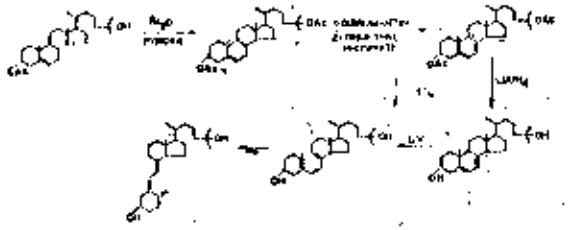

FIGURE 1: Synthesis of 25-hydroxycholecalciferol from 25-hydroxycholesterol.

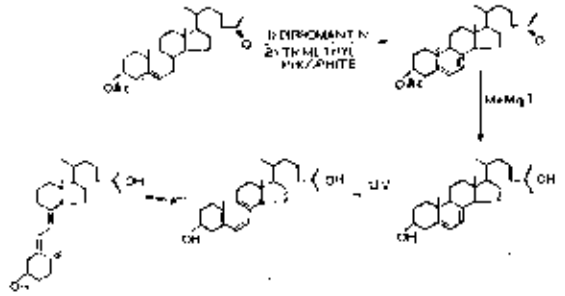

FIGURE 2: Synthesis of 25-hydroxycholecalciferol from 25-ketonorcholesterol.

dry Skelly B was added to 50 mg of dibromantin and reacted as in method A. The dehydrobromination was also performed as in method A, by adding the brominated material in 0.5 ml of xylene with 0.3 ml of rinse to 0.15 ml of trimethyl phosphite in 0.5 ml of xylene. The product isolated from this step was adsorbed onto a column of 20 g of neutral alumina (grade I) in chloroform. Elution with chloroform and collection of 5.5-ml fractions gave, in fractions 10–14, 39 mg (as indicated by ultraviolet spectra, ϵ_{262} 11,000) of the 5,7-diene. Gas liquid partition chromatography indicated the presence of the starting material, 3β-acetoxy-26-norcholest-5-en-25-one, as the only contaminant. The infrared spectrum of the diene showed ν_{max} 1730 and 1712 cm^{-1}, corresponding to the carbonyl absorption of the acetate and methyl ketone groups, respectively. The nuclear magnetic resonance spectrum displayed δ 0.63 (C_{18}-H_3), 0.95 (C_{19}-H_3), 2.02 (OAc), and 2.12 ppm (C_{26}-H_3).

The material (245 mg) prepared as above and containing 180 mg of the 5,7-diene as indicated by ultraviolet spectrum was dissolved in 3.5 ml of dry benzene and added to the Grignard reagent prepared by the addition of 0.24 ml of methyl iodide in 2.5 ml of ether to 72 mg of magnesium turnings. This was refluxed for 2.5 hr and then left 18 hr before adding to cold ammonium chloride solution. The benzene extract of this was dried and evaporated and the residue was triturated with Skelly B giving 212 mg of an amorphous powder shown by ultraviolet analysis and gas liquid partition chromatography to consist of 140 mg of cholesta-5,7-diene-3β,25-diol, the remainder being 25-hydroxycholesterol. This material was used as such for conversion into 25-hydroxycholecalciferol as follows.

Irradiation of Cholesta-5,7-diene-3β,25-diol. All irradiations were carried out in 400 ml of ether contained in a jacket around a double-walled, water-cooled, quartz immersion well. An Hanovia high-pressure quartz mercury vapor lamp, Model 654 A, was ignited for 1 min before placement in the immersion well for the times indicated below. During irradiation, the ether was flushed continuously with nitrogen and stirred vigorously.

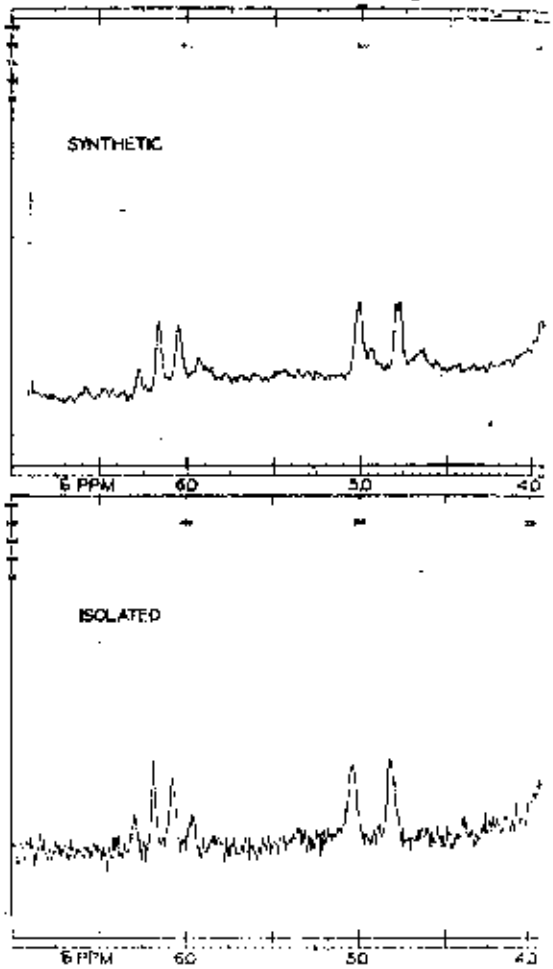

FIGURE 3: Downfield nuclear magnetic resonance spectra of synthetic and isolated 25-hydroxycholecalciferol.

The mixture (106 mg) prepared as in method B and containing 70 mg of the 5,7-diene was irradiated for 3.5 min. The products were applied in ether-Skelly B (1:1) to a multisorb column (Fischer and Kabara, 1961) prepared from 14 g of heat-activated silicic acid (Bio Rad silicic acid, HA, −325 mesh, California Corp. for Biochemical Research, Los Angeles, Calif.). The column was eluted with a convex gradient obtained by running ether into a 250-ml mixing chamber initially filled with ether-Skelly B (1:1) (Brock and Ling, 1954). Fractions of 2.8 ml were collected. Ultraviolet analysis and gas liquid partition chromatography indicated the presence of 25-hydroxyprecholecalciferol only in fractions 32–33, while the latter fractions contained 25-hydroxycholesterols, 25-hydroxycholesterol, and a small amount of unconverted 5,7-diene. Fractions 33–38 were combined, giving 11 mg, and after storage at room temperature in ether under hydrogen for 7 days, the material was [illegible]

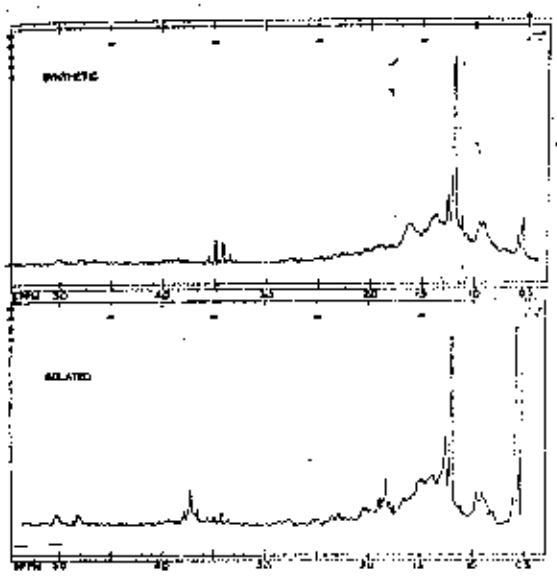

FIGURE 4: Nuclear magnetic resonance spectra of synthetic and isolated 25-hydroxycholecalciferol. Note that $\Delta = 0.58$ ppm peaks are due to tetramethyl-silane internal standard.

in the form of 25-hydroxycholecalciferol, ultraviolet max 265 mμ (ε 18,000); nuclear magnetic resonance C_{21}-H_3, δ 0.90 (J = 8 cps); C_{18}-H_3, 0.54; $C_{26,27}$-H_6, 1.22; C_{19}-H_2, 4.80 and 5.00; $C_{6,7}$-H, 5.97 and 6.25 ppm (J = 12 cps). The nuclear magnetic resonance spectra of the isolated and synthetic 25-hydroxycholecalciferols are shown in Figures 3 and 4 and it is readily apparent that they are identical.

The mass spectra of the synthetic and isolated compounds are shown in Figure 5 leaving no doubt that they are identical. A molecular weight of 400.3340 (calcd for $C_{27}H_{44}O_2$, 400.3341) was determined by high-resolution mass spectrometry which gave characteristic fragments at *m/e* 271.2059 (calcd for $C_{19}H_{27}$, 253.1956).

Those fractions containing 25-hydroxytachysterol and unchanged 5,7-diene were combined, together with similar fractions from the irradiation of a second batch of material as above, and were reirradiated for a further 3.5 min. Chromatography of the products as described above yielded, after equilibration, a further 8 mg of 25-hydroxycholecalciferol.

Irradiation for 2.5 min of 15 mg of the 5,7-diene prepared by method A yielded 2.4 mg of 25-hydroxypre-cholecalciferol, and reirradiation of the 25-hydroxytachysterol and unchanged 5,7-diene, followed by chro-

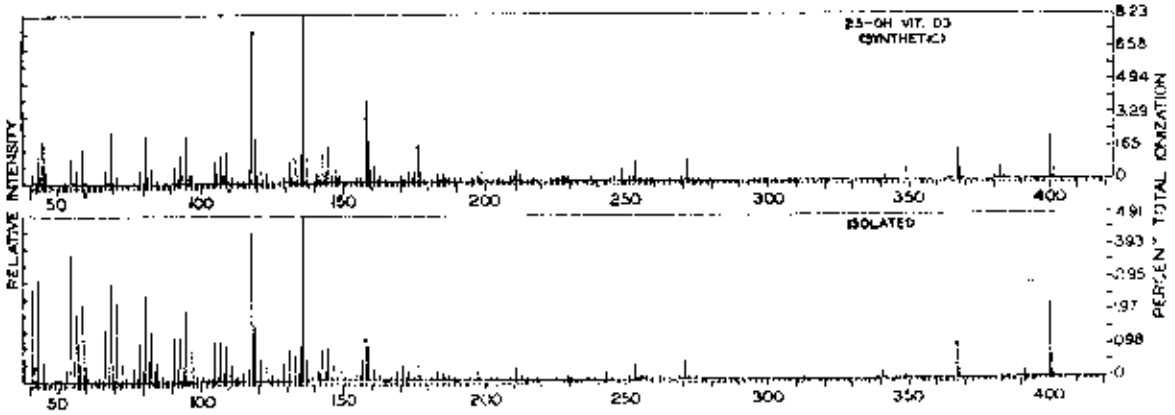

FIGURE 5: Mass spectrum of synthetic and isolated 25-hydroxycholecalciferol.

TABLE I: Comparison of Intestinal Calcium Transport Responses of Rats on a Vitamin D Deficient Diet to an Intravenous 0.25-μg Dose of Either Synthetic 25-Hydroxycholecalciferol or Isolated 25 Hydroxycholecalciferol as Compared with Cholecalciferol.[a]

	^{45}Ca Serosal/^{45}Ca Mucosal		
		25-Hydroxycholecalciferol	
hr after Administration	Cholecalciferol	Synthetic	Isolated
Control	1.24 ± 0.05	1.59 ± 0.17[b] (4)[c]	1.24 ± 0.05 (12)
4	1.01 ± 0.12	2.31 ± 0.50 (5)	1.78 ± 0.35 (4)
6	1.32 ± 0.10	2.33 ± 0.31 (4)	2.62 ± 0.44 (4)
10	2.01 ± 0.32		

[a] The rats were prepared and the everted gut sac assay performed as described by Blunt *et al.* (1968c). [b] Standard deviation. [c] Number of rats in each group.

TABLE II: Serum Calcium Response of Rats on a Low Calcium, Vitamin D Deficient Diet to a 2.5-μg Intravenous Dose of Synthetic 25-Hydroxycholecalciferol or Isolated 25-Hydroxycholecalciferol as Compared with Cholecalciferol.[a]

hr after Administration	Serum Ca (mg/100 ml)		
		25-Hydroxycholecalciferol	
	Cholecalciferol	Synthetic	Isolated
0	3.8 ± 0.11	3.75 ± 0.23[b] (6)[c]	3.8 ± 0.23
8	3.8 ± 0.14	6.4 ± 0.5 (7)	5.7 ± 0.24
12	5.0 ± 0.44	7.36 ± 0.12 (6)	7.1 ± 0.41
16	6.6 ± 0.47		

[a] The rats were prepared and the assay performed as described by Blunt *et al.* (1968c). [b] Standard deviations. [c] Number of rats in each group.

matography as before, yielded a further 0.4 mg of the desired product.

Biological Activity of Synthetic 25-Hydroxycholecalciferol. All assays were performed as described previously (Blunt *et al.*, 1968c) and only the results are presented here. In the line test assay in rats, the synthetic 25-hydroxycholecalciferol assayed at the level of 55-60 I.U./μg as previously reported for the isolated 25-hydroxycholecalciferol. Table I demonstrates the ability of a 0.25-μg intravenous dose of 25-hydroxycholecalciferol to stimulate calcium transport in isolated, everted, rat intestinal sacs after 4 and 6 hr, times at which no response to a similar dose of vitamin D_3 have been observed. Note that vitamin D_3 has no effect until 10 hr while 25-hydroxycholecalciferol both isolated and synthetic was effective within 4 hr. Table II shows that 25-hydroxycholecalciferol is able to stimulate bone resorption as indicated by a rise in serum calcium concentration within 8-12 hr following an intravenous dose of 2.5 μg of either isolated or synthetic 25-hydroxycholecalciferol to D-deficient rats maintained on a low calcium diet.

Discussion

This communication reports the synthesis of 25-hydroxycholecalciferol in over-all yields of 3.6% from 26-norcholest-5-en-25-on-3β-yl acetate and 3.2% from 25-hydroxycholesteryl acetate. Clearly, the synthetic material is identical in all respects with the 25-hydroxycholecalciferol isolated from porcine plasma. The synthetic material mimics the action of the isolated metabolite in curing rickets in rats, and in stimulating both calcium transport in intestine and bone calcium mobilization more rapidly than vitamin D_3. The physical data, including ultraviolet, gas–liquid partition chromatography, nuclear magnetic resonance, and mass spectrum, are all identical for the synthetic and isolated materials, the latter's constants having been reported earlier (Blunt *et al.*, 1968b).

Using the knowledge that there is a photochemical equilibrium between tachysterol$_3$ and precholecalciferol (Fieser and Fieser, 1959), the 25-hydroxytachysterol$_3$ obtained from the irradiations of cholesta-5,7-diene-3β,-25-diol in this work was reirradiated, yielding further amounts of the 25-hydroxyprecholecalciferol. It is felt that this technique could be used more exhaustively than applied in the work reported here. Such a study here was hindered by the presence of 25-hydroxycholesterol in the 5,7-diene prepared by method B, for, in the chromatographic system used in this work, this contaminant was not separated from 25-hydroxycholecalciferol.

The synthesis of 25-hydroxycholecalciferol by method B is particularly applicable to a synthesis of tritiated 25-hydroxycholecalciferol, since the label may be incorporated *via* the Grignard reaction introducing the 26-methyl group, the penultimate step of the synthesis. This method is currently being utilized in this laboratory, with results to be published shortly. The availability of such labeled material will enable the study of vitamin D metabolism to continue in greater detail.

Acknowledgment

We are grateful to Dr. Tatsuo Suda and Mrs. Yoko Tanaka for performing the biossays, and Dr. C. J. Sih of the School of Pharmacy, University of Wisconsin for the generous gift of some 26-norcholest-5-en-25-on-3β-yl acetate. We are also grateful to Dr. H. K. Schnoes of the Department of Biochemistry for recording the mass spectra, through the generosity of Professor Burlingame, University of California, Berkeley, and to Miss Martha Petrie and Dr. P. Bender, Chemistry Department, University of Wisconsin, for some of the nuclear magnetic resonance measurements.

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25-Hydroxycholecalciferol. A Biologically Active Metabolite of Vitamin D_3*

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ABSTRACT: Milligram quantities of a polar metabolite of vitamin D_3 have been isolated in pure form from the plasma of pigs fed 250,000 IU of vitamin D_3 per day for 26 days. This metabolite is the major biologically active component of the peak IV metabolite fraction reported earlier. A combination of ultraviolet spectra, gas liquid partition chromatography and mass spectrometric and nuclear magnetic resonance spectrometric analyses have unequivocally established its structure as 25-hydroxycholecalciferol. Its biological activity has been set at 1.4 that of vitamin D_3 in its ability to cure rickets in rats.

It has long been assumed that unaltered vitamin D_3 (I) is responsible for its metabolic and physiologic manifestations. The first demonstration of biologically active metabolites of vitamin D appeared from this laboratory in 1964 (Norman *et al.*, 1964). It was, however, the work of Lund and DeLuca (1966) which first demonstrated unambiguously that a polar metabolite of vitamin D designated as peak IV was possibly the metabolically active form. Thus they were able to show large amounts of the peak IV metabolite fraction in bone, liver, and blood serum of rats. Upon estimation of its biological activity it was evident that it was at least as active and perhaps more active than vitamin D itself. Furthermore, it was the predominant form of vitamin D in the target tissues (intestine and bone) after truly physiologic doses (10 IU) of the vitamin (DeLuca, 1967). The metabolite was also demonstrated in human plasma (DeLuca *et al.*, 1967; Avioli *et al.*, 1967), chicks (Drescher *et al.*, 1968), and porcine plasma (present report). Other experiments have shown that vitamin D is converted into this material before its action in promoting intestinal absorption of calcium can be observed (DeLuca, 1967). Finally, the metabolite stimulates the intestinal absorption of calcium and mobilization of bone much as does vitamin D itself (Morii *et al.*, 1967) providing strong evidence that it is in fact the metabolically active form of the vitamin. Finally, that this metabolite is the primary or only form associated with intestinal nuclei, the believed site of vitamin D action, has been unequivocally demonstrated by Stohs and DeLuca (1967).

The biologically active component of the peak IV metabolite fraction has now been isolated in milligram quantities in pure form from the plasma of pigs maintained on high vitamin D_3 rations. Its chemical structure has unambiguously been shown to be 25-hydroxycholecalciferol (II) and its biological activity shown to be greater than that of vitamin D_3 itself. A preliminary report of this work has recently appeared (Blunt *et al.*, 1968).

Methods and Results

General Procedures. Ultraviolet spectra were recorded with a Beckman DB-G spectrophotometer. Nuclear magnetic resonance spectra were recorded in $CDCl_3$ solutions in a Varian Associates Model HA-100 spectrometer coupled to a time-averaging computer, the latter being necessary because of the small amount of metabolite isolated. Tetramethylsilane was used as the internal standard. Data are recorded as δ in parts per million, relative to tetramethylsilane ($\delta = 0$). Gas liquid partition chromatography was performed in an F & M Model 402, using 4 ft × 0.25 in. glass columns packed with 3% W-98 on 80–100 mesh Diatoport S. High-resolution mass spectra were recorded with an

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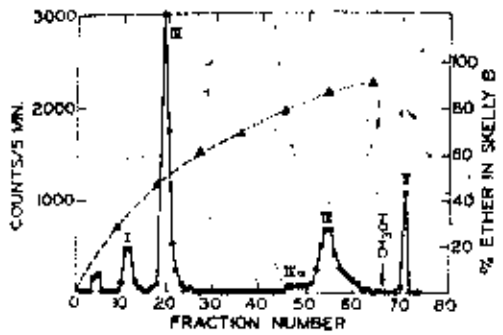

FIGURE 1: Silicic acid chromatography of vitamin D and its metabolites. Peaks III and I are vitamin D and its esters respectively, peaks IVa and V are unidentified, and peak IV is 25-hydroxycholecalciferol.

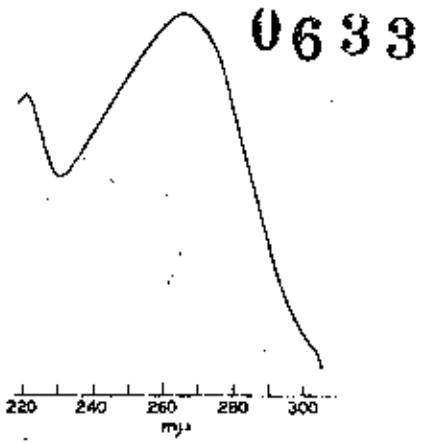

FIGURE 3: Ultraviolet spectrum of 25-hydroxycholecalciferol in ether.

A.E.I. MS-9 mass spectrometer coupled to a Scientific Data Systems Sigma-7 computer.

All radioactivity measurements were made in a Packard Tri-Carb liquid scintillation counter, Model 3000, and automatic external standardization was used to determine efficiency. Radioactive samples were counted in a scintillator solution which consisted of 2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene) per L. of toluene.

Preparation of Extract Containing Labeled Vitamin D_3 and Its Metabolites. To determine the optimum time after dosing a hog with vitamin D_3 so as to recover the greatest amount of peak IV metabolites, a 50-lb hog was given a dose of 0.5 mg of random-labeled [^{3}H]vitamin D_3 (Norman and DeLuca, 1963) (5.7 mCi/mmole) in 1 ml of ethanol intravenously. A preliminary experiment on serial blood samples taken at various times revealed that optimum concentrations of peak IV metabolites appeared at 24 hr after dosing. Accordingly, the same hog was given a further 2-mg dose of [^{3}H]vitamin D_3 in 1 ml of ethanol intravenously 72 hr after the original 0.5-mg dose. Twenty-four hours later, the blood was removed under Nembutal anesthesia. Blood (1.2 L) was collected, giving 800 ml of serum.

To provide a convenient means for extracting the vitamin D and its metabolites from large volumes of plasma a method involving the precipitation of serum proteins with ammonium sulfate was investigated. Aliquots (0.5 ml) of the radioactive serum were saturated to varying degrees with $(NH_4)_2SO_4$. The protein precipitates were removed by centrifugation and extracted with methanol–chloroform (Bligh and Dyer, 1959) and the whole extracts were counted. The results showed that 65% saturation with $(NH_4)_2SO_4$ effectively precipitates virtually all of the radioactivity.

The remainder of the serum was either extracted directly with methanol chloroform or precipitated by 65% saturation with $(NH_4)_2SO_4$, followed by extraction of the precipitate. The original dose (2.6%) of [^{3}H]D_3 was recovered as peak IV metabolites representing 42% of the radioactivity in the plasma. Of this, 60% was 25-hydroxycholecalciferol.

Isolation of 25-Hydroxycholecalciferol (II). A 100-lb hog was maintained on normal rations supplemented with 100,000 IU of vitamin D_2 (Delsterol, Vita Plus Corp., Madison, Wis.) daily for 3 days before collection of a blood sample. The serum from this sample was extracted with chloroform–methanol and separated as before (Lund and DeLuca, 1966) into the vitamin D and peak IV fractions which were submitted to assay for vitamin D activity by the rat line test method (U. S. Pharmacopeia, 1955). This assay indicated 4 IU/ml due to vitamin D and 2 IU/ml due to peak IV. The same hog was maintained on a supplemented diet (100,000 IU of D_2 daily) for 4 months (weight gain, 300 lb) after which time serial samples assayed for 10 IU/ml of serum due to vitamin D and 5 IU/ml due to peak IV. Hogs weighing 200 lb and maintained on normal diet supple-

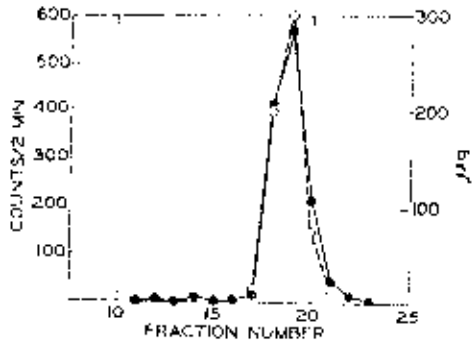

FIGURE 2: Partition chromatography of peak IV from Figure 1.

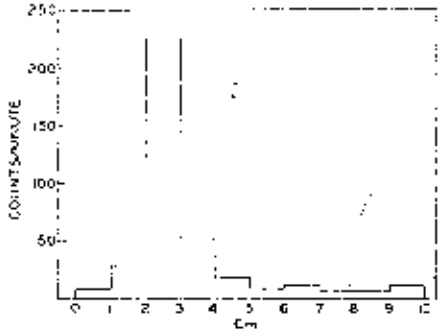

FIGURE 4: Thin-layer chromatography of 25-hydroxycholecalciferol on silica gel G.

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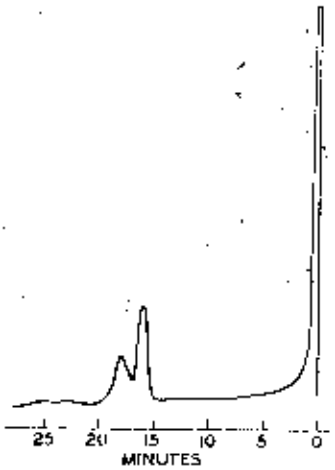

FIGURE 5: Gas–liquid partition chromatogram of 25-hydroxycholecalciferol. The larger peak is 25-hydroxypyrocholecalciferol (VI), while the smaller is its isopyro isomer (IV). Injection and column temperature 240°, helium flow rate 70 cc/min at 50 psi. Sensitivity 10 × 8.

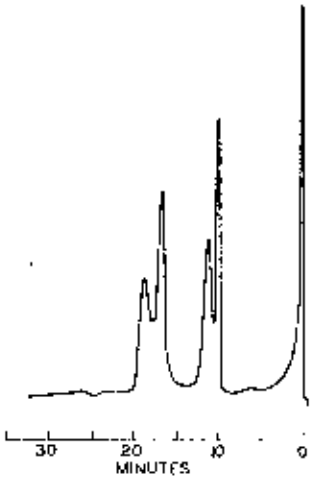

FIGURE 6: Gas–liquid partition chromatography of 25-hydroxycholecalciferol as in Figure 4, but with vitamin D_3 added.

mented with 250,000 IU of D_3 daily for 10 days gave blood serum which assayed for 14 IU/ml of total vitamin D activity, consisting of 6 IU/ml due to vitamin D and 8 IU/ml due to peak IV. Finally, four hogs (210–240 lb) were maintained on the 250,000 IU of D_3 daily supplement for 26 days. Serial samples assayed for 20 IU/ml of serum total D activity, consisting of 8 IU/ml due to vitamin D and 12 IU/ml due to peak IV. The blood collected from these four hogs was treated with 1.6 l. of 0.1 M sodium oxalate, giving 6.8 l. plasma. Sufficient ammonium sulfate was added to the plasma to achieve 65–70% saturation. The precipitate was allowed to form at 4° for 3 days, collected by centrifugation for 25 min at 15,000 rpm in a Sharples AS-16-P centrifuge, and extracted with methanol chloroform. The total extract was made up to 50 ml with Skelly B. This extract assayed for a total of 100,000 IU of vitamin D activity by a rat line test assay (U. S. Pharmacopeia, 1955).

About 400,000 dpm of the radioactive extract from the 50-lb hog was combined with 20 ml of the Skelly B extract from the four hogs. This was applied to a silicic acid (25 g) adsorption column for chromatography (Lund and DeLuca, 1966). The column was eluted with an ether–Skelly B gradient, obtained by running 400 ml of 85% ether–Skelly B from a holding chamber into a 250-ml mixing chamber initially containing 250 ml of Skelly B. Following the collection of 36 11-ml fractions, 100% ether was placed in the holding chamber, and an additional 30 11-ml fractions were collected. Methanol was then applied directly to the column. Figure 1 shows the radioactivity profile of the column eluate, and the ether–Skelly B gradients obtained. It is seen that the originally described peak IV has been resolved into at least three components, of which the major one retains the designation peak IV. Peak IV and the minor preceding peak IVa are eluted by high concentrations of ether in Skelly B, whereas peak V is eluted by methanol. Occasionally, a small distinct peak appeared immediately after peak IV, and was labeled peak IVb. It is emphasized that the size of these peaks represents the amount of metabolites 24 hr after a single dose of [^{3}H]D_3 and has no reflection on the amount of metabolites present after 26 days of daily dosage of unlabeled vitamin D_3. Other chromatographic systems developed in this laboratory have also demonstrated the heterogeneity of peak IV, and these will be reported later, together with the biological activity data for the minor peaks (G. Ponchon and H. F. DeLuca, unpublished data). Fractions 51–60 from Figure 1 were combined and applied to a partition column, constructed as follows. Celite (20 g) was mixed with 15 ml of a stationary phase (80% methanol 20% water, equilibrated with an equal volume of Skelly B). About two-thirds of this was packed in a 1-cm diameter column. The sample was applied to the column in a small quantity of the mobile phase (the Skelly B equilibrated with methanol water) and the column eluted with mobile phase, with 5-ml fractions being collected. Figure 2 shows the radioactivity profile, and also the weight of the metabolite, as determined from its ultraviolet spectrum in ether (Figure 3).

The radioactive homogeneity of the peak material was further demonstrated on thin-layer chromatography

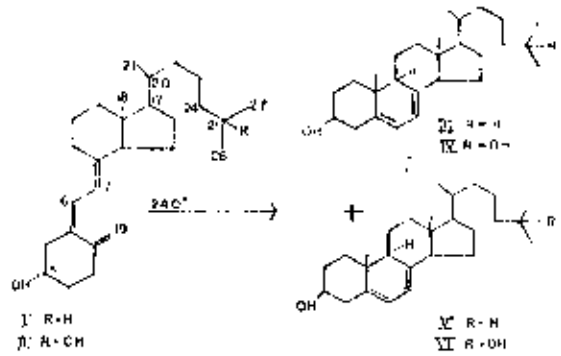

FIGURE 7: Structures for cholecalciferol (vitamin D_3) (I), 25-hydroxycholecalciferol (II), pyrocholecalciferol (V), isopyrocholecalciferol (III), and their respective 25-hydroxy derivatives, VI and IV.

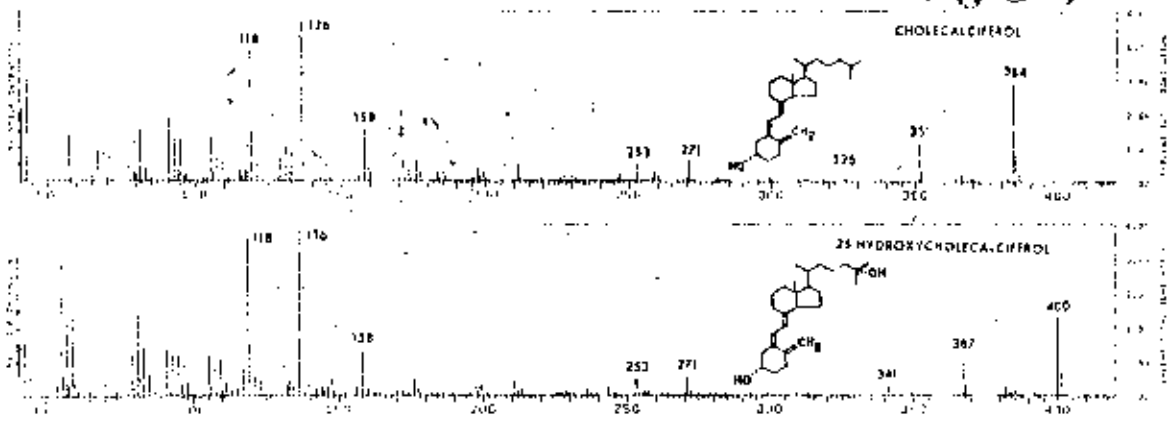

FIGURE 8: Mass spectra of cholecalciferol and 25-hydroxycholecalciferol.

using silica gel G as support and 4% methanol in chloroform as the developing solvent. Figure 4 shows the location of the radioactivity confined to a very narrow region. Further, visualization (with 50% H_2SO_4 followed by heating) of a plate to which 10 μg of metabolite was applied and developed with 6% methanol in chloroform displayed one spot only, R_F 0.64, compared with 25-hydroxycholesterol, R_F 0.54. Gas chromatography (see later) of the peak material indicates for it a high purity (Figure 5).

The remainder of the 50 ml of Skelly B extract was combined with a further quantity of radioactive extract and applied in two portions to silicic acid columns followed by partition chromatography of the peak IV as just described. In this manner, 1.3 mg of pure metabolite was obtained.

Identification of Peak IV as 25-Hydroxycholecalciferol. The peak IV material exhibited a ultraviolet absorption maximum in diethyl ether at 265 mμ (Figure 3) as for vitamin D. When subjected to gas–liquid partition chromatography, two peaks only appeared (see Figure 5). Vitamin D_3, when chromatographed under the same conditions, also gave rise to two peaks, representing pyro- and isopyrocholecalciferol (V and III) (see Figures 6 and 7), the products of thermal rearrangement of vitamin D_3. Collection of the peaks from the metabolite and from vitamin D_3 and measurement of their ultraviolet spectra showed the first peaks of each pair to have identical spectra (λ_{max} 274, 284, and 296 mμ). Similarly, the second peaks of each pair had identical spectra (λ_{max} 274, 284, and λ_{inf} 294 mμ).[1] Clearly then, the metabolite has a similar structure to that of vitamin D_3, particularly in the region of the triene system. Accordingly, an extinction coefficient of 18,200, as for vitamin D, is assumed for the ultraviolet absorption maximum of the metabolite. The high-resolution mass spectra of the vitamin and its metabolite provided information on the difference between the two compounds (Figure 8). The molecular weight of the metabolite was 400.3343 ($C_{27}H_{44}O_2$), *i.e.*, a cholecalciferdiol. The presence of a peak at *m/e* 271.2053 ($C_{19}H_{27}O$) in the spectra of both compounds indicated the location of the second hydroxyl group of the metabolite on the side chain, since the fragment *m/e* 271 arises by loss of side chain through cleavage of the C_{17-20} bond. Further, a peak at *m/e* 59.0492 (C_3H_7O) in the spectrum of the metabolite, but not in the spectrum of the vitamin, could arise by cleavage of the C_{21-25} bond, with the hydroxyl group attached to C_{25}. A detailed study of the mass spectra of these, and other related compounds, will be the subject of a forthcoming publication. The location of the hydroxyl group at C_{25} was verified by the 100-Mcps nuclear magnetic resonance spectrum of the metabolite (Figure 9), which exhibited a strong singlet peak at δ = 1.20 ppm, as in 25-hydroxycholesterol (Figure 10), and an absence of the doublet at δ = 0.87 ppm (J = 6.5 cps) due to the secondary $C_{26,27}$-methyl groups as found in cholecalciferol (Figure 11). The peaks at δ = 0.58 ppm are due to the tetramethylsilane internal standard. The C_{18}-H_3 resonance is seen at δ = 0.54 ppm, and the doublet due to the C_{21}-H_3 group is at δ = 0.93 ppm (J = 5 cps) for both the metabolite and the vitamin. Further, the downfield regions for the vitamin and the metabolite are shown in Figure 12, and are seen to be identical. The peaks at δ = 4.81 and 5.03 ppm are from

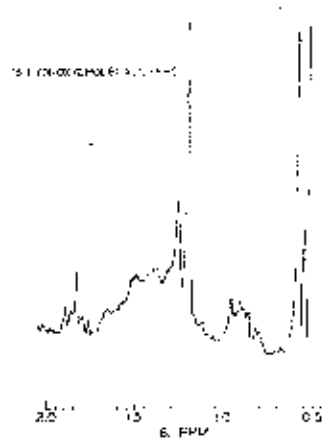

FIGURE 9: Nuclear magnetic resonance spectra (at 100 Mcps) of 25-hydroxycholecalciferol.

[1] These data are identical with those supplied by Professor E. R. H. Jones in a personal communication, but differ from data reported elsewhere (Fieser and Fieser, 1959).

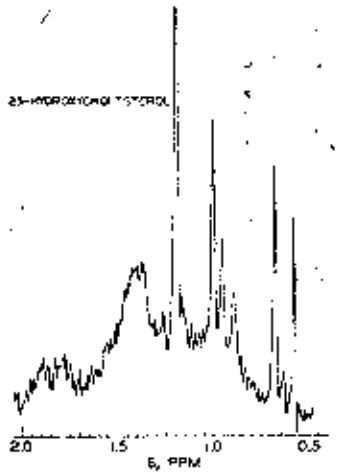

FIGURE 10: Nuclear magnetic resonance spectra (at 100 Mcps) of 25-hydroxycholesterol.

FIGURE 11: Nuclear magnetic resonance spectra (at 100 Mcps) of cholecalciferol.

the $C_{19}H_2$ group, and the doublets at $\delta = 6.02$ ppm ($J = 11.5$ cps), and $\delta = 6.24$ ppm ($J = 10.5$ cps) are due to the protons at the 6 and 7 positions.

Thus, a complete identification of the major metabolite as 25-hydroxycholecalciferol has been made.

Biological Activity of 25-Hydroxycholecalciferol. Upon completion of its isolation and identification, the 25-hydroxycholecalciferol was dissolved in Wesson oil and bioassayed in rats for vitamin D activity by the U. S. Pharmacopeia line-test method (1955). Several independent assays were carried out at the Wisconsin Alumni Research Foundation and in our own laboratory. The samples were made up according to their ultraviolet absorbance at 265 mμ assuming an extinction of 18,200. Routinely the 25-hydroxycholecalciferol gave a value of 56–60 IU/μg or 1.4 times that of vitamin D_3.

Discussion

A number of metabolites of vitamin D are now known to exist (DeLuca, 1967). In his early work, Kodicek reported the existence of breakdown products of vitamin D but could find no biologically active metabolites (Kodicek, 1956, 1958). In this laboratory, at least three biologically active metabolites and a number of inactive metabolites were unequivocally demonstrated (Lund and DeLuca, 1966). One of these proved to be an ester of long-chain fatty acids with the 3-OH of the vitamin D_3 (Lund *et al.*, 1967). Fraser and Kodicek (1968) have studied the ester fraction and shown that palmitic, stearic, oleic, and linoleic are the predominant fatty acids found in this combination. However, exactly what is the physiologic importance of the ester fraction remains unknown inasmuch as its concentration remains low regardless of vitamin D dosage. Up to the present time all other metabolites of the vitamin remained unidentified although a sulfate ester of vitamin D has also been reported (Higaki *et al.*, 1965). In addition the existence of glucuronides of vitamin D and its metabolites have been reported but have not been positively identified as yet (Avioli *et al.*, 1967).

This report demonstrates unequivocally the existence of 25-hydroxycholecalciferol as a biologically active metabolite of vitamin D_3. First, the triene structure of the parent vitamin was shown in the metabolite by its ultraviolet spectrum which was identical with that of vitamin D_3. This structure was supported by the generation of pyro and isopyro forms (VI and IV) on gas liquid partition chromatography of the metabolite and by the downfield region of the nuclear magnetic resonance spectrum. The mass spectrum demonstrated a molecular weight of 400, or a calciferdiol. Fragmentation analysis clearly showed the extra oxygen in the side chain, probably at the 25 position. Unequivocal proof for 25-hydroxycholecalciferol was provided by its nuclear magnetic resonance spectrum, in which the doublet due to the 26,27-methyl groups coupled with the 25-H was shifted to a singlet corresponding to a substitution of OH for H at the 25 position.

The biological significance of the 25-hydroxycholecalciferol is still under study; however, there is a strong possibility that it represents the metabolically active form of vitamin D. A complete study of its biological activities is currently nearing completion and will be the subject of a forthcoming report. It is nevertheless clear that on a weight basis the 25-hydroxy compound is more effective in curing rickets in rats than is the un-

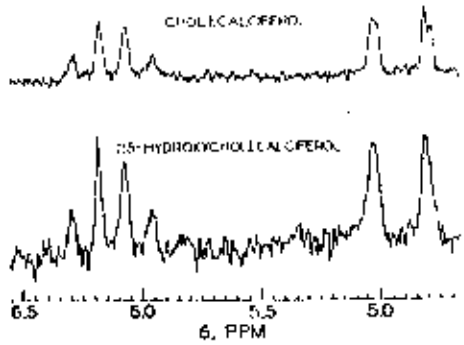

FIGURE 12: Nuclear magnetic resonance spectra (at 100 Mcps) of cholecalciferol and 25-hydroxycholecalciferol.

altered vitamin. In previous work it was shown that the peak IV metabolites from rats mimicked the action of vitamin D in raising serum calcium (bone mobilization) and in increasing intestinal transport of calcium (Morii *et al.*, 1967). Furthermore, the metabolite acted more rapidly than did vitamin D itself. The 25-hydroxycholecalciferol represents the biologically active component of the peak IV metabolite fraction previously used and although not tested directly as yet, it seems almost certain to possess these properties. Other experiments have shown that the peak IV metabolites appear in the target tissues before any biochemical changes due to vitamin D are known to occur and that after truly physiological doses of the vitamin, the peak IV fraction is the predominant form of the vitamin in the body. Finally, the major if not sole form of vitamin D in at least one of the believed subcellular sites of vitamin D action is in the peak IV fraction. Although the evidence for the 25-hydroxycholecalciferol as the metabolically active form of the vitamin is highly suggestive, final proof of such a role must rest with the isolation of the biochemical system in which it functions.

Of greatest importance to the successful isolation was the achievement of high levels of metabolite in the plasma of the pigs. Clearly, large single doses of vitamin D_3 were not sufficient. However, chronic doses of large amounts of vitamin D resulted in metabolite activities as high as 12 IU/ml of plasma. Modification of the silicic acid chromatography to extend the diethyl ether gradient also resulted in unexpected purification over that achieved with all other solvent systems attempted. Finally, the partition column developed for the metabolite effected a high degree of purification resulting in essentially pure material. Purity was established by gas liquid partition chromatography, by thin-layer chromatography, by ultraviolet spectra, and by nuclear magnetic resonance data.

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Regulation by Calcium of *In Vivo* Synthesis of 1,25-Dihydroxycholecalciferol and 21,25-Dihydroxycholecalciferol

0705

(rat/vitamin D/dietary Ca/intestine/serum/kidney)

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ABSTRACT **Tritiated 1,25-dihydroxycholecalciferol accumulates in several tissues, to an extent that varies with dietary calcium content. 12 hr after the administration of 325 pmoles of tritiated 25-hydroxycholecalciferol to rats, as the dietary and serum calcium concentrations increase, the amount of 1,25-dihydroxycholecalciferol is diminished and the concentration of 21,25-dihydroxycholecalciferol increases. This correlation is especially evident in rats given vitamin D_3. In vitamin D-deficient rats, the repression of 1,25-dihydroxycholecalciferol formation occurs with a diet containing 3% calcium and 20% lactose. The results suggest that the production of 1,25-dihydroxycholecalciferol, believed to be the metabolically active form of vitamin D in the intestine, is responsible for the adaptation of calcium absorption to low dietary concentrations of calcium.**

A metabolite of vitamin D believed to be the metabolically active form in the intestine has recently been isolated in pure form from intestine and unequivocally identified in this laboratory as 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) (1, 2). Simultaneously, Lawson *et al.* provided evidence for this structure with a partially purified material from kidney homogenates (3). This metabolite acts more rapidly than 25-$(OH)D_3$ to initiate intestinal calcium transport (4-6). The kidney is the site of synthesis of 1,25-$(OH)_2D_3$ from 25-$(OH)D_3$ (7); this observation was confirmed by Gray *et al.* (8). Other experiments have provided strong evidence that 1,25-$(OH)_2D_3$ is the metabolically active form of vitamin D in the intestine (9-11). It, therefore, seemed possible that the concentration of 1,25-$(OH)_2D_3$ in the serum and intestinal mucosa plays an important role in the adaptation of calcium absorption to the concentration of calcium in the diet (12).

This report demonstrates that dietary calcium concentration has a profound effect on the *in vivo* production of 1,25-$(OH)_2D_3$ and 21,25-$(OH)_2D_3$. Furthermore, these alterations in metabolite balance correlate with changes in serum calcium, but not serum phosphate concentration. An independent study has also shown that dietary strontium has an equally marked effect on the production of 1,25-$(OH)_2D_3$ and 21,25-$(OH)_2D_3$ from 25-$(OH)D_3$ (J. Omdahl and H. F. DeLuca, manuscript in preparation).

Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 21,25-$(OH)_2D_3$, 21,25-dihydroxycholecalciferol; 25-$(OH)D_3$, 25-hydroxycholecalciferol.

* To whom all inquiries should be addressed. I. T. B. is a Richard Hartenstein fellow and recipient of a Wellcome Research Travel Grant.

MATERIALS AND METHODS

Preparation of rats and diets

Weanling, male albino rats were obtained from the Holtzman Co., Madison, Wis., and were housed individually in hanging wire cages. They were given food and water *ad libitum*. The diet provided was the vitamin D-deficient diet described by Suda *et al.* (13) containing 0.47% Ca and 0.30% P.

The calcium content was varied by addition or deletion of $CaCO_3$, with compensatory changes in the sugar (glucose monohydrate) content. Similarly, where indicated, lactose was added at the expense of the glucose. Diets containing Ca at 0.02%, 0.20%, 0.47%, 2.0%, and 3.0%—and 3.0% Ca plus 20% lactose—were used. Rats were fed the diets for 3 weeks and were then killed to obtain tissue samples.

A daily oral dose of 1 unit of vitamin D_3 in 0.05 ml of cottonseed oil–soybean oil (Wesson) was given to half the animals after 1 week on their vitamin D-deficient diet and was continued for the remaining 2 weeks.

Administration of [^{3}H]25-$(OH)D_3$ and preparation of extracts

325 pmoles of tritiated 25-$(OH)D_3$ was administered intrajugularly in 0.05 ml of 95% ethanol with mild ether anesthesia. The rats were fasted at this time, but water continued to be available *ad libitum*. 12 hr later the rats were killed by decapitation, and blood serum was collected. The upper 50 cm of small intestine was quickly removed, flushed with ice-cold saline, slit open, and the mucosa was scraped off with a glass slide. The kidneys and livers were removed. The fore- and hind-limbs were stripped of muscle, the long bones were split, and the marrow was discarded. All tissues were stored at −16°C until they were extracted with chloroform and methanol (14). In early experiments, the tissue from 3 to 4 animals fed the same diet was combined before extraction, while in later experiments the serum calcium concentrations of the rats were analyzed for similarity before pooling of tissues. In other cases, the tissues from each animal were analyzed individually.

Radioactive metabolites were separated on a 2 × 15 cm column containing 10 g of Sephadex LH-20 equilibrated with 35% Skellysolve B (petroleum ether, b.p. 67–68°C) in 65% chloroform (15). The tissue extracts were applied to the column in 1 ml of the same solvent mixture and eluted with a further 190 ml. Recovery of radioactivity from the columns varied from 90 to 105%.

The elution position of 1,25-$(OH)_2D_3$ on this column has been established (2, 8). The less-polar metabolite was identified as 21,25-$(OH)_2D_3$ by cochromatography. A sample of the

TABLE 1. *Regulation by calcium of the metabolism in vivo of [^{3}H]25-(OH)D$_3$ to polar metabolites*

	Diet calcium (%)	No. of animals	Average weight (g)	Average serum calcium (mg/100 ml) Before 25-(OH)D$_3$	12 hr after 25-(OH)D$_3$	% Recovered ^{3}H in serum 1,25-(OH)$_2$D$_3$	24,25-(OH)
Vitamin D-deficient	0.02	4	87	3.8	5.2	15.8	1.
	0.02	4	83	(3.0)*	5.5	16.5	0.
	0.47	3	147	(5.2)	7.5	18.0	0.
	2.0	4	153	(8.3)	8.7	14.0	0.
	3.0	3	103	9.1	9.3	15.6	1.
	3.0 + 20% lactose	1	145	9.6	9.4	3.1	7
	3.0 + 20% lactose	1	112	9.8	9.4	1.0	7
	3.0 + 20% lactose	1	115	9.6	9.8	0.7	10.
1 Unit of vitamin D daily	0.02	4	97	(4.5)	5.4	14.0	1.
	0.02	4	101	4.4	5.2	14.5	1.
	0.3	3	165	7.0	8.7	15.7	1.
	0.17	4	187	9.3	9.6	5.0	5
	0.47	4	167	(9.2)	9.5	2.5	5.
	2.0	3	141	...	9.7	0.8	10.
	3.0 + 20% lactose	1	120	9.8	8.9	1.1	11.
	3.0 + 20% lactose	1	110	9.9	8.6	1.5	1.

* Figures in brackets are representative serum calcium concentrations of animals on identical diets from another series of experime

metabolite was obtained by column chromatography on Sephadex LH-20 of a kidney extract obtained from rats fed the 3% calcium-20% lactose plus vitamin D diet (see Fig. 2A). The rats had received an injection of [^{3}H]25-(OH)D$_3$ 12 hr before they were killed. Authentic [^{3}H]24,25-(OH)$_2$D$_3$, isolated and identified as described (16), was mixed with this metabolite and the mixture was chromatographed on a Celite liquid-liquid partition column specifically designed for 24,25-(OH)$_2$D$_3$ isolation and capable of completely resolving the polar metabolites of 25-(OH)D$_3$ (ref. 16; see Fig. 2B).

Chemicals

Crystalline vitamin D$_3$ was a gift of the N. V. Philips-Duphar Co., Weesp, The Netherlands. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. [26,27-^{3}H]25-(OH)D$_3$ was synthesized by the method of Suda *et al.* (17) and had a specific activity of 1.3 Ci/mmol.

Analytical procedures

Radioactivity was measured with a Packard Tri-Carb liquid-scintillation spectrometer, model 3375, equipped with automatic external standardization (Packard Instrument Co., LaGrange, Ill.).

In all but the first group of experiments, a small sample of blood was obtained from the tail vein of each animal just before it was injected with [^{3}H]25-(OH)D$_3$. This, as well as a sample of the blood obtained at the end of the experiment, was analyzed for calcium, in the presence of 0.1% LaCl$_3$, by atomic absorption spectrometry.

Serum inorganic phosphorus was measured with a Technicon autoanalyzer by the method of Fiske and Subbarow (18).

RESULTS

Fig. 1 shows the profiles of radioactive metabolites separated on Sephadex LH-20 from serum extracts obtained from rats fed the various diets, with and without vitamin D. As expected, the daily supplement of one unit of vitamin D failed to prevent vitamin D deficiency (Table 1); this low dose was chosen to minimize dilution of radioactive metabolites and to provide a small amount of 25-(OH)D$_3$ as a possible inducer for the 24-hydroxylase enzyme in the kidney (9, 10). The comparat

TABLE 2. *Appearance of polar metabolites of [^{3}H]25-(OH)D$_3$ in various tissues as a function of dietary calcium concentratio*

		Vitamin D-deficient 0.02% Ca	0.47% Ca	2.0% Ca	Vitamin D-supplemented 0.02% Ca	0.47% Ca	2.0%
Intestinal mucosa	1,25-(OH)$_2$D$_3$	48	60	64	46	8	8
	24,25-(OH)$_2$D$_3$	2	3	2	3	5	8
Kidney	1,25-(OH)$_2$D$_3$	29	32	28	30	6	3
	24,25-(OH)$_2$D$_3$	1	1	1	1	14	14
Bone	1,25-(OH)$_2$D$_3$	21	25	20	25	5	2
	24,25-(OH)$_2$D$_3$	1	1	1	2	7	8
Serum	1,25-(OH)$_2$D$_3$	17	18	14	17	3	1
	24,25-(OH)$_2$D$_3$	1	1	1	1	9	10

The values represent the percentage of the total radioactivity recovered as the indicated metabolite. The remaining radioactivity in each case was [^{3}H]25-(OH)D$_3$.

weights of the vitamin D-deficient and vitamin D-supplemented animals (Table 1), however, demonstrate a growth effect of the vitamin even at this low concentration in the diet.

In serial experiments with the vitamin D-deficient rats fed diets with 0.02–2.0% Ca, 14–18% of the total radioactivity in the serum was consistently recovered as 1,25-$(OH)_2D_3$. However, in the rats given vitamin D, the recovered radioactivity as 1,25-$(OH)_2D_3$ was 17% with 0.02% dietary calcium; this figure decreased to 2.5% with 0.47% dietary calcium and to 0.8% with 2.0% dietary calcium. It was of great interest that another metabolite, 21,25-$(OH)_2D_3$, appeared in rats fed the vitamin D-supplemented diets. This less polar metabolite was identified by cochromatography on the liquid-liquid partition column (Fig. 2*B*).

In rats given vitamin D, the suppression by dietary calcium of 1,25-$(OH)_2D_3$ production correlates with an increase in serum calcium concentration, whereas the suppression of 21,25-$(OH)_2D_3$ production correlates with a decrease in serum calcium content. With very high concentrations of dietary calcium (Table 1, 3% calcium plus lactose; ref. 19), the production of 1,25-$(OH)_2D_3$ is inhibited in vitamin D-deficient rats as well.

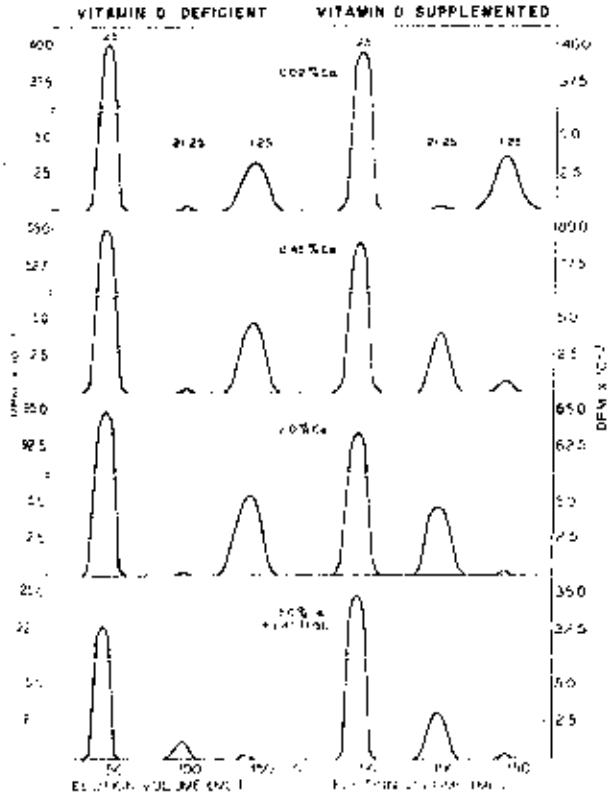

FIG. 1. Sephadex LH-20 chromatographic profiles of serum extracts from rats on diets with different calcium contents. Weanling rats were maintained on the indicated diets for 3 weeks. Half the animals on each diet were given 1 unit of vitamin D_3 each day for the last 2 weeks of the experiment. Rats were given 325 picomoles of [^{3}H]25-$(OH)D_3$ intrajugularly, 12 hr before they were killed. Serum was collected for chromatographic analysis from the different numbers of rats used in each group; this accounts for the apparent differences in total radioactivity shown on the ordinate. 25 = 25-$(OH)D_3$; 21,25 = 21,25-$(OH)_2D_3$; 1,25 = 1,25-$(OH)_2D_3$.

The relationship between 1,25-$(OH)_2D_3$ accumulation in serum and the serum calcium concentration appears to involve an abrupt rather than a gradual change. At serum calcium concentrations above 9.0–9.6 mg/100 ml, production of 1,25-$(OH)_2D_3$ is suppressed and 21,25-$(OH)_2D_3$ is enhanced, whereas below this range of concentrations, the synthesis of 1,25-$(OH)_2D_3$ appears to be unaffected by calcium concentration and the synthesis of 21,25-$(OH)_2D_3$ is suppressed. Similar correlations of metabolite patterns with the serum calcium concentration were found in the other tissues analyzed (Table 2).

It also is apparent (Table 1) that when the initial calcium concentration of the serum is low, a distinct rise of from 1 to 2 mg of calcium per 100 ml of serum occurs in response to the 325-pmole dose of 25-$(OH)_2D_3$, whereas when the initial serum calcium concentration is normal, no further rise occurs.

Finally, there is no correlation between inorganic P concentrations of serum and production of the metabolites (Table 3). These results suggest that it is calcium *per se*, or possibly the hormones that control the serum calcium concentration, that regulates the synthesis of 1,25-$(OH)_2D_3$ and 21,25-$(OH)_2D_3$.

DISCUSSION

Our results demonstrate a clear relationship between the amount of 1,25-$(OH)_2D_3$ appearing in tissues after the injection of [^{3}H]25-$(OH)D_3$ and the serum calcium concentra-

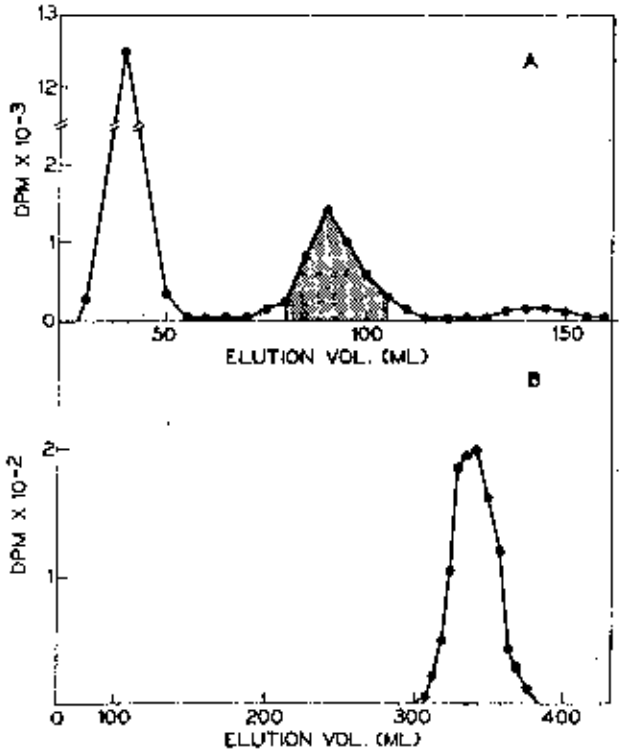

FIG. 2. *A*. Sephadex LH-20 chromatographic profile of kidney extracts from rats on a diet of 3% calcium plus lactose for 3 weeks. The rats were given 1 unit of vitamin D_3 daily for the last 2 weeks. The fractions in the *shaded area* were pooled for cochromatography, described below in *B*.

B. Liquid-liquid partition column cochromatography of 1,000 dpm of the metabolite isolated in *A* (*above*) together with 600 dpm of pure 21,25-$(OH)_2D_3$ isolated by Suda *et al.* (16). The chromatographic system used is that described by the same investigators.

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TABLE 3. *Lack of correlation between the concentration of inorganic phosphorus in serum and the production of 1,25-$(OH)_2D_3$ and 21,25-$(OH)_2D_3$ from [^{3}H]25-$(OH)D_3$*

Diet	Serum calcium (mg/100 ml)	Serum P_i (mg/100 ml)	% Radioactivity recovered from serum as 1,25-$(OH)_2D_3$	21,25-$(OH)_2D_3$
3% Ca	9.1	3.7	15.6	1.0
0.45% Ca + D	9.5	9.8	2.5	8.6
0.3% Ca + D	7.5	10.5	15.7	1.0
0.02% Ca + D	4.4	9.9	14.5	1.4

tion at the time of injection. In young rats, the synthesis of 1,25-$(OH)_2D_3$ is maximal in hypocalcemia and is almost completely repressed in the presence of normal or high concentrations of serum calcium. This relationship is evident in the presence or absence of vitamin D. Coincident with the repression of 1,25-$(OH)_2D_3$ synthesis in normocalcemic animals is an equally dramatic appearance of 21,25-$(OH)_2D_3$.

Experimental work by Fairbanks and Mitchell (20), Rottensten (21), and Nicolaysen *et al.* (22) in the period from 1935 to 1943 established that young rats absorb calcium more efficiently after a period on a diet low in calcium concentration (0.15–0.2%) than after a similar period on a diet high in calcium concentration (0.8–1.25%). Calcium absorption was measured by these authors either as a difference in carcass calcium content and/or by calcium-balance studies. Serum calcium concentrations were not reported.

Nicolaysen *et al.* proposed that the adaptation mechanism is regulated by some endogenous factor (22). This factor or factors was shown to be vitamin D-dependent and to influence the velocity with which calcium passes through the mucosa of isolated intestinal loops. The calcium-adaptation phenomenon is a feature of young rather than old rats; neither sexual maturity nor the presence of the thymus gland are important (22).

Kimberg *et al.* (23), using an everted gut-sac technique as a measure of calcium absorption, confirmed Nicolaysen's observations regarding the vitamin D-dependence and expanded them to show that the adaptation mechanism remains after removal of the pituitary, thyroid, parathyroid, or adrenal glands. Our data strongly suggest that the serum calcium concentration, or factors that correlate with the serum calcium concentration—such as parathyroid hormone or calcitonin concentration—have a controlling influence on the rate of synthesis *in vivo* and on the tissue concentrations of 1,25-$(OH)_2D_3$.

In view of the strong evidence that 1,25-$(OH)_2D_3$ is the metabolically active form of vitamin D in the intestine (9–11), and that its concentration is regulated by the dietary calcium concentration, we suggest that 1,25-$(OH)_2D_3$ is the "endogenous factor" of Nicolaysen *et al.* (22). The presence of 1,25-$(OH)_2D_3$ is obviously vitamin D-dependent and even slight hypocalcemia stimulates its synthesis. Slight hypocalcemia undoubtedly occurs in the young growing animals that were fed a diet low in calcium concentration, and the hypocalcemia probably causes increased 1,25-$(OH)_2D_3$ synthesis. Calciferol 901 also may remove calcium from the blood, causing slight hypocalcemia. This tendency to slight hypocalcemia and, hence, 1,25-$(OH)_2D_3$ production would persist until the [illegible] are fully calcified or until adequate calcium intake could [illegible] come these needs. Thus, 1,25-$(OH)_2D_3$ meets the criter[illegible] Nicolaysen *et al.* (22) for the endogenous factor.

There is a strong possibility that the concentration of [illegible] factors, such as parathyroid hormone or calcitonin, [illegible] regulate serum calcium concentration, may be the [illegible] regulators of 1,25-$(OH)_2D_3$ synthesis. In this regard, the [illegible] vation of Kimberg *et al.* (23) that thyroparathyroide[illegible] does not abolish the adaptation of calcium absorp[illegible] dietary calcium concentration may be relevant. Ho[illegible] more direct studies on the interplay of these hormone[illegible] 1,25-$(OH)_2D_3$ synthesis are in progress.

The 21,25-$(OH)_2D_3$ seen in the normocalcemic animal[illegible] not be involved directly in the adaptation mechanis[illegible] notable that this metabolite activates bone mineral re[illegible] tion and has slight activity in intestinal calcium tr[illegible] (16). That the kidney has the highest concentration [illegible] $(OH)_2D_3$ suggests a renal function for this compound [illegible] 2). For example, it may play a protective role by pre[illegible] hypercalcemia by decreasing the tubular reabsorption [illegible] calcium, or it may be involved in tubular phosphate re[illegible] tion.

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FAT-SOLUBLE VITAMINS[1,2]

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Vitamin A

Determination.—Spectrophotometric, colorimetric, or biological methods are used for the determination of vitamin A (1). In the two former methods, a preliminary saponification is generally required and often chromatographic purification.

If the vitamin is present exclusively in the form of all-*trans* vitamin A, it is possible as well to determine the vitamin after conversion to anhydrovitamin A (2). However, natural sources of vitamin A generally contain several different vitamin-A isomers; moreover, isomerisation has been found to occur in products prepared from the all-*trans* vitamin (3). Since the conversion of the different vitamin-A isomers to anhydrovitamin does not give the same increase in extinction, it will be possible only in exceptional cases to apply the anhydro method without a preceding separation of the isomers (4); however, it may be used in connection with chromatographic separation for the identification of the isomers in the collected fractions (5). The transient blue color resulting from the reaction between vitamin A and antimony trichloride, which is widely used for colorimetric determination, is followed, when the antimony trichloride concentration is low, by a more stable red color, which can also be used for determination purposes (6). Chlorhydrins also produce colored reaction products with vitamin A; the reaction with glycerol chlorhydrin is the most sensitive, although those with 1-2- and 1-3-propylene chlorhydrin are most convenient because the colors produced are more stable and these obey Lambert-Beer's law (7). A colorimetric method based on the reaction with phosphotungstic acid may be mentioned (8). Also the colorimetric assay of vitamin-A preparation is complicated by the presence of isomers, since the different isomers differ in biological potency. The relative biopotency of different products may be determined, however, on the basis of the antimony trichloride method combined with maleic anhydride treatment (9).

Adsorption chromatography is generally used for chromatographic isolation of vitamin A, since it is convenient both for routine separation from other substances (10) and for the more complicated isolation of the different isomers (5). In routine separation, the losses of vitamin A are small; for instance, when using weakened alumina, the losses are of a magnitude of 2 to 3 per cent (11). Incidentally, it seems to be possible to reduce the losses by the

[1] The survey of the literature pertaining to this review was concluded in September, 1960.

[2] The following abbreviations are used: $NADH_2$ for nicotinamide-adenine dinucleotide, reduced form; FAD for flavin-adenine dinucleotide.

addition of arachis oil, which, because of its content of tocopherol, has an antioxidative action (12). Partition chromatography may also be used for the purification of vitamin A (13, 14), but this method is probably more widely used for the determination of other fat-soluble vitamins.

Particular difficulties are encountered in the determination of vitamin A in vitaminized fodder mixtures, firstly because vitamin A is generally added in small quantities and, therefore, is difficult to isolate in sufficiently pure condition, and secondly because coated vitamin-A preparations are used in which the individual particle contains such a large amount of vitamin that only a few particles will be present in the sample drawn for the analysis. Since proteins are generally used for the coating, methods that begin by extraction with light petroleum (15) cannot be used. The whole fodder sample must be subjected to saponification to ensure complete extraction (16). Collaborative examinations by this method have shown low reproducibility (17), which, statistically, has been accounted for by the above-mentioned difficulty of distribution of the coated particles in the fodder (18). The remedy must be to use larger analytical samples (19). The magnitude of the sample required is still open to discussion (19, 20).

Collaborative examinations have also shown lack of reproducibility in margarine (21). In some countries, sesame oil is added to margarine to serve as a tracer. It has been found that separation of vitamin A from the sesamin and sesamolin of sesame oil requires a special chromatographic technique (22).

The presence in various natural products of vitamin-A aldehydes, which have biological potency, calls for a method (23, 24, 25). The determination, however, is complicated by the impossibility of applying saponification, and results hitherto do, in fact, show some discrepancies. This may in part be explained by an unwanted reaction between vitamin-A aldehyde and acetone when the latter substance is used for the extraction (26).

The recognition of vitamin-A acid as a substance active in the organism (27) also calls for an analytical method. With antimony trichloride, vitamin-A acid produces an absorption band at 373 mμ, but no description of the isolation and separation of this substance from other substances that react with antimony trichloride has apparently been published as yet.

Utilization.—A number of investigations on the extent to which the utilization of vitamin A depends on the route of administration seem, on the whole, to show agreement. De Man *et al.* (28) investigated the utilization of large doses and used the amount stored in the liver as the criterion. With oral administration the utilization was better in aqueous dispersions than in oily solutions. The utilization of vitamin A in aqueous dispersions was equally good with oral and intramuscular administration and somewhat better with intravenous administration. Only a small amount from an oily solution injected intramuscularly is stored in the liver. Christensen *et al.* investigated the value of large doses for the storage in the liver (29), as well as the clinical effect of smaller doses (30), and found results that agreed with those just

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mentioned, except that the clinical effect is asserted to be the same in case of intramuscular and oral administration of oily solutions of vitamin A. The explanation must be that the amount of vitamin A accumulated at the site of injection is able to serve as a vitamin depot. Kring & Lund (31) have made the same observation, but they mention that the administration of a few large doses is under all circumstances less economical than smaller, daily doses, because vitamin-A destruction increases with the magnitude of the deposits. However, when the object is to transfer vitamin A from mother to offspring, intramuscular or subcutaneous administration of an oily solution is unsuitable (32). Sobel *et al.* (33) investigated the absorption of vitamin through the skin and found the utilization to be considerably lower than in the case of oral administration. When vitamin A was administered in this way, maximum utilization was obtained from aqueous emulsions.

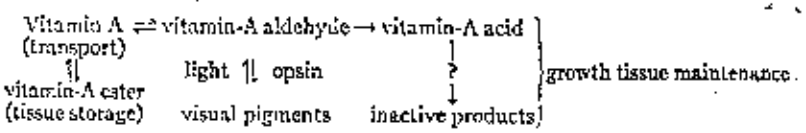
When vitamin A is mixed in feedstuffs, utilization will likewise depend on the type of vitamin preparation used. In this connection, interest has centered on the utilization of coated products, and most investigations are based on measurements of the amount stored in the liver following the administration of a large dose (34 to 38). These investigators (34 to 38) agree that in the animals examined (rats and chickens) the utilization of vitamin A in gelatin-coated products and in aqueous dispersions was equally good and considerably better than in oily solutions or products coated with fat. The composition of the feedstuff, other than the vitamin-A content, also influences the utilization. Alfalfa, for instance, contains one or more factors that improve vitamin-A absorption, and some of these factors may be obtained by aqueous extraction (39). Also the contents of vitamin E (40) and of protein (41) in the feedstuffs have a certain influence on the utilization.

The conversion of provitamins into vitamin A, which in case of oral administration normally takes place in the intestinal wall, can presumably take place in most organs and tissues (42), although this does not apply to all species of animals (42, 43). It has been possible *in vitro* to demonstrate the conversion of carotene in duodenum and in liver homogenate (44). Carotene, vitamin-A ester, β-ionone, and vitamin-A aldehyde were isolated in corresponding experiments with intestine homogenates (45). The presence of the aldehyde agrees with the scheme of Glover *et al.* for carotene conversion (46). The presence of β-apo-8′-carotenal in nature may also be mentioned as evidence of this course of the reaction, but the lower biological potency of this substance and of β-apo-8′-carotenic acid as compared with β-carotene suggests that conversion of the β-carotene molecule into two vitamin-A molecules may also take place (47). Like vitamin A, carotene is better utilized when administered in the form of aqueous dispersions than of oily solutions (49). When carotene is administered together with its natural carrier, vegetable material, the utilization will depend not only on the nature of the vegetable (50) but also on the processing of the latter. Silaging results in a considerable decrease in carotene utilization (51), which may possibly be accounted for by the destruction of the soluble carotene-protein complex

(52) in the vegetable (53). The protein content of the feedstuff also seems to influence carotene utilization (54).

When considering that the ability in animals to utilize carotene varies from species to species, it is understandable that the Vitamin Subcommission of the International Union of Pure and Applied Chemistry concludes a report on the expression of the potency of β-carotene in terms of vitamin A by recommending that this problem be referred to the World Health Organization (55).

Physiological function.—Lack of vitamin A produces a number of deficiency symptoms, whose interdependency and relation to the function of the vitamin are as yet not very well-known. The action of the vitamin in the organism depends on at least two properties of the vitamin which are to some extent mutually independent. Dowling & Wald (27) confirmed the observation made by Van Dorp & Arens (56) that vitamin-A acid is able to maintain growth in rats that are fed a diet devoid of vitamin A. At the same time, they showed that vitamin-A acid is unable to ensure the formation of the visual pigments. On the basis of this, they suggest the correlation shown in the reaction between the vitamin-A derivatives and their metabolic functions:



Vitamin-A acid cannot be converted into vitamin-A aldehyde in the organism and, thus, cannot take part in the formation of the visual pigments nor result in storage of vitamin A in the liver. It is remarkable that the depletion of the vitamin store in rats fed a vitamin A-free diet is quite independent of whether or not vitamin-A acid is given. This suggests that the amount of vitamin A metabolized is independent of the extent of its function in the organism; were this not the case, there could be no agreement with the above reaction scheme or with other observations of vitamin-A losses in the organism (57). Even when very large doses were given, Dowling & Wald were unable to demonstrate the presence of vitamin-A acid in the tissues [cf. also Sharman's investigations (58)]. Following oral administration of vitamin-A acid, Sharman was unable to demonstrate the presence of this substance in the intestinal wall, but he observed an increase in the content of substances with lower absorption maxima. This seems to indicate that it is not vitamin-A acid but rather a derivative that is active in the organism. A question that now presents itself is whether it is possible to ascertain the presence of this substance in the organism in any considerable concentration.

The ability of vitamin-A acid to maintain growth in rats on a vitamin A-deficient diet made it possible for Dowling & Wald to investigate night blindness in extreme stages. They had ascertained previously that a linear relationship existed between the content of rhodopsin in the eye and the log-visual threshold (measured by electroretinography) (59). They then showed

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that this relationship applied until the rhodopsin content had decreased to practically nil. Hubbard *et al.* (60) examined the conversion of rhodopsin when subjected to light, and they have presented the following reaction scheme for vertebrates:

Rhodopsin (with 11-*cis* retinene) ⇌ lumirhodopsin (with all-*trans* retinene) → meta-rhodopsin → all-*trans* retinene + opsin

The foregoing applies to the visual pigment of the rods, but the bleaching of the pigment (iodopsin) isolated from the cones occurs in a corresponding manner (61). The conversion of rhodopsin into lumirhodopsin has further been examined by Yoshizawa & Kito (62). A brief survey of the known importance of vitamin A to vision has been given by Fisie (63).

It is possible to maintain normal vision in rats by means of much smaller doses of 11-*cis* vitamin A than of all-*trans* vitamin A, whereas, with respect to growth, the potency of 11-*cis* vitamin A is considerably less than that of all-*trans* vitamin A (64). In this connection, it may also be mentioned that astaxanthin (65) and the corresponding 3-hydroxy-4-keto-retinene (66) maintain growth and normal vision in the animals but cannot maintain normal reproduction in either female or male rats.

Dowling & Wald's investigations of night blindness showed that vitamin-A deficiency not only resulted in destruction of rhodopsin but also of opsin, and, consequently, in partial degeneration of retinal tissue. Mention is made of the possibility that vitamin A has a similar influence on other types of tissue (59). Later investigations have ascertained that the degeneration of retinal tissue is not prevented by ingestion of vitamin-A acid (27). Whether it is also impossible to replace vitamin A by vitamin-A acid in other tissues is still open to discussion. At present it appears that further investigation of the influence of vitamin-A acid and 3-hydroxy-4-keto-retinene on the different vitamin A-deficiency symptoms might throw new light on the function of vitamin A.

Wolf and co-workers (67, 68) found that the synthesis of glycogen from acetate, lactate, and glycerol was depressed in vitamin A-deficient rats. Ingestion of cortisone re-established the power of gluconeogenesis, whereas deoxycorticosterone was without influence. It thus appears that vitamin A takes part in the biosynthesis of the cortical hormones. *In vitro* experiments, however, seem to exclude the possibility of any direct influence of vitamin A on the synthesis, although van Dyke *et al.* (48) have recently found that the conversion of cholesterol into corticosterone in adrenal homogenate from vitamin A-deficient animals was stimulated by the addition of vitamin-A acid, vitamin-A aldehyde, and vitamin-A ester. Other investigations also confirm the association of vitamin A with the steroid hormones (69, 70). Administration of progesterone, but not of pregnenolone, was able to ensure normal oestrus in vitamin A-deficient animals and resulted in an increase in the number of living newborn animals. In male rats, administration of progesterone delayed the development of vitamin A-deficiency symptoms (71). It is obvious that vitamin A influences the conversion of pregnenolone into progesterone and, presumably, several links in the biosynthesis of the steroid hormones and, consequently, the establishment of a certain balance between the latter (73). Vitamin A also seems to have a direct influence on one or more of the stages of the synthesis of cholesterol from squalene (72). However, in rats that were fed large doses of vitamin A, there was an increase in the weight of the adrenals and an increased excretion of 17-keto-steroids with the urine (74). Other investigations have also dealt with the relationship between cholesterol and vitamin A. In male, but not in female, rats it was possible to observe a reduction in the storage of vitamin A in the liver when cholesterol was given together with vitamin A, but administration of cholesterol had no influence on the vitamin A already stored in the liver (75). In humans with increased cholesterol content in the blood, a reduction has been found to result from ingestion of large quantities of vitamin A (76). It is difficult to correlate these observations with the influence of vitamin A on the cholesterol synthesis. In a great number of publications, Morton and co-workers have accounted for the significance of vitamin A to the occurrence of substance SC and substance SA in animal organs. These investigations resulted in the discovery of ubiquinone (SA) (77) and ubichromenol (SC) (78). In vitamin-A deficiency, the content of these substances in various organs will be increased. It is possible that this is caused by the reduction in the steroid synthesis resulting from vitamin-A deficiency (72).

Lack of vitamin A results in reduced basal metabolism because of reduced thyroxine secretion (79), but the administration of large doses of vitamin A also results in a reduced basal metabolism (74, 79). Farmand (80) suggests that this effect is not caused by reduction in the function of the thyroid but by the increased turnover of thyroxine in the tissues.

Vignais (81) found in vitamin-A deficiency an increase in the activity of $NADPH_2$[3] cytochrome-*c* reductase in liver mitochondria to about twice the normal value, whereas in thyroidectomized rats the activity of this enzyme system is reduced. Further investigations are necessary to ascertain the connection between vitamin A and the thyroid hormone.

A thorough description of the influence of vitamin A on the development of tissues has been given by Fell (82). Vitamin-A deficiency manifests itself in the development of bones through disturbances in the function of the epiphyseal plate, which results in serious skeletal deformities. It was also possible to stop the development of the bones in *in vitro* experiments in which

[3] Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464–65 (November 5, 1960); *Science*, 132, 1548–60 (November 25, 1960)], nicotinamide-adenine dinucleotide, reduced form ($NADH_2$) has been substituted for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form ($NADPH_2$), for triphosphopyridine nucleotide, reduced form (TPNH).

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foetal bones were incubated with large quantities of vitamin A. The matrix of cartilaginous tissue contains mucopolysaccharides, i.e., chondroitin sulphuric acid, and it has been ascertained that the presence of large quantities of vitamin A disturbed the absorption of sulphate. In experiments with pigs, Trape *et al.* (83) found increased $S^{35}O_4$ absorption in vitamin-A deficiency. With an increase in the vitamin-A supply, the absorption of $S^{35}O_4$ decreased but again increased when very high vitamin-A doses were reached. When administering overdoses of vitamin A to rats, Berdjis (84) found an increase of the parathyroid glands with histological alterations which were explained as hyperplasia. The effect of subcutaneous administration of parathyroid extract on bone formation was similar to the consequences of hypervitaminosis A. Through *in vitro* experiments with tibia from newborn rats, it was shown, however, that the effect of parathyroid hormone was less pronounced and the cartilage was not affected, whereas vitamin A resulted in almost total resorption of terminal cartilaginous tissue.

Vitamin-A deficiency results in the formation of a keratin layer in epithelial tissue, as in normal epidermis (82), whereas as a result of hypervitaminosis A the normal cornification of vagina in rats during the oestrum will not take place (85), and the normal keratinization of skin from chicken foetuses can be prevented in *in vitro* experiments (82). Redfearn & Strangeways (86) found in normal epithelial tissue only —SH groups, but in hyperkeratinized tissue they found both —SH and —SS— groups, in agreement with the theory that keratinization is accompanied by an oxidation of cystine to cystine. Reduced cystine content in the skin of rats suffering from vitamin-A deficiency has also been observed (87). *In vitro* experiments with embryonic chicken skin by Pelc & Fell (87a) showed that vitamin A reduces the deposit of cystine and increases the DNA concentration throughout the tissue, but incorporation of tyrosine is reduced only in surface cells. In control experiments without vitamin A, keratinization was accompanied by reduced sulphate deposits in surface tissue. Incorporation of $S^{35}O_4$ into mucopolysaccharides in colon homogenates from vitamin A-deficient rats is decreased, but the ability for SO_4-uptake is restored by vitamin A *in vitro*. The effect of vitamin A is attributable to its influence on the synthesis of 3'-phospho-adenosine-5'-phosphosulfate (91a). In goats on a vitamin A-deficient diet, keratinization of kidney epithelium with calcium deposition was observed. It is stated (88) that vitamin-A deficiency is only a predisposing factor in the formation of urinary stones.

Perhaps the importance of vitamin A for the development of tissues manifests itself most drastically through the influence of this vitamin on the development of the foetus, the viability of the young, and congenital deformities in the latter (89, 90, 91). In the discussion of the physiological functions of vitamin A, the only deformities of interest are those that may be considered to be the specific results of vitamin-A deficiency and not merely the consequences of a general disturbance of the development of the foetus. However, by far the greatest number of such congenital deformities have

also been observed following diets deficient in other accessory food factors (91).

Vitamin D

Determination.—Chemical assay of vitamin D, which has high biological potency, is rendered difficult by the fact that it is frequently present in very low concentrations in the materials to be tested. It may be extremely difficult to isolate it in sufficiently pure condition from the large quantities of the other substances with which it occurs. In the case of products containing synthetic vitamin D, other substances formed by the irradiation will impede the determination. In this case, however, good agreement may be obtained between chromatographic-colorimetric and biological determinations (92). In the case of natural vitamin-D sources and multivitamin products, it is chiefly the presence of vitamin A that causes difficulties; this is particularly true because vitamin A will often be present in concentrations a hundred times, or more, that of the vitamin D. The presence of even a small quantity of vitamin A or of decomposition products of vitamin A in the final test solution will impede both spectrophotometric and colorimetric antimony trichloride determinations.

Vitamins A and D can be separated in several ways. By treatment with talc (magnesium polysilicate) activated by boiling with hydrochloric acid, it is possible to remove vitamin A and enable colorimetric determination (93). Chromatographic separation may be effected on superfiltrol (94), if required after a special activating process (95), on the condition that the weight ratio of vitamin A to vitamin D does not exceed 200. However, if the substances concerned are not pure synthetic vitamins, vitamin D will not be completely separated from interfering substances (95, 96), which may necessitate a correction of the colorimetric measurement. Separation may also be obtained by repeated chromatography, first on magnesium oxide-hyflo-supercel mixture and subsequently on alumina. In this case, however, a special blank determination is required to correct for the influence of interfering substances on the colorimetric measurement (the "inhibitor method") (97). Recovery values of 89 to 106 per cent of added vitamin D were obtained by applying this method to fortified evaporated milk (98).

The difference in the solubilities of vitamins A and D allows for separation by partition chromatography. Polyethylene powder with a butanol-methanol-water mixture as the mobile phase (99) or Celite impregnated with polyethylene glycol 600 with iso-octane as the mobile phase (100) are examples. Separation may also be made by modifications of the paper chromatographic method (101, 102). All in all, at the moment a combination of partition chromatography and adsorption chromatography may be considered to be the most convenient for the isolation of vitamin D. Even so, it is hardly possible in the case of low-potency vitamin-D preparations to purify the material sufficiently to permit direct spectrophotometric or

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nous tissue a substance has been found which is not present in corresponding tissue from rachitic rats, but which is formed when vitamin D is administered (131). The ability to mineralize cartilaginous tissue is only recovered, however, about two days after the administration of vitamin D (132). At the same time, the matrix gives off large quantities of a substance that shows an ability to bind calcium *in vitro*, possibly chondroitin sulphate. Reduced ability to synthetize glucosamine from glutamine and glucose-6-phosphate is found in cartilaginous tissue from rachitic rats (133). *In vivo*, however, a drop in the content of hexosamine in the cartilaginous tissue is observed when vitamin D is given to rachitic rats [compare the above observation with respect to the chondroitin sulphate given off by the matrix]. The difference in the epiphyseal cartilage in rachitic and non-rachitic rats may also be demonstrated by a special staining technique (134). It is reasonable to assume that the influence of vitamin D on the calcium absorption may be explained by the fact that the vitamin affects the ability of the intestinal tissue to absorb and transfer calcium.

The effect of vitamin D on bone is differential insofar as the addition of this vitamin impedes development of certain parts of the skeleton, while furthering the development of others without altering the relative contents of minerals (125). The content of alkaline phosphatase in the blood, which is increased in rachitis, is assumed to be of importance in the deposition of calcium and phosphate. However, Gran & Eeg Larsen (135) were unable to confirm the theory that vitamin D stimulates the activity of alkaline phosphatase. It had previously been ascertained that vitamin D increased the citrate concentration in certain tissues and in the urine and lessened the conversion of citric acid to α-keto-glutaric acid. In agreement with this, tibial cartilaginous tissue from rachitic rats on incubation with citrate showed reduction of the oxygen consumption and increase of the α-keto-glutarate concentration (136). The difference between rachitic and non-rachitic groups in this case was in the phosphate intake and not in the vitamin-D intake. On the whole, many experiments on rachitis have been based on rats fed an experimental diet which, for the purpose of producing a rachitic condition, was not only deficient in vitamin D, but also in calcium and phosphorus. Such experiments are not immediately relevant to the function of vitamin D. In epiphyseal cartilage from rachitic rats, Meyer *et al.* (137) found reduction not only of the citrate oxidation but also of the citrate synthesis. By incubation of palatal mucosa with succinate or malate, Zar & Irving (138) found an increase in the oxygen consumption after addition of phosphorylated vitamin D, i.e., a somewhat different influence on the citric acid cycle than that demonstrated in the case of cartilaginous tissue. Incidentally, it is not very probable that this influence is the result of direct action of vitamin D on the enzyme system in the citric acid cycle. By means of cortisol it is possible to prevent this action of vitamin D without influencing the anti-rachitic effect (139).

VITAMIN E

Chemistry, analysis, and occurrence.—Continued investigations of the natural tocopherols have shown that we should now add to the list of methyl-substituted tocols a new series of substances which differ from the tocol derivatives by having three double bonds in the C-2 side chain (140, 141). Two substances of this series have been found to date. Thus, natural ε-tocopherol, which may be isolated from wheat bran, is not, as hitherto assumed, identical with 5-methyl tocol but is an unsaturated analogue of β-tocopherol (140). ζ-Tocopherol, which occurs in palm oil and wheat bran, is similarly an unsaturated analogue of α-tocopherol (5,7,8-trimethyl tocol), whereas the ζ-tocopherol (ζ_2), which occurs in rice, is apparently, as originally assumed, identical with 5,7-dimethyl tocol (141). Final proof of the structure of these substances in the form of a total synthesis is still lacking.

The interest in paper-chromatographic methods of tocopherol analysis is still considerable. The Society for Analytical Chemistry has published a detailed description of such a method on the basis of the work performed by The Vitamin E Panel (142). The method is based on two-dimensional paper chromatography and includes such preliminary steps as extraction, saponification, separation of sterols by freezing from methanol solution, and purification on floridin earth. This seems to be the most convenient method for determination of the individual tocopherols, but the great number of precautions required would appear to render the method less suitable for routine quantitative analysis. This method, or modifications of it, has been used for the determination of tocopherols in margarine (143), oats (144), and animal tissue (145). Green (146) investigated the tocopherol pattern in young, growing plants of maize, wheat, barley, and pea and found mostly α-tocopherol during the period of vigorous growth and cellular differentiation, which suggests that a conversion of the non-α-tocopherols of the seed in the resting stage to α-tocopherol may take place during the development of the plants.

Complete separation of the known tocols is not possible by the conventional two-dimensional paper-chromatographic method, since β- and ε-tocopherol do not separate from γ- and η-tocopherol, respectively. However, this problem has been solved by Marcinkiewicz & Green (147), who describe a complete separation and determination of β-, γ-, δ-, ε-, and η-tocopherols by paper chromatography of the nitroso derivatives. Separation on the basis of a coupling reaction between tocopherols with free 5-position and diazotized dianisidine was not feasible (148).

A spectrophotofluorometric method for the determination of vitamin E (149) and a new color reaction with crystals of 2-hydroxy-3-methyl-1,4-naphthoquinone and cysteine hydrochloride (150) have been published. Descriptions are also given of modifications of some of the conventional methods for vitamin-E determination in plasma (151), milk (152), and grass (153) and for the determination of free α-tocopherol in oil seeds (154). In

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whole wheat, 80 per cent of the total vitamin-E content has been found in the ground outer endosperm (155), although the germ is generally assumed to have the greatest content of this vitamin.

Bioassay and biopotency.—Friedman *et al.* (156) have modified and standardized the rapid and simple method of bioassay for vitamin E which depends upon the *in vivo* action on diaturic acid-induced hemolysis of rat erythrocytes. The relative activities of the racemic and optically active forms of α- and γ-tocopherols and esters of these tocopherols have been examined, and they are in close agreement with the activities determined by the officially accepted rat anti-sterility tests (157). The same method has been used by Sharman & Richards (158) for the examination of the vitamin-E potency of bread prepared from flour bleached with chlorine dioxide and of bread enriched with wheat germ. The results agree well with those found by chemical determination of tocopherol contents in bread.

Green *et al.* (159) examined the relative biological activities of the known tocols and of the two above-mentioned natural α- and β_2-tocopherols. The examination of the substances was based either on the effect on the respiratory decline in necrotic rat liver following intraportal administration (159) or on the *in vivo* and *in vitro* effect on hemolysis (160). The *in vitro* effect of the individual tocopherols showed considerably smaller differences than the *in vivo* effect, a fact which suggests that the biological specificity is partially lost in the *in vitro* experiments. On the other hand, the results show fairly good agreement between the activity measured after intraportal injection and after oral administration, which indicates that the biological selectivity that determines the relative activities of the tocopherols is not solely dependent upon the intestinal walls, as generally assumed.

The curative effect of intravenous administration of α-tocopherolhydroquinone on muscular dystrophy has been re-examined and confirmed (161). The disuccinate of the hydroquinone had a very small effect, however, because of either slow hydrolysis of the ester or rapid excretion or metabolism.

The enzymatic role of vitamin E.—The role of α-tocopherol in the electron transport system has evoked considerable interest. Several reviews on this topic have been published; in particular, mention should be made of those of Slater (162) and Nason (163, 164).

The theory that tocopherol functions as a cofactor in the cytochrome-*c*-reductase system in the respiratory chain, as suggested by Nason and co-workers, apparently cannot obtain sufficient experimental support. Attempts to restore the activity in iso-octane-extracted enzyme preparations show that the reactivation does not show specific dependency on α-tocopherol. Weber and co-workers (165, 166, 167), Marinetti *et al.* (168), and others (169, 170, 171) found that a great number of substances, chiefly of lipid nature, were active; for example, vitamin K, ubiquinone, phytol, neutral fat, Tween 80, and certain hydrocarbons. Pollard & Bieri (172) have further shown that physical processes such as freeze-drying, lyophilization, and centrifuging

restore the activity in the iso-octane-extracted enzymatic preparations. These results, together with a direct demonstration of the inhibiting effect of iso-octane and other hydrocarbons (167, 172), suggest that the inactivation is an experimental artifact. Thus, residual iso-octane adsorbed on the surface of the enzyme can cause the observed inhibition (173).

Subsequently, the Nason group (174, 175, 176) described experiments with aged cytochrome-*c*-reductase preparations with or without iso-octane extraction and found that α-tocopherol had a specific reactivating effect, whereas tocopheryl acetate, tocopheryl quinone, and other lipids, which were active in the solvent-inhibited preparations, had no effect in this case. Pollard & Bieri (177), in duplicating these experiments, found, on the contrary, that not only tocopherol but also certain antioxidants and phenolic compounds were able to reactivate cytochrome-*c*-reductase preparations after ageing, and they postulated that the activity-depressing effect of ageing was caused by a formation of lipid-peroxides.

Another essential point in connection with the possible enzymatic role of tocopherol is the question of whether tocopherol metabolites are present in the enzyme preparations. Bouman & Slater (178) and Donaldson & Nason (179) have submitted results which indicate that tocopherol in certain tissues is present chiefly as tocopheryl quinone. Slater (162), however, emphasized that the quinone, which following acid reduction is determined as α-tocopherol, might be ubiquinone, and this later proved to be the case (180). Martius & Costelli (181) stated that, following administration of labelled α-tocopherol to rabbits, they could isolate from the liver mitochondria a metabolite which on acid reduction yielded α-tocopherol but which resembled trimethylphytylbenzoquinone rather than α-tocopherylquinone. It does not appear probable that the metabolite in this case could be identical with ubiquinone, since Morton & Phillips (182) and Moore (183) have shown that neither intake nor deficiency of vitamin E has any influence on the ubiquinone concentration in the tissues and Alaupovic & Johnson (184), after administration of C^{14}-labelled D-α-tocopherylsuccinate, were able to isolate only non-radioactive ubiquinone. Consequently, it does not seem possible at the moment to ascribe to tocopherol per se a role as electron carrier.

Schwarz (185) has drawn attention to a new class of tocopherol derivatives with benzoquinone structure which had previously been isolated by Simon *et al.* (186) from urine by means of C^{14}-labelled tocopherol. By means of liver-slice preparations in a Warburg substrate, they showed that *in vitro* addition of the Simon metabolite prevented the respiratory decline observed in necrotic livers from rats on a *Torula* yeast diet deficient in vitamin E and factor 3 (187). α-Tocopherol had no effect in these experiments and could only prevent the respiratory decline when added to the diet or given intravenously before the extirpation of the liver. In liver homogenates from necrotic animals suffering from deficiency symptoms, no decrease in the oxidation was observed when glucose was the substrate and ATP and diphosphoryridine

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nucleotide were added (188). On the other hand, Corwin & Schwarz (189) have recently described another homogenate with α-keto-glutarate or succinate as substrate: it showed a decrease in oxidation which could be prevented by *in vitro* addition of α-tocopherol, the tocopherol metabolite, diphenyl-*p*-phenylenediamine, menadione, and methylene blue.

Biochemical alterations in vitamin-E deficiency.—There was no change in the $NADH_2$ cytochrome-*c*-reductase activity in liver mitochondria from rats fed a vitamin E-deficient diet (190). Increased succinic acid-cytochrome-*c*-reductase activity may be observed, however (191), particularly during the last part of the deficiency condition (192). The mitochondrial stability is concurrently lessened, and Corwin & Lipsett (191) assume that the apparent increase in the activity is due to increased accessibility to the substrate for the enzyme. This is in agreement with the results found by Zalkin & Tappel (193), who demonstrated increased *in vivo* lipid peroxidation in liver mitochondria from vitamin E-deficient rabbits; they are of the opinion that tocopherol stabilizes the content of unsaturated lipids in the cells against oxidative destruction and thereby maintains the structural integrity at the subcellular level.

Investigations of the creatine metabolism in vitamin-E deficiency have been continued. The creatinuria and the low content of creatine in the muscles of monkeys suffering from vitamin-E deficiency is accompanied by an increased content of DNA and RNA in bone marrow and of DNA in muscles (194). By examination of liver homogenates, the synthesis of creatine from glycocyamine and methionine was found to be normal (195). Fitch & Dinuing (196) gave vitamin E-deficient rabbits and rats supplements of P^{32}-phosphate and found an increased turnover of intracellular inorganic phosphate in skeletal muscles, but the results did not show clearly whether the phosphorylation of creatine and ATP was reduced or normal. Other investigations, however, seem to show that the phosphorylation of creatine is reduced. Carpenter *et al.* (197) have shown that the transfer of phosphate from creatine phosphate to glucose-1-phosphate is reduced in vitamin-E deficiency in spite of normal glycolysis and phosphoglucomutase activity. Moreover, Nesheim *et al.* (198) have found that the contents of active and total phosphorylase and glycogen in striated white breast muscles are reduced in chicks fed a vitamin E-free *Torula* yeast diet.

Glycine in some way seems to play a role in the dystrophic condition which results from vitamin-E deficiency (199). Tallan (200) had previously shown that in muscle extracts from dystrophic rabbits the contents of most of the free amino acids are unchanged, whereas the glycine content is greatly reduced. Smith & Nehoyaran (199) found reduced phosphatase activity in vitamin E-deficient rabbits, which could be prevented by a supplement of 1 per cent glycine to the diet. This deficiency condition is characterized by considerable amino aciduria (201, 202) in which the glycine content in the urine is not increased (202). The most significant increase in the content of

free amino acids in the urine is observed in the case of 1-methyl histidine, which represents 75 per cent of the total amino acid content and which, incidentally, is the earliest sign of the deficiency, occurring even earlier than the creatinuria (203). Moreover, glycine, anserine, and carnosine are also present in diminished quantities in dystrophic muscles, presumably because a reduction of the synthesis of the substances occurs (204). However, the decrease in anserine content is neither rapid enough nor sufficiently large to account for the increased excretion of methyl histidine (203).

Antioxidant relationships.—The relative antioxidant activities of the known tocopherols have been investigated by Lea & Ward (205). As an empirical rule, activity decreases in the order: monomethyl tocol, dimethyl tocol, trimethyl tocol; and in the order: 8-methyl tocopherol, 7-methyl tocopherol, 5-methyl tocopherol. There are, however, a few exceptions.

Investigations of the vitamin-E activity of chemical antioxidants have been continued. Ethoxyquin (Santoquin; 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline) prevents both encephalomalacia and exudative diathesis as well as the *in vivo* peroxidation of liver lipids in chicks on vitamin E-free diet (206). If the effect of the antixoidant in this experiment is the result of a protection of the vitamin E in the body, the vitamin-E requirements must be extremely low. Using diphenyl-*p*-phenylenediamine, Draper *et al.* (207) found not only a preventive but also a curative effect on a severe vitamin-E deficiency as measured by the fetal resorption in rats. This suggests that it is a direct biological effect, not caused by vitamin-E sparing.

Contrary to these results, Shull *et al.* (208) could not prevent muscular dystrophy in vitamin E-deficient guinea pigs by means of diphenyl-*p*-phenylenediamine, ethoxyquin, butylated hydroxy-toluene, or 2,5-di-*t*-butyl hydroquinone; nor could they prevent an increase in the cholesterol content in plasma and skeletal muscles. Methylene blue prevented the brown discoloration of uterus and degeneration of testes in rats on vitamin E-free diet, but it had no influence on the hemolysis of erythrocytes in the dialuric acid test (209).

An antagonistic action between tocopherol and unsaturated fatty acids may be observed. This appears to explain the fact that cod liver oil cannot prevent symptoms of vitamin-E deficiency, although it contains 100 μg. α-tocopherol per gm., which is four to 12 times greater than the vitamin-E requirement in a diet containing lard instead of cod liver oil (210). Dam *et al.* (211, 212) found that an addition of polyunsaturated fatty acids to a vitamin E-free diet causes a considerable aggravation of exudative diathesis and encephalomalacia in chicks. A supplement of various fatty acids to a fat-free bakers' yeast diet gave increased frequency of liver necrosis in rats, the frequency increasing with the degree of unsaturation (213). With linolenic acid as supplement, liver necrosis was observed in all of the animals, and it is suggested that in vitamin-E deficiency autoxidation of unsaturated lipids may take place in the tissues and that this is responsible for the lesions of the

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tissues. Bieri *et al.* (214) are of the opinion that the anti-vitamin-E activity of *Torula* yeast, besides depending on the mineral substances, is also caused by the content of unsaturated fatty acids in this yeast. This agrees well with results found by Tappel & Zalkin (215, 216), who demonstrated *in vivo* peroxidation in the liver of vitamin E-deficient rabbits and increased lability of isolated liver mitochondria measured by the $NADH_2$ cytochrome-*c*-reductase activity. It is assumed that vitamin E primarily acts as an antioxidant and thereby protects the structural integrity and enzymatic activity of the mitochondria.

Vitamin E and selenium.—This subject has been dealt with in detail by Schultze in Volume 29 (1960) of the *Annual Review of Biochemistry* and has therefore been omitted from the present review.

VITAMIN K

Analysis, chemistry, and biological activity.—A new color reaction with xanthane hydride has been proposed by Schilling & Dam (217) and has been used for the determination of vitamin K_1 in alfalfa after chromatography on secondary calcium phosphate (218). For products with lower concentrations and high contents of interfering substances, e.g., related benzoquinones (219), the purification involved is hardly sufficient. Lev (220, 221) has examined the vitamin-K requirements of rumen strains of *Fusiformis nigrescens* and has thereby opened up the possibility of a microbiological assay method; however, the technique is not yet suitable for routine work.

An excellent survey of the chemistry and biochemistry of vitamin K has been published by the Isler group (222). By total synthesis and comparison with natural vitamin K_2 isolated from putrefied fish meal, the structure of vitamin K_2 has been established as 2-methyl-3(all-*trans*-farnesylgeranylgeranyl)-1,4-naphthoquinone; i.e., at position 3 there is a side chain with 35 carbon atoms consisting of seven isoprene units (223). According to Isler's nomenclature, the substance is denoted as vitamin $K_{2(35)}$. In addition, a number of other vitamin-K_2 compounds were synthesized, having side chains with 10, 15, 20, 25, and 30 carbon atoms, respectively. Several of these substances occur in nature. In putrefied fish meal, Isler *et al.* (223) found also small amounts of vitamin $K_{2(30)}$; i.e., the substance that Doisy had assumed to be the natural vitamin K_2. Kruse Jacobsen & Dam (224) isolated from various bacterial strains three different types of vitamin K, among which was vitamin $K_{2(35)}$. The vitamin K-active naphthoquinone isolated by Brodie *et al.* (225) from *Mycobacterium phlei* could not, however, be identified as belonging to the vitamin-K_2 series.

Natural vitamin K_1 with 20 carbon atoms in the side chain shows greater biological activity than analogues with either more or fewer carbon atoms (226). On the other hand, in the vitamin K_2 series the natural vitamin $K_{2(35)}$ was less active than analogues with 20, 25, and 30 carbon atoms; $K_{2(25)}$ showed the highest activity. Geometrical isomers of vitamin $K_{2(20)}$

and $K_{2(20)}$ were likewise tested on vitamin K-deficient chicks. 6′,7′-Mono-*cis* isomers in which the *cis* double bond is near the naphthoquinone ring were considerably less active than, for example, the 10′,11′-mono-*cis* compounds or the all-*trans* isomers. Martius & Esser (227) administered C^{14}-labelled menadione to chicks and rats and isolated radioactive vitamin K, which in countercurrent distribution of the bis-(*p*-bromobenzoyl) derivative behaved as vitamin $K_{2(20)}$. The authors consider it probable that all vitamin K-forms after oral administration are converted into menadione and then add the 20-C atom chain.

Comparisons between the biological activities of vitamin K_1 and menadione have been performed by means of the prothrombin test applied to

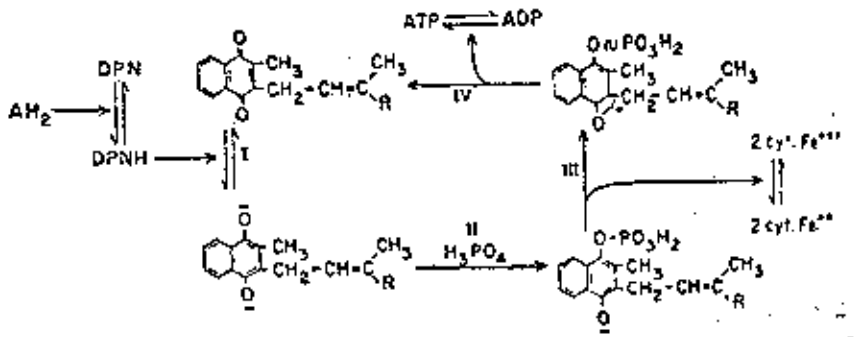

FIG. 1. Proposed mechanism for the action of a napthoquinone monophosphate in oxidative phosphorylation (235).[5]

chicks (228, 229, 230). The sodium bisulphite compound of menadione was found to be 1.5 times as potent as vitamin K_1 (230).

The enzymatic role of vitamin K.—Several articles surveying the literature on the enzymatic role of vitamin K, especially in oxidative phosphorylation, have been published [see Martius (231, 232); Dam & Søndergaard (233)].

Harrison (234) and Brodie & Ballantine (235) have formulated a probable reaction scheme (Fig. 1) of the oxidative phosphorylation: according to this scheme, the naphthoquinone has two functions: as electron carrier between two coenzymes in the respiratory chain and as catalyst for the generation of energy-rich phosphate bonds. In the first stage of the reaction, the naphthoquinone is reduced to hydroquinone by the transfer of electrons from $NADH_2$, presumably by an FAD-linked enzyme (236) corresponding to the vitamin-K reductases isolated by Martius & Märki (237) and Wosilait

[5] According to a personal communication from Arnold F. Brodie, recent investigations indicate that chromanol phosphates and not quinol phosphates as stated in the scheme are intermediates. A ring closure takes place between the side chain at position 3 and the oxygen atom at position 4.

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(238) from calf and dog liver, which are inhibited by dicumarol. The hydroquinone is phosphorylated and then oxidized, with cytochrome as the electron acceptor while giving off energy-rich phosphate to ADP, probably with a highly unstable compound, the enol phosphate ester, as an intermediate.[5] In a model compound experiment using a monophosphorylated naphthoquinone (mono-ethyl menadiol monophosphate) and with ADP as the only phosphate acceptor, Brodie & Davis (239) demonstrated the formation of ATP. This required the addition of both particulate and supernatant fractions from a cell-free *Mycobacterium phlei* extract. The particulate fraction contained dehydrogenases, cytochromes-*c*, -*b*, and -*a*. Kinetic studies indicate that the supernatant fraction contains at least two protein components, one for oxidation and one for phosphorylation (240).

Irradiation of bacterial extracts with light at 360 mμ destroys a vitamin K-like substance which is necessary for oxidative phosphorylation without destruction of the apoenzymes (235, 241, 242). Addition of vitamin K or closely related homologues (241, 242, 243) is specific for the reactivation of both oxidation and phosphorylation. Reactivation of the oxidation alone may be effected with riboflavin phosphate, FAD (241), or naphthoquinones substituted in either position 2 or 3, e.g., lapachol or menadione (235). With succinate as substrate, an unknown component in the supernatant liquid is required for the reactivation (241).

Reduced menadione is rapidly oxidized in the presence of respiratory chain preparations according to a first-order reaction scheme (244). The reaction is inhibited by cyanide (244, 245). Also cyclic photophosphorylation is catalyzed by vitamin K, presumably with an alternative pathway catalyzed by flavin mononucleotide (246). The latter pathway is dependent on the presence of NADP and is more sensitive to inhibition with dinitrophenol and *o*-phenanthroline than the former. Both pathways are inhibited by *p*-chloromercuribenzoate but not by antimycin-A. The oxidative phosphorylation of glutamate and succinate in liver mitochondria from vitamin K-deficient chicks could not be correlated with the prothrombin time in experiments by Beyer & Kennison (247); this is contrary to previous results of Martius & Nitz-Litzow (248).

Vitamin-K requirements.—Investigations of vitamin-K requirements are being continued. In rats on vitamin K-free diet and prevented from coprophagy, the prothrombin time increases but becomes normal when 1 μg. menadione or vitamin K₁ per 100 gm. of body weight is administered (249). Mameesh & Johnson (250) found that 5 μg. per rat per day was sufficient to prevent hemorrhage and prolonged prothrombin time, irrespective of whether the animals were coprophagous. In the case of turkeys on a vitamin K-free diet, 0.8 mg. menadione per lb. feed or a single oral dose of 6 to 8 μg. per 100 gm. body weight was required to maintain normal prothrombin time (251).

Addition of substances such as sulphaquinoxaline, nicarbazin, arsanilic acid, aureomycin, or arsonic acid to a vitamin K-free diet administered to

chicks prolonged the clotting time and caused hemorrhage (252). Menadione counteracted the increase in the prothrombin time but did not influence the hemorrhagic condition. In experiments with chicks on a vitamin K-free diet, Griminger (253) found that when a single dose of about 50 mg. sulphaquinoxaline was given orally with varying doses of menadione, one mole of menadione was sufficient to counteract the inhibiting effect of more than 800 moles of sulphaquinoxaline. Chicks with coecal coccidiosis are assumed to have an increased vitamin K requirement (254). The magnitude of this increase in the requirements hae not been finally determined (255).

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Enrichment and Fortification of Cereals and Cereal Products with Vitamins and Minerals

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Clinton L. Brooke

Enrichment of cereal products with B vitamins and minerals became feasible in the late 1930's, as the vitamins became commercially available and prices declined. The bread and flour enrichment program, inaugurated in the United States in 1941, was followed by programs to enrich corn meals and grits, rice, and other products. White flour and corn meals are enriched at the mill by metering a mixture of vitamins and iron into the flour stream. Enriched bread may be made with flour enriched at the mill, or the enriching ingredients may be added at the bakery in powdered or tablet form. Corn grits and farina are enriched through admixture of vitamin-containing, rinse-resistant particles or a powdered vitamin-iron mixture. Rice is enriched with premix kernels of rice which have been coated or impregnated with vitamins and iron and made resistant to washing losses, or with a powder which adheres to the rice, but is not resistant to washing. Flour enriched at the mill is preferred for macaroni and noodle products made by continuous processes. Breakfast cereals are usually enriched at several stages of processing, by separating enrichment ingredients and adding the heat-labile thiamine to the toasted product by spray application. The development of dry forms of vitamin A has expanded the scope of cereal enrichment. A flour enrichment program recently instituted in the Near East includes vitamin A in powdered form, as well as thiamine, riboflavin, niacin, and iron.

The purpose of this paper is to tell how cereal products are enriched and fortified—to explain how the vitamins and minerals are combined and incorporated in wheat flour and bread, corn meals and grits, durum flour for macaroni products, rice, and breakfast cereals.

The history of cereal enrichment and fortification—the story of the steps that have been taken to correct nutritional deficiencies through the supplementation of basic foods with vitamins and minerals—has been well covered in a number of publications.

Harris (1959) has published a comprehensive survey of food supplementation with vitamins. In a series of publications, the Committee on Cereals, Food and Nutrition Board (1944a, 1944b, 1946, 1947, 1948, 1950, 1958) kept pace with developments in the cereal enrichment program from its inception in 1941 until 1958.

The early history of enrichment and recent developments in relation to the world food situation were reviewed in several papers included in the symposium on enrichment presented by the American Association of Cereal Chemists (1966).

ENRICHMENT OPERATIONS IN THE FLOUR MILL

Flour is enriched at the mill by metering a vitamin-mineral mixture into the stream of flour flowing to the packing bins or by incorporating a weighed quantity of the enrichment mixture into flour in a batch mixing operation. The rate of addition in large mills is usually 1 ounce of mixture per 100 pounds of flour. Small mills prefer to add a less concentrated mixture at the rate of ½ ounce per 100 pounds of flour.

National Center for Chronic Disease Control, U. S. Public Health Service, Bethesda, Md. 20014

When enrichment of flour at the mill began in mid-1941, each miller had to make his own enrichment mixture by adding carefully weighed quantities of thiamine, niacin, and iron to a given quantity of flour and mixing thoroughly to produce a more or less uniform premix. By mid-1942, the miller was relieved of the troublesome task of making premixes through the introduction, by several manufacturers, of accurately compounded, finely milled enrichment mixtures, with wheat or corn starch as the carrier for the thiamine, niacin, and iron.

Definitive formulas for flour enrichment mixtures were recommended to the milling industry and its suppliers by the Millers National Federation (1944). Riboflavin had been included in the enrichment formula late in 1943 when an adequate supply became available. These formulas include a safety factor of 10%, intended to cover fluctuations in the rate of flow of the flour stream, and to ensure that the enriched flour conforms to the Standard of Identity for Enriched Flour (1955).

The feeders used to dispense the enrichment mixtures are dry chemical feeders, often referred to as powder feeders. Because the feeder is a volumetric and not a gravimetric device, and because the bulk density of the enrichment mixture will vary, the rate of feed must be monitored frequently by intercepting and weighing the quantity of enrichment mixture delivered in a period of several minutes. The output of the feeder is either dropped into the flour conveyor by gravity or picked up in an air stream and blown into the flour moving along in the conveyor.

If as little as ¼ ounce of enrichment mixture is to be uniformly distributed throughout 100 pounds of flour, a ratio of 1 in 6400, the blending operation between the point of addition and the storage bin is of vital importance. Whether the flour is conveyed by mechanical means or by

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air, the conveyor must be long enough and must be constructed so as to ensure uniform distribution of the enrichment ingredients in the finished product. Screw conveyors for blending usually are equipped with cut flights to facilitate distribution.

Since the levels of the three B vitamins and iron are carefully controlled by the manufacturer of the enrichment mixture, a check of the enriched flour for a single ingredient gives reasonable evidence that the other three are present in the correct amounts. Many mills make use of a quick, roughly quantitative test for riboflavin. A flattened, wetted wedge of enriched flour on a glass or metal strip is examined in the dark under ultraviolet light. The intensity of fluorescence exhibited by the wetted particles of riboflavin provides an approximation of the level of enrichment. Where quantitative analyses are made, thiamine is most often checked as the index ingredient.

In the United States, little interest has been shown in adding the optional ingredients, calcium and vitamin D, to flour at the mill.

ENRICHMENT OF BREAD

Several years prior to the introduction of enriched flour, a few large bakeries experimented with nutritional supplementation of bread through the use of high-B_1 yeast, synthetic thiamine, and high-vitamin fractions of wheat. By the time the mills began to enrich flour in mid-1941, the baker had at his disposal other methods of incorporating the B vitamins and iron in his bread, mainly the use of enriched yeast. This was a compressed baker's yeast, containing thiamine, niacin, and iron in such quantities that when the yeast was used at the customary level, the resulting bread would contain the amounts of enrichment ingredients required by the Standard of Identity for Enriched Bread (1955). The thiamine in enriched yeast was somewhat lower in cost than synthetic thiamine, since the final step of synthesis was completed in vivo in the yeast from synthetic intermediates added to the fermenter. For the next two years, enrichment of bread was accomplished chiefly through the use of this type of yeast, not only because it was cheaper to enrich by this method, but also because it eliminated the necessity of purchasing a special flour for the production of enriched bread.

A third method of enriching bread at the bakery level, also introduced in 1941, was the enrichment tablet or wafer. Like the enrichment mixture used in the mill and the enriched yeast used in the bakery, the tablet supplied only thiamine, niacin, and iron because riboflavin was not yet available in sufficient quantity. During the next two years, the use of tablets found increasing favor with bakers as a simple, accurate, and selective method of enriching only the flour that was being made into dough for enriched bread. The tablets, consisting of the amounts of enrichment ingredients required for units of 50 or 100 pounds of flour with a rapid-swelling starch as the carrier, were allowed to disintegrate in a portion of the water used to make the dough, and the resulting suspension was added during the mixing of the dough.

The inclusion of riboflavin in the enrichment formula, beginning October 1, 1943, brought about rapid changes in methods of enriching bread. During the first two years of enrichment, 1941 and 1942, approximately 80% of the bread being enriched utilized yeast as the source of the vitamins and iron. The remaining 20% was enriched by means of vitamin mixtures, mostly in the form of tablets. Only a negligible proportion of the bread was made from flour enriched at the mill. During this time, however, practically all flour used in home baking was being enriched at the mill.

With the introduction of riboflavin, the yeast manufacturers found it impractical and uneconomical to add this vitamin to their product. Not only did it impart a highly undesirable orange-yellow color to their creamy-white product, it also was not well assimilated by the yeast cells, and an excessive amount was lost in the waste liquor.

The cost of enrichment had been declining steadily as vitamin manufacturers expanded their production. The addition of riboflavin halted the decline, but only briefly. Bakers use milk in their breads, usually in the form of nonfat milk solids, and milk is a good source of riboflavin. If the baker used enriched flour and also used nonfat milk solids in his dough, the resulting bread contained more riboflavin than required by the Standard of Identity for Enriched Bread. Accordingly, the manufacturers of enrichment tablets made it possible for the baker to take credit for the riboflavin in his milk powder by offering two types of tablets, one for bread made with less than 3% and the other for bread with more than 3% milk powder. This adjustment to the baker's requirements and the resulting saving, plus the fact that the use of the tablet eliminates the need to purchase a special flour for enriched bread, have made the enrichment tablet the preferred medium for enrichment throughout the baking industry. Flour enriched at the mill finds little application in commercial baking operations.

Instead of enrichment tablets, some bakers are finding it convenient to use enriched salt, in which the enrichment ingredients are combined with salt in such proportions that the amount of salt commonly used in bread dough will carry with it the amounts of vitamins and iron needed for bread meeting the requirements of the Standard of Identity for Enriched Bread. Enrichment mixtures are also combined with other ingredients which are added to the dough in small but fairly constant amounts. Such combinations may be packaged in water-soluble edible film and the packet allowed to disintegrate in the dough water.

Although calcium and vitamin D were included as optional ingredients in the Standards of Identity for Enriched Flour and Enriched Bread, little interest was shown in these as enrichment ingredients until 1953, when one large bakery chain began adding calcium and soon thereafter included vitamin D. Other bakers followed suit, and for several years a large share of the enriched bread produced in this country included the optional ingredients. From a promotional point of view, the bakers found little value in these new supplements, and one by one the bakers discontinued their use.

The feasibility of including three other nutrients in the enrichment program has been discussed by Rubin (1966) who points out the probable need for supplementation of the American diet with vitamin E, vitamin B_6, and ly-

sine, which could be added to flour either at the mill or at the bakery. Rubin also mentions the development of stabilized beadlets of vitamin A in a form suitable for flour enrichment.

Trials of vitamin A beadlets as an ingredient of a flour enrichment mixture including B vitamins and iron were made in December 1966 in a pilot installation in a flour mill in Amman, Jordan, as the first step in a nationwide fortification program. Brooke (1968) has reported that after the feasibility of adding vitamin A to the national diet through the medium of wheat flour had been demonstrated, the Technical Executive Committee recommended to the government's Higher Nutrition Council that the 10 or 12 large mills producing about 80% of Jordan's wheat flour be required to purchase and install enrichment feeders. It was further recommended that the government of Jordan purchase and distribute to the mills, as needed, the quantities of vitamin-mineral mixture required for fortification of all of their white flour. This recommendation was made not only for public health reasons, but also for reasons of economy, to prevent unjustified increase in the prices of flour and bread. So far as is known, this was the first time vitamin A in dry form had been added to flour in a commercial milling operation. Unfortunately, the program was interrupted by the war in the Middle East.

ENRICHMENT OF CORN MEALS AND GRITS

Experience gained in the enrichment of wheat flour at the mill through the addition of vitamins and iron made it apparent that corn meals could be enriched in the same manner. In the southeastern states, where high consumption of corn meals and grits was paralleled by a high incidence of pellagra, methods were developed for the enrichment of locally ground corn products as well as those shipped in from other sections of the United States.

Mandatory enrichment of degerminated corn meal, a finely ground meal from which most of the bran and germ have been removed, began in South Carolina in 1943, two years after the inception of the wheat flour enrichment program. Within the next few years, North Carolina, Georgia, Alabama, and Mississippi also adopted legislation requiring that corn meals and grits be enriched to the same levels as white flour. Legislation in these five southeastern states was followed by the promulgation of Standard of Identity for Enriched Corn Meals and Grits (1955) covering products moving in interstate commerce.

Mixtures for the enrichment of corn meals differ slightly in formulation from those used for enriching wheat flour. They are added to the corn meal on stream, using the same types of powder feeders and mixing conveyors as for wheat flour.

The enrichment of corn grits, which are made by coarse grinding of the endosperm of the corn kernel, presented several problems. Grits were usually sold in bulk, and most housewives in the southern states washed the grits before cooking, discarding the wash water. Consequently, the finely powdered vitamins and iron had to be added in such a form that they would not separate from the granular grits in the dry state or be lost when the grits were rinsed before cooking. The problem was solved by the development of rinse-resistant premixes, consisting either of grits granules coated with enrichment ingredients and protected with an insoluble coating, or of simulated grits granules made from a corn meal dough in which the vitamins and iron had been incorporated. The cost of these premixes, which were added to the grits at the rate of 2 ounces per 100 pounds, was much higher than that of a powdered enrichment mixture. In recent years, however, grits have been marketed mostly in printed paper bags and cartons instead of unprinted cotton sacks. This has made it possible to print a statement on the container, warning the housewife not to wash, as washing removes valuable ingredients. With the washing requirement removed, the costly rinse-resistant type of premix is no longer needed, and grits can be enriched with a specially formulated powdered mixture which adheres to the granules and does not settle out in the package.

ENRICHMENT OF MACARONI PRODUCTS

Macaroni, spaghetti, vermicelli, noodles, and many other alimentary pastes are commonly classed together as macaroni products. These products are made from flours and semolinas milled from durum wheat. In the early years of enrichment, when the B vitamins were high priced and in short supply, it was felt that it would be wasteful to enrich macaroni products, because as much as 50% of the added thiamine, 40% of the niacin, and 30% of the riboflavin might be lost in cooking and draining.

By 1946, five years after the beginning of flour and bread enrichment, the cost of vitamins had dropped to a level low enough to permit establishment of a Standard of Identity for Enriched Macaroni Products (1955) which took into account the losses to be expected in cooking and draining and prescribed levels of thiamine, riboflavin, niacin, and iron in the uncooked product, anticipating that the cooked product would contain the same levels per pound as enriched flour.

Formulas were written for an enrichment mixture to be added on stream at the mill and for an enrichment wafer to be added, in suspension, during mixing of the dough. Most macaroni plants have continuous mixers and presses, so the only possible way to make enriched macaroni is to use flour enriched on stream at the mill. An enrichment tablet was made available for the small plants operating on the batch basis, but was discontinued within a few years.

ENRICHMENT OF RICE

Rice millers began experiments with the application of vitamins to rice in the early years of the enrichment program, and vitamin-fortified rice was on the U. S. market several years before the Standard of Identity for Enriched Rice was established (1958).

The first process for making a premix for the enrichment of rice was developed in Manila by an American company. This process consisted of impregnating rice kernels with thiamine, niacin, and iron, then covering the impregnated kernels with a rinse-resistant coating. The premix kernels were blended at local rice mills with ordinary white rice in the ratio of 1 to 200, to produce enriched rice

providing the same levels of vitamins and iron as enriched flour. Rice enriched with premix kernels was used in the Bataan experiment in the Philippines during 1948–50, which gave dramatic proof of the importance of enrichment.

A second process for making rice premix by coating rice kernels with vitamins and iron and protecting the added nutrients with a rinse-resistant coating was developed several years later and placed in production in two plants on Formosa in 1958 to provide premix for the rice consumed by the Chinese Nationalist Army as well as for civilians. This second process for making premix kernels differed from the first by including riboflavin. Consequently, the vitamin-covered kernels were distinctly yellow in color, and when rice carrying one pound of these yellow kernels in each 200 pounds was cooked, yellow splotches appeared in the cooking pot. This presented no problem in the Army mess kitchens where the cooked rice was invariably stirred thoroughly before serving, but the native housewife was at first so suspicious of the yellow spots that she dug them out with the tip of a spoon and discarded them. Some years later, Chen and Tung (1955) reported that not only had the consumer's suspicion of the yellow spots been removed, but that some said the yellow color gave a sense of security.

Enrichment of rice by admixture of rinse-resistant premix kernels is an effective and practical method for those parts of the world where rice, the most important food, is often contaminated in transit and in storage and is invariably washed before cooking. The premix kernel method seals the enrichment ingredients on the rice kernels in such a way that the vitamins and iron are not released and dispersed until the rice is cooked.

Some success has been achieved with a premix consisting of simulated rice kernels, made by extruding a dough consisting of enrichment ingredients and a suitable binder through a short-goods die in a macaroni press.

For white rice marketed in printed cartons or bags, enrichment with a finely powdered vitamin and iron mixture, so formulated that it will adhere to the surfaces of the kernels and will not sift out, has proved very satisfactory, but the ease with which such a mixture can be washed off the rice poses a problem.

The Standard of Identity for Enriched Rice (1958) stipulates, in effect, that powder-enriched rice packed in consumer packages must bear the statement "To retain vitamins do not rinse before or drain after cooking." However, this statement is not required for precooked rice packed in consumer packages labeled with directions which, if followed, will avoid washing away or draining off enriching ingredients.

When the Standard of Identity for Enriched Rice was published, it required the same levels of thiamine, riboflavin, niacin, and iron as for enriched flour, corn meals and grits, and macaroni products after cooking. However, the requirement for riboflavin was stayed "until final action should be taken disposing of the objections after public hearing thereon." This exception was made because several of the large rice millers objected to the slight yellowish tint that the riboflavin imparted to their polished white rice. More than nine years have passed, and no hearing has been held. With one or two notable exceptions, rice millers in the United States do not include riboflavin in their enrichment mixtures.

Whereas in the premix kernel method of enrichment, the vitamins and iron are applied to only 0.5% of the total weight of the rice, in the powder method, a mixture of vitamins and iron with a suitable carrier is applied on stream to the entire body of rice. But this is not simply a matter of blending one powdered substance with another; it is necessary literally to rub the vitamins and iron into the surfaces of the kernels by passing the stream of rice through a slowly rotating baffled cylinder (trumbol) or a sequence of cylinders where the attrition of kernel on kernel serves to work the enrichment ingredients into the surfaces of the kernels. This treatment eliminates segregation in the bag or package.

ENRICHMENT AND FORTIFICATION OF BREAKFAST CEREALS

The bewildering array of breakfast cereals now on the grocery shelves and the variety and range of their label claims make it difficult to generalize about the fortification of these products. The only breakfast cereal that can be labeled "Enriched" is farina which is a hot cereal—i.e., one that requires cooking. A Standard of Identity for Enriched Farina (1955) requires the same enrichment levels for enriched farina as for the other enriched products mentioned in the foregoing. Farina may be enriched with a premix made in the same way as corn grits premix, or a powder may be used, as with corn meals.

The numerous ready-to-eat or cold cereals are usually fortified at least to whole-grain levels in respect to thiamine, niacin, and iron, but an increasing number are boosting both the levels and the number of vitamins and minerals added during processing. Dietary labeling claims for fortified cereals are expressed both in milligrams and in percentages of minimum daily requirements supplied by a 1-ounce serving.

Some of these products supply, in a 1-ounce serving, one third of the minimum daily requirements of a half-dozen vitamins and one or more minerals; others supply one half; and several supply a full 100%, including in the list vitamin B_6 and vitamin B_{12}. The vitamins are added at different stages of processing, depending on the physical and chemical characteristics of the substance. Heat-stable vitamins and the minerals may be added to the mixture of raw cereal, flavoring, and other supplements before cooking. The heat-labile vitamins, such as thiamine, are usually sprayed on the toasted product as it emerges from the oven.

COST OF ENRICHMENT

In 1956, the year that marked not only the fifteenth anniversary of enrichment but also the twentieth anniversary of the synthesis of thiamine, the American Institute of Baking (1956) published a booklet of letters from eminent scientists, testifying to the genius of R. R. Williams, the man who took the great stride toward making enrichment possible. The following is taken from the tribute paid to Williams and to the flour and bread enrichment

program, in the letter from the dean of American cereal chemists, C. H. Bailey.

"It is one thing to discover scientific truths through research; it is another thing to translate them into services to the human population, and particularly without disturbing normal routines or adding to the cost of living. Yet that dual function was discharged in the instance of the flour and bread enrichment program. It not only involved making essential nutrients available in adequate quantities, but did so without altering the delectable qualities of the foods involved, and with a minimum of inconvenience to the processor, and of cost to the consumer."

It is instructive and heartening to the chemist, nutritionist, and food technologist to compare enrichment costs in 1941 and 1967.

In 1941, the cost of enriching 100 pounds of flour at the mill was 17 cents; in 1967, it is two cents. In 1941, the baker could enrich 10 pounds of bread for 1 cent; in 1967, he can enrich 57 pounds of bread for 1 cent.

With these figures before us, and knowing as we do that enrichment of cereal products can be accomplished with a minimum of inconvenience, we can agree heartily with Bailey's statement.

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THE DETERMINATION OF VITAMINS AND CAROTENOIDS IN FATS

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I. Introduction

In natural fats and oils, the presence of the vitamins A, E, and D is to

be expected. In the vegetable kingdom, vitamin A as such seems not to be present, but in all vegetable oils carotenes and/or carotenoids are found. Animal fats may also contain these pigments in small quantities.

In enrichment or re-vitamination, the biologically active substances mentioned above are added to the products during processing. We are here concerned with chemically uniform substances, as opposed to vitamins which occur naturally.

The assay of the particular components of a mixture of natural vitamins is laborious and in general should be conducted in laboratories with a scientifically well-informed staff. In many cases the problem is still completely unsolved. When analysing a mixture of this kind, it is evident that the vitamin activity of the specific components of a mixture cannot be calculated from the specific activity of a standard substance according to the molecular weight; it can be determined only in animal experiments. This must be considered when compiling the results of the analysis. Table I contains a list of substances possessing vitamin activity in the A, E, and D groups which are principally expected to occur in fats and oils. As regards the most important active substances in the vitamin A, E, and D groups, Table II contains data on their biological activity relative to a standard substance.

In general, the standard preparations mentioned in Table II serve only to standardize biological methods. For chemical methods of assay, any preparation can be used which satisfies the standard analytical requirements.

When assessing the numerical values given in Table II, it is essential to note that these relate to the biological efficacy of the compounds after oral application to the test animal. Besides their biological activity, the tocopherols are of interest for their potential as antioxidants, although this does not parallel the biological activity. Moreover, the esters of the tocopherols possess no antioxidant properties, and the racemic tocopherols equal the optically uniform tocopherols in activity. In general, β- and γ-tocopherols have more pronounced antioxidant properties than α-tocopherol. They differ from substrate to substrate and must be determined specifically in each case[38, 39].

Finally, for vitamin D activity, distinction must be made between the biological activity determined in a rat test or in a chicken test. In the rat test, both vitamin D active compounds possess the same biological activity. This activity is standard for the use of compounds in human nutrition. In the chick test the biological activity of vitamin D_2 can be neglected. For the use of vitamin D in the nutrition of poultry, the result of the chicken test is standard. To distinguish, the vitamin D

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content is declared in International Units corresponding to a rat or a chick test[30] (I.U. by chick assay or I.U. by rat assay).

TABLE I

The Most Important Members of the Vitamin A, D, and E Groups

Vitamin A
all-*trans*-Vitamin A_1 alcohol, retinol[a], vitamin A
all-*trans*-Vitamin A_1 acetate
all-*trans*-Vitamin A_1 palmitate
all-*trans*-Vitamin A_1 aldehyde, retinal[a], retinene
2-*cis*-Vitamin A_1 alcohol, 13-*cis*-vitamin A_1 alcohol, neo-a-vitamin A_1 alcohol
2-*cis*-Vitamin A_1 acetate, etc
all-*trans*-Vitamin A_2 alcohol, 3',4'-dehydroretinol[a]
all-*trans*-Vitamin A_2 acetate, etc.

Vitamin E[b]
d-α-Tocopherol[a], 5,7,8-trimethyltocol
dl-α-Tocopherol[a], 5,7,8-trimethyltocol
d-α-Tocopheryl acetate[a], 5,7,8-trimethyltocyl acetate
dl-α-Tocopheryl acetate[a], 5,7,8-trimethyltocyl acetate
β-Tocopherol[a], 5,8-dimethyltocol
γ-Tocopherol[a], 7,8-dimethyltocol
δ-Tocopherol[a], 8-methyltocol
α_1-Tocopherol, α-tocotrienol, 5,7,8-trimethyltocotrien-3',7',11'-ol, ζ_1-tocopherol
δ_1-Tocopherol, δ-tocotrienol, 5,8-dimethyltocotrien-3',7',11'-ol, ε-tocopherol

Vitamin D
Vitamin D_2, ergocalciferol[a]
Vitamin D_3, cholecalciferol[a]

[a] According to the nomenclature proposed by IUPAC[32a].
[b] For nomenclature of the tocopherols, see Pennock and others[31].

As for the carotenes and carotenoids, the presence of the substances given in Table III must be expected. The analyst's task is made even more difficult in that not only pure substances are used for colouring but also plant extracts which contain carotenes and carotenoids. These extracts often cease to correspond to the natural substances, for they have been altered extensively by the extraction and working up processes. This has been investigated by McKeown[40, 41, 42] in the case of the "annatto-extracts" which are often applied industrially.

Several of the carotenes and carotenoids given possess provitamin A activity. These can, in fact, be converted partly into vitamin A in the animal body. The most important compound possessing provitamin A

TABLE II

Activity of Specific Forms of Vitamins

Vitamin A
Definition: 1 I.U. of vitamin A corresponds to the activity of 0.344 μg of all-*trans*-vitamin A_1 acetate[64a]

Substance	Activity of 1 μg (in I.U.)
all-*trans*-Vitamin A_1 alcohol	3.33
all-*trans*-Vitamin A_1 acetate	2.91
all-*trans*-Vitamin A_1 palmitate	1.82
all-*trans*-Vitamin A_1 aldehyde	3.06
2-*cis*-Vitamin A_1 alcohol	2.66
all-*trans*-Vitamin A_2 alcohol	1.33

Vitamin E
Definition: 1 I.U. of vitamin E corresponds to the activity of 1 mg of synthetic racemic vitamin E acetate

Substance	Activity of 1 mg (in I.U.)
d-α-Tocopherol	1.49
d-α-Tocopheryl acetate	1.36
dl-α-Tocopherol	1.10
dl-α-Tocopheryl acetate	1.00
d-β-Tocopherol	0.4
dl-β-Tocopherol	0.3
d-γ-Tocopherol	0.2
dl-γ-Tocopherol	0.15
d-δ-Tocopherol	0.016
dl-δ-Tocopherol	0.012

Vitamin D
Definition: 1 I.U. of vitamin D corresponds to the activity of 1 mg of a standard solution of vitamin D_3 in vegetable oil[68]

Substance	Activity of 1 μg (in I.U.)
Vitamin D_2	40
Vitamin D_3	40

TABLE III

The Most Important Carotenes and Carotenoids[a]

Source	Constituents
Palm oil	β-Carotene α-Carotene
Other vegetable oils	β-Carotene α-Carotene Cryptoxanthin Lutein Zeaxanthin Lycopene etc.
Beef tallow	β-Carotene α-Carotene
Butter	β-Carotene α-Carotene
Annatto	Labile norbixin and its decomposition products[8]
Synthetic compounds	β-Carotene β-Apo-8′-carotenal β-Apo-8′-carotenoic acid ethyl ester Canthaxanthin

[a] See refs. 40, 41, 42.

activity is β-carotene. As the amount of β-carotene which is converted into vitamin A varies under different circumstances, the relative activity of β-carotene must be determined conventionally. For this IUPAC[32] states that 1 μg of β-carotene corresponds to 1 I.U. of vitamin A. English legislation (Ministry of Food[45]) gives the following relationship for margarine: 0.75 μg of β-carotene is equivalent in activity to 1 I.U. of vitamin A. For β-carotene which is dissolved in fat and oil, Swiss legislation[46] defines the activity of 0.66 μg of β-carotene to equal 1 I.U. of vitamin A. This approaches very closely the theoretically expected relationship of 0.6 μg of β-carotene to equal 1 I.U. of vitamin A. This relationship is widely used in Germany[10]. English legislation most probably based their value on the assumption that the vitamin A titre of natural sources such as palm oil consists of the theoretically

possible vitamin A activity of pure β-carotene plus the vitamin A activity of other low-potency substances contained therein; the β-carotene conversion factor is therefore corrected accordingly.

Accepting the relationship that the activity of 0.6 μg of β-carotene is equivalent to 1 I.U. of vitamin A, the remaining carotenoids possess the following activities (Table IV).

TABLE IV

The Vitamin A Potency of Carotenes and Carotenoids

Substance	1 μg corresponds to
β-Carotene	1.66 I.U.
β-Apo-8′-carotenal	1.2 I.U.
α-Carotene	0.9 I.U.
β-Apo-8′-carotenoic acid ethyl ester	0.4 I.U.
Canthaxanthin	0
Bixin, norbixin, etc.	0

Almost nothing is known about the occurrence in fats and oils of the last fat-soluble vitamin, vitamin K_1. Furthermore, no chemical methods exist for assaying this vitamin in these substances. Hence it can be dismissed from a discussion of the specific vitamins.

Finally, two fat-soluble forms of water-soluble vitamins must still be mentioned: the fatty acid esters of ascorbic acid possess good antioxidant properties and are therefore added to oils that are sensitive to oxidation (cf. Somogyi and Kündig-Hegedüs[56]). Their vitamin C activity is equal to that of ascorbic acid on a molar basis[17]. Vitamin B_6 tripalmitate can be used for the vitamination of fats and oils. This preparation, however, is not yet widely used. As yet, there are no standard methods for analysing either compound. An assay method for ascorbyl palmitate has been published by Sedláček[54] and by Strohecker[69].

II. Isolation from Fats and Oils of Vitamins, Carotenes, and Carotenoids

In order to assay the above substances, which are nearly always present in minute quantities, they are usually concentrated and separated from substances which may interfere in the estimation. The following techniques are suitable for this purpose:

(1) saponification and isolation of the unsaponifiable material,

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(2) freezing out of the major components of fats and oils, and isolation of the substances contained in the mother liquors,

(3) partition between two solvents.

Of these, saponification is the most destructive method. When it is used, there is a risk of destroying the vitamins present. After saponification, from the total vitamins the amount of esters of vitamin C originally present, vitamin B_6, vitamin A, and vitamin E can no longer be determined.

Vitamin K is destroyed under alkaline conditions.

If vitamin D is saponified under hot conditions, it is converted to a certain extent into the previtamin D forms; with prolonged heating further decomposition products occur[63].

In the presence of catalysts, isomerization products of vitamin A active substances, carotenes, and carotenoids, may be formed.

Tocopherols, ascorbic acids, and to a lesser extent, compounds possessing vitamin A and vitamin D activity, are sensitive to oxidation under alkaline conditions. This may be prevented either by the exclusion of oxygen or by the addition of antioxidants.

A general working method cannot be given here. The procedure used must be adapted to suit the type of substrate and the vitamin to be determined.

The second technique is suitable for the isolation of esters of vitamin A and vitamin E, vitamin K, and certain carotenes and carotenoids. A working method for the isolation of vitamin K has been given by Vuilleumier, Probst, and Brubacher[65]. In this method, a suitable amount of fat is taken up in acetone and the acetone solution is cooled in a mixture of acetone and dry ice. After the majority of the material has precipitated, the mother liquor is passed through a precooled, sintered-glass filter and further processed. The working method must be modified in each case, so that the type and quantity of the solvent and the temperature of freezing out can be varied.

The third technique is suitable for the isolation of ascorbic acid esters[51] and for canthaxanthin[65], for example. In this method, the fat or oil is dissolved in petrol ether and is extracted with aqueous methanol or ethanol. The substance to be assayed goes into the alcohol phase. Subsequently, the concentrated fractions are mostly purified by column chromatography. For details about the chromatographic equipment and the apparatus used for evaporation, see Vuilleumier and others[65]. In many cases, the isolated material can be directly subjected to thin-layer chromatography, at least where only a semi-quantitative assessment is required.

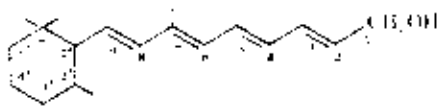
As all fat-soluble vitamins are sensitive to light, their isolation and subsequent assay must be conducted under exclusion of the respective radiation. Suitable measures for this are discussed in the article by Vuilleumier and others[65], mentioned above.

III. Special Methods

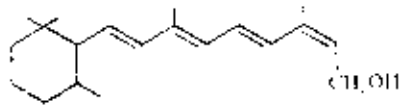
1. VITAMIN A

A. Chemistry

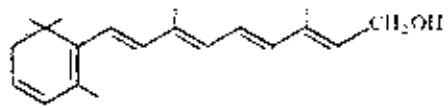
The formulae of the most important molecules with vitamin A activity are the following:



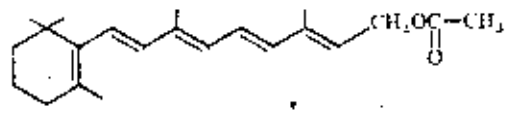
all-*trans*-Vitamin A_1 alcohol



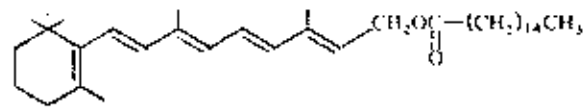
2-*cis*-Vitamin A_1 alcohol (neo-a-vitamin A_1)



all-*trans*-Vitamin A_2 alcohol

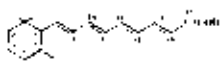


all-*trans*-Vitamin A_1 acetate



all-*trans*-Vitamin A_1 palmitate

In carotene chemistry of the following numbering system is also used:



B. Assay of Vitamin A in Oily Concentrates[31]

Directions for the assay of vitamin A in oily concentrates were published by IUPAC in 1959, based on an investigation in which thirty-seven laboratories participated[15]. In this method, the oily concentrates are saponified and the unsaponifiable fraction is isolated. The unsaponifiable fraction is then dissolved in propan-2-ol and the u.v. absorption spectrum of this solution is determined. Depending upon the result of this measurement, the oily concentrates are separated into two categories. The first category consists of test samples whose maximum absorption lies between 323 nm and 327 nm, whereby the ratio of the extinctions measured at 300 nm and 325 nm should not exceed a value of 0.73. All samples which fail to fulfil these requirements are placed in the second category.

With samples of the first category, measurements are made at 310, 325, and 334 nm and a correction is made according to the Morton–Stubbs principle[46]. Samples in the second category are purified chromatographically and the purified samples are then tested to determine whether the requirements for the first category are now fulfilled. If so, the purified samples are assayed spectrophotometrically; if not, the samples must be further purified.

In order to calculate the content of vitamin A, the $E^{1\%}_{1cm}$ value measured at 325 nm, with adequate correction, is multiplied by 1830. A value for the vitamin A content is thus obtained in International Units (I.U.) per gram of the material investigated. The factor 1830 corresponds to the value determined by a number of collaborating investigators in 1951[9] for pure all-*trans*-vitamin A alcohol in propan-2-ol. The assay is conducted as follows:

(1) *Samples in Category* 1

Apparatus. A precision spectrophotometer is required whose wavelength scale can be calibrated. Mercury lines of 302.25 nm, 313.16 nm, and 334.15 nm are suitable for calibrating the instrument. The calibration is indispensable, since in order to make the correction according to the principle of Morton and Stubbs[46] measurements must be made in the steep sloping parts of the vitamin A absorption curve. Hence a slight mistake in the regulation of the wavelength results in a large error when the respective extinction is determined.

Reagents. Ethanol: 95 per cent (v.v).

Propan-2-ol (isopropanol): this must fulfil the following criteria of spectral purity: when measured in a 1 cm quartz cuvette against distilled water, the extinction may not exceed 0.01 between 350 and 320 nm, or 0.05 at

300 nm. *Diethyl ether:* this must be free of peroxides and stabilizers. The commercial ether can be purified by distillation over pellets of potassium hydroxide, after which the distillate is shaken with a 10 per cent solution of ferrous sulphate and allowed to stand over this solution. Before use, the ether is separated and redistilled over potassium hydroxide pellets.

Potassium hydroxide solution: 20 g potassium hydroxide (85 per cent pure, analytical grade) is dissolved in distilled water allowed to cool to room temperature, and then made up to 40 ml with distilled water.

Phenolphthalein solution: 1 per cent solution in 95 per cent ethanol.

Procedure. All the operations to be described should be conducted on the same day and the fractions containing vitamin A should not be exposed directly to light.

Preparation of the unsaponifiable fraction (according to the directions given in U.S.P. XV[62]): An exactly weighed amount of fat or oil which should not exceed 1 g and should contain at least 500 I.U. of vitamin A, is placed in a saponification flask. This is heated under reflux with 30 ml of ethanol in an all-glass apparatus until the alcohol boils, and 3 ml of a 50 per cent potassium hydroxide solution is added to the boiling solution through the reflux condenser. After being allowed to saponify for thirty minutes under reflux, the solution is brought to room temperature and mixed with 30 ml of water. The solution is then transferred into a pear-shaped (Squibb-type) separating funnel and extracted four times each with 30 ml of diethyl ether. After combining the ether extracts, these are washed carefully with 50 ml of distilled water. They are washed for a second and a third time with vigorous shaking until the water no longer gives a colouration with phenolphthalein solution. After these washing operations the phases should separate cleanly. When the separation is clean, the lower phase appears brilliantly clear. The washed ether extract is then transferred into a 250 ml calibrated flask and brought up to volume with diethyl ether.

Spectrophotometric measurement: An aliquot of the ether solution of the unsaponifiable fraction is concentrated in a stream of inert gas or in a vacuum. Immediately after removal of the solvent, propan-2-ol is added, the solution is transferred to a calibrated flask, and brought up to volume. The calibrated flask should have about 10 I.U./ml.

The extinction of the solution is now measured at 300, 310, 323, 325, 327, and 334 nm and tested to determine whether the criteria of samples of the first category are fulfilled.

Calculation of the vitamin A content: From the values of the extinctions measured at 310, 325, and 334 nm, the corrected value for the

extinction at 323 nm is calculated according to the following formula:

$$E_{323}\,(\text{corrected}) = 6.815\,E_{325} - 2.555\,E_{310} - 4.260\,E_{331}$$

The corrected $E^{1\%}_{1cm}$ value ($E^{1\%}_{1cm,325}$(corrected)) is calculated from the values of the weight of the sample, the dilutions, and the corrected extinction (E_{325} (corrected)) and, when multiplied by 1830, gives the content of vitamin A in I.U./g.

If the uncorrected $E^{1\%}_{1cm}$ value does not deviate more than ±3 per cent from the corrected $E^{1\%}_{1cm}$ value, the uncorrected value should be used to calculate the vitamin A content. If the corrected value is greater than 1.03 times the uncorrected value, the corrected value must be redetermined. When the discrepancy occurs again, the Morton–Stubbs correction cannot be used and the fat or oil in question cannot be analysed by the present method.

Limits of error: The limits within which the results lie with a probability of 95 per cent, determined by different laboratories for the same sample, is about ±8 per cent of the average value found for the sample.

Comments. Using the correction according to the principle of Morton and Stubbs[48], it is assumed that the irrelevant absorption runs linearly in the region between 310 and 334 nm. This hypothesis has been made by Morton and Stubbs in their investigation of cod liver oils. However, it may not be justified for other materials and should be checked for each specific case. Actually, if the material under investigation contains *cis–trans* isomers of vitamin A_1 alcohol, vitamin A_2 alcohol or its *cis–trans* isomers, the deviations from the actual vitamin A content cannot be eliminated by the Morton–Stubbs correction. When the vitamin A content is calculated by using a conversion factor of 1830, it may appear too high because of the presence of other substances which possess vitamin A activity. See examples in Table V.

Table V contains the data relevant to ethanolic solutions of different substances with vitamin A activity. The data relevant to isopropanolic solutions are basically the same.

The condition that the absorption maximum of the mixture to be analysed must lie between 323 and 327 nm harbours the danger that significant amounts, at least of compounds from the vitamin A_2 group, are not included in the assay.

(2) *Samples in Category 2*

Apparatus. In addition to a precision spectrophotometer having the specifications described in the previous section (p. 615), equipment for

TABLE V

$E^{1\%}_{1cm,325}$ Values, Biological Activity, and Conversion Factors, for all-*trans*-Vitamin A_1 Alcohol, 2-*cis*-Vitamin A_1 Alcohol, and all-*trans*-Vitamin A_2 Alcohol (Solvent Ethanol)

	$E^{1\%}_{1cm,325}$	Biological activity I.U./µg	Conversion factor
all-*trans*-Vitamin A_1 alcohol	1800[49]	3,330,000[a]	1850[50]
2-*cis*-Vitamin A_1 alcohol	1685[50]	2,660,000[a]	1579[50]
Vitamin A_2 alcohol	1060[50]	1,330,000[a]	1255[49]

[a] By definition.

column chromatography is required. Any apparatus is suitable which allows chromatography under pressure. The column should be 240 mm high and 5 mm wide (i.d.).

The chromatography can be conducted as follows: Small fractions are collected in test-tubes as described in Fig. 1. Aliquots of these fractions are used for checking vitamin A by the Carr–Price reaction. A second rather more difficult method is to follow the course of the chromatography by fluorescence. A Philips 125 watt mercury vapour lamp with a Wood's glass bulb and a housing fitted with Schott light filters Bg 12, Wg 2 (each 2 mm thick), and Ug 2 (1 mm thick) is suitable

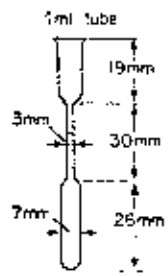


Fig. 1. Collecting vessel for vitamin A analysis according to Boldingh and others[9].

for providing the fluorescent excitation of the vitamin A. Fig. 2 presents an adequate device[18].

Reagents. Ethanol: 95 per cent (v/v).

Absolute ethanol: not less than 99.5 per cent of ethanol (v/v).

Benzene: (analytical grade). For the analysis, the benzene must be redistilled.

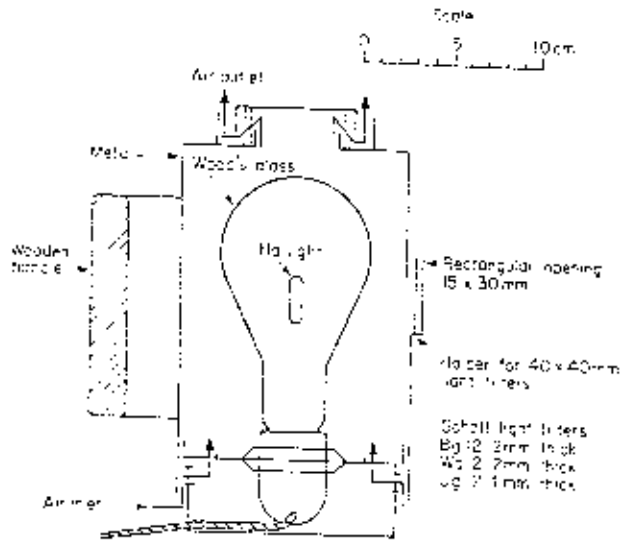


Fig. 2. Lamp house for mercury vapour lamp according to Brunius [18].

Propan-2-ol: according to the specifications for samples of the first category (see p. 615).

Diethyl ether: (*a*) for the extraction: according to the specifications for samples of the first category (see p. 616); (*b*) for the chromatography: the diethyl ether under (*a*) is treated with anhydrous sodium sulphate (analytical grade) to remove the moisture, and redistilled over potassium hydroxide pellets immediately before use.

Light petroleum: boiling range 40–60°C. It is advisable to use light petroleum which is suitable for spectroscopy. Normal light petroleum can be purified by percolation through norite or silica gel. Immediately before use it must be distilled once more.

Potassium hydroxide solution: 50 per cent, according to the specifications for samples of the first category (see p. 616).

Phenolphthalein solution: 1 per cent solution in 95 per cent ethanol.

Antimony(III)chloride solution: 20 g of crystalline antimony(III)chloride is dissolved in chloroform so that the total volume is 100 ml, and the solution filtered, if necessary.

Alumina: crystalline alumina trihydrate produced by the Bayer process is suitable.* Not more than 25 per cent of this alumina should pass

* Obtainable, for example, through The British Aluminium Company Ltd., Norfolk House, St. James's Square, London, S.W.1., under the name Dried Hydrate of Alumina. Special Product 901.

through a sieve of 300–325 mesh (opening: 0.05 mm). The alumina is sieved through 150 mesh (opening: 0.1 mm) and the fraction that has passed through the sieve is activated by heating uniformly in a furnace at 800 ± 20°C for at least five hours.

The alumina is allowed to cool in an empty desiccator. A certain quantity of water is added, and the alumina is either ground with the water in a mortar or shaken up with the water in a flask closed with a glass stopper. The mixture is then allowed to stand for at least 24 hours in a tightly closed container. Immediately before use, the alumina is shaken. The amount of water used for deactivation of the alumina should be such that the vitamin A is eluted by 9–15 ml of a mixture of light petroleum and diethyl ether (80:20–76:24, v/v). If 15 ml of this mixture does not suffice to elute the vitamin A, more water must be used for deactivation of the alumina; if the vitamin A is eluted by less than 9 ml, too much water has been used for deactivation. Measured by means of a loss in weight upon drying at 800°C, a water content of 4–5 per cent seems adequate.

When a suitable alumina is prepared, it can be kept in an atmosphere of constant humidity such as over a saturated solution of ammonium chloride.

Finally, all commercial forms of alumina can be used providing the above specifications are fulfilled and it is non-acidic.

Examples of suitable commercial products are:

Alumina Brockmann; 1st activation stage
E. Merck A.G., Darmstadt, Germany.

Alumina Camac 507 C
Chemie-Erzeugnisse und Adsorptionstechnik, Muttenz, Switzerland.

Alumina Giulini; 1st activation stage
Gebrüder Giulini GmbH, Ludwigshafen am Rhein, Germany.

Alumina Woelm neutral
Woelm, Eschewege, Germany (distributed in America by Alupharm Chemicals, Box 755, New Orleans, La., U.S.A.).

If necessary, before deactivation these products must be passed through a 150 mesh sieve or heated to 800°C.

Procedure. An extract of the unsaponifiable fraction is prepared as previously described (p. 616) and chromatographed over alumina using one of the two techniques mentioned (see under "Apparatus"). The technique of observing the chromatographic separation under u.v. light is simpler and quicker to conduct, but harbours the danger that if the assay is only carried out occasionally, without having been practised

extensively, the vitamin A zone may be exposed to u.v. light for too long a period causing a certain amount of the vitamin to be destroyed. In particular, the following procedure is advised.

An aliquot of the ether extract of the unsaponifiable fraction is evaporated to dryness either in a stream of inert gas or *in vacuo*. A few mls of absolute ethanol or a mixture of equal volumes of absolute ethanol and benzene are then added immediately and the solvent is again removed as described above. By this method, the last traces of moisture can be removed. If necessary, the procedure must be repeated several times. A few mls of light petroleum are then added and also evaporated in order to remove the last traces of alcohol. This is repeated. The residue is then dissolved in light petroleum and, after transferring the solution to a calibrated flask, diluted with light petroleum, so that the solution contains between 100–200 I.U. of vitamin A/ml.

The chromatographic tube is now filled, first by adding light petroleum and then alumina. By applying a slight pressure, the excess solvent is removed until the surface of the alumina is just covered with the solvent. 2 ml of the extract containing vitamin A is then added at the top of the column and allowed to infiltrate under pressure. Elution is then conducted first with 5 ml of light petroleum containing 4 per cent (v/v) of diethyl ether, then with 5 ml of light petroleum containing 8 per cent (v/v) of diethyl ether, and so forth with 5 ml aliquots of light petroleum containing 12, 16, 20, 24, 28, 32, and 36 ml of diethyl ether, respectively.

If the Carr–Price reaction is used to follow the course of the chromatography, the eluate is collected in 1 ml fractions as soon as the elution mixture contains 16 per cent (v/v) diethyl ether. Alternate fractions are mixed thoroughly by blowing a current of air through them, and then about 0.2 ml of this mixture is pipetted off. To this, ten drops of antimony(III)chloride solution are added and the presence of vitamin A is indicated by the resulting blue colouration. In order to determine the limiting fractions exactly, the respective fractions are still further tested. Then after a thorough mixing (by blowing through air), exactly 0.5 ml of each is removed and transferred into a suitable round-bottomed flask.

If u.v. light is used to follow the course of the chromatography, the column is illuminated with the u.v. lamp for a short period as soon as the eluant mixture containing 16 per cent (v/v) diethyl ether is used, and the position of the zone containing vitamin A is determined. Chromatography is then carried out with the further eluant mixtures

and from time to time the u.v. light is switched on for the least possible duration. As soon as the base of the vitamin A fraction is 2 cm above the tip of the column, the receiver is changed and chromatography is conducted carefully until the entire fraction containing vitamin A has been eluted. The receiver must be covered with black paper to protect it from ultraviolet radiation.

The content of vitamin A is assayed as for samples of the first category; the eluate containing vitamin A is evaporated to dryness and then mixed with propan-2-ol. In calculating the result, it must be noted that in samples analysed by the first chromatographic technique, only half of the eluate is further analysed.

Limits of error: the range within which the results lie with a probability of 95 per cent, determined by different laboratories for the same sample, is about ± 12 of the average value found for the sample.

Comments. The method proposed by IUPAC[31] cannot be used in the following cases: to assay samples which, after chromatography, fail to fulfil the criteria required for inclusion in the first category; samples whose extinction value at 325 nm, corrected with the Morton Stubbs principle, exceeds 1.03 times the uncorrected extinction value; or samples whose content of vitamin A is less than 500 I.U./g. In these cases either the spectrophotometric method must be discarded and replaced by another principle or the chromatographic purification must be extended so that after chromatography on alumina a second chromatography is conducted, or that other adsorbents are chosen.

C. Other Principles to Assay Vitamin A

(1) *Carr–Price Reaction*

Antimony(III)chloride reacts with solutions containing vitamin A under formation of intensely blue compounds. The *Schweizerisches Lebensmittelbuch*[33] recommends this method of estimating vitamin A in the section on the assay of vitamins in foods (Hausheer and others[28]). The reaction is specific to the extent that only compounds having a system of at least three conjugated double bonds react with the Carr–Price reagent. In addition to members of the vitamin A group, such systems occur in carotenes, carotenoids, and members of the vitamin D group. These compounds therefore must be eliminated by chromatography. However, the chromatography need not be so rigorously conducted as in the spectrophotometric method of determining unchanged vitamin A. A suitable method for the assay of vitamin A in margarine and fresh butter is given below (p. 626).

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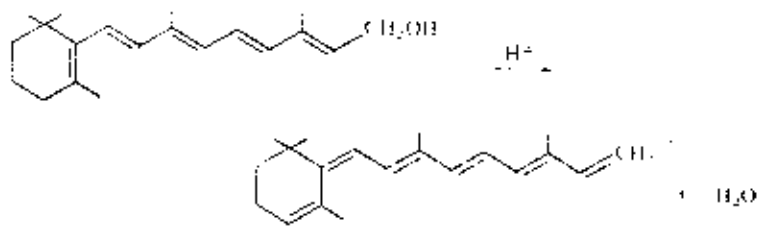
(2) *Dehydration According to Budowski and Bondi*

A second direct method of spectrophotometric assay is given by Budowski and Bondi[9]. The method is based upon the conversion of vitamin A_1 alcohol by acid catalysts into anhydrovitamin A_1:



Anhydrovitamin A_1 shows a characteristic absorption spectrum, with absorption maxima at 377 and 399 nm. The maximum at 399 nm, in fact, is suitable for the spectrophotometric assay because at this wavelength no absorption interference occurs which would not be eliminated by the preparation of a suitable control value.

Description of the method for products containing high or low concentration of vitamin A

Apparatus. A spectrophotometer having a monochromator or filter which allows measurements at 399 nm.

Reagents. Benzene: analytical grade, completely anhydrous, freshly distilled.

Aqueous sodium hydroxide: 0.5*N*.

p-*Toluenesulphonic acid:* 15 mg of *p*-toluenesulphonic acid monohydrate is heated under reflux with 100 ml of benzene until it is completely dissolved. Then 10 ml of solvent is boiled off in order to remove all the moisture. The solution is allowed to cool in a moisture-free atmosphere, and made up to 100 ml by adding dry benzene. Each time before use, 10 per cent of the solvent must be distilled off and, after cooling, replaced by dry benzene. The glass apparatus used to prepare the reagents must be scrupulously dry.

Procedure. The unsaponifiable fraction of a fat or oil is isolated by the method described on p. 616. However, any standard method may be used which is suitable for the isolation of vitamin A. The unsaponifiable fraction has then to be carefully freed from traces of moisture as described on p. 621 and dissolved in dry benzene so that 1 ml of the solution contains between 5 and 50 I.U. of vitamin A. Two millilitres of this benzene solution are transferred into a 10 ml calibrated flask.

The flask is then filled to the mark with the *p*-toluenesulphonic acid reagent and the contents are immediately mixed thoroughly. After one minute, the contents are transferred to a separating funnel which is then filled with 10 ml of 0.5*N* sodium hydroxide. The mixture is shaken for one minute and then the benzene phase is centrifuged in order to separate the aqueous phase completely.

The extinction of this solution is now measured at 399 nm; a blank is used consisting of one part by volume of the benzene solution of the unsaponifiable fraction and four parts by volume of dry benzene.

In order to calculate the content of vitamin A, it is advisable to use a U.S.P. standard preparation and to saponify different quantities of this by the same technique and to convert them into anhydrovitamin A. The extinction values obtained serve to construct a standard curve; this should be a straight line and should pass through the zero point of the coordinate system. With strict adherence to the working directions given by Budowski and Bondi, the content of vitamin A per ml of benzene solution can be calculated from the formula

$$\text{vitamin A content (I.U./ml)} = \frac{E_{399}}{0.0122}$$

E_{399} indicates the extinction measured at 399 nm against the respective blank value, and 0.0122 corresponds to the increase in the extinction for 1 I.U./ml. It should be kept in mind that the amount of vitamin A converted into anhydrovitamin A depends on the reaction time and on the other conditions. Great care must be taken that all glass vessels which contact the catalyst and the reaction mixture are absolutely dry, otherwise the velocity of the reaction will be greatly reduced.

If this method is used to determine vitamin A, interference from decomposition products (such as occur by autoxidation of vitamin A palmitate) is only slight, so that even in the presence of such substances an assay is possible without the need for chromatographic separation. The method is slightly more sensitive than the spectrophotometric method of determination of unchanged vitamin A at 325 nm and slightly less sensitive than the Carr–Price reaction. Systematic investigations have not yet been conducted on the behaviour of isomers of vitamin A_1 alcohol, vitamin A_2 alcohol or its isomers, but it is expected that all these compounds react positively to the catalyst.

The presence of small amounts of carotenoids does not introduce a significant systematic error. However, if an unfavourable ratio exists between the carotenoid content, a marked error can occur because carotenoids can likewise be converted by acid catalysts.

D. Purification of Vitamin A Fractions by Chromatographic Procedures

For purification by chromatography, in addition to approximately neutral alumina as described before, alkaline aluminium oxide, secondary calcium phosphate, and magnesia may also be used as adsorbents. Finally, the purification of fractions containing vitamin A can be conducted with the aid of partition-chromatographic techniques.

Chromatography on secondary calcium phosphate is especially suitable for the separation of 2-*cis*-vitamin A alcohol from all-*trans*-vitamin A, and for the separate determination of both vitamin A active components (see also Bro-Rasmussen, Hjarde, and Porotnikoff[15]; Barnholdt and Hjarde[7]; and Vuilleumier, Probst, and Brubacher[60]).

Boldingh and Drost[19] purify the fraction containing vitamin A using first a column which contains almost neutral alumina and then using a second column filled with alkaline alumina. This is prepared in the following way: a crystalline alumina trihydrate (see p. 619) is activated for 6 hours at 750–800°C and passed through a 180 mesh sieve. The part which has passed through the sieve is then deactivated with 2 per cent water. Equal parts of this alumina and a 10 per cent solution of sodium carbonate are mixed and allowed to stand for one hour at room temperature. The mixture is then dried in a vacuum oven, first at a temperature of 30–40°C until most of the water has evaporated, and then at 100°C (15–20 mm Hg). Twenty-gram portions are decanted off into bottles which are closed in such a way that no moisture can enter. A special column is used for the chromatography which allows both adsorbents to be used in the same column.

This method is officially used in a modified form in the United Kingdom for margarine[45]. However, it is not contained in the collection of methods of the Analytical Methods Committee[35]. Proposals for the requisite chromatographic equipment are given by Turner[61] and the Analytical Methods Committee[3]. Also, the AOAC[4] proposes for the determination of vitamin A in margarine a combination of chromatography on neutral and basic alumina. If the eluate containing vitamin A also contains carotenoids, it is further purified over magnesium oxide.

Better separations than the named adsorption-chromatographic procedures are attained by partition-chromatographic techniques. Such a technique was perfected by Wiss and Gloor[62], who used a solution of chloroform in heptane as stationary phase, a mixture of propan-2-ol and water as mobile phase, and Hostalen W* as carrier. Wilkie and others[64] used isooctane as mobile phase, polyethylene

* Obtainable from Farbwerke Hoechst A.G., Frankfurt am Main.

glycol 600* as stationary phase, and Celite 545† as carrier material. Similar directions are given by Härtel[27]. With a technique of this type, it is possible to separate not only vitamin A from other components such as vitamin D, vitamin E, and vitamin K, but also to separate the fraction containing vitamin A active substances into its components. However, these techniques are generally too difficult for routine use. It is to be expected that it will be possible to standardize partition-chromatographic techniques in such a way that they can be successfully used for routine analysis (see also ref. 43).

E. Determination of Vitamin A in Butter and Margarine

If butter or margarine is analysed by the method of AOAC[4], whereby the product is saponified wet and then chromatographed over neutral alumina, it is sometimes difficult to obtain a fraction which is sufficiently pure for spectrophotometric determination. If, despite this, the vitamin A content is determined on the basis of the extinction measured at 325 nm, the result may be expected to deviate considerably from the actual content[43].

On this basis, with equal justification, an assay can be made using the Carr–Price method. This assay is more specific than the assay using the extinction measured at 325 nm. The chromatographic purification of the extract may thus be simplified. Positive discrepancies are to be expected in the presence of *cis-trans* isomers of vitamin A, of large amounts of decomposition products of vitamin A, and of large amounts of carotenoids, e.g. cryptoxanthin, which are difficult to separate from vitamin A. The latter can be partly eliminated by the use of a correction value for yellow pigment (see also, for example, ref. 4).

Moreover, the extraction of the saponified margarine or butter sample according to the method of AOAC offers considerable difficulties because of the formation of emulsions. An improved method, which is suitable for routine assay, was therefore offered by Deutsch and others[22].

In the following, a modified method is described which has proved suitable for routine laboratory use (see also ref. 65).

Apparatus. For carrying out the Carr–Price reaction in the given manner of working, a spectrophotometer is required which allows the extinction to be measured within 5 seconds after addition of the Carr–Price reagent at 610–620 nm. Suitable, for example, are the Lumetron, Model 401 A, of the Photovolt Corporation, New York 16; the Beckmann Spectrophotometer, Model B, with test-tube holder, and the

* Obtainable, e.g., from Carbide and Carbon Chem. Co.
† To be obtained from Johns-Manville Products Corp. New York 16.

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Spectronic 20 of Bausch and Lomb Optical Co., Rochester 2, New York, U.S.A.

Moffit and others[46] cite a device which permits the extinction to be registered automatically.

In addition to the normal laboratory glassware, a chromatographic apparatus (as in Fig. 3) is needed, a rotatory evaporator as shown in Fig. 4 (obtainable from W. Büchi, Flawil, Switzerland), or a similar model and a burette for the Carr Price reagent (Fig. 5).

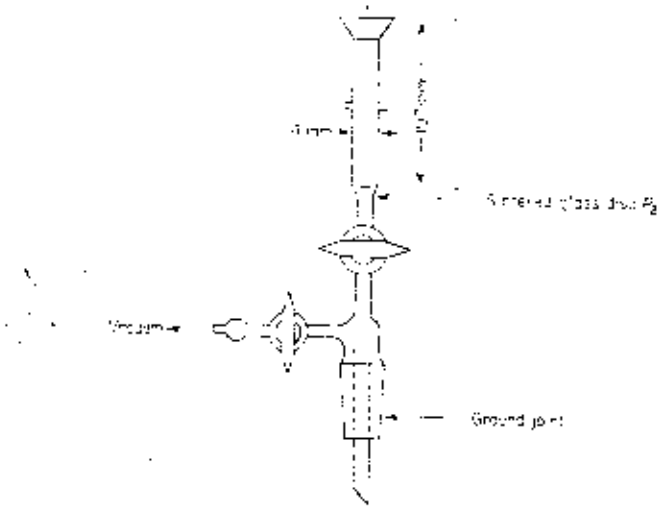

Fig. 3. Chromatographic column.

Reagents. Diethyl ether, free of peroxides and dry.
Benzine: boiling range 90–115°C, freshly distilled.
Eluant mixture: 15 parts diethyl ether + 85 parts benzine (b.p. 90–115°C).
Chloroform: analytical grade.
Alumina: 50 g of highly activated alumina (e.g. one of the qualities given on p. 620) is mixed in an Erlenmeyer flask having a ground-glass stopper with 6 ml of distilled water. The stopper is immediately inserted and the contents are shaken until all the lumps have disintegrated. It must be allowed to stand for at least an hour at room temperature before use. To test the activity, a chromatography is conducted with 100–200 I.U. of pure vitamin A alcohol as described later. The total quantity must be eluted with 50 ml of eluant mixture.
Ethanol: at least 95 per cent (v/v).

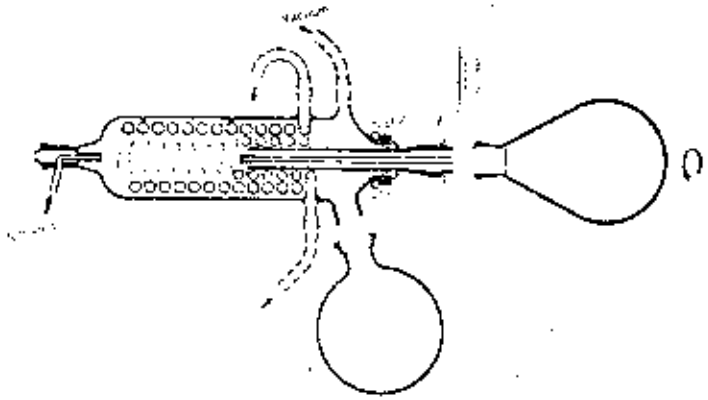

Fig. 4. Rotatory evaporator (Rotavapor R., W. Büchi, Flawil SG, Switzerland).

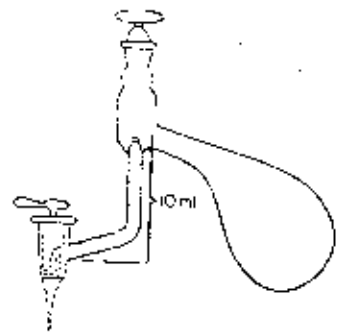

Fig. 5. Burette for antimony(III)chloride reagent.

Ethanolic sodium hydroxide: mix immediately before use: 10 ml of 30 per cent sodium hydroxide with 90 ml of ethanol and 200 mg of *p*-aminophenol.
Maize starch: before use, put pure maize starch in a desiccator; after evacuating the desiccator, fill it with nitrogen, and repeat this at least once.
Antimony(III)chloride solution: 22 g of antimony(III)chloride is dissolved by heating in 100 ml of chloroform. After cooling, it is filtered over highly active neutral alumina into a bottle of dark glass. It is kept over alumina and filtered shortly before use.

Procedure. 2 ml of butter or margarine is melted and taken up in 23 ml of a mixture of 85 parts benzine (b.p. 90–115°C) and 15 parts diethyl ether.

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This solution is filtered over a 4–5 cm high column of alumina followed by rinsing with a further 50 ml of benzine–diethyl ether mixture. The filtrate is concentrated in a rotatory evaporator under vacuum. The residue is brought into a crystallizing dish (diameter: 8 cm; rim height: 4 cm) with about 30 ml of diethyl ether. After mixing with 15 ml of ethanolic sodium hydroxide, several quartz boiling stones are added. The crystallizing dish is placed in a waterbath at 80 °C, so that it stands on the base and does not float in the water. The soap solution is allowed to concentrate to a syrupy consistency (at least 10 minutes). During this time, a 10 ml measuring cylinder is filled with maize starch up to the 8 ml mark. After the soap solution has sufficiently thickened, the maize starch is added. The soap residue and the starch are processed with a spatula to a mass that is as homogenous as possible. The starch adsorbate is once again placed in the waterbath until the odour of alcohol is no longer detectable (maximum: 20 minutes). The resulting lumps are ground with the spatula to a fine powder and added quantitatively to a centrifuge tube having a ground-glass stopper (content about 40 ml).

The contents are mixed with 25 ml of diethyl ether and shaken for 1 minute. After centrifuging, the ether solution is decanted into a 250 ml evaporation flask, and the extraction with 25 ml of fresh diethyl ether each time is repeated three times. The combined ether extracts are concentrated *in vacuo* and taken up in 10 ml of benzine (90–115 °C).

In a chromatographic column (see Fig. 3) a 10 cm high alumina layer is moistened with benzine so that the alumina is covered with the liquid for about 1–2 cm. The residue taken up in benzine is added to the column. It is now precluted under vacuum with 40 ml of benzine. This eluate is discarded. After changing the collecting dish, the vitamin A is eluted under vacuum with 50 ml of the eluant mixture of benzine and diethyl ether. The eluate is concentrated to dryness at 50 °C in a rotatory evaporator (see p. 628). The residue is taken up in chloroform such that 5–15 I.U. of vitamin A are contained in 2 ml of solution.

Two millilitres of the chloroform solution are placed in a test-tube, which can be used as a cuvette in the respective spectrophotometer. Out of a burette (Fig. 5), 10 ml of the antimony(III)chloride solution is rapidly added and the corresponding blue colour is measured at 620 nm within 5 seconds. A mixture of 10 ml of the reagent and 2 ml of chloroform is used as a blank.

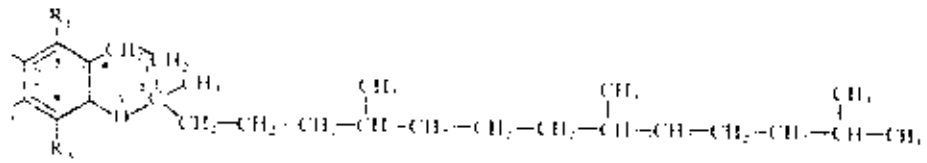
The evaluation occurs using a calibration curve or with the aid of an internal standard.

2. VITAMIN E

A. Chemistry

Naturally occurring tocopherols belong to the tocol series or to the tocotrienol series.

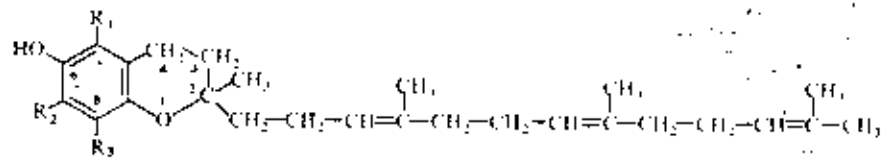
The general formula of the tocols is the following:



Examples are

α-tocopherol (5,7,8-trimethyltocol); $R_1 = R_2 = R_3 = CH_3$
β-tocopherol (5,8-dimethyltocol); $R_2 = H$; $R_1 = R_3 = CH_3$
γ-tocopherol (7,8-dimethyltocol); $R_1 = H$; $R_2 = R_3 = CH_3$
δ-tocopherol (8-methyltocol); $R_1 = R_2 = H$; $R_3 = CH_3$

The general formula of the tocotrienols is the following:



Examples are

ζ-tocopherol (5,7,8-trimethyltocotrienol): $R_1 = R_2 = R_3 = CH_3$
ε-tocopherol (5,8-dimethyltocotrienol); $R_2 = H$; $R_1 = R_3 = CH_3$

All tocopherols may be in the form of esters. The most common of these is the acetate.

B. General Remarks

The analysis of vitamin E in fats and oils takes a different form according to the problem in question.

The actual physiological vitamin E activity of a fat or oil may be sought. This determination can only be seriously conducted with the aid of animal experiments. In such animal experiments, it must be ensured that the vitamin E requirement of the experimental animals is not varied by the type and amount of the free and bound fatty acids

present in the fat. The biological testing should therefore be conducted with the aid of the unsaponifiable fraction of a fat or oil. From Table II, it can be seen that α-tocopherol and its derivatives are by far the most important compounds with regard to biological activity. It is therefore justifiable, in place of the total vitamin E activity, to determine only the part caused by α-tocopherol. Such a method has the advantage that the analysis can be conducted chemically, that it is reproducible, and that it can be performed in a routine fashion.

As regards the question of the biological vitamin E activity of an oil or fat, it may be of interest to know which part of the activity emanates from free tocopherols or from added α-tocopherol esters or from both. From a physiological and nutritional point of view the esters of α-tocopherol, i.e. essentially α-tocopheryl acetate, are much more important than free α-tocopherol. The latter can be destroyed either during storage, during processing, or in the gastrointestinal tract, so that its vitamin E activity is lost. α-Tocopheryl acetate is stable under most conditions. Ames and Tinkler[1] worked out a method for the determination of tocopheryl acetate in feedstuffs. It has not yet been determined, to what extent this method can be used for fats and oils enriched with α-tocopheryl acetate; it is possible that it can be used after freezing out the major part of the neutral fats (see p. 645).

If, however, the antioxidant properties and not the physiological activity of vitamin E are being discussed, the tocopherols must be separated and determined singly. Jolly[33] has described a suitable method. See also the Analytical Methods Committee[6]. When using this method, the tocopherols must be isolated after saponification. Any α-tocopheryl acetate which is present cannot therefore be differentiated from free tocopherol, whose antioxidant potential is therefore overestimated.

If the tocopherols are determined together without being previously saponified, as Mehlenbacher[44] proposes, this error is indeed avoided. However, the presence of interfering substances even with this method leads mostly to a positive systematic error. In spite of this disadvantage, such a method is well suited for the characterization of fats and oils; only in this case the expression "total tocopherols" should be avoided and should be replaced by the expression "total Emmerie–Engel positive substances".

C. *Assay of Total α-Tocopherol*

Vuilleumier, Brubacher, and Kalivoda[64] have described a method of analysis which takes the following stages:

(*a*) saponification of the sample with the addition of antioxidants.

(*b*) chromatography of the unsaponifiable fraction on alumina, using β-apo-8′-carotenal as monitoring pigment.

(*c*) removal of β-apo-8′-carotenal and other disturbing substances with the aid of Fuller's earth.

(*d*) assay of α-tocopherol by the Emmerie–Engel method.

(*e*) thin-layer chromatographic control.

Apparatus. For conducting the analysis, in addition to the usual laboratory material, chromatographic tubes of the size 21 × 300 mm and 18 × 300 mm are required. Also a photoelectric instrument which allows the extinction of a solution at 520 nm to be determined. For this, instruments are suitable which have a filter or monochromator. Finally, the usual equipment for thin-layer chromatography is necessary.

Reagents. Ascorbic acid solution: dissolve 3 g of ascorbic acid in 94 per cent methanol and make up to 100 ml.
Absolute methanol: analytical grade.
Absolute ethanol: analytical grade.
Saturated potassium hydroxide solution: mix about 12 g of KOH with 8 ml of water and warm slightly; allow the undissolved solid to settle and to form a layer at the bottom.
Diethyl ether: peroxide free, see p. 616.
Benzine: medium boiling range (60–90°C).
Benzene: analytical grade.
Chloroform: analytical grade, stabilized with ethanol.
Trichloroethylene.
Eluant mixture I: 60 vol. benzine 60–90°C + 40 vol. benzene.
Eluant mixture II: 85 vol. benzine 60–90°C + 15 vol. diethyl ether.
β-Apo-8′-carotenal solution: dissolve about 5 mg of β-apo-8′-carotenal in 0.5 ml of chloroform and dilute with benzine (60–90°C) to 50 ml (store in a refrigerator).
Iron(III)chloride solution: dissolve 0.2 g of $FeCl_3 \cdot 6H_2O$ analytical grade in 100 ml of absolute ethanol.
α,α′-Dipyridyl solution: dissolve 0.5 g of α,α′-dipyridyl in 100 ml of absolute ethanol.
Concentrated hydrochloric acid: analytical grade.
Tin(II)chloride: analytical grade.
Filter aid (Celite, Dicalite-Speedex).
Silica gel G for thin-layer chromatography after Stahl (Merck 7731).
Silica gel for chromatography: mix 500 g of silica gel 0.05–0.2 mm (Merck 7734) or silica gel G (Merck 7731) thoroughly by stirring with 1 litre of 3N hydrochloric acid, and allow to stand for 15 minutes. Suck the acid through a large sintered-glass filter of porosity 3, and wash the

residue afterwards with water. Resuspend it in water and refilter until the water used for washing is free of chloride, i.e. until at the most a light cloudiness results from the addition of silver nitrate. Dry the purified silica gel overnight at 120°C.

Alumina, deactivated: mix 100 g of alumina with 12 ml of water in a 250 ml flask with a ground-glass stopper. Seal immediately and shake until all the lumps have disintegrated. Keep the deactivated alumina well sealed for at least an hour before use. (Alumina Merck 977 or a type of equal value is suitable for the preparation; neutral products of activation stage I are also suitable.)

Fuller's earth XS for the analysis of vitamin E (British Drug Houses). For immediate use, the following procedure is conducted: suspend 8 g of Fuller's earth and 0.8 g of tin(II)chloride in 20 ml HCl in a 50 ml beaker and heat for 2 minutes up to boiling. Decant the hydrochloric acid, shake up with 10 ml of absolute ethanol for three successive times, and discard the suspended finer particles. Use the last suspension in alcohol immediately for preparing the column. If a certain store of Fuller's earth must be prepared, the following procedure is carried out: heat to the boil 200 g of the earth and 12.5 g of tin(II)chloride with 500 ml of hydrochloric acid and boil it for a short time. Then suck the hydrochloric acid off through a large sinter-glass filter (Schott 63) and wash the earth three times, each time with 400 ml of absolute ethanol, and five times, each time with 200 ml of benzene. While removing the acid, the earth must not be allowed to become dry. Keep the earth in a brown flask with a glass stopper under benzene. Under these conditions, it is stable for about 4 weeks without loss of activity.

Anhydrous sodium sulphate.

(a) *Saponification of the Specimen with the Addition of Ascorbic Acid*

Mix 1 g of the fat or oil to be assayed with 10 ml of ascorbic acid solution and 10 ml of methanol in a round-bottomed flask, and then add through the reflux condenser 1 ml of a saturated potassium hydroxide solution and keep boiling for 10 minutes. Place the flask rapidly in a freezing bath and cool the contents to −7°C. Subsequently, add 20 ml of water and rinse the contents of the flask with 30 ml of water into a separatory funnel. Extract the first time with 100 ml of diethyl ether and a second time with 50 ml of diethyl ether, and then add a further 50 ml of water to the aqueous phase and extract for a third time with 50 ml of diethyl ether. Combine the three ether extracts and wash them with water until there is no further reaction to phenolphthalein. Then, add 40 ml of benzine (60–90°), and dry with anhydrous Na_2SO_4. Pipette 1

ml of apocarotenal solution into the solution and, after filtration through a glass filter covered with Na_2SO_4, distil off the solvents, bring to dryness, and take up in 1–2 ml of benzine (60–90°).

Comments. The alkaline saponification of substrates which contain tocopherols succeeds only rarely without losses of tocopherols, since these substances are very sensitive to oxidizing agents in alkaline media. Therefore the potassium hydroxide should not be added until the methanolic solution is boiling well and until a cushion of steam has formed over the solution. The present method in which ascorbic acid is used as antioxidant originates from Krukovsky[30]. Good results have been attained using this method in the analysis of milk fat. Moore and Tosic[47] use pyrogallol for the same purpose. Burnell and others[20] use *p*-hydroxyacetanilide (see also Vuilleumier and others[53] where further references can be found).

It seems that for the success of the analysis the type of antioxidant used is not as crucial as the specific procedure for the saponification. It is important primarily that the warm saponification solution comes into contact with air for as short a time as possible. For this reason, it is advantageous to cool the solution immediately after saponification.

(b) *Chromatography of the Unsaponifiable Fraction on Alumina Using β-Apo-8′-carotenal as Monitoring Pigment*

Put in a chromatography tube (diameter: 21 × 300 mm) terminating in a capillary, some benzine 60–90°C, and regulate the percolation to about 2 drops per second by pressing a small pad of cotton wool tightly into the narrow end of the tube. Then pour in a free-flowing suspension of deactivated alumina in benzine (at best out of a separatory funnel), until the deposited alumina has reached a height of 10 cm. It is very important that the Al_2O_3 column is uniformly packed, and this can be attained by turning and tapping the tube whilst it is being filled. Allow the benzine to run off until a few mm above the surface of the column (take care that the column is not allowed to run dry), and with a dropper pipette add the solution of the unsaponifiable fraction to the top of the column, rinse with a few ml of benzine until the β-carotene fraction has been eluted. If no β-carotene is observable, develop with at least 60 ml of benzine before converting to the eluant mixture I. Run with the eluant mixture I the apocarotenal zone with the α-tocopherol to the base and collect quantitatively (eluate 1) but without tolerance.

If the remainder of the tocopherols has to be determined, then elute with 80 ml of eluant mixture II (eluate 2). By performing this operation, the following must be observed: after receiving eluate I, the receiving

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dish must be changed immediately and the eluant mixture I which still remains above the column in the chromatography tube should be removed and replaced by eluant mixture II.

Evaporate eluates 1 and 2 to dryness in the rotatory evaporator and take them up in 5 ml of benzene.

Comments. In addition to α-tocopherol, eluate 1 still contains ζ_1-tocopherol (5,7,8-trimethyltocotrienol or α_3-tocopherol).

The biological activity of ζ_1-tocopherol is only known approximately (Bunyan and others[20]) and is about 20 per cent of that of α-tocopherol. It is therefore important to separate ζ_1-tocopherol in a later step, so that the vitamin E activity is not overestimated. Eluate 2 contains β- and γ-tocopherol, and probably also the corresponding trienols (β_3- and γ_3-tocopherol, respectively ε- and γ-tocopherol), possibly also δ-tocopherol and the corresponding trienol. However, there are no systematic investigations of this. Of all tocopherols, δ-tocopherol is retained the strongest by the alumina column; for an account of this see ref. 37.

Other adsorbents can be used in place of alumina (e.g. a mixture of zinc carbonate, alumina, and celite—Bieri and others[8]; or secondary magnesium phosphate—Bro-Rasmussen and Hjarde[14], Bunnell and others[27]). The working technique given here has the advantage that the addition of a monitoring pigment allows the fraction containing α-tocopherol to be separated sharply, but has the disadvantage that the β-apo-8′-carotenal added must subsequently be separated again.

(c) *The Removal of β-Apo-8′-carotenal and other Interfering Substances by means of Fuller's Earth*

Put the Fuller's earth suspended in ethanol and activated with $SnCl_2$ and HCl in a chromatography tube (18 × 300 mm) having a capillary end. Then rinse with absolute ethanol and wash with three portions of absolute ethanol, each of 20 ml. Rinse the column twice each time with 20 ml of benzene. If the Fuller's earth is stored in benzene, then the column can be filled directly with an appropriate portion. With a third portion of benzene add to the column 0.5 g of filter aid, and with a fourth portion 1 g of silica gel. In all these operations, the column must not be allowed to run dry. Add the eluates 1 and 2 quantitatively each to the top of a separate column, change the collecting dish immediately, and rinse the tocopherols through the column with 80 ml of benzene. The apocarotenal is retained by the Fuller's earth and appears as a blue zone at the head of the column (similar to xanthophylls from eluate 2). During the elution of the

tocopherols, this blue zone may not *surpass* the first third of the column of Fuller's earth. Evaporate the benzene eluates to dryness on the rotatory evaporator and take the residue up in 20 ml of absolute ethanol (solution used for measurement). Filter this alcoholic solution through a layer (about 0.5 cm high) of Celite filter aid on dry sintered glass. This filtration must always be conducted as a security measure.

(d) *Estimation of α-Tocopherol or the Remaining Tocopherols after Emmerie Engel*

Make an aliquot of the filtered solution (20–80 μg of tocopherol), in the present example 5 ml, up to 8 ml with absolute ethanol, and mix with 1 ml of a 0.5 per cent α′-pyridyl solution. After addition of 1 ml of $FeCl_3$ solution, start the stop-watch, mix the reaction mixture thoroughly, fill into the measuring cuvette (1 cm sheet thick), and place the cuvette holder as quickly as possible in the photometer. This should occur 30–45 seconds after addition of $FeCl_3$. Exactly 2 minutes after addition of the $FeCl_3$ solution, measure the extinction at 520 nm against absolute ethanol.

Determine the blank value in the same way with 8 ml of absolute ethanol and 1 ml of each of the reagent solutions.

Calculate the tocopherol content on the basis of a standard curve and the determined extinction.

Comments. The reaction between α-tocopherol and iron(III)chloride after Emmerie and Engel[24] is still today the most commonly used method to determine tocopherol. The blank value of the reagent measured against pure ethanol should not give an extinction higher than 0.05–0.06. Otherwise, the ethanol must be freed carefully from reducing impurities. Instead of using the Emmerie–Engel reaction, Lambertsen and others[37] have analysed the solution directly by measuring the extinction at the maximum absorption of α-tocopherol.

(e) *Thin-layer Chromatographic Control*

Five millilitres of the alcoholic solution of tocopherols (solution to be measured) are concentrated on the rotatory evaporator and the residue is taken up in the least possible volume of chloroform. The solution is placed as a stroke or as two starting points on a silica gel thin-layer plate. A solution of *dl*-α-tocopherol (0.5–1 mg/ml in $CHCl_3$) serves for comparison or in the case of the remaining tocopherols β- or γ-tocopherol. When applied as a stroke, the test and control solutions are made such that they overlap slightly, otherwise a mixed starting spot is applied. Chloroform is normally used as solvent. For the spraying reagent

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a 0.5 per cent solution of α,α'-dipyridyl in ethanol and a 0.2 per cent solution of iron(III)chloride in ethanol (to be kept in the dark) are used. Before use, equal volumes of both solutions are mixed. The sprayed chromatogram may only show tocopherols. Particular attention should be paid to interfering substances near to the solvent front. Spots which only appear after a prolonged time period need not be considered.

If Emmerie–Engel positive spots appear, the result of the analysis cannot be used. The α-tocopherol content, however, can be estimated on the basis of the relative size of the spots.

The testing of the α-tocopherol fraction for the presence of ζ_1-tocopherol can be performed with the remaining part of the test-solution. When the vitamin E content of the investigation material is so low, it is generally more favourable to carry out the analysis in duplicate, in order to have more tocopherol solution available. The test-solution is applied several times in spot form: one spot is mixed with *dl*-α-tocopherol, another with ζ_1-tocopherol (or if this is not obtainable with an α-tocopherol fraction from wheat bran (α- + ζ_1-tocopherol)). The chromatography is conducted horizontally with trichloroethylene as solvent[18] and lasts about 3 hours. The duration is affected by the thickness of the paper which dips into the solvent. The approximate position of the tocopherol spots can be determined by simultaneously chromatographing about 10 μg of β-apo-8'-carotenal. By visually comparing the spot size after spraying with Emmerie–Engel reagent, it is generally possible, after a little practice, to determine the ratio of the tocopherols in 10 per cent stages. For a more exact determination it is necessary to chromatograph simultaneously mixtures of known proportion or to planimeter the spots.

D. Other Methods of Assay of α-Tocopherol

Similar methods of analysis have been proposed by various other authors. For example, the assay method of Bunnell and others[20] consists of the following stages:

(*a*) saponification
(*b*) chromatography on Florex XXS
(*c*) chromatography on secondary magnesium phosphate
(*d*) determination by Emmerie–Engel.

That of Lambertsen and others[37] consists of

(*a*) saponification
(*b*) chromatography on alumina
(*c*) paper-chromatographic purification
(*d*) spectrophotometric determination of α-tocopherol.

That of the Analytical Methods Committee which is also described by Jolly[21] consists of

(*a*) saponification
(*b*) removal of steroids by freezing out
(*c*) chromatography on Fuller's earth
(*d*) paper-chromatographic separation of the tocopherols
(*e*) determination of the tocopherols by Emmerie–Engel.

The latter analytical method is especially suitable to determine the remaining tocopherols; this is necessary to evaluate the antioxidant behaviour. It must, however, be stated that the described technique of two-dimensional paper chromatography requires significantly more experimental skill than the methods which only require column and thin-layer chromatography.

Molecular distillation can be used instead of saponification (according to Quaife and Harris[22]). This method is less destructive and allows a better separation of the remaining tocopherols from the other interfering substances, but necessitates the use of a suitable apparatus. Recently, analytical methods have also been described in which the tocopherols are determined by gas chromatography[43]. In this type of analytical process, a column-chromatographic purification must still be conducted before the final determination.

E. Assay of the Total Emmerie–Engel Positive Substances

In order to determine the total tocopherol content, Mehlenbacher[44] recommends a method described by Stern and Baxter[58].

In this method, 2 ml of an alcoholic solution of the fat or oil containing 100–600 μg of the total tocopherols is added to an opaque 25 ml calibrated flask. The alcohol used must be distilled over potassium hydroxide and potassium permanganate.

One millilitre of a 0.2 per cent alcoholic $FeCl_3 \cdot 6H_2O$ solution is added, and next 1 ml of a 0.5 per cent alcoholic α,α'-dipyridyl solution, and then filled up with purified ethanol. After mixing thoroughly the extinction of the solution is determined exactly at 520 nm after 10 minutes, whereby a corresponding preparation lacking the sample to be analysed is used as a blank value (reagent blank value). The tocopherol content is deduced from a standard curve, which is constructed under the same conditions using pure α-tocopherol instead of the sample. Since the various tocopherols give a colouration of different intensity, the value obtained has the same significance as a chemical constant, without giving information on the actual content of the tocopherols. A discussion of the notion of chemical constants is given

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by Baltes[6]. If solely a mixture of α-, β-, γ- and δ-tocopherols is present, and if α-tocopherol constitutes about 30 per cent (soyabean oil), then the value obtained can be multiplied by 0.91. In this case, a good estimation of the total tocopherol content is obtained. The use of such conversion factors, however, should be limited to cases in which the composition of the tocopherol mixture is known and in which no other Emmerie–Engel positive substances are present in the mixture.

3. VITAMIN D

A. General Remarks

Vitamin D in fat and oil should be assayed by biological means, particularly if vitamin A or by-products of the irradiation process of ergosterol and dehydrocholesterol are present. If the biological method is used, the recommendations given by IUPAC[20] should be taken into account.

It is only possible to assay vitamin D by chemical means in high concentrates. Even in this case, it is advisable to check from time to time the chemical method by means of the biological method. Under favourable conditions, preparations which contain only small amounts of vitamin D can also be assayed chemically. Often, in routine work for control purposes, this has to be done. However, in this case, the biological checking of the method is indispensable.

A simple procedure to perform such a routine analysis is described by Hausheer and others[28]. If concentrates have to be assayed, in general a method described by U.S.P. XVI is used. Recently, Bolliger and König[31] have published a more specific method using thin-layer chromatography.

B. Determination of Vitamin D after U.S.P. XVI

Apparatus. In addition to the equipment for performing chromatographic separations on columns, a spectrophotometer is required, which measures the extinction of a solution at 550 nm and 500 nm.

Reagents. Hexane: optically pure when measured in a 1 cm cell at 300 nm. The hexane should have a value of absorbance of not more than 0.070 using air as the blank.

Ethylene dichloride (1,2-dichloroethane): this is purified by passage through a column of granular (10–200 mesh) silica gel.

Potassium hydroxide: an aqueous solution of 780 g/l.

Cottonseed oil: this should meet the requirements described in the U.S.P. XVI, pp. 183 and 911.

Antimony(III)chloride solution: two stock solutions are prepared as follows:

Solution *A*:

The total content of a 100 g bottle of dry crystalline antimony(III) chloride is put into a flask containing 350 ml of ethylene dichloride. The antimony(III)chloride is allowed to dissolve and 2 g of anhydrous alumina is added. After mixing, the suspension is filtered through paper into a flask, ethylene dichloride is added to make a total volume of about 440 ml. After mixing, the solution should have a value of absorbance of not more than 0.070 measured in a 2 cm cell at 500 nm using ethylene dichloride as the blank.

Solution *B*:

100 ml of acetyl chloride is mixed under the hood with 400 ml of ethylene dichloride.

Before use, 45 parts of solution *A* are mixed with 5 parts of solution *B*.

Isooctane: 99.5 per cent, optically pure.

Chromatographic siliceous earth: (e.g. Celite 545 of Johns-Manville).

Polyethylene glycol 600 (manufactured by Carbide and Carbon Chemical Co.).

Fuller's earth: the suitability of a specific earth has to be checked for each new batch by performing a chromatography as indicated below (according to Osadca and others[39], Florex XXS 60–100 mesh of the Floridin Co., Tallahassee, Fla., U.S.A., is a suitable product).

Ethanol: 95 per cent.

Sodium sulphate: Na_2SO_4, 10 H_2O analytical grade.

Anhydrous sodium sulphate: Na_2SO_4.

Inhibitor: equal parts of acetic acid anhydride and ethylene dichloride are mixed.

Benzene: analytical grade, crystallizable.

Procedure

(a) *Saponification*

Weigh an amount of fat or oil corresponding to 10,000 I.U. of vitamin D in a round-bottomed flask and add 2 ml of a solution of vitamin A in cottonseed oil in case the sample does not contain enough of this vitamin, so that the total amount is about 3,000 I.U. of vitamin A. This addition is necessary in order to have a pilot band in the subsequent chromatography. In case the sample contains enough of this vitamin, add only 2 ml of cottonseed oil. Subsequently, add for each gram of fat (sample plus cottonseed oil) 2.5 ml of potassium hydroxide solution, but not less than 15 ml, and 50 ml of ethanol and heat the mixture on the

steambath under reflux for 20 minutes. If the sample size exceeds 5 g, the heating time should be expanded to 30 minutes. In case this time does not suffice to saponify the sample completely, more potassium hydroxide solution should be added and the heating should be continued.

After saponification, cool the contents of the saponification flask and transfer it quantitatively to a 500 ml separatory funnel. Rinse the saponification flask with 50 ml of water in small portions and add these to the soap solution.

Add 4.5 g of crystalline sodium sulphate decahydrate and 150 ml of hexane. Shake the separatory funnel vigorously for 2 minutes and, after separation, transfer the aqueous layer to a second separatory funnel and extract again with two 50 ml portions of hexane. Combine the extracts and reject the aqueous layer.

Wash the combined extracts with 50 ml portions of water (test with phenolphthalein), dry with anhydrous sodium sulphate, filter, and evaporate to dryness at a temperature that should not exceed 40 °C. Take up the residue in 10 ml of hexane.

Comments. When conducting the saponification according to the conditions described in the U.S.P. XVI, part of vitamin D is changed into previtamin D and part of it is destroyed completely. For this reason, it is necessary to treat the standard vitamin D preparation with the same saponification procedure when establishing the standard curve.

(b) *Purification by Chromatography*

1st column

Preparation of the column: Transfer 125 ml of isooctane to a screw-capped, wide-mouth bottle and add 25 g of siliceous earth. Close the bottle and shake it until a slurry is formed. Now add 10 ml of polyethylene glycol 600 drop by drop with vigorous mixing. Close the bottle with a new screw-cap and shake again vigorously for 2 minutes. Pour half of the slurry into a chromatographic tube (inside diameter: 25 mm; the tube should be closed by a plug of glass-wool, a sintered-glass disc or a Teflon stopcock). Wait until the siliceous earth has settled, then apply gentle suction and add the remainder of the slurry in small portions, packing each portion with a suitable instrument.

When a solid surface has formed, remove the vacuum and add about 2 ml of isooctane.

Procedure: Wait until the isooctane has just disappeared into the surface of the column and immediately transfer 2 ml of the extract

dissolved in hexane on to the top of the column and allow it to enter the column. Wash the walls of the column with two portions of hexane, 2 ml each, and finally elute the vitamin D using portions of hexane, 5–10 ml each, until a total volume of maximum 100 ml of hexane has been added to the top of the column. Reject the first 20 ml of the effluent and collect the rest in a round-bottomed flask. From time to time, irradiate the column shortly with u.v. light (see p. 621) to trace the vitamin A band. When the front of this band appears about 5 mm from the bottom, discontinue the chromatography and evaporate the collected effluent in a rotatory evaporator at 40 °C. Dissolve the residue in 10 ml of hexane.

2nd column

Preparation of the column: For the second chromatography apply a tube consisting of three sections. The top section has an inside diameter of 18 mm and a height of 140 mm, the inside diameter of the middle section is 6 mm and the length 250 mm. The last section consists of a capillary tube about 50 mm long. Put a plug of glass-wool at the end of the middle section and fill it with 3 g of Fuller's earth under gentle suction.

Procedure: Transfer the residue dissolved in 10 ml of hexane to the top of the column. Allow it to enter, then rinse the flask with 10 ml of hexane. Just as the solution of the residue disappears into the surface, add the rinsing to the top of the column and let it enter until 1 ml of hexane covers the surface of the column. Discard the effluent, bring small portions of benzene to the top of the column and apply gentle suction until a total volume of 75 ml is used. Evaporate the effluent *in vacuo* at a temperature of not more than 40 °C and dilute the residue with ethylene dichloride to a volume of 5 ml.

Comments. There are many cases where the chromatography by means of siliceous earth polyethylene glycol and Fuller's earth does not separate vitamin D from interfering byproducts. In these cases, a third chromatography has to be performed between the two chromatographies described. For this, the absorbent of choice is alumina. A suitable procedure was published by Osadca and De Ritter[50]. With this procedure, the analysis of an oily concentrate for feeding purposes gave a value of 484 (± 27 per cent) I.U. of vitamin D per g of concentrate. For the same sample, the biological test gave 446 (± 21 per cent) I.U. for the rat and 514 (± 14 per cent) I.U. for the chicken (cf. ref. 29). It remains to be seen whether the modification of Osadca and De Ritter may be applied generally to assay vitamin D in fats and oils or whether

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it is to be restricted to oily concentrates similar to the sample mentioned above.

(c) *Determination*

The vitamin D content of the chromatographically purified solution is performed according to the indications of Osadca and De Ritter as follows:

To a 2.0 ml aliquot of the solution add rapidly 5 ml of antimony(III) chloride (a similar burette to that shown in Fig. 5 may be of use) and read the absorbance in a 1 cm cell at 500 nm exactly 45 seconds after addition of the reagent against a blank consisting of 2 ml of ethylene dichloride and 5 ml of antimony(III)chloride solution. Exactly 45 seconds later (or a total of 90 seconds after addition of the antimony(III) chloride reagent) read the absorbance again at 550 nm against the same blank.

To another 2.0 ml of aliquot of the solution add 1 ml of a mixture of equal parts of acetic anhydride and ethylene dichloride (as a colour inhibitor) and 5.0 ml of antimony(III)chloride reagent. Again measure the absorbance at 500 and 550 nm at 45 and 90 seconds, respectively, after addition of the antimony(III)chloride reagent using the solution described above as blank.

Calculation of the corrected absorbance:

A — absorbance without inhibitor at 500 nm
B = absorbance with inhibitor at 500 nm
C — absorbance without inhibitor at 550 nm
D — absorbance with inhibitor at 550 nm

The corrected absorbance can be expressed as follows:

$$E_{corr} = (A - 1.14\,B) - 0.67\,(C - 1.14\,D)$$

(d) *Evaluation*

For the evaluation establish a standard curve by means of an external standard by using different amounts of vitamin D dissolved in ethylene dichloride. To 2 ml of these solutions, add rapidly 5 ml of antimony(III) chloride solution and read the absorbance in a 1 cm cell at 500 nm exactly 45 seconds after addition of the reagent against a blank consisting of 2 ml of ethylene dichloride and 5 ml of antimony(III)chloride solution.

The values of absorbance found are represented in a coordination system and serve to establish the standard curve. This curve is linear

in the range of 500–1000 I.U. of vitamin D ml and goes through point zero. To check one or two points of the curve should be reevaluated from time to time, especially if new reagent solutions are used.

Comments. It is suitable to use a standard vitamin D preparation saponified by the same procedure as mentioned above.

The theory for the above-mentioned correction formula is given by Wilkie[67]. The factor 1.14 has its origin in the fact that the volume of the inhibited solution is 8 ml, whereas the volume of the non-inhibited solution is only 7 ml.

4. CAROTENES AND CAROTENOIDS

A. General Remarks

In Table III it is shown that in fats and oils of animal or vegetable origin a number of carotenes and carotenoids occur. Only slight differences exist in the physical and chemical behaviour of these compounds, so that it is difficult to separate them by physical or chemical means. An exact analysis of a mixture of carotenes and carotenoids should only be undertaken by a laboratory with a staff of well-trained analysts with a high scientific background. General rules, which have to be observed in separating and determining carotenes and carotenoids, are described by Brubacher and Vuilleumier[16], Goodwin[25], Jensen[34], Stahl and others[57], and Zechmeister[71]. In practice, one must be content to analyse only certain fractions according to the specific problems. In general, such fractions contain a whole series of carotenes and carotenoids besides the leading carotene or carotenoid.

When evaluating the analysis, these byproducts are treated as identical with the main carotenes or main carotenoids. In this case, the result of an analysis has the character of a chemical constant (see ref. 6). It does not express the real content of a certain carotene or carotenoid but characterizes the fat or oil in question.

If the biological vitamin A value of a complex mixture of carotenes and carotenoids is of primary interest, only a biological test can give a true answer (therapeutic or prophylactic growth test with the vitamin A depleted rat). If the colour of a certain fat or oil rather than its biological value is the primary interest, this should be measured directly by means of colour tables or measurement of the reflectance.* Only in cases where a single carotene or carotenoid was added to a more or less colourless basic fat or oil, can the analysis be performed

* EDITOR'S NOTE. Compare the chapter by M. Naudet and E. Sambuc on p. 45.

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in such a way that the result answers exactly the question of the biological vitamin A value of this fat or oil and, in certain cases, even characterizes the colour of the material.

Several conventional methods exist for analysing certain fractions of carotenes or carotenoids; the most important ones are described by Vuilleumier and others[63], by Booth[12], by the Association of Official Agricultural Chemists[1], by the *Schweizerische Lebensmittelbuch*[28, 64] and in the British Margarine Order[15].

The problem of the analysis of β-carotene is treated by Booth in a very thorough and critical manner.

B. Isolation of Carotenoids

The three procedures mentioned on pp. 612 and 613 are suitable for the isolation of carotenoids. An example for saponification will be given in the next section.

If the carotene-carotenoid mixture is isolated by freezing out, the following procedure may be used.

Isolation of a Carotenoid Mixture by Freezing Out

Weigh 20 g of fat in a round-bottomed flask and dissolve in 100 ml of acetone. Cool the flask in a mixture of acetone and dry ice, until the majority of the fatty material is frozen out. Filter the mother liquor through a precooled sintered glass (for details see Vuilleumier and others[63] and redissolve the residue in 100 ml of acetone, cool again, and filter. Evaporate the combined filtrates in a rotatory evaporator (see Fig. 4) in such a manner that a small quantity of solvent remains in the flask. Take up the residue in light petroleum, dry with anhydrous sodium sulphate and after filtration make up with light petroleum to the desired volume.

In a few cases, the carotenoid can be extracted quantitatively by partition. For instance, canthaxanthin can be analysed by this procedure in coloured fats.

Extraction of Canthaxanthin (4,4-diketo carotene)

Dissolve 5 g of fat in 100 ml of diethyl ether free of peroxides, dry the solution with anhydrous sodium sulphate, and filter in a round-bottomed flask. Evaporate the solvent and take up the residue in 50 ml of n-hexane. Transfer the solution into a separatory funnel of 250 ml content and shake it vigorously with 50 ml of 95 per cent methanol. Allow the layers to separate and collect the methanolic layer. Repeat the extraction of the hexane layer four times, each time with 50 ml of

95 per cent methanol. Combine the methanolic extracts, evaporate the solvent, and take the residue up in light petroleum.

C. Purification by Chromatography

In general, the mixture of carotenes and carotenoids reproduced by one of the three procedures mentioned above has to be purified at least by one chromatography.

Hausheer and others[25] suggest the following procedure for the estimation of β-carotene:

Determination of β-Carotene in Fats and Oils

Apparatus. A spectrophotometer is required which enables the absorbance of a solution between 350 nm and 500 nm to be measured. If such an instrument is not available, an instrument without monochromator but with a filter for light of 450 nm can be used. Such an instrument would be sufficient to assay β-carotene but would not be able to ensure that the fraction measured consists of β-carotene or another dyestuff.

In addition to the usual laboratory material, an apparatus to carry out the column chromatographies is required and a rotatory evaporator (see Figs. 3 and 4).

Reagents. Hydroquinone solution: dissolve 20 g of pure 1,4-benzohydroquinone in 100 ml of absolute ethanol.

Methanolic potassium hydroxide solution: dissolve 18 g of potassium hydroxide in 10 ml of distilled water and make up to 100 ml with methanol.

Methanol: pure, absolute; if necessary, purify by distilling 10 l of methanol over 5 g of potassium permanganate and 10 g of potassium hydroxide.

Benzine: boiling range 90°-115°C.

Alumina: the alumina has to be highly active; alumina "Merck" according to Brockmann of the activity grade 1 (E. Merck A.G., Darmstadt, Germany) or alumina "Giulini", activity grade 1 (Gebrüder Giulini GmbH, Ludwigshafen am Rhein, Germany), can be used without further treatment. It must, however, be kept in tightly closed glass flasks.

Elution mixtures: mixtures of diethyl ether and benzine are prepared as follows:

	A	*B*	*C*	
diethyl ether	5	25	50	ml
benzine	95	75	50	ml

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Diethyl ether, free from peroxides, absolute, for preparation see p. 619.

Procedure

(a) *Saponification*

Weigh approximately 5 g of the sample, containing about 20 μg of carotene, exactly into a round-bottomed flask, and add 2 ml of hydroquinone solution and 15 ml of methanolic potassium hydroxide. Reflux for 15–20 minutes in a waterbath at 65–70 C and shake from time to time until the solution is clear. Pour 10 ml of methanol through the condenser in order to cool the contents of the saponification flask and transfer the soap solution quantitatively to a 250 ml separatory funnel. Rinse the saponification flask with 40 ml of methanol *in toto* and combine the rinsings with the soap solution. After the contents of the separatory funnel are cooled, extract them with 100 ml of benzine (90–115 C). After adding 10 ml of distilled water, leave the mixture for 10–20 minutes in the dark until separation of the layers is completed. Then transfer the benzine layer into a second separatory funnel and wash it with one tenth of its volume of distilled water. Evaporate an aliquot part of the benzine solution corresponding to about 15 μg of carotene under vacuum, and take the residue up to 10 ml of benzine for chromatography.

(b) *Chromatography*

Fill the chromatography tube to a height of about 10 cm with alumina. Run benzine (90–115 C) through until the alumina layer is covered by about 1 cm of solvent. Pour the carotene solution prepared for chromatography on the alumina and wash the column with 40 ml of benzine. Replace the collecting flask by a clean one and fractionate the various carotene isomers by elution in the following way:

(1) Fraction obtained with about 100 ml of elution mixture *A* contains *cis* isomers of β- and α-carotenes.

(2) Fraction obtained with about 50 ml of elution mixture *B* contains *trans*-β-carotene and possibly *trans*-α-carotene.

(3) Fraction obtained with about 50 ml of elution mixture *C* contains γ-carotene.

Evaporate the eluate (2) containing all-*trans*-β-carotene under vacuum and take the residue up in a quantity of benzine to achieve a solution of approximately 2 μg of carotene/ml.

(c) *Measurement and calculation*

Measure the absorbancy of the obtained solution between 452 and

454 nm, and take the maximum value. Calculate the β-carotene of this solution by means of the following formula:

$$C = \frac{E}{d} \cdot \frac{1000}{2560} = \frac{E}{d} \cdot 0.391$$

where C = β-carotene content expressed as mg all-*trans*-β-carotene/100 ml of the measured solution.

E = maximum value for absorbancy in the range of 452 to 454 nm

2560 = $E^{1\%}_{1\,cm}$ value of pure all-*trans*-β-carotene at the same wavelength

d = width of the spectrophotometer cell in cms.

After chromatography, make sure that the measured fraction contains essentially β-carotene. This can be done by measuring an absorption spectrum and comparing it with a standard spectrum. Such a spectrum is shown in Fig. 6. From this figure it can be seen that, in a solution

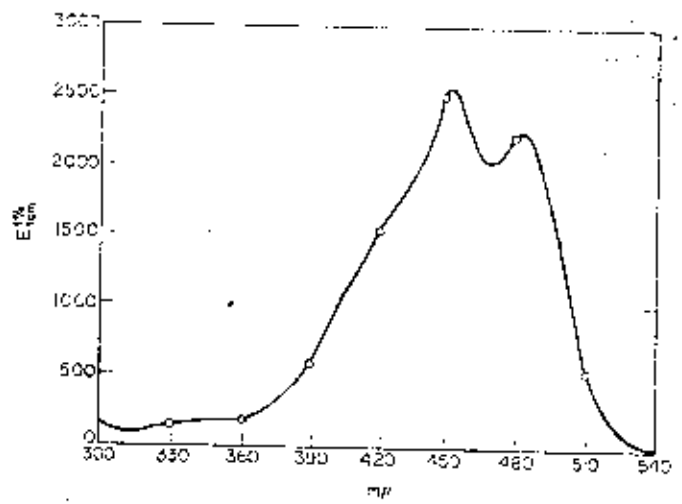

Fig. 6. Absorption curve of all-*trans*-β-carotene in light petroleum.

of β-carotene in benzine, there are two maxima of absorption (452–454 nm and 480–482 nm, respectively) and one minimum (468–470 nm). Since the position of the maxima depends to a great extent on the

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solvent, and since the use of simple photometers causes systematic errors, it is recommended to determine the curve of absorption in every case under the given laboratory conditions.

If the benzine solution of the unsaponified fat already shows an absorption curve nearly identical with that of pure β-carotene, it is sufficient, above all in the case of control analyses, to measure the benzine solution of the unsaponified fat. In that way, all carotenes and carotenoids present are checked in the same manner and expressed in absorption equivalents of β-carotene.

A discussion of the method by Hausheer and others is contained in the article by Vuilleumier and others[65]. The chromatography with highly active alumina has to be carried out under strict conditions in order to yield reproducible values. Therefore, it is recommended to chromatograph by means of partially deactivated alumina, followed by a second chromatography using another adsorbent. Since, in many cases, the analysis includes an assay of both vitamin A and β-carotene, it is advisable to choose an activity of alumina and an elution mixture which allow the separation of vitamin A from β-carotene quantitatively. For this purpose, the procedure described on p. 626 is suitable. However, a wet saponification process is preferable, since with higher temperatures, carotenes and carotenoids may be destroyed or isomerized. For the wet saponification, the method described on p. 616 can be used, for example. If it is uncertain whether the carotenoid in question is destroyed by saponification, it is best to use the freezing-out method. When chromatographing on deactivated alumina, the total β-carotene is eluted with the first 40 ml of light petroleum. This fraction contains, besides β-carotene, α-carotene and further carotenoids of similar polarity. In most cases it is sufficient to measure the absorbancy of this fraction at the absorption maximum as recorded in the Official Methods of the AOAC[4] or in the British Margarine Order[46].

After elution of the β-carotene, continue to elute with a mixture of light petroleum and benzene. With a ratio of 8 : 2, the lycopene fraction is washed out; with a ratio of 6 : 4 a yellow band leaves the column, the nature of which is not known, and, at the same time, β-apo-8′-carotenal and possibly β-apo-8′-carotenoic acid ethyl ester, if the initial extract was not saponified under these circumstances. The carotenoids of the annatto extract, canthaxanthin, lutein, and zeaxanthin remain in the top section of the column.

β-Apo-8′-carotenal can be determined by means of a derivative of 2-diphenylacetylindane-1,3-dione-1-hydrazone (see ref. 65) and β-apo-8′-carotenoic acid ethyl ester by saponification and measurement of

the absorbance of the light petroleum solution of the unsaponifiable before and after saponification at the absorption maximum.

To analyse the β-carotene fraction, it is necessary to rechromatograph on magnesia. The column for this is prepared as follows:

Suspend a mixture of two parts (by weight) of magnesia (British Drug House, Poole, England) and one part (by weight) of Celite filter aid in light petroleum to produce a free-flowing suspension.

Fill a chromatographic tube with light petroleum to a height of about 4 cm. Shake the magnesia suspension vigorously and pour into the tube in portions. Allow the suspension to settle by gently vibrating the tube. Apply a slight nitrogen pressure to every portion. The compact column, ready for use, should be 6–7 cm high and completely free of bubbles. Add the light petroleum solution which contains the β-carotene fraction to the top of the column and develop with a mixture of light petroleum and diethyl ether, beginning with pure petroleum and adding increasing amounts of diethyl ether, until a ratio of 95 : 5 is reached. α-Carotene moves as a yellow band in front of the rose-coloured β-carotene band. After elution, the content of α- and β-carotene, respectively, is determined by means of the absorbance measured at the absorption maximum.

To determine lutein, zeaxanthin, and canthaxanthin, the alumina column is allowed to run dry under nitrogen. Thereafter, the coloured zones are cut out and eluted with a suitable solvent; canthaxanthin, for example, with a mixture of chloroform–acetone (1 : 1), and xanthophylls with ethanol.

The eluate is evaporated by means of a rotatory evaporator, the residue is taken up in a little light petroleum and adsorbed on a short column (1–2 cm high) of magnesia/Celite (2 : 1) and immediately eluted with chloroform–acetone (1 : 1) until the effluent is colourless. This is evaporated and taken up again in a little light petroleum.

This step is necessary only when greater amounts of chlorophylls are present. If this is not the case, chromatography can take place immediately. The solution is transferred quantitatively on to a magnesium oxide column with several portions of light petroleum. The canthaxanthin must remain adsorbed at the top of the column and is only developed by adding graded amounts of acetone according to the following scheme:

about 5 ml of petrol ether with 5 per cent acetone,
about 5 ml of petrol ether with 10 per cent acetone,
about 5 ml of petrol ether with 15 per cent acetone,
about 5 ml of petrol ether with 25 per cent acetone.

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In this way, the xanthophylls are eluted, whereas the canthaxanthin remains on the column.

As soon as the canthaxanthin fraction is separated distinctly and 2–3 cm away from the bottom of the column, it is allowed to run dry under nitrogen (about 0.2 atü). The compact column of magnesium oxide Celite is removed by tapping the inverted tube with a glass rod. The canthaxanthin fraction is cut out and eluted immediately in a glass filter (G 4) with acetone chloroform (1 : 1). Continue the evaporation until the suspension is not quite dry and take it up with a suitable volume of cyclohexane. If the extract contains annatto, it remains on the top of the column as a brownish red band. With regard to the analysis of annatto extracts, see McKeown[40, 41, 42].

To obtain data on the content of carotenes and carotenoids, the absorbance of suitable dilutions can be measured at the absorption maximum and by means of the $E^{1\%}_{1cm}$ values. The concentrations of these dilutions can be calculated. Table VI contains the corresponding data of a number of compounds.

TABLE VI

Absorption Maxima and $E^{1\%}_{1cm}$ Values of a Few Carotenes and Carotenoids in Light Petroleum

Substance	Max. nm	$E^{1\%}_{1cm}$
β-Carotene	452–454	2560
β-Apo-8′-carotenal	455	2700
β-Apo-8′-carotenoic acid ethyl ester	444	2600
Canthaxanthin	464–467	2200
Lycopene	472	3450

A second possibility consists of measuring all absorbancies at 450 nm and assuming an average $E^{1\%}_{1cm}$ value of 2500. For this procedure, the fractions should be dissolved in light petroleum. Under these conditions the value of 2500 corresponds to the $E^{1\%}_{1cm}$ value of β-carotene. These figures therefore do not indicate the amount of the actual carotenoid or carotene but rather the amount of β-carotene which would lead to the same value of absorbance.

In general, this procedure is preferable since it permits a balance to be established and losses occurring in the course of the analysis to be checked.

Further details regarding the chromatographic separation of carotenes and carotenoids may be found in the paper by Vuilleumier, Probst, and Brubacher in the *Handbuch der Lebensmittelchemie*[55].

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PHARMACOLOGY

RECEPTORS FOR 1α,25-DIHYDROXYVITAMIN D_3 IN INTESTINE: PROPERTIES AND UTILIZATION IN AN ASSAY FOR THE STEROL. Peter F. Brumbaugh* and Mark R. Hussler* (SFOX: Rubin Pressler) Dept. of Biochemistry, Arizona Medical Center, Tucson, Arizona 85724.

Specific binding of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3 was observed in the cytosol and chromatin fractions of chick small intestinal mucosa, *in vitro*. The cytosol contains a macromolecule with a high affinity (K_d=2.2x10^{-9}M) for 1α,25-$(OH)_2$-D_3 and has a limited number of binding sites which are saturated at low hormone concentrations (≈10^{-9}M). The macromolecule-sterol complex is sensitive to pronase, but not nucleases, and sediments at 3-3.5S in 0.1M KCl-sucrose gradients.

1α,25-$(OH)_2$-D_3 remains bound to the cytosol fraction at 0°C in whole intestines, *in vitro*, and transfer of 1α,25-$(OH)_2$-D_3 to the nuclear chromatin occurs at 37°C. This temperature dependence of hormone localization is also evident in intestinal mucosa homogenates and reconstituted cytosol-chromatin systems, indicating that the cell membrane and nuclear membrane are not involved in the temperature mediated movement of hormone receptor complex to the chromatin.

The interaction of [^{3}H]1α,25-$(OH)_2$-D_3 with both the cytosol and chromatin components can be reversed only by much higher concentrations of unlabeled 25-hydroxyvitamin D_3 (150 fold excess), 1α-hydroxyvitamin D_3 (450 fold excess) and vitamin D_3 (>60,000 fold excess). The specificity and high affinity of these receptors has permitted their use in a competitive protein binding assay for 1α,25-$(OH)_2$-D_3 in human plasma. (Supported by USPHS Grant AM15781).

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Radioreceptor Assay for 1α,25-Dihydroxyvitamin D_3

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Abstract. *A competitive protein binding assay with a sensitivity of 80 picograms has been developed for 1α,25-dihydroxyvitamin D_3, the hormonal form of vitamin D_3. 1α,25-Dihydroxyvitamin D_3 displaced tritiated hormone from a cytosol-chromatin receptor preparation isolated from chick small intestine, providing a simple assay for the hormone. The concentration of 1α,25-dihydroxyvitamin D_3 in human plasma, as determined by this assay, is approximately 6 nanograms per 100 milliliters; in patients with renal disease the concentration of this kidney-produced hormone is significantly lower.*

Vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 (25-OH-D_3) (*1*) and subsequently to 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3] (*2, 3*). The latter conversion occurs exclusively in the kidney (*4*) and produces what is considered to be the hormonal form of the vitamin. 1α,25-$(OH)_2$-D_3 is the most active and fastest acting metabolite of vitamin D in stimulating both intestinal calcium transport and bone mineral mobilization (*5*).

Previous detection of 1α,25-$(OH)_2$-D_3 has been carried out only in experimental animals, and there is little information available on the occurrence of this hormone in humans. Defects in the production of this hormone may explain abnormal calcium metabolism in diseases such as chronic renal failure. There is also evidence that parathyroid hormone is involved in the regulation of the renal synthesis of 1α,25-$(OH)_2$-D_3 (*6*), suggesting that abnormal production of parathyroid hormone may affect calcium homeostasis by altering the circulating level of 1α,25-$(OH)_2$-D_3. In order to study the role of 1α,25-$(OH)_2$-D_3 in calcium metabolism in normal humans and to study its possible relation to metabolic bone disease, we have developed a sensitive, competitive protein binding assay capable of detecting circulating amounts of the hormone.

The assay depends upon the binding of 1α,25-$(OH)_2$-D_3 to its chromatin receptor in the small intestine (*7*). This receptor is saturable and binds 1α,25-$(OH)_2$-D_3 with high affinity ($K_d \approx 10^{-9}M$). The receptor is specific for 1α,25-$(OH)_2$-D_3, and the radioactive hormone is displaced from its binding site only by much higher concentrations of 25-hydroxyvitamin D_3 (150-fold excess) and vitamin D_3 (> 20,000-fold excess) (*8*). Moreover, 1α,25-$(OH)_2$-D_3 binds initially to a cytosol receptor protein, and this sterol receptor complex migrates into the nucleus, via a temperature-dependent process, where it associates with the chromatin (*8*). In practice the receptor system is prepared by isolating Triton X-100-washed chromatin from intestines of rachitic chicks (*7*) and reconstituting it with the 100,000*g* supernatant from a centrifuged homogenate of the same intestine. A 200-μl portion of the receptor system (containing 100 μg of DNA) was added to a small tube containing nitrogen-dried sterol or

Table 1. Concentration of 1α,25$(OH)_2$-D_3 in plasma of normal individuals and in patients with disorders in calcium metabolism; S.D., standard deviation.

Group	Patient (treatment)	Calcium (mg/100 ml)	1α,25-$(OH)_2$-D_3* (ng/100 ml ± S.D.)	Average of 1α,25-$(OH)_2$-D_3 (ng/100 ml ± S.D.)
Normal	20 (none)	10.5–11.7†	4.1–8.5†	6.4 ± 1.2
Renal disease	1 (no dialysis)	6.5	3.1 ± 0.4	
	2 (dialysis 1.3 yr)	12.2	2.9 ± 0.4	
	3 (dialysis 3.5 yr)	8.5	2.3 ± 0.6	2.6 ± 0.5‡
	4 (dialysis 1.7 yr)	11.8	1.9 ± 0.3	
	5 (dialysis 0.7 yr)	11.0	2.6 ± 0.2	
Postsurgical hypoparathyroid§	1 (surgery 1970)	9.5	3.3 ± 0.5	
	2 (surgery 6/1973)	8.6	6.7 ± 0.4	4.7 ± 1.8‖
	3 (surgery 1968)	10.1	4.1 ± 0.2	
Presurgical primary hyperparathyroid	1 (no surgery)	12.6	12.3 ± 0.6	
	2 (surgically confirmed adenoma)	15.5	11.4 ± 0.7	
Rachitic chicken		5.3	1.8 ± 0.6¶	
Normal chicken		11.4	10.1 ± 1.2¶	

* Represents the average of four assays; corrected for losses occurring in purification sequence. † Range for 20 normal volunteers. ‡ Significantly different from normal, $P < .005$. § All patients received 100,000 international units of vitamin D_2 daily. ‖ Significantly different from normal, $P < .10$. ¶ Represents two separate assays on pooled plasma from 14 chickens each.

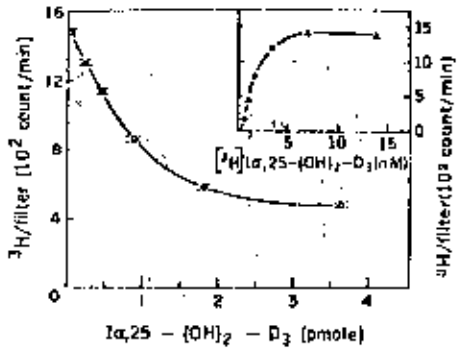

Fig. 1. Standard curve for 1α,25-(OH)$_2$-D$_3$. A sample of 5 nM [^{3}H]1α,25-(OH)$_2$-D$_3$ was incubated with increasing amounts of nonradioactive 1α,25-(OH)$_2$-D$_3$ and reconstituted cytosol-chromatin receptor system. The amount of bound tritrated compound is plotted as a function of the amount of nonradioactive hormone in the incubation mixture. [^{3}H]1α,25-(OH)$_2$-D$_3$ (9.8 c/mmole) was produced, in vitro, by a modification of the method of Lawson *et al.* (*3*). Homogenates of kidney from 4-week-old rachitic chicks were prepared and mixed with a phosphate buffer and a reduced nicotinamide adenine dinucleotide phosphate–generating system (*9*). [^{3}H]25-Hydroxyvitamin D$_3$ (10 nmole) (Amersham/Searle) was added to this reaction mixture in 1 ml of ethanol, and incubation was carried out under air at 37°C with gentle shaking for 1 hour. Lipids were extracted with a mixture of chloroform and methanol (1:2, by volume) (*9*). The [^{3}H]1α,25-(OH)$_2$-D$_3$ was purified by column chromatography on silicic acid, Sephadex LH-20, and Celite. The final radiochemical purity of the [^{3}H]1α,25-(OH)$_2$-D$_3$ as determined by chromatography on Celite was greater than 98 percent. Nonradioactive 1α,25-(OH)$_2$-D$_3$ was prepared as described (*9*). Each point on the standard curve represents the average ± standard error of the mean of the three assays. (Inset) Saturation of reconstituted cytosol-chromatin receptor system with [^{3}H]1α,25-(OH)$_2$-D$_3$. Increasing amounts of [^{3}H]1α,25-(OH)$_2$-D$_3$ (9.8 c/mmole) were incubated with the receptor system for 20 minutes at 25°C. The amount of bound [^{3}H]1α,25-(OH)$_2$-D$_3$ per filter was then determined. Each point represents the average of three determinations.

sterol extract and incubated for 20 minutes at 25°C. The chromatin was then harvested on glass fiber filters (Gelman type A), with the use of a 30-place Millipore manifold. The filters were washed with five portions (8 ml) of 1 percent Triton X-100 in 0.01*M* tris-HCl, *p*H 7.5, to remove unbound sterol. Control experiments without chromatin indicated that this procedure results in greater than 98 percent removal of free sterol from the filters. The specifically bound [^{3}H]1α,25-(OH)$_2$-D$_3$ was then extracted from the filters with methanol and chloroform (2:1), and the radioactivity was counted (38 percent efficiency) in a standard liquid scintillation counting solution (*8*).

In the competitive binding assay (Fig. 1) the receptor becomes filled when the concentration of hormone is 5 nM. This concentration of radioactive 1α,25-(OH)$_2$-D$_3$ is then used in the presence of increasing concentrations of nonradioactive 1α,25-(OH)$_2$-D$_3$ to create an isotope dilution-standard curve for the amount of nonradioactive hormone. Nearly linear competition results in the range of 0 to 1 pmole of added sterol, and the assay is sensitive enough to detect 0.20 pmole (83 pg).

The assay was then used to determine the concentration of 1α,25-(OH)$_2$-D$_3$ in human plasma. Heparinized blood was collected and lipids were extracted in methanol and chloroform (2:1) (*9*). 1α,25-(OH)$_2$-D$_3$ was isolated from the lipid extract by successive chromatography on a silicic acid column and two Sephadex LH-20 columns (*10*). The recovery of 1α,25-(OH)$_2$-D$_3$ after plasma extraction and chromatography was determined by initial addition of [^{3}H]1α,25-(OH)$_2$-D$_3$ (2500 count/min) to each plasma sample, and the assay results were corrected for losses of tritium. Recoveries ranged from 50 to 75 percent. Purified extracts of plasma were assayed as described.

Concentrations of 1α,25-(OH)$_2$-D$_3$ in plasma, as measured by the above assay, are shown in Table 1. The average concentration from 20 normal humans (6.4 ± 1.2 ng/100 ml) corresponds to 1.5×10^{-10}*M* 1α,25-(OH)$_2$-D$_3$, and is 1/300 that of its immediate precursor, 25-hydroxyvitamin D$_3$ (*11*). The concentration of 1α,25-(OH)$_2$-D$_3$ in the plasma of patients with chronic renal failure is less than 1/2 the normal concentration. This finding suggests that decreased production of the hormone may play a role in the osteodystrophy and calcium imbalance associated with kidney disease.

Hypoparathyroid patients appear to have slightly lower levels of 1α,25-(OH)$_2$-D$_3$ than normal in spite of the fact that they are under treatment with large doses of vitamin D$_2$ (Table 1). Presumably, in these patients the primary circulating vitamin D metabolites are derivatives of vitamin D$_2$, rather than vitamin D$_3$ as in normal individuals (*12*). Therefore, the results on patients receiving large supplements of vitamin D$_2$ must be viewed with caution because the relative affinity of the chick intestinal receptor for 1α,25-(OH)$_2$-D$_2$ and 1α,25-(OH)$_2$-D$_3$ is not known at present. Two hyperparathyroid patients had approximately twice the normal concentration of 1α,25-(OH)$_2$-D$_3$. These results suggest that parathyroid hormone may be involved in production of 1α,25-(OH)$_2$-D$_3$ in humans.

As a control for the human study, plasma from rachitic and normal chickens was assayed. The apparent concentration of 1α,25-(OH)$_2$-D$_3$ in the rachitic chick was 1.8 ng per 100 ml of plasma which is 1/6 that in the normal chicken (Table 1). The value in the rachitic chick either represents a true concentration of 1α,25-(OH)$_2$-D$_3$ in this vitamin D state or is a measure of nonspecific competition in the binding assay. In order to further confirm that displacement of the tritiated hormone was caused by 1α,25-(OH)$_2$-D$_3$, human plasma extracts which had been purified by successive silicic acid and two Sephadex LH-20 columns were treated with periodic acid (*13*) or were further purified by Celite chromatography. These procedures eliminate possible interference by 25,26-dihydroxyvitamin D$_3$, a circulating metabolite of vitamin D$_3$ which is not resolved from 1α,25-(OH)$_2$-D$_3$ by silicic acid or Sephadex LH-20 chromatography (*10*). Portions of plasma extracts were assayed after each step of the purification scheme (Table 2). Essentially identical plasma concentrations of 1α,25-(OH)$_2$-D$_3$ were determined with these additional purification

Table 2. Plasma 1α,25-(OH)$_2$-D$_3$ concentrations (nanograms per 100 ml) at various stages of purification as determined by radioreceptor assay. The values shown are corrected for losses occurring in each purification step.

Sample	Purification scheme			
	A*	B†	C‡	D§
1	8.3	7.3		
2	10.9	7.4		
3	8.2	7.2		
4	5.0	4.2	4.3	
5	11.9	8.4	8.3	
6	7.5	6.0	6.4	
7	6.5	1.9		2.1
8	7.7	6.0		6.0
9	6.8	6.5		6.2

* Silicic acid column and one Sephadex LH-20 column. † A plus additional Sephadex LH-20 column. ‡ B plus Celite liquid-liquid partition column. § D plus successive treatment with periodic acid and final Sephadex LH-20 column.

steps. However, an interfering substance was removed by the second Sephadex LH-20 column (Table 2, scheme B), demonstrating that at least three successive column purifications are required before assaying 1α,25-$(OH)_2$-D_3 from human plasma by this method.

This radioreceptor assay has shown for the first time that the hormonal form of vitamin D_3, 1α,25-$(OH)_2$-D_3, is detectable in humans. The use of the intestinal chromatin receptor system to detect very low levels of 1α,25-$(OH)_2$-D_3 further confirms the high affinity, specificity, and physiologic importance of this receptor for 1α,25-$(OH)_2$-D_3. Application of the nuclear hormone receptor complex to the measurement of 1α,25-$(OH)_2$-D_3 represents a unique approach to the assay of sterol hormones. Moreover, the use of filters to adsorb the chromatin associated receptor during the washing away of unbound sterol is a novel method of separating free sterol from that which is bound to macromolecular components. This assay may be used to study the regulation of the metabolism of 25-OH-D_3 to 1α,25-$(OH)_2$-D_3 in humans and experimental animals. Finally, the utility of the assay for diagnosis of disorders in calcium homeostasis, such as hypoparathyroidism and renal osteodystrophy, prior to possible treatment with 1α,25-$(OH)_2$-D_3, is clear. Involvement of the hormone in hyperparathyroidism and neoplastic hypercalcemia may also be determined.

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13. A purified plasma sample was treated for 10 minutes with 10 ml of periodic acid (1 mg/ml) in a mixture of water and ethanol (67 : 33). This procedure converts 24,25-dihydroxyvitamin D_3 and 25,26-dihydroxyvitamin D_3 to the corresponding aldo- and keto- derivatives (unpublished observations).
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The Role of The Fat Soluble Vitamins A and D in Nutrition

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FOREWORD

THERE are a number of reasons for including a [illegible] of the present status of both vitamins A and D together in nutrition. In the first place, they are both fat soluble vitamins. Secondly, their absorption from the alimentary tract is influenced to a considerable degree by those factors which interfere with fat absorption generally. Thirdly, they both occur not uncommonly in combination in natural sources. Their extremely important role in nutrition is of course unquestioned.

An attempt has been made to outline the major role that these two fat soluble vitamins have achieved in the field of nutrition. For the most part only those facts have been presented that have stood the test of time and have achieved general recognition. Contributions of a controversial character which still remain primarily in the field of speculation have received only passing mention or have not been included.

The generally accepted facts about vitamins A and D are [illegible] contributions of a sufficiently brilliant character and indicative of the march of medical progress in the field of nutritional research.

Early History of the Role of Vitamins in Nutrition

There are two main groups of alimentary constituents. In the first group are those substances which yield energy as well as the building stones for the creation of tissue. In the second group are the protective substances. Vitamins belong to this second group. They yield neither energy nor do they enter into the chemical structure [illegible] of the living cell, yet without their presence such transformations of food into energy or structure are impossible. They are organic compounds essential for growth and function. They therefore make possible by their presence the transformation of energy as well as the metabolism of the living cell.

Evidence of the existence of vitamin deficiency states is to be found in the writings of antiquity. The skeletons of prehistoric man show changes which resulted from a deficiency of vitamin D (rickets) as well as a deficiency of vitamin C (scurvy).

By the middle of the sixteenth century, the value of the juice of orange and lemon was discovered as a cure of scurvy, [illegible] the value of fresh vegetables [illegible] antiscorbutic. These remedies were [illegible] by Lind in 1757.

[illegible] the earliest attempt to [illegible] recognized as a vitamin [illegible] (lack of vitamin A) was in the use of [illegible] to counteract night blindness. [illegible] were recommended for this condition by the old Greek, Roman and Arab physicians. The ancient Hebrews recommended fish bile for this condition.

Further evidence of the value of fish livers is to be found in the writings of the middle ages and the use of fish liver oils was later strongly recommended by Schutte in 1824. Additional evidence linking night blindness with faulty nutrition is to be found in the work of Hoefer (1657) and Von Bergen (1754).

As will be more fully elaborated later, modern clinical and experimental procedures have verified fully the value of liver and particularly of fish liver as an excellent source of vitamin A.

The original belief was that certain foods exerted their beneficial effect by overcoming toxic material present in the organism which was the actual cause of the disease. It was only later that the concept arose that it was an absence in the dietary of a substance essential to the life process which was responsible for the nutritional disorders. Thus when the diet consisted of proteins, fats, carbohydrates, water and minerals, the animals died. Numerous experimental efforts were made which eventually proved that the life of an animal was impossible if fed only with the dietary constituents then recognized. Lunin, however, showed that when fresh milk was added to the same diet the animals survived. This experiment indicated that in addition to the recognized constituents of the diet, there were unknown substances without which life was impossible.

In 1897 came the epoch making report of Eijkman who proved experimentally that beri-beri was due to a food deficiency present in the rice bran and that this substance capable of curing the disease could be extracted from the bran by means of water or alcohol. This was followed by the work of Holst and Frolich in 1907, who demonstrated experimentally that scurvy was similarly a food deficiency disorder.

Eventually the name vitamin was given to these compounds. *(Funk, 1912)*. Ultimately, as is well known, many more vitamins were discovered and their chemical nature established. In many cases the artificial synthesis of these vitamins was thus made possible.

The first experimental evidence of the importance of a fat soluble substance in the maintenance of normal nutrition was that of Steppe in 1909. He subjected milk-bread to extraction with alcohol and ether. When mice were fed on this extracted bread, they died. If, however, the extracted material was fed with the bread, the mice showed normal growth. Evidently a substance soluble in both alcohol and ether had thereby been removed, essential to life itself.

Hopkins in 1912 showed the importance for the

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growth of rats that were fed on a diet of purified food substances, of the addition of a small amount of milk or in its place, the alcohol soluble portion of the milk. Carefully controlled experimental research was thus getting on the trail of an essential though as yet unrecognizable factor present in milk which was necessary to the life of the organism.

A further approach to the solution of the mystery was afforded by the work of McCollum and Davis and of Osborn and Mendel which appeared in 1913. These investigators showed that on a diet of purified casein, carbohydrates and various salt mixtures, life could sometimes be maintained but that normal growth was seriously hampered.

McCollum and Davis *(McCollum, E. V., and Davis, M., The Necessity of Certain Lipins in the Diet During Growth. J. Biol. Chem. 15:167, 1913)* showed that after the cessation of growth in the experimental animal, prompt resumption of such growth occurred with the introduction into the diet of an ether extract of butter or of egg, after such substances had originally been eliminated from the diet. They obtained these results in about thirty animals and concluded that these extracts contained an organic substance in the absence of which, the animal showed cessation of body growth. These results were never obtained when lard or olive oil were substituted for the ether extract of either butter or egg. This led these workers to the conclusion that an accessory food factor was present in the ether extracts of these substances.

This important accessory food factor was found to be present in butterfat. Osborn and Mendel also showed on the basis of their experiments *(Osborn, T., and Mendel, L. B., The Relation of Growth to the Chemical Constituents of the Diet. J. Biol. Chem., 15:311, 1913)* that the mere supply of an ample amount of energy yielding food was not sufficient to promote normal growth but in addition the diet required substances of the nature of chemical activators. They demonstrated the superiority of "protein-free milk" over diets consisting of artificial salt mixtures in order to maintain growth. Milk, however, proved to be adequate not only for maintenance but for growth of the animal. They showed that the essential accessory factor in milk necessary for growth was present in the butter-fat.

This fat soluble growth promoting substance became known as vitamin A. In 1914 Osborn and Mendel demonstrated its presence in cod liver oil. Attempts were made to isolate vitamin A and to determine its chemical structure. It was found that vitamin A was present in the unsaponifiable fraction of natural fats known as "sterols," the most representative members of which are cholesterol and phytosterol. The cholesterol occurs in animal fats and the phytosterol in vegetable fats. Most of the studies in the effort to isolate vitamin A have concerned the animal fats. Drummond (1914) separated vitamin A from cholesterol. He was able to concentrate the vitamin A of cod liver oil a thousand times. This purified substance obtained by Drummond appeared to be mainly an unsaturated sterol.

THE CHEMISTRY OF V[illegible]

The history of the isolation of vit[illegible] to 1826 when Wackenroder fir[illegible] pigment occurring in plants [illegible] because he obtained it from carr[illegible] structure of carotene was estab[illegible] 1907. He showed that the provitamin A [illegible] a simple five carbon atom [illegible] the formula of isoprene.

Experimentally the conversion [illegible] to vitamin A has been shown in a number [illegible] Thus Steenbock in 1919 *(Steenbock, H. [illegible] vs. Yellow Corn and A Probable [illegible] Fat-Soluble Vitamins and Yellow [illegible] Science 50:352, 1919)* showed that [illegible] min A in rats could be supplied by [illegible] green leafy material, and that [illegible] varied with the amount of carotene present [illegible]

In that article Steenbock [illegible] results which followed his attempt [illegible] rear their young when the food [illegible] white corn. Yellow corn on the [illegible] normal growth and reproduction [illegible] given white corn, the young [illegible] months with evidence of a vitamin [illegible] indicated by xerophthalmia. Thus [illegible] shown the relationship of yellow pigment [illegible] the occurrence of the fat soluble vitamin.

Osborn and Mendel had previ[illegible] *(Osborn, T. B. and Mendel [illegible] tions of the Influence of [illegible] Jour. Biol. Chem. 21:379, [illegible]* the yellow color of oils to the [illegible]

These facts led Steenbock [illegible] soluble vitamine may be [illegible] or a closely related compound [illegible] because of its occurrence in [illegible] other substances rich in "carotin" [illegible] ment related to the fat soluble [illegible]

Although Steenbock [illegible] closely related to vitamin A, [illegible] *(Karrer, P., Helfenstein, A., [illegible] stein, A. Über die Konst[illegible] carotins, Helvet. chim. [illegible]* Moore, *(Moore, T. [illegible] chem. J. 25:275, 19[illegible])* [illegible] actual forerunner of vitamin [illegible] provitamin A.

Moore showed that [illegible] of carotene to the [illegible] of the colorless vitamin A [illegible] the conclusion that the [illegible] cursor of the vitamin. [illegible] reserves of the body were [illegible] tive reaction for vitamin A [illegible] amination of the [illegible] experiments on the [illegible] monstered as [illegible] fat was [illegible] minimal [illegible] [illegible]

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[illegible] the presence of unchanged [illegible] indicating that the [illegible] not occur in the course of [illegible] took place after the absorption [illegible]

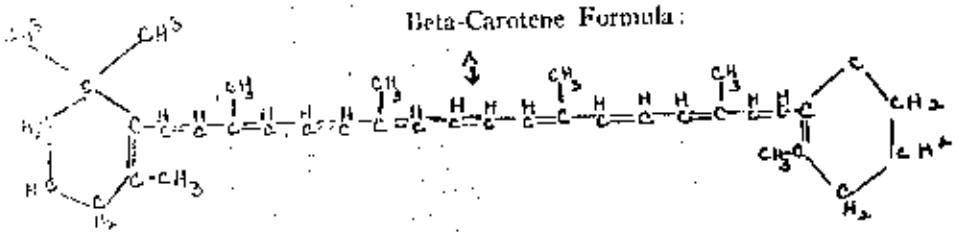
[illegible] the liver as the organ in [illegible] of carotene to vitamin A took [illegible] work of Buckley (1930) who [illegible] of parenchymatous degeneration [illegible] this organ had a deep yellow [illegible] excessive amount of carotene. When [illegible] amounts of crystalline carotene [illegible] large amounts of vitamin A are normally stored in the liver oils of cattle and only traces of carotene, the pathologic condition of the liver was evidently the etiologic factor in the failure of the conversion of carotene to vitamin A. Another evidence of the role of the liver in this process is the fact that most of the vitamin A is to be found in the liver and only comparatively small quantities are present throughout the rest of the body. The liver is therefore not only the organ primarily concerned with the conversion of carotene to vitamin A but it is also the essential storage place for this vitamin.

This conversion of the provitamins A to vitamin A is believed to be caused by an enzyme, carotenase, apparently present in the liver (*Olcott, H. S. and [illegible] Carotenase, Transformation of Carotene [illegible] in Vitro. J. Biol. Chem. 91:185, 1931*). [illegible] showed that carotene [illegible] vitamin A by incubating it with [illegible] aqueous extract of liver. [illegible] agents responsible for this change [illegible] was destroyed by heat.

[illegible] variation in the ability of [illegible] animals to convert the provitamins [illegible] A. Fortunately carnivorous animals [illegible] quantity of vitamin A contained [illegible] are essentially independent of the necessity of converting carotene into vitamin A.

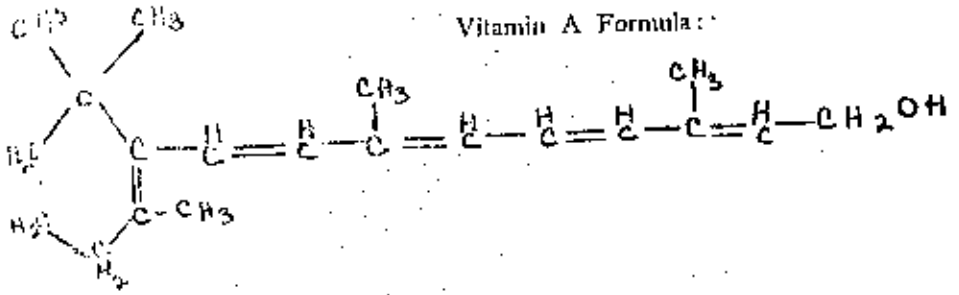
A number of scientific investigators were perplexed by the fact that the yellow-red plant pigment, carotene, exhibits vitamin A potency although the vitamin A of fish liver oils is colorless. Palmer (*Palmer, L. The Chemistry of Vitamin A and Substances Having a Vitamin A Effect. J. A. M. A. 110:1718, 1938*) showed that of the yellow pigments in plants there were four that could be converted into vitamin A, namely, alpha, beta and gamma carotene and cryptoxanthin. In the process of conversion to vitamin A, these yellow pigments become almost colorless. The manner in which this change occurs chemically is shown by the following formulae as demonstrated by Karrer in the transition of plant carotene to the vitamin A of fish liver oil.

Beta-Carotene Formula:



In the liver there is a hydrolytic process which breaks up the formula into identical molecules of vitamin A of the following chemical structure:

Vitamin A Formula:



Alpha and gamma carotene and cryptoxanthin however are supposed to produce only one molecule of vitamin A from each molecule of the provitamin. This is due to the difference in chemical structure of these particular provitamins.

If the formula of vitamin A is studied it is noted that it has an alcohol group (-CH₂OH), resulting from retention of part of the water in the hydrolytic cleavage of beta-carotene. This alcohol group is capable of forming esters when another organic group replaces the OH group. The variation in fish oils from the standpoint of vitamin A potency is in part attributable to the presence of the ester form instead of the alcohol group.

Although the chemical relations involved in the change from the carotenoids to vitamin A have been shown, the reverse procedure, namely, the conversion of vitamin A to carotene has not been accomplished.

Vitamin A is apparently the only vitamin which occurs as a result of animal metabolism, although it

provitamins are entirely the result of plant metabolism.

The conversion of the provitamins, carotene and cryptoxanthin, into vitamin A in the liver occurs through the intermediary of an enzyme as previously stated. The thyroid gland may also play a role in this process. Some of the experimental evidence to sustain this claim has been obtained on thyroidectomized goats. Under normal conditions with the thyroid intact, the goat converts carotene to the colorless vitamin A and the milk produced is white in color. When the thyroid has been removed, the milk is yellow in color.

Further evidence suggesting a relationship of the thyroid to the ability of the organism to convert carotene to vitamin A is shown in the work of Clausen and McCoord *(Clausen, S. W. and McCoord, A. B. The Carotinoids and Vitamin A of the Blood. J. Pediat. 13:635, 1938)*. They found that large amounts of thyroxin lowers the vitamin A content of the liver. In most cases of hyperthyroidism, the plasma carotene showed high values. The vitamin A content of the plasma, however, was low in all cases.

Besides vitamin A which is found only in salt water fish and mammals, other similar substances have been described such as vitamin A_2 *(Fidisbury, J. R., Morton, R. A. and Simpkins, G. W. Possible Vitamin A_2, Nature. 140:231, 1937)*. Vitamin A_2 is present in fresh water fish in addition to vitamin A. Small amounts of Vitamin A_2 however have been found in halibut liver oils in addition to vitamin A. It is rarely demonstrable in cod liver oils and was never detected in whale liver oils.

Vitamin A_2 has not been isolated in pure form nor has its exact chemical constitution been determined. Evidence, however, of the relationship of vitamin A and vitamin A_2 is to be found in the fact that when carotene is fed to fish such as the perch, both of these vitamins are synthesized, thus indicating that carotene is also a precursor of vitamin A_2 *(Morton, R. A. and Creed, R. H. Conversion of Carotene to Vitamin A_2 by Some Fresh-water Fishes. Biochem. J. 33:318, 1939)*.

There is no evidence according to Wormall *(Wormall, A. Annual Reports On the Progress of Chemistry for 1938, 35:232, 1939)* that vitamin A_2 plays any significant role in the nutrition of the mammal although biologically it may be the equivalent of vitamin A_1. Experimentally it has been shown that the livers of rats fed with a concentrate containing vitamin A_2 will on examination be found to contain this vitamin.

A method of distinguishing between vitamin A and A_2 has been recommended by Greenberg and Popper *(Greenberg, R. and Popper, H. The Differentiation Between Vitamin A_1 and A_2 Fluorescence Microscopy. Proc. Am. Physiol. Soc., 129:367, 1940)* and depends on the variation in the nature of fluorescence of the two. Thus vitamin A shows a green fluorescence and vitamin A_2 exhibits a reddish fluorescence. They found that in salt water fish a considerable amount of this green fluorescence characteristic of vitamin A [illegible] demonstrable in the fat droplets of the [illegible] of the liver. When exposed to ultra violet [illegible] this characteristic green fluorescence fades. A [illegible] condition is to be found in the mammal. In the [illegible] of fresh water fish, the green fluorescence [illegible] but in its place one can demonstrate a yellowish-[illegible] fluorescence distributed in a similar manner. [illegible] fades on irradiation with ultra violet light. [illegible]cence microscopy therefore offers not only a [illegible] distinguishing between vitamin A and vitamin A_2 [illegible] also enables us to determine the distribution of the two vitamins in various potential sources and demonstrates the fact that vitamin A is present in the [illegible] of salt water fish, while vitamin A_2 is to be found in the livers of fresh water fish. Since it is the vitamin A that plays the predominating role in mammalian physiology, it indicates the importance of such a [illegible] as halibut liver oil coming from a salt water [illegible] When vitamin A was fed to the rat, fluorescence microscopy was able to demonstrate its presence within the lumen of the stomach and also within the [illegible] and the wall of the small intestine. From there vitamin A could be traced by way of the epithelial [illegible] and lymphatics to larger lymphatics, the main site of absorption being in the jejunum. Within several [illegible] after feeding vitamin A to rats showing a deficiency state, the green fluorescence could be demonstrated in the Kupffer and adjacent epithelial cells of the liver as well as in the adrenal glands and ovaries.

The origin of the precursors of vitamin A is [illegible] found in plants, chiefly as carotene. These are [illegible] scured by the green coloring matter of the plant, chlorophyl. Although there are apparent exceptions there is a fairly parallel relationship between the [illegible]ness in carotene, and the amount of chlorophyl [illegible]tion in green leaves. Following the ingestion of leafy substances, the carotenes are [illegible] altered by the body into vitamin A. [illegible] needed reaches the tissues of the body and [illegible] is stored. Chickens and cows are able to [illegible] the provitamins of grasses and [illegible] tion into vitamin A containing foods. [illegible] sources of this vitamin in [illegible] manner, namely eggs and milk. [illegible] min A are therefore to be found [illegible] yellow vegetables, eggs, milk [illegible] as butter, cream cheese and [illegible]

The most important source of vitamin A [illegible] oils obtained from fish. This is [illegible] of vitamin A in the animal organism [illegible] in the liver.

The exact origin of the large [illegible] in fish livers is not clearly known. [illegible] of fish ingest plankton which [illegible] A may contain its precursors. [illegible] transformed into vitamin A and [illegible] liver. The large reserves of vitamin [illegible] may also be due to the fact that the [illegible] astaxanthin which is the pigment [illegible] minate crustacea which are [illegible]

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[illegible] Acantlin appears to be closely [illegible] vitamin A and fish may possess the peculiar [illegible] of utilizing this substance and converting it into [illegible] A. Whatever the method of transformation, [illegible] it is that the fish liver oils are an [illegible] source of the fully synthesized vitamin A [illegible] *The Constitution and Physiological* [illegible] *of Carotene and Allied Pigments. Chemistry and Industry* [illegible] 1931).

Vitamin A can be readily isolated from the liver oils by means of the vacuum distillation process. It was first crystallized (*Holmes, H. N. and Corbet, R. E. The Isolation of Crystalline Vitamin A. J. Am. Chem. Soc.* [illegible] 12, 1937). Isolated crystalline vitamin A from fish liver oils appears as pale yellow needles. Assays on the crystals of various fish liver oils showed variations between about 2,000,000 [illegible] 3,000,000 International Units per gram. It is destroyed by ultraviolet light. It is fat-soluble but insoluble in water.

Vitamin A is lost in the presence of oxygen because it is oxidized. Oxidation processes generally cause a reduction in the physiologic activity of the vitamin. Because of the difficulty in the determination of vitamin A potency in commercial preparations, methods are employed to prevent oxidation. Various antioxidants may therefore be added to the vitamin A preparations or in the case of the liver oils, they are sealed in vacuo.

The Carr-Price test (*Carr, F. H. and Price, E. A. Chemical Reactions Attributed to Vitamin A. Proc. Soc. Exp. Biol.* [illegible] 1926) is ordinarily employed for the determination of vitamin A. To a solution of vitamin A in chloroform is added a 20 to 25% solution of antimony trichloride in chloroform. A blue color develops which reaches its maximum intensity [illegible] and then begins to fade promptly. The color is measured in a colorimeter and the value may then be checked against a standardized solution of [illegible]. Values obtained in this manner correspond fairly accurately with the method of biological assay. The Carr-Price colorimeter method is not applicable to the determination of the synthetic preparations of vitamin A.

Although the pure crystalline vitamin A is available, it has no great commercial significance because concentrates of vitamin A may be obtained which are clinically satisfactory. Halibut liver oil is one of the principal commercial sources of vitamin A. In order to preserve the vitamin A in the fish livers, they are stored [illegible] and [illegible] conditions. Otherwise the vitamin will be rapidly destroyed.

[illegible] of biological assay of determining the [illegible] vitamin A preparations as recommended by [illegible] is as follows: Young rats [illegible] stopped because of a deficiency of [illegible] to gain weight by adding vitamin [illegible] are then compared with those [illegible] an International Standard [illegible] This standard as supplied by [illegible] Health Organization is a beta-[illegible] which is the equivalent of one International Unit of Vitamin A. The preparation of the carotene employed was made from carrots by the method of Willstatter.

The adaptation to darkness of the eye after being exposed to bright light is also used as a means of determining the minimum amount of vitamin A essential to the organism (*Evans, P. C. and Zentmire, J. Clinical Method for Determining Moderate Degrees of Vitamin A Deficiency. J. A. M. A. 102:892, 1934*) (*Feldman, J. B. Instrument for Qualitative Study of Dark Adaptation. Arch. Ophthalmol. 18:821, 1937*). This test depends on the fact that night blindness is one of the earliest manifestations of vitamin A deficiency, a point which will be discussed more fully later. Such eyes when examined may be free of any retinal pathology and yet by testing the individual for dark adaptation, early evidence of a deficiency state may be demonstrated. Various methods have been devised for testing the degree of night blindness by the use of adaptometers (*Hecht, S. and Mandelbaum, J. Relation Vitamin A and Dark Adaptation. J. A. M. A. 112 1910, 1939*) (*Pett, L. B. Vitamin A Deficiency. II. Prevalence and Importance as Shown by New Test. J. Lab. Clin. Med. 25:149, 1939*).

Clinically the dark adaptation phenomenon is familiar to us all. Thus on coming from the sunlit outside into a dark room it may take a long time before objects become clear to the field of vision. If one walks into the dimly lighted theatre, from the brightly illuminated street it may take quite some time before one is able to recognize either the audience or any vacant seat. After sometime however, the various objects in the theatre take shape and one may see fairly clearly the faces of the nearby people. This process is described as dark adaptation.

When attempts are made to determine the degree of dark adaptation, various methods of a fairly exact character are employed in order to determine the minimum amount of light which may be visible after definitely stated periods of time.

Another biologic method, the clinical significance of which will be discussed more fully later, depends on the demonstration of pathologic alterations in the appearance of the vaginal smear, the result of a deficiency of vitamin A.

Evans (*Evans, H. M. and Bishop, K. S. On An Invariable and Characteristic Disturbance of Reproductive Function in Animals Reared on a Diet Poor in Fat Soluble Vitamin A. Anat. Rec. 23:17, 1922*) showed that in the female, vitamin A deficiency leads to cornification of the vaginal mucous membrane. This may be shown by the study of a vaginal smear. When however, ample vitamin A is added, the vaginal smear picture becomes normal.

Finally the vitamin A concentration of the blood has been recommended as the most subtle procedure in the determination of the earliest evidence of subclinical deficiency. (*Varbringh, M. E. and Dann, W. J. Dark Adaptometer and Blood Vitamin A Measurements in a North Carolina Nutrition Survey. J. Nutrition 22:597-607, Dec. 1941*) (*Bodansky, O., Lewis, J. M. and Haig, C. The Comparative Value of the Blood*

Plasma Vitamin A Concentration and the Dark Adaptation as a Criterion of Vitamin A Deficiency. Science 94:370-371, Oct. 17, 1941). Diminution in the vitamin A concentration of the blood, may indicate a subclinical state of vitamin A deficiency long before there is any detectable evidence by the dark adaptation method.

THE MODE OF ABSORPTION OF VITAMIN A AND THE FACTORS INVOLVED

There are a number of factors which may produce a vitamin A deficiency state because of their interference with the absorption of the vitamin even when administered in normal amounts.

Bile and the Absorption of Vitamin A

The absorption of vitamin A is disturbed in the presence of jaundice, indicating the importance of bile *(Greaves, J. D. and Schmidt, C. L. A. The Utilization of Carotene by Jaundiced and Phosphorus Treated Vitamin A Deficient Rats. Am. J. Physiol. 111:502, 1935) (Greaves, J. D. and Schmidt, C. L. A. On the Absorption and Utilization of Carotene and Vitamin A in Choledochcolonostomized Vitamin A Deficient Rats. Am. J. Physiol. 111:492, 1935).* In their experiments, Greaves and Schmidt used the vaginal smear technique as a means of determining the absorption of carotene and vitamin A. An internal biliary fistula was established by anastomosing the bile duct to the upper part of the ascending colon, by means of a small silver cannula inserted into the bile duct which was then sewed into the colon.

Those animals that were not operated but were maintained on a vitamin A deficient diet responded within a few days to carotene therapy administered either orally or subcutaneously. The rats subjected to the side-tracking operation failed to respond to the oral administration of carotene, although a therapeutic response resulted from subcutaneous injection.

The evidence indicated that the presence of bile in the small intestine was important for the alimentary absorption of carotene. Vitamin A however, in the form of halibut liver oil was absorbed from the alimentary tract when administered orally even in the absence of bile, although less effectively under such conditions *(Greaves, J. D. and Schmidt, Studies on the Vitamin A Requirements of the Rat. Am. J. Physiol. 116:456, 1936).*

The reason for the disturbance of absorption of vitamin A in the absence of bile in the small intestine is apparently due to the fact that bile is essential for the digestion of fat. In the absence of bile, fat digestion is therefore seriously disturbed. Since vitamin A is fat soluble, its absorption will therefore also be materially affected by those same factors which impede the digestion and absorption of fat.

In this connection, the case of Elschnig described in 1899 and referred to in Blegvad's article *(Blegvad, O. Xerophthalmia, Keratomalacia and Xerosis Conjunctivae. Am. J. Ophth. 7:89, 1924)* is of considerable clinical interest. This was the case of a woman fifty-one years of age who was icteric. Physical examination also showed evidence of an enlarged liver and gall bladder and the presence of keratomalacia. Autopsy revealed a carcinoma of the bile duct with [illegible] preventing the entrance of bile into the [illegible] keratomalacia appeared to be the result of [illegible] fat absorption because of the absence of bile in the intestinal tract.

Not only is there a disturbance in the absorption of vitamin A when fat digestion is incomplete because of the lack of bile, but a similar interference [illegible] result from a deficiency of pancreatic lipase. *([illegible] S. W. Limits of the Anti-Infective Fat [illegible] A (Carotene) J. A. M. A. 101:1381, 1933)* an enzyme also essential for the digestion of fat.

In this connection it is of interest that Breese and McCoord *(Breese B. B. Jr. and McCoord, A. B. [illegible] min Absorption in Celiac Disease. J. Pediat. 15:[illegible], 1939)*, found deficient absorption of vitamin A in a case of cystic fibrosis of the pancreas.

The Absorption of Carotene as Compared with Vitamin A

The superiority of the active vitamin A as compared with carotenes from the standpoint of absorption was shown by Clausen and McCoord *(Clausen, S. W. and McCoord, A. B. The Carotinoids and Vitamin A of The Blood. J. Pediat. 13:635, 1938).* [illegible] showed that when young infants are fed parsley [illegible] or solutions of carotene in oil, there is a slow rise in the level of plasma carotinoids. The maximum level may not be reached before at least twenty-four hours. When however, large doses of halibut liver oil as a source of vitamin A are administered, examination of the plasma shows a maximum concentration in [illegible] hours.

This experimental evidence would indicate the superior value of active vitamin A containing [illegible] particularly when an attempt is being made to [illegible] come as promptly as possible a [illegible] deficiency of this vitamin.

Effect of Short-Circuiting Operations on Vitamin A Absorption

Short circuiting of ingested food interfering [illegible] with digestion and absorption was [illegible] blindness in the case described by [illegible] man *(Wilbur, E. L. and Eusterman, [illegible] Night Blindness. J. A. M. A. 102:[illegible]).* [illegible] case a gastro-colic fistula had [illegible] gastric surgery. Most of the [illegible] passed directly into the colon. [illegible] occurs primarily in the small intestine [illegible] seriously interfered with. When [illegible] was repaired so that small bowel [illegible] tion again became possible, the [illegible] peared, owing to the effective [illegible]

Vitamin A Absorption and [illegible]

The fact that vitamin A is [illegible] intestinal tract, showing maximum [illegible] blood in three to five hours [illegible] the efficiency of intestinal absorption [illegible] this test, the vitamin A in the [illegible] is administered by [illegible] vitamin A concentration of the [illegible]

[illegible] In the presence of coeliac disease, Chesney and McCoord (*Chesney, J. and McCoord, A. B. Vitamin A of Serum Following Administration of Halibut Oil in Normal Children and in Chronic Steatorrhea. Proc. Soc. Exptl. Biol. Med. 31:887, 1934*) observed that the rise of vitamin A in the serum was considerably less than in normal children. With clinical improvement in the condition, there was a marked rise in the vitamin A content of the serum.

Breese and McCoord (*Breese, B. B. Jr. and McCoord, A. B. Vitamin A Absorption in Celiac disease. J. Pediat. 15:[illegible], 1939*) studied the absorption of vitamin A in two cases of coeliac disease. After a basal blood determination was made, the child suffering from the disease was given fish liver oil in quantity furnishing 7,000 U.S.P. units per kilogram of body weight. Blood determinations were then made 2, 4, 6, 9, 12, and 24 hours later. These tests showed that the child with coeliac disease was unable to absorb vitamin A even after treatment, with the same efficiency as the normal child.

In coeliac disease, the serum and plasma may be colorless because of the diminished amount of carotene. The vitamin A content may also show a marked diminution in affected by the ingestion of halibut liver oil because of poor absorption from the alimentary tract. Thus a diagnostic procedure in the establishment of the presence of coeliac disease is the demonstration of a failure of a rise in the vitamin A of the plasma after the ingestion of halibut liver oil. Such a finding would lend support to the diagnosis in addition to the presence of increased fat in the stool and a "flat" blood sugar curve.

That absorption of vitamin A is disturbed in the presence of coeliac disease was also shown by Heymann (*Heymann, W. Absorption of Carotene, Am. J. Dis. Child. 51:273, Feb., 1936*).

Fat and Vitamin A Absorption

The relation of fat to vitamin A absorption in different individuals when the same amount of the vitamin is ingested, is indicated by the work of Wilson, Das Gupta and Ahmad (*Wilson, H. E. C., Das Gupta, S. M. and Ahmad, Bashir. Studies on the Absorption of Carotene and Vitamin A in the Human Subject. Indian J. M. Research. 24:807-811, Jan., 1937*). When the diet contained no fat, only fifty percent of the carotene ingested by the human subject was absorbed. When, however, a moderate amount of fat was included in the diet, the absorption of the carotene from the alimentary tract was increased to about ninety per cent.

All of these factors indicate the importance of a [illegible] supply of vitamin A well above that essential for the maintenance of ordinary health, in order to make sure that accidental factors will not interfere with the sufficient utilization of vitamin A, not only for the [illegible] needs but for ample storage during periods of unconsolidated depletion.

Mineral Oil in Relation to Vitamin A Absorption

That mineral oil may interfere with the absorption of vitamin A is indicated by the work of Dutcher, Ely, and Honeywell (*Dutcher, R., Ely, J. O., and Honeywell, H. E. Vitamin Studies XV. Assimilation of Vitamins A and D in Presence of Mineral Oil. Proc. Soc. Exper. Biol. and Med., 24:953-955, June, 1927*). These authors demonstrated in experiments on rats that mineral oils possess the power of dissolving vitamin A and depleting foods of this vitamin. Thus in one group of rats in which the stored supply of vitamin A had been depleted, a curative dose of butter fat was administered mixed with mineral oil, in the proportion by weight of 2.5 of mineral oil to 1.0 of butter fat. Xerophthalmia developed and death occurred in three cases. On removing the mineral oil, xerophthalmia disappeared and growth recurred.

Similiar results on the action of mineral oil in removing fat soluble vitamins from food have been noted in the experimental observations of Burrows and Farr (*Burrows, M. T., and Farr, W. K. The action of Mineral Oil per Os on the Organism. Proc. Soc. Exp. Biol. and Med., 24:719-723, April, 1927*).

Jeans showed as a result of animal experiments, that when mineral oil is ingested some of the vitamin A is carried away with it in the feces, thus further verifying the work of other observers. (*Jeans, P. C. Vitamin Deficiency in Childhood, Minnesota Med. 16:683, Nov., 1933*). When the amount of vitamin A administered is therefore barely sufficient a deficiency of vitamin A may develop. This however, may be readily overcome by the administration of larger amounts of vitamin A. He described the case of a child with deficient dark adaptation which could not be cured even though fish liver oil was administered for several months. When she was deprived of the mineral oil which she had been taking, her vision promptly became normal.

Vitamin A Absorption and Infections

It has also been shown that the rate of absorption of carotene is diminished in infants as a result of some infections (*Heymann, W. Absorption of Carotene. Am. J. Dis. Child. 51:273, Feb., 1936*). Heymann showed a greatly diminished rate of absorption of carotene in pneumonia, grippe and sepsis. Although healthy children showed excellent absorption when carotene was administered in oil, the infants suffering from the infectious diseases showed a marked decrease of absorption amounting to one-half that of the healthy infant. These infections therefore appeared to interfere with the normal intestinal absorption of carotene.

Vitamin A Absorption and Tuberculosis

Among a group of 197 tuberculous patients, Getz, Hildebrand and Finn (*Getz, H. R., Hildebrand, G. B. and Finn, M. Vitamin A Deficiency In Normal and Tuberculous Persons, As Indicated by the Biophotometer. J. A. M. A. 112:1308-1311, 1939*) found that 53 percent showed evidence of vitamin A deficiency. In the non-tuberculous group only 11 per cent showed such vitamin A deficiency. The degree of vitamin A deficiency in the tuberculous individual appeared to parallel the seriousness of the pulmonary involvement. This evidence of vitamin A deficiency was present in spite of the fact that the diet apparently

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contained an amount of vitamin A sufficient for individuals in normal health and even though 40 percent of the patients received an additional supply of vitamin A as fish liver oil, not in excess of 30,000 units a day. This indicated that even in the presence of the added vitamin A intake the amount was insufficient for the prevention of a deficiency state. Larger doses of vitamin A therefore appeared to be essential not only in some non-tuberculous individuals but particularly in individuals suffering from tuberculous infection. Doses up to 200,000 units a day might be necessary in refractory cases in order to obtain a normal biophotometric response.

These are therefore added reasons for the addition not only of large amounts of vitamin A during health, but also of making certain that an ample supply is available in chronic disease. When prompt absorption of vitamin A is desired, this may be accomplished more effectively through the administration of natural containing sources of the actual vitamin A such as milk, egg and fish liver oils than through the carotenes.

Vitamin A Absorption and Other Disorders

The absorption of vitamin A may also be disturbed in other disorders. Thus Abels, Gorham, Pack and Rhoads (*Abels, J. C., Gorham, A. T., Pack, G. T. and Rhoads, C. P. Metabolic Studies in Patients [illegible] Cancer of the Gastro-Intestinal Tract I: [illegible] min A Levels in Patients with Malignant [illegible] Disease, Particularly of the Gastro-Intestinal [illegible] Clin. Investigation 20:749-761, Nov. 1941*) [illegible] *J. C., Gorham, A. T., Pack, G. T. and Rhoads [illegible] Metabolic Studies in Patients with Gastro-[illegible] Cancer: III The Hepatic Concentrations of [illegible] A, Proc. Soc. Exper. Biol. and Med. [illegible] Nov., 1941*) found a diminution in the plasma [illegible] vitamin A in 86 percent of patients suffering [illegible] cancer of the gastro-intestinal tract.

The low plasma level of vitamin A in such patients was shown not to be due to an inability of the liver to store vitamin A.

Page and Bercovitz (*Page, R. C. and Bercovitz, Z. The Absorption of Vitamin A in Chronic [illegible] ative Colitis. Amer. Jour. Digestive Diseases, [illegible] May, 1943*) studied twenty-five patients with chronic ulcerative colitis. Following the oral administration of 100,000 U.S.P. units of vitamin A in the form of [illegible] oil concentrates, they found a lower plasma vitamin [illegible] level than in normal controls. The disturbance may be best explained on the basis of disturbed absorption associated with diarrhoea.

(To be continued in May 1944 Issue)

Autonomic Pharmacology and Therapeutics of The Gastro-Intestinal Tract

By

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THE exigencies of human life require that individuals react properly to their external environment, and also that the cells composing the organs of the individual act harmoniously and do not thwart one another (i.e. react properly to the internal environment). The cerebrospinal nervous system insures the optimal exterior conditions for life, and enables the individual through his sense organs, skeletal system and voluntary muscles to change his external environment. The effect of the autonomic nervous system is to adjust the internal functions of the body, such as blood circulation, respiration, digestion and excretion so that they are coordinated for the benefit of the body cells. The metabolic operations of the body incident to the demands of everyday life cause certain changes in the internal environment of the body, i.e., changes in pH, body temperature and osmotic pressure of the blood, and therefore require adjustments by the organs of the body. These adjustments are made by the autonomic nervous system.

There is one fundamental anatomical difference between the cerebrospinal or "voluntary" nervous system and autonomic or "involuntary" nervous system. The fibers of the autonomic nervous system do not run directly to their effector organs, but are interrupted at a break or synapse. Many of these synapses combine to form a ganglion. The post-ganglionic [illegible] come from these ganglia and then directly innervate the effector organs, i.e., stomach or other organs.

However, the central nervous system and the autonomic nervous system can not be so [illegible] completely separated. For example, it [illegible] known [illegible] most autonomic functions are under [illegible] system influence. Pleasurable emotions [illegible] ceral functions, while unpleasant [illegible] them. It has also been shown that there are [illegible] of cells in the hypothalamus, medulla, and [illegible] that control autonomic function. [illegible] vegetative centers. Some of the [illegible] by these centers are the contraction [illegible] uterus, the regulation of body temperature, the [illegible] ination of water and other functions [illegible] studied.

Autonomic activity, as a rule, [illegible] those functions which are carried [illegible] consciousness. Therefore, [illegible] endocrinological and other activities [illegible] this system. Most of the organs [illegible] nomic nervous system are innervated [illegible] pathetic and parasympathetic [illegible] In general these divisions have [illegible] balancing actions, [illegible] these innervated organs. [illegible] always an inhibitor or [illegible]

* Lecture delivered on May 17, 1943 in a Post-Graduate course in Gastroenterology sponsored by the Kings County Medical Society and under the charge of Dr. Samson A. Seley.

[illegible] On the basis of a single experiment [illegible] mixtures of equal volumes of two plasmas of different bilirubin content, with different proportions of direct-reacting bilirubin, Coolidge[7] concluded that the differences in reactivity of direct- and non-direct-reacting bilirubin are dependent on structural rather than catalytic factors. However, as also pointed out by Castex, Lopez Garcia and Zelasco[9], the quantities of direct-reacting bilirubin in these samples of serum was practically identical, a fact which may have been responsible for the failure to obtain a significant difference between the actual and theoretical proportions of direct-reacting bilirubin in the mixture. The latter, authors' study of mixtures of equal parts of serum from (a) patients with obstructive jaundice, (b) a normal subject and one with "hepatosis", (c) patients with "hepatosis" and (d) a patient with obstructive and one with hemolytic jaundice. They found that the actual proportion of direct-reacting bilirubin was distinctly higher than the theoretical in each instance except in the case of the mixture of samples from two patients with "hepatosis".

The data reported here suggest that whether or not there is a structural difference between direct-reacting and non direct-reacting forms of bilirubin, the capacity of the former for reacting with the van den Bergh reagent in aqueous acid depends to some extent upon other factors in the serum. Although the quantity of albumin may be significant in this connection, similar changes were observed in the presence of varying and constant albumin concentrations in the case of both obstructive and hemolytic jaundice serum.

Summary

When samples of serum from patients with obstructive and hemolytic jaundice are mixed in varying proportions with one another or with normal serum or concentrated normal plasma, the actual proportion of direct-reacting bilirubin in the mixture does not coincide with the theoretical, calculated from the quantities present in the components of the mixture. With decreasing bilirubinemia in such mixtures, the actual rose progressively above the theoretical in the case of hemolytic jaundice serum and fell progressively below the latter in the case of obstructive jaundice serum. These findings suggest that the capacity of serum bilirubin for reacting with the van den Bergh reagent in aqueous acid is dependent, in part at least, on factors in the serum other than the bilirubin molecule or the nature of its combination with albumin.

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The Role of The Fat Soluble Vitamins A and D in Nutrition

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(Continued from April Issue)

THE ROLE OF THE LIVER IN VITAMIN A

[illegible] whether the storage of vitamin A may be in [illegible] supplying the needs of the animal during periods of depletion is indicated by the work of Davies and Moore (*Davies, A. W. and Moore, T. Vitamin A and Carotene. The Influence of the Vitamin A Reserve on the Length of the Depletion Period in the Young Rat. Biochem. J. 31:172, 1937.*) They showed that in the rat fed with brilliant liver oil as a source of vitamin A yielding high reserves of the vitamin, weight increase would continue for several months after stopping its administration. In those rats having a low reserve of vitamin A, growth ceased at the end of five weeks, after such depletion.

The amount of vitamin A stored in the liver is less at birth than in the adult (*Ellison, J. B. and Moore, Thomas. Vitamin A and Carotene: The Vitamin A Reserves of Human Infant and Child in Health and Disease, Biochem. J. 31:165, Jan., 1937*). Ellison and Moore examined the livers of about two hundred children under fifteen years of age at autopsy, that had

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died either as a result of accident or disease. Their results confirmed the work of other observers that the vitamin A reserves in young infants were very low. The amount of vitamin A in the liver increased with age and is affected by the vitamin A content in the diet.

The importance of possessing an ample reserve of vitamin A above the actual requirements of ordinary health is also suggested by the work of Moore (*Moore, Thomas. Vitamin A and Carotene. The Vitamin A Reserve of the Adult Human Being in Health and Disease. Biochem. J. 31:155, Jan., 1937*). He obtained 1,000 specimens of livers from patients who died of all types of diseases and the vitamin A content was determined by the antimony trichloride method. For a determination of the vitamin A reserve of the normal adult, those livers were studied of patients who died as a result of accident and not of disease. The median average was 220 International Units per gm.

The typical human reserve of vitamin A based on the physiological requirements of the rat is sufficient to support life for about six months. He found low reserves of vitamin A in individuals who died of septic diseases and he believed that such vitamin A deficiency might be a predisposing cause in some cases in the development of infection. This confirmed the work of Wolff who found subnormal reserves in erysipelas, phlegmons, sepsis and chronic suppurations. Moore also found low reserves in patients dying of chronic nephritis. The vitamin A reserve in these individuals was sufficient for only three weeks as compared with the reserve in the healthy subject which was sufficient for six months.

His work also supported the observations of Wolff that very low reserves, often none at all, were found in the presence of cirrhosis of the liver. The low reserves found in some cases of cirrhosis of the liver is evidently a result and not a cause of the liver injury. In the presence of a failing myocardium, disturbance of the liver because of congestion may be considered as a cause of the low vitamin A reserve.

In puerperal infection, the failure of the liver to store vitamin A occurred even when these patients were treated with large amounts of vitamin A concentrate. Also in cases of chronic nephritis with low vitamin A in the liver, there was nevertheless a preponderance of unconverted carotene suggesting also that there was an impairment in the ability of the liver to convert the carotene into vitamin A. In a significant proportion of cases, however, vitamin A deficiency appeared to be a contributory factor in the susceptibility to the development of infectious disease.

The role of the liver as the great storehouse of vitamin A has been demonstrated experimentally in many ways. As the result of such storage, the organism may be furnished with an ample supply of vitamin A during periods of depletion.

It is an important fact that the body is able to store a sufficient amount of vitamin A to meet its nutritional needs for a long time. This storage potentiality thus provides a great factor of safety during periods of vitamin A depletion, and during periods of shortage, the organism may not readily exhibit obvious organic derangements. The subclinical state of vitamin A deficiency may continue for a long time before physical changes eventually become apparent.

The importance of the liver as a storehouse for vitamin A in the rat is shown by the fact that excluding the fat and skin of the animal, ninety percent of the vitamin A is to be found in the liver. It contains about 200 to 400 times the amount of the vitamin as compared with muscle tissue. On the basis of feeding experiments with fresh skeletal muscle, kidney, lung and liver tissue of rats as a source of vitamin A in the maintenance of the growth curve of the experimental rat, Sherman and Boynton found that the liver was forty times as rich in vitamin A as the muscle (*Sherman, H. C. and Boynton, L. C. Quantitative Experiments Upon the Occurrence and Distribution of Vitamin A in the Body, and the Influence of the Food. J. Am. Chem. Soc. 47:1646-1653, 1925*). When a larger amount of vitamin A was included in the diet of the rat, the concentration of vitamin A was increased [illegible] to ten times.

Baumann, Riising and Steenbock (*Baumann, C. A., Riising, B. M. and Steenbock, H. The Absorption and Storage of Vitamin A in the Rat. J. Biol. Chem. 107:705-715, 1934*) showed that about 95% of the total vitamin A was stored in the liver of the rat. Small amounts were also found in the kidneys and lungs. None was found in the brain, blood, muscle [illegible] in the alimentary tract. However, when the vitamin A of the liver was absent none of the vitamin was found in any other tissues. The stores of vitamin A in the newborn were very low. This amount gradually decreased until the third week following which there was a rapidly increased storage. The daily addition of [illegible] drops of halibut liver oil to female rats during the [illegible] five days of pregnancy caused a tremendous increase of vitamin A in the liver. When the liver of the newborn was examined there was also a considerable increase in the amount of stored vitamin A as compared with the newborn in the case where the mother had not received this additional amount of vitamin A.

When the lactating mother was fed an increased amount of vitamin A in the form of halibut liver oil there was also an increase in the vitamin A of the liver of the suckling. Only a percentage of the vitamin A [illegible] absorbed, this varying between 15 to 7[illegible] on the [illegible] of the observations of various [illegible] (*Davis, [illegible] Moore 15%; McCoord and Luce-Clausen 25 to [illegible]*). Baumann, Riising and Steenbock noted that 20[illegible] the ingested vitamin A was recovered in the [illegible]. There is therefore considerable loss of ingested vitamin A. Fecal excretion accounted for only a small [illegible] of this loss. Much of the vitamin A ingested is [illegible] destroyed within the alimentary tract.

Further evidence of the importance of the [illegible] vitamin A storage is shown by the fact that this [illegible] is seriously affected in the presence of liver [illegible] Greaves and Schmidt (*Greaves, J. D. and [illegible] C. L. A. On Absorption and Utilization of [illegible] and Vitamin A in Cholecystectomy [illegible] A Deficient Rats. Am. J. Physiol. [illegible] (Greaves, J. D. and Schmidt, C. L. A. [illegible]*

[illegible] *Jaundiced and Phosphorus Treated* [illegible] *Am. J. Physiol. 111:502,* [illegible] showed that although vitamin A could [illegible] the intestine of the jaundiced rat [illegible] vitamin A, when given orally, nearly all of [illegible] to respond when carotene was administered [illegible] vitamin A. These experiments indicated the [illegible] of the animal to convert carotene to vitamin A [illegible] presence of jaundice. The jaundice had been produced experimentally by ligating and sectioning the [illegible] bile duct.

They also produced liver damage through the administration of phosphorus to rats which had been kept on a vitamin A deficient diet for six to seven days. The [illegible] of the animal to transform carotene to vitamin A [illegible] diminished in the presence of the liver disease which had been produced by the phosphorus. The experimental work further emphasized the importance of the normal liver as the organ in which the transformation of carotene into vitamin A mainly occurs.

Haig, Hecht and Patel *(Haig, C.; Hecht, S. and Patel, A. L., [illegible] Vitamin A and Rod-cone Dark Adaptation in Cirrhosis of the Liver, Science 87:534, 1938)* showed that patients with cirrhosis of the liver were susceptible to the development of poor dark adaptation. These patients could be aided materially in recovery from this condition through the administration of large doses of vitamin A. This is evidently due to the inability of the cirrhotic liver to properly convert the carotenoids into vitamin A. This would therefore be an added reason for the inclusion of vitamin A, itself, such as is present in fish liver oils, in preference to the provitamins usually present in the ordinary diet. A disturbed dark adaptation has also been noted in individuals on limited diets over long periods of time in the treatment of peptic ulcer or gall-bladder disease. The inclusion of a vitamin A concentrate should therefore be a valuable addition to the therapy.

The effect of liver pathology on the ability of the liver to store vitamin A is shown in the studies of Haig and Post. *(Haig, C. and Post, J. Vitamin A Concentration in Rat Liver During Recovery from CCl4 Cirrhosis. Proc. Soc. Exper. Biol. and Med. 48:710, Dec., 1941).* Carbon tetrachloride was administered to nineteen rats over a period of two months in order to render the liver cirrhotic. There was a definite diminution in the vitamin A concentration of these livers as compared with the livers of a controlled group of rats. This inability of the livers of the animals that had been subjected to the administration of carbon tetrachloride to concentrate vitamin A normally was all the more remarkable since the examination of the livers was made three months after cessation of the poison. In spite of the fact that the histologic changes in the livers were of a minimal character there was nevertheless marked interference with the ability of these livers to maintain a normal reserve of vitamin A.

Ralli, [illegible] and Roberts *(Ralli, E. P., Bauman, E. and Roberts, J. B. The Plasma Levels of Vitamin A [illegible] Ingestion of Standard Doses: Studies in Normal Subjects and Patients with Cirrhosis of the Liver. J. Clin. Investigation 20:709-713, Nov., 1941)* administered 100,000 U.S.P. units of vitamin A to five patients with cirrhosis of the liver and one case of acute catarrhal jaundice. The rise in the plasma level of vitamin A was much less in these patients than in a group of normal individuals to whom a similar amount had been administered.

One patient with cirrhosis of the liver was fed 300,000 U.S.P. units of vitamin A daily. Following this the plasma level of the vitamin increased from 40 U.S.P. units per hundred cc. to 118 U.S.P. units per hundred cc. at the end of three days. The presence of a low plasma level of vitamin A in individuals with cirrhosis of the liver confirms the observation that there is a lowered concentration of vitamin A in the livers of such patients.

Vitamin A was never detected in the urine of normal human beings even when fed with large amounts of the vitamin, nor was it ever found during pregnancy. However in some diseases such as pneumonia and chronic nephritis, vitamin A was found in the urine. In pneumonia the daily excretion of the vitamin in the urine at times amounted to 3,000 International Units. It is in pneumonia and chronic nephritis, the two diseases in which there may be a large output of vitamin A in the urine, that the liver, at autopsy, shows a low reserve of vitamin A *(Lawrie, N. R., Moore, T. and Rajagopal, K. R. Excretion of Vitamin A in Urine. Biochem. J. 35:825-836, 1941).*

Popper and Steigmann *(Popper, Hans and Steigmann, Frederick. Causes of Drop of Plasma Vitamin A Level in Liver Disease. Proceedings of the Central Society for Medical Research. J. A. M. A. 121:1413, April 24, 1943)* found that the plasma vitamin A levels were zero in almost all cases of cirrhosis of the liver associated with jaundice as determined by means of the examination of specimens of liver obtained by biopsy and examined chemically as well as by fluorescence microscopy. Similar values were found in sixty-six percent of the cases of hepatitis, secondary to malignant obstructive jaundice. In some cases a zero level of vitamin A was found in cirrhosis of the liver unassociated with jaundice, in cases of acute hepatitis as well as in secondary hepatitis with incomplete obstruction. The reduced levels of vitamin A in the blood were apparently the result of impaired liver function.

THE RESULTS OF DEFICIENCY OF VITAMIN A

Eye Disease

IN ADDITION to the stimulation of growth which is a nonspecific characteristic of vitamin A, it has an important function in relation to the maintenance of normal vision; and one of the earliest manifestations of its absence is the development of night blindness.

The relation of vitamin A to visual function is a subject of considerable importance. The pigments in the retina of the eye are present in the rods and cones. The pigment in the rods is known as visual purple or rhodopsin. The pigment in the cones is known as vi-

ual violet. The important fact as regards vitamin A is that it is present in both these pigments. When rays of light reach the retina, the visual purple is bleached, the rhodopsin being altered to a yellowish material known as retinene. The retinene then stimulates the optic nerve and the brain records the visual image.

In order for vision to continue, retinene must be changed back to the original visual purple or rhodopsin. For this conversion to take place vitamin A is essential. Vitamin A is also essential for the regeneration of the visual violet present in the cones *(Hecht, S. and Mandelbaum, J. Relation Between Vitamin A and Dark Adaptation. J. A. M. A. 112 1910, 1939)*. From this description the significance of vitamin A for the maintenance of visual acuity can be readily understood. A deficiency of vitamin A will interfere with the rapidity with which retinene or visual yellow can be reconverted into rhodopsin or visual purple. Under such circumstances there would be a definite lag in the adaptability of the eye once exposed to light to the reestablishment of its visual acuity. A deficiency in the amount of vitamin A by disturbing the maximum integrity of the visual purple will also reduce the effectiveness of vision when light is dim and ultimately lead to complete night blindness.

The close relationship of vitamin A to the retina is shown by Yudkin, Kriss and Smith *(Yudkin, A. M., Kriss, Max and Smith, A. H. Vitamin A Potency of Retinal Tissue. Am. J. Physiol. 97:611, July, 1931)*. They extracted the fat from the retina and showed this to be very rich in vitamin A. They obtained pigs eyes from the slaughter house and these were freed from all recognizable surrounding fat. After discarding the fluids in the eye, the retinae were removed, dried and ground in a mortar. About 1 gm. of the dried retinae was obtained from one hundred eyes. The arsenic trichloride test was strongly positive. After signs of ophthalmia were obtained in rats on a vitamin A free diet, they were fed weighed quantities of the retinae. The dried retina proved to be a potent source of vitamin A as indicated by the fact that the eyes were restored to normal within a comparatively short time. When fifty mgm. of the dried retinae was fed to the animals, the ocular symptoms disappeared in three to seven days. When thirty mgm. of the substance was administered, evidence of eye disease disappeared in five to eleven days. Normal growth was restored and no evidence of the changes produced by [illegible] ficiency were found at autopsy.

This work confirmed the findings of [illegible] who experimented with the retinae of [illegible].

The close interdependence of the [illegible] purple on the presence of vitamin A has been [illegible] demonstrated by Wald *(Wald, George. Vitamin A [illegible] Eye Tissues. J. Gen. Physiol. 18 [illegible], July, [illegible])* indicating that vitamin A is directly involved [illegible] chemical synthesis of visual purple. [illegible] 1929 and as described above, Yudkin, Kriss and [illegible] had shown that animals suffering from avitaminosis [illegible] could be cured by the addition of material obtained from the retina, it did not necessarily prove that [illegible] substance was actually vitamin A, since the carotenoids might have yielded a similar result. [illegible] the [illegible] color obtained when ether extracts of the pig [illegible] are treated with arsenic trichloride, is not an indication that the substance responsible for the reaction [illegible] vitamin A since this may occur with carotenoid pigments. Based on the fact that the absorption [illegible] vitamin A is different from any natural carotenoid [illegible] was possible for Wald to show that [illegible] retinal and pigment layers obtained from the eye [illegible] frogs, sheep, pigs and cattle, were actually vitamin [illegible] and not one of the natural carotenoids. The [illegible] gram of the antimony trichloride reaction obtained [illegible] an extract of the retina of the ox was compared with that obtained in the reaction with [illegible] the two spectra were found to be identical.

Wald was also able to obtain [illegible] produced xerophthalmia in rats by feeding [illegible] quantities of retinal substance daily for a period [illegible] fourteen days.

An interesting finding was that the proportion [illegible] vitamin A obtained from the dried retinal and [illegible] layers of the eye was thirty five times [illegible] the liver, an extremely high concentration at [illegible] which is most sensitive to the [illegible] deficiency. It is evidence indicating [illegible] portance of vitamin A for the integrity of the [illegible] purple essential for protection against [illegible] No trace of carotene was ever demonstrated in [illegible] retinae and pigment layers of any [illegible] the possible exception of hepaxanthin [illegible] therefore to note that some [illegible] always [illegible] companies the vitamin A of [illegible]

(To be continued in [illegible])

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to potato, while a reaction is obtained to both white and sweet potato in case 3 in the 1st and 2nd tests. Reactions were obtained to both of these potatoes in case 6 in the 2nd and 3rd tests. Although no reaction was obtained to sweet potato in cases 1, 4, 8, which gave reactions to white potato.

Citrus Fruits:

Orange, no reaction in case 1, 3, 7, 9, while reactions were obtained to orange in case 2, 3rd test; case 4, 2nd test; case 5 at each test; case 6, 2nd test; case 8, 2nd test; and case 10, 2nd test.

Grapefruit is unreactive in cases 1, 2, 4, 5, 6, 8, 10, while grapefruit shows a reaction in case 3, test 1, 2 and 3, but not in the other two tests made in this case. Grapefruit gave a reaction in case 7, 3rd test and case 9, 2nd test.

Lemon gave a reaction in case 3 on the 5th test. Grapefruit gave reactions in case 3 in test 1, 2 and 3, although on test 4 there was no reaction to any of the citrus fruits. Lemon gave reaction in case 8 at the 4th test, while the orange was the only other citrus fruit in this case which gave reaction on the 2nd test. Thus we see that in all these tests, the three citrus fruits, orange, grapefruit and lemon, have not, at any one time, or in any one case, shown a reaction to all of these in any one test or any one case.

In looking over these discussions regarding the [illegible] ferent reactions to different foods, [illegible] glancing at the table, *Foods to which [illegible] sitive at different times*, that within [illegible] constitution, there can be a change [illegible] tain foods, to which he might become [illegible] definitely allergic into a considerable [illegible] between tests. No explanation is [illegible] rather the effort to record the results of [illegible] ducted tests.

CONCLUSIONS

1. Foods are a factor in arthritis cases.
2. Changes in cutaneous reactions to food to which a patient is allergic can take place.
3. The interval of time elapse between tests of these arthritic cases varied from one year one month to eight years and eight months.
4. The time of relief between symptom [illegible] varies from one year one month to eight years and four months.
5. The average relief from symptoms being [illegible] years and four months.

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The Role of The Fat Soluble Vitamins A and D in Nutrition

Continued

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NEW YORK, N. Y.

THE EXTENT OF VITAMIN A DEFICIENCY AS DETERMINED BY VISUAL ADAPTATION

BY MEANS of a photometer, Jeans (*Jeans, P. C. Vitamin Deficiency in Childhood, Minnesota, Med. 16:688, Nov., 1933*) showed the large percentage of children in whom diminished visual adaptation was present as an indication of vitamin A deficiency. His work was conducted as follows: The child was first put in a dark room for ten minutes, then the child was exposed to bright light for three minutes. During the next ten minutes, the child's visual adaptability was again tested. Recovery with improvement in visual adaptability resulted from the administration of ample amounts of vitamin A over a period of several weeks. Although the work has been criticized and the increased adaptability blamed on the practice resulting from repetitions of the test with the photometer, this of course does not alter the fact of the therapeutic significance of the administration of ample amounts of vitamin A in the rehabilitation of visual adaptability.

Jeans and his co-workers (*Jeans, P. C. and Zentmire, Z. The Prevalence of Vitamin A Deficiency Among Iowa Children. J. A. M. A. 106:996-997, 1936*) (*Jeans, P. C., Blanchard E. and Zentmire, Z. Dark Adaptation and Vitamin A. A New Photometric Technic. J. A. M. A. 106:151-152, 19[illegible]*) [illegible] that 26 percent of a group of children [illegible] tricts and 53 percent of a village group of children [illegible] Iowa showed evidence of vitamin A deficiency [illegible] termined by the photometric test for dark adaptation. In an urban group of children, the percentage [illegible] between 56 and 79 percent depending on the normal level. Normal dark adaptation developed when [illegible] ample amounts of vitamin A. Halibut liver oil and carotene in oil were the sources of vitamin A that were used.

Moreover, Getz, Hildebrand and [illegible] (*Getz, H. [illegible] Hildebrand, G. B. and Finn, M. Vitamin [illegible] Deficiency In Normal and Tuberculous Persons. [illegible] the Biophotometer. J. A. M. A. 112:[illegible]*) found a shortage of vitamin A [illegible] apparently normal individuals. The [illegible] vitamin A was found to be particularly [illegible] tuberculous. In a series of tuberculous [illegible] culous individuals exhibiting evidence of vitamin A deficiency by means of the biophotometer [illegible] of the condition was obtained by the administration of massive doses of vitamin A in the form [illegible] liver oil. In a group of six persons [illegible] units a day of vitamin A in [illegible] produce any amelioration of the condition [illegible]

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[illegible] was obtained by the [illegible] a day. In those cases [illegible] failed to produce a sufficient [illegible] vitamin A per day in the [illegible] oil was successful. This amount [illegible] until a favorable response was [illegible] was then reduced to 100,000 units [illegible]

[illegible] were given 2,000,000 units of vitamin [illegible] in the form of halibut liver oil. Except [illegible] there were no other pathologic [illegible]

[illegible] of the prevalence of vitamin A deficiency [illegible] by Jeghers (*Jeghers, Harold. The [illegible] of Vitamin A Deficiency in [illegible] on Its Experimental Production [illegible] 109:75[illegible], Sept. 4, 1937*). [illegible] medical students for evidence of [illegible] through a study of the dark adaptation [illegible] of the biophotometer. Thirty-five [illegible] evidence of vitamin A deficiency [illegible] another twelve percent showed [illegible] deficiency. The cause was to [illegible] habits of the students which [illegible] other adults in the general population [illegible] the worst of these diets con[illegible] units of vitamin A daily, it was [illegible] normal requirements necessary to [illegible] evidence of any disturbances due to [illegible] Every student with one exception [illegible] showed a normal dark adaptation [illegible] a daily intake of 4,000 International Units A [illegible] percent increase above this amount is recommended in order to obtain optimal [illegible] This of course, applies to adults under normal conditions.

[illegible] results were obtained in the care of students exhibiting hemeralopia. Vitamin A in the form of halibut liver oil was added in addition to milk, butter, [illegible] and vegetables. The best results were obtained after the daily oral administration of 70,000 units of vitamin A for two weeks, followed by the [illegible] of 25,000 units of the vitamin [illegible] was cured.

[illegible] and Harris (*Maltro, M. K. and Harris, L. J. Vitamin A Deficiency Among School Children in London and Co. Ariaga, Lancet 2:1009-1014, Oct. 30, 1937*) showed the frequency of vitamin A deficiency particularly among the underprivileged. In the examination of [illegible] children among the poor there was evidence of a vitamin A deficiency varying from twenty-[illegible] percent. Contrasted with the results [illegible] examination of the underprivileged [illegible] that among 30 boys educated in [illegible] not a single case of vitamin [illegible]

[illegible] group of 22 adults whose [illegible] 20 and 30, they found only five [illegible] abnormal state. In a [illegible] elementary school children [illegible] not a single [illegible] state of vitamin A deficiency was found. In both of the groups of adults however, and also among the better nourished group of children there were a number of cases exhibiting a moderate degree of hypovitaminosis A.

Eight drops of halibut liver oil or three teaspoons of cod liver oil daily for two to four weeks produced striking changes in practically all of the cases showing evidence of vitamin A deficiency.

Those children showing mild evidence of vitamin A deficiency became normal when treated with six drops of halibut liver oil a day for two weeks.

Other Disturbances of the Eye Due to Vitamin A Deficiency

Kruse (*Kruse, H. D. Methods of Detecting Mild Cases of Vitamin A Deficiency, Science 95:623, June 19, 1942*) stated that in many individuals who showed no impairment of visual adaptation, evidence of vitamin A deficiency could be demonstrated by gross or microscopic changes in the conjunctivae which disappeared following the administration of vitamin A.

In man serious changes in the eye as a result of the deficiency of vitamin A, other than those directly related to light perception, have been studied with great care. In the canthi small patches develop resulting from cornification of the epithelium and bacterial proliferation (Bitot's spots). The cornea as a result of infection may be completely destroyed. The conjunctival epithelium also becomes infected and undergoes destruction. Pillat has described pigmentation of the eye apparently related to melanin as an evidence of vitamin A deficiency. (*Pillat, A. Does Keratomalacia Exist in Adults? Arch. Ophth. 2:256, 399, 1929*).

When the vitamin A potentialities of the organism are exhausted and there is no replenishment, the animal loses weight, and becomes sickly. Many of the animals then develop serious eye disease. The lids become swollen. There is increased sensitivity to light. Soon there is a catarrhal inflammation of the conjunctivae. The discharge becomes purulent and bloody, the lids sticky and the eye closed. Eventually the cornea is also affected and blindness sets in. Before the final stages of the disease, however, cure is possible by the administration of adequate quantities of vitamin A. In this manner the vitamin A potency of any food may be determined, and experimentally accurate quantitative gradations of vitamin A content may be established as measured by the effect of the food to be tested on the rate of growth of the animal.

Early History of Xerophthalmia

Historically, the role of vitamin A deficiency in the development of xerophthalmia is of considerable interest. Among the earliest descriptions of xerophthalmia is that of Fischer in 1846 and of Arlt in 1851, although the first minute description of the disease was that of Albrecht v. Graefe in 1866 (*Blegvad, O. Xerophthalmia, Keratomalacia and Xerosis Conjunctivae. Am. J. Ophth. 7:89, 1924*). In that year Gama Lobo described keratomalacia in Brazilian children. He believed the condition to be one of cellular atrophy due to bad nutrition. He called the disease "ophthalmia Brasiliana." The earliest signs of xerophthalmia were described by Bitot in 1863, on the basis of a study [illegible]

twenty-nine cases which occurred in a foundling hospital in Bordeaux. Similar descriptions of xerosis conjunctivae as well as hemeralopia and the more severe cases of keratomalacia were described by Blessig in Petrograd in 1866. He noted that the disease occurred most commonly during the lenten season and he attributed the disorder to a disturbance of nutrition because of the fasting.

Mori noted that although the disease was not uncommon in children, it was practically unknown on the coast where good fish was available. Schiele in 1907 also noted among the cases of keratomalacia in Russia that cod liver oil was curative.

The treatment of hemeralopia with liver has been known since early antiquity and is recommended as a therapeutic measure in "Eber's Papyrus" (1500 B. C.). The use of raw ox liver was recommended by Hippocrates. Treatment of xerophthalmia with liver was also common in Japan and China.

There is ample clinical evidence of the seriousness of vitamin A deficiency as a result of food shortages during various periods in history. One of the most serious was that which developed in Denmark during the first World War. Owing to the insistent demand of the Germans for butter-fat from the Danes, the infants in Denmark received only skimmed milk. Since the vitamin A is present in the butter fat of the milk, these infants being deprived of this essential vitamin, showed a high mortality rate (21%). Blindness was common and many of the infants that survived showed permanent evidence of the effect of the deficiency.

The Effect of Vitamin A Deficiency on the Mucous Membrane

Wolbach and Howe (*Wolbach, S. B. and Howe, P. E. Tissue Changes Following Deprivation of Fat Soluble A Vitamin. J. Exper. Med., 42:573, 1925*) in their histopathologic studies of the effect on the various tissues resulting from deprivation of vitamin A showed that the essential lesion was a replacement of the various epithelia by stratified squamous keratinizing epithelium. He found these changes to occur in the nares, the nasal septum, the larynx, trachea and bronchi.

Similar changes were noted in the submaxillary glands, the parotid glands, the accessory salivary glands and at times in the pancreatic ducts. The stomach, liver, the small and large intestine were not affected. In the genito-urinary tract the changes were noted in the bladder, ureter, pelvis of the kidney, in the uterus and oviducts, the epididymis, prostate, and seminal vesicles. The epithelium of the kidney and of the seminiferous tubules is not affected.

Epithelial changes were noted in the conjunctiva, the ducts of the Meibomian glands, the cornea, lacrimal gland, Harderian gland and the extra orbital gland. The thymus showed small cysts filled with desquamated keratinized epithelium.

As a result of these changes, there is a disturbance in the functional behavior of the affected regions. Because of the loss of cilia, the trachea is not properly cleared of extraneous material. In addition, the substituted epithelium is less resistant to warding off infection. Vitamin A deficiency may thus lead to an impairment of "the body's first line of defense" in resisting infection. A similar result takes place in the conjunctiva because of the destruction of the cells which secrete mucus. The metaplasia also leads to an occlusion of the ducts leading from the involved glandular structures. This results not only in an inability of the involved gland to carry on normal secretory functions but also makes it susceptible to infection.

Inflammatory lesions of the alimentary tract and of the urinary tract were described by Mellanby (*Mellanby, E. Nutrition and Disease. London, Boyd and Oliver, 1934*) as occurring in many of the experimental animals maintained on a diet deficient in vitamin A.

Mellanby found the following distribution of lesions in young rats maintained on a vitamin A deficient diet. The larger percentage showed infection of the genito-urinary tract, including pyelonephritis, cystitis, renal and bladder calculi, hydronephrosis as well as dilatation of the ureters and distention of the bladder. The next common complication was the development of abscesses in the floor of the mouth with suppurating glands in the neck. Next in frequency was xerophthalmia. In addition some of the animals showed acute inflammatory changes of the stomach, the small and large intestine, disease of the middle ear and accessory sinuses.

An analysis of clinical evidence indicated that when the diet was devoid of vitamin A or carotene, human beings also developed lesions similar to those in the experimental animals. In the presence of deficiency of vitamin A, human beings may be more susceptible to certain types of infection. Under such conditions of a vitamin deficient state their resistance to infection may be increased by the addition of vitamin A to the diet.

Evidence of the deleterious effect of vitamin A deficiency in the alimentary tract has been shown in the experiments of Tilden and Miller on monkeys. The injurious effect often proved fatal, before any gross evidence of damage to vision (*Tilden, E. B. and Miller, E. G., Jr. The Response of the Monkey (Macacus Rhesus) to Withdrawal of Vitamin A from the Diet. J. Nutrition 3:121-139, 1930*).

Tilden and Miller kept monkeys on a practically completely vitamin A deficient diet [illegible] found a diseased condition of the [illegible] The severity of the disorder varied in different [illegible] though gross lesions of the intestine [illegible] cases there was a small area of ulceration of the [illegible] Histologic study showed the presence of [illegible] ative colitis, the ulcers being demonstrated [illegible] microscopic study. It is interesting to [illegible] was an absence of xerophthalmia, [illegible] of the lesions most readily produced in [illegible] when vitamin A starved. Evidence of [illegible] plasia in some of the organs, however, [illegible] trachea and salivary glands was [illegible] delicacy of the monkey [illegible] possible that the monkey [illegible]

[illegible] opportunity for the de- [illegible]

[illegible] the effect of vitamin A de- [illegible] of the alimentary tract and [illegible] in the number of goblet [illegible] of the diminution in the amount of [illegible] of the alimentary tract [illegible] effects produced by [illegible] with capillary bleeding. Thus the [illegible] blood in the stool might be an early [illegible] of a vitamin A deficiency state (*Mar[illegible] The Interrelationship of Vitamin A and Gl[illegible] Held in Mucin Metabolism. Science 35:41.*

Skin [illegible] Resulting from Vitamin A Deficiency

Among the various changes that have been described as resulting [illegible] vitamin A deficiency are those found in the [illegible] first is dry and slightly rough, followed by the [illegible] appearance of eruptive lesions in local [illegible]. The lesions first appear on the antero-lateral [illegible] of the thighs or in the postero-lateral [illegible] then spreading to other regions. [illegible] the free resembles acne. This skin lesion [illegible] by Frazier and Hu was amenable to vitamin A [illegible] improvement in the secretory function [illegible] the gradual disappearance of the eruptive [illegible].

A [illegible] of the relationship of vitamin A to [illegible] lesion is indicated by the fact that it was [illegible] associated with the classical ocular manifestation of the disease. Both these lesions disappear [illegible] therapy. The main type of cutaneous [illegible] was the keratotic follicular papule. In addition [illegible] of excessive keratinization [illegible] of warty plaques on the knuckles and [illegible].

Histopathologic studies were made of specimens of the skin lesions. These showed that the pathologic process [illegible] due to increased keratinization of the epithelium of the hair follicles. As a result there was an obstruction of the hair follicles. The damaged follicles showed a moderate infiltration with lymphoid cells. The minute skin changes appeared to be similar to those occurring in the tissues of the eye as a result of vitamin A deficiency. The skin lesion was most common in those over fifteen years of age and infrequent in younger individuals (*Frazier, C. N. and Hu, C. Nutr[illegible] According to Age of Cutaneous [illegible] Vitamin A Deficiency. Arch. Derm. [illegible] 1935*).

In addition to the changes in color of the conjunctivae, due to an increase of the melanin, Pillat (*Pillat, A. Dec[illegible] Adults? Arch. Ophth. [illegible]*) [illegible] observed peculiar grayish yellow [illegible] skin in some of his vitamin [illegible] patients. The skin in addition is rough [illegible] gave the impression as if [illegible] with coarse powder. Scaling [illegible]. The roughness of the skin [illegible] to the [illegible] of sebum as well as to the presence of horny cells in large numbers. The skin may be edematous and swollen. Because of the lack of sebum, the skin may be covered with hundreds of comedones. Secondary infection of the comedones and hair follicles frequently occurs and leads to the development of follicular abscesses, similar to the condition found in simple acne.

"In cases in which nourishment rich in vitamin A is given, these abscesses and the deepest pockets dry up in wonderfully short time without any surgical treatment." Pillat also noted extreme dryness of the hair in these cases with the scalp covered with dandruff. The hair showed a tendency to become gray and fall out. The nails showed furrows which peeled off in small particles.

Straumfjord (*Straumfjord, John V., Lesions of Vitamin A Deficiency, Northwest Medicine 41:7, 289, July, 1942*) "observed in a large number of patients the effect upon various clinical conditions of a daily oral intake of 100,000 units of Vitamin A. A large number of patients with follicular hyperkeratosis have been followed, many over a period of four years. . . . The response to Vitamin A administration is extremely variable. In some patients the lesions disappeared in a few months, while in others they are still present, although greatly diminished after a four year period of continuous daily oral intake of 100,000 units of Vitamin A."

The relationship of vitamin A deficiency to lesions of the skin is also suggested by the work of Rapaport, Herman and Lehman (*Rapaport, H. G., Herman, H. and Lehman, E. Treatment of Ichthyosis with Vitamin A. Jour. Pediatrics 21:733, Dec., 1942*). They treated six patients with ichthyosis by means of the oral administration of 60,000 to 200,000 International Units of vitamin A per day over a period of several months. Some of the patients also received 100,000 units of vitamin A injected intramuscularly two or three times a week over a period of several months.

Improvement was reported in the condition of the skin lesion. The texture of the skin became more normal, with disappearance of cracks and fissures as well as a marked diminution of pruritis. An impaired dark adaptation which was coincidentally present also showed marked improvement with the vitamin A therapy.

The Teeth as Affected by Vitamin A Deficiency

Wolbach and Howe have described metaplastic changes of the enamel organ with replacement by stratified squamous epithelium. Because of the destruction of the normal enamel, the teeth assume a chalky appearance produced by the dentin. Apparently it is the incisor teeth which are most seriously involved as a result of the deficiency state.

Orten, Burn and Smith (*Orten, A. N., Burn, C. G. and Smith, A. H. Effects of Prolonged Chronic Vitamin A Deficiency in the Rat With Special Reference to Odontomas. Proc. Soc. Exper. Biol. and Med. 36:82, 1937*) showed that the incisor teeth as a result of Vitamin A deficient diet developed a progressive loss of the normal orange pigment, with opacity and

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distortion of shape. In those animals that died, the cause in most cases was tracheal obstruction from a muco-purulent plug. They were also found to have evidence of otitis media, sinusitis and calculi in the urinary bladder.

Most of the animals that survived 365 days on a low vitamin A diet, not only showed changes in the incisor teeth, but also tumor growth (odontomata) replaced the alveolar structures of the upper and lower jaws. Most of the incisor teeth were distorted or replaced by these tumor growths.

Renal Calculi and Vitamin A Deficiency

The formation of renal calculi in the experimental animal has been attributed to a diet deficient in vitamin A (*Steiner, M., Zuger, B. and Kramer, B. Production of Renal Calculi in Guinea Pigs by Feeding Them a Diet Deficient in Vatimin A. Arch. Path. 27:104, 1939*). Steiner, Zuger and Kramer showed that in guinea pigs, on a vitamin A deficient diet, autopsy revealed changes in the urinary tract. The ureters were enlarged and thickened in thirty-one of the thirty-five animals that had survived for more than fourteen days. These alterations were due to thickening and reduplication of the mucosa. Desquamation of the epithelium was a common finding. In nine of the thirty-five animals there were calculi in the ureter or renal pelvis. The first microscopic evidence was a hyperplasia of the ureteral mucosa. This was followed by a metaplasia of the mucosa to a stratified keratinized epithelium. The large plaques of desquamated epithelial cells acted as foci for the development of calculi. The inorganic material of the calculus was calcium carbonate. Wolbach, however, doubts the etiologic relationship and believes that factors other than the absence of normal amounts of vitamin A are responsible for this development. When such calculi do occur in the experimental animal, it is believed that they form about masses of desquamated epithelium resulting from the metaplastic process. There is no proof at present that vitamin A has any effect on the occurrence of renal calculi in man.

Jewett, Sloan and Strong (*Jewett, H. J., Sloan, L. L. and Strong, G. H. Urolithiasis. J. A. M. A. 121:566-568, Feb. 20, 1943*) studied twenty patients with urolithiasis for evidence of vitamin A deficiency by means of the dark adaptation method as well as the vitamin A content of the blood.

In addition the respiratory and urinary tracts were examined at autopsy in seventy-eight patients with urolithiasis for evidence of epithelial metaplasia. They showed that there was no evidence to indicate that in the human being vitamin A deficiency was present in urolithiasis.

Pregnancy and Vitamin A

The possible role of pregnancy in being associated with a diminution in vitamin A is indicated by the work of Edmond and Clemmesen (*Edmond, Carsten and Clemmesen, S. On a Deficiency of A Vitamin and Visual Adaptation. London, Oxford Univ. Press, 1936*). They showed on the basis of disturbed visual adaptability that about fifty percent of pregnant women in Copenhagen municipal hospital suffered from vitamin A deficiency.

In addition to the evidence of disturbed visual adaptation in pregnancy, it was also detected in women who had recently given birth and in cases of hepatitis. Excluding pregnancy and organic eye disease, they found evidence of vitamin A deficiency by the dark adaptation method in five to six percent of the general run of medical patients.

They stated that evidence of the effectiveness of the intramuscular administration of vitamin A concentrate liver oil preparations could be noted in a couple of minutes, and after oral administration in less than twelve hours.

A more serious relationship of vitamin A deficiency to the reproductive function has been indicated by Evans and Bishop (*Evans, H. M. and Bishop, K. S. On An Invariable and Characteristic Disturbance of Reproductive Function in Animals Reared on a Diet Poor in Fat Soluble Vitamin A. Amer. Jour. Phys. 58:165, 1922*). On a diet poor in vitamin A, they showed that among the earliest manifestations even in the presence of good growth and apparent health, the female rat suffers from an abnormality of the ovaries, determined by histological changes in the vaginal smear. These changes can be correlated with the growth, maturation and rupture of the Graafian follicle. On a diet low in vitamin A and yet nevertheless sufficient to permit normal growth there is an interference with the ability of the Graafian follicles to mature and rupture. These abnormalities may be detected by examination of the vaginal smear showing the development of cornified epithelial cells.

A possible relationship of vitamin A deficiency to sterility was shown by Evans (*Evans, H. M. Testes for Degeneration Due to Inadequate Vitamin A in Cases Where Vitamin D is Adequate. Jour. Nutr. 99:477, Jan., 1932*). He maintained animals on a diet low in vitamin A but adequate in vitamin D and found that they became sterile in three months. A histological study of the tests showed the cause of the sterility to be due to the development of marked degenerative changes.

Mason (*Mason, K. E. Fetal Death, Prolonged Gestation and Difficult Parturition in the Rat as Results of Vitamin A Deficiency. Am. J. Anat. 55:[illegible], 1934*) showed that the method of examining the vaginal smear for a determination of vitamin A deficiency is much more sensitive than that of other criteria such as growth or the development of scurvy in the rat. The evidence of cornification of the vaginal epithelium is long before either of these other criteria. The value of the method lies therefore in the detection of comparatively mild degrees of vitamin A deficiency which can be definitely determined.

When pregnant rats were kept on a low vitamin A intake and the vaginal cornification period started without pregnancy, the animals frequently failed to carry to term. This reproductive disturbance was in the majority of cases due to vitamin A deficiency. When ample vitamin A was given, normal pregnancy took place to term and delivery.

[illegible] of the disturbance in the reproduction [illegible] as a result of vitamin A deficiency can parallel [illegible] of the vaginal cornification. When the [illegible] occurred in vitamin A deficient rats, [illegible] changes in the uterine epithelium with [illegible] infection of the maternal decidua and of the [illegible]. Pronounced vitamin A deficiency [illegible] may lead to death in utero and re[illegible] the fact that the maternal decidua be[illegible] the nutrition of the fetus is seriously [illegible]. The cornification of the vaginal epi[illegible] may increase the difficulty of labor.

[illegible] that vitamin A is important in the [illegible] of the normal structure of the vaginal epithelium, Simpson and Mason (*Simpson, J. W. and Mason, K. E. A New Concept of Senile Vaginitis, Am. J. Obst. and Gyn. 32:125, July, 1936*) treated cases of senile vaginitis by the administration of vitamin A in the form of liver oil. An improved state of the vaginal epithelium occurred in those cases in which there had previously been a dietary deficiency of vitamin A. In these cases of senile vaginitis which were [illegible], the [illegible] of the increased amounts of vitamin A in the form of fish oils was followed not only by [illegible] of the vaginal epithelium but also by relief of symptoms.

Vitamin A and the Nervous System

McCarthy (*McCarthy E. Nutrition and Disease, London, Gyll and Oliver, 1934*) noted the nutritional influence of vitamin A deficiency on the nervous system of young dogs. These animals showed evidence of incoordination and stiffness of the hind legs which ultimately became very weak. In addition, the animals appeared as if dazed and mentally affected. By the addition of such sources of vitamin A as fish liver oil, these changes could be prevented. Even after the incoordination had developed, marked improvement might result from the addition of vitamin A to the diet. Not only was the pathologic process in the nervous system arrested but some degree of cure was also possible. The evidence of changes in the spinal cord was substantiated by histopathologic studies.

That the effects of a vitamin A deficiency state in producing degenerative changes in the spinal cord was not limited to the dog was indicated by the fact that similar findings were obtainable when the experimental animals were young rabbits, rats and birds.

Varying degrees of demyelinization in the optic nerve and the trigeminal nerve were also noted when the diet was deficient in vitamin A.

In some cases of retro-bulbar neuritis in children cure was obtained through the addition of the vitamin A of fish liver oil.

Disturbances in the nervous system due to vitamin A deficiency have been described in the experimental animal by Irving and Richards (*Irving, J. T. and Richards, M. B. Early Lesions of Vitamin A Deficiency, J. Phsiol. 94:307, 1938*). Of 141 rats fed on a vitamin A free diet 108 showed degenerative changes in the medulla. In those animals in which no such changes were found, the reason seemed to be that they were killed too soon after being on this diet. Some of these pathological changes occurred in animals killed at a time when growth and apparent health were still maintained. Thus subtle degrees of vitamin A deficiency might be responsible for organic changes in the nervous system. A comparable group of animals on a vitamin A free diet to which adequate amounts of carotene or vitamin A had been added showed no such pathologic changes. (To be continued in July 1944 issue)

Hypoglycemic Reaction With Convulsions In Ascariasis (Case Report)

By

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I HAD the opportunity to see the case of a young boy [illegible] when we first were inclined to classify as hypoglycemic epilepsy (1). The case-report is in brief as follows:

J. & S., age 9. Family history irrelevant. First convulsion at the age of 8 months when he had bronchial pneumonia. Since then, he had several attacks of convulsions when he had fever. Now and then a round worm was passed. No pathologic findings on the first physical examination. Presence of phimosis was noted. On December 1, 1942, he was brought to the hospital again. He now and then in the meantime had been complaining of [illegible] spells with choking in the throat and excessive [illegible] which forced him to drink, for [illegible] large amounts of water. He had a craving for food all the time, and turned pale each time when he could not satisfy his hunger at once. The boy reported that he always felt the spells coming; they were more prone to recur after exertion. The day before admission he vomited, then turned pale and moaned; finally, he went into a convulsion with jerking of arms, loss of consciousness, rolling of eyes upward, rigidity of the body, and opisthotonos. This fit lasted for about five minutes. He felt drowsy and sleepy thereafter. There was another similar spell in the evening on the day of admission. Physical examination again did not reveal any evidence of organic disease. Sella turcica was of normal appearance on x-ray. No scars or evidence of previous tongue-bites found. No increase of urinary output in 12-hour period. Blood sugar (venous blood, method of Folin-Wu) fasting A. M.: 85 mgs. %, before noon 50 mgs. %.

[illegible]

...an Pharmacological Department, University of Lund, Sweden.

The Effect of Vitamin D on the Absorption of Inorganic Phosphate.

By

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With the technical assistance of

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Numerous balance experiments have shown that the fecal excretion of both Ca and P may be increased in vitamin D deficiency. The mechanism of this disturbance was studied in detail by NICOLAYSEN (1937 a and b). The addition of Ca to a basal diet that was essentially free of both Ca and P was found in rats to produce an increased excretion not only of Ca but also of P in the feces. On the other hand, when P was added instead of Ca it seemed to be completely absorbed, whether it was given as inorganic phosphate, glycerophosphate, or casein, the fecal Ca and P being unaffected. In both types of experiments, and even if the basal diet was given without any addition, vitamin D was found to reduce the amounts of Ca and P in the feces. It was concluded that vitamin D affected primarily the absorption of Ca (including the reabsorption of endogenous Ca), the action on the absorption of P being indirect: when the amount of Ca in the intestinal tract was reduced as a consequence of increased absorption, less Ca phosphate was precipitated, and more phosphate was thus absorbed.

There are certain difficulties in this interpretation. When the fecal P is plotted against the fecal Ca, the curve of the vitamin D treated animals is found to lie definitely below that of the non-treated animals (Figure 1; the data are averages taken from Tables I and II, NICOLAYSEN 1937 a; values from animals said

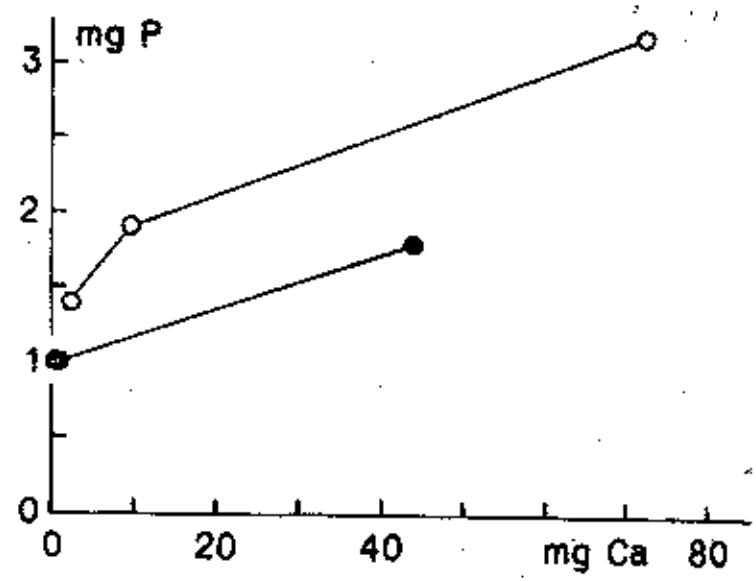

Fig. 1. The Daily Excretion of P in Relation to the Daily Excretion of Ca in the Feces of Vitamin D Deficient Rats (Circles) and Rats Treated with Vitamin D (Dots).
The 3 values of either curve were obtained on Ca intakes of 2.3 mg, 15 mg, and 90 mg daily. The data are taken from NICOLAYSEN (1937 a).

to be in an early stage of vitamin D deficiency were omitted, since in these animals there was no effect of vitamin D on the fecal P). The conclusion that the influence of vitamin D on the absorption of P is referable to a reduced amount of Ca in the intestinal tract therefore seems hardly justified.

COHN and GREENBERG (1939) reported that in rats the absorption of P^{32}, given as inorganic phosphate, was increased but little by vitamin D. The tracer was given 1 hour after the vitamin. At this short interval an effect on the absorption can hardly be expected (compare LINDQUIST 1952).

Below some experiments are given in which the action of vitamin D on the absorption of inorganic phosphate is further studied.

Experimental.

Two experimental series with different diets were studied. In the first series the Ca "free" diet described in an earlier paper (CARLSSON 1952) was used. It contained 0.04 per cent Ca and 0.5 per cent P. In the second series $CaCO_3$ had been added to this diet so that the Ca

content was 2.0 per cent. The growth of animals kept on the former diet is increased by vitamin D, whereas on the latter diet there is no such effect, the daily increase in body weight being about 2 grams both in the presence and in the absence of vitamin D.

Three-week-old rats were transferred to either of these diets. After another 3 weeks in the first series and 5 weeks in the second series Ca^{45} and P^{32} were given. About half of the animals in either series received calciferol. In the first series some of the animals were given 2 doses of calciferol, viz. 11 and 3 days, respectively, before the administration of the isotopes. The remaining animals in the first series and all the animals in the second series received a single dose of calciferol 3 days before the isotopes were given. Each dose contained 500 I. U. of vitamin D, given orally in 0.1 ml peanut oil. The values obtained for Ca^{45} and P^{32} absorption were practically the same whether 1 or 2 doses of calciferol were given. In the second series about half of the animals were parathyroidectomized (RICHTER and BIRMINGHAM 1941) 16 days before the administration of the isotopes. The remaining animals in the series were sham operated, i.e., the parathyroids were exposed but not removed.

The isotopes were administered in doses of a few microcuries. Solutions containing Ca^{45} in Ca lactate and P^{32} in Na_3PO_4 were mixed. The freshly prepared mixture containing a fine precipitate of Ca phosphate was given by stomach tube. The dose of P was 2.5 mg throughout. In the first series Ca was given in a dose of 5 mg (which is almost equivalent to 2.5 mg P), 10 mg, or 20 mg. In the second series all the animals received 5 mg Ca. The volume of fluid administered was 1.5 ml.

The urine and feces of each animal were collected separately. The rats were killed 18 hours after the isotopes were given. The Ca^{45} and P^{32} in the gastrointestinal tract + feces were estimated. The percentage absorption of either isotope was said to be 100 — (per cent of dose in gastrointestinal tract + feces). For further technical details, see CARLSSON (1951, 1952).

Results.

The results of the first experimental series, in which the animals were kept on the Ca "free" diet, are given in Figure 2. The *percentage* absorption of Ca was fairly independent of the amount of Ca administered, in agreement with earlier findings (CARLSSON 1951); it was almost doubled after vitamin D administration. The absorption of P, which was given in a dose of 2.5 mg throughout, was but slightly affected by the amount of Ca administered at the same time. It was increased by vitamin D, although somewhat less than the Ca absorption. The increase in P absorption produced by vitamin D could not be attributed to a reduced

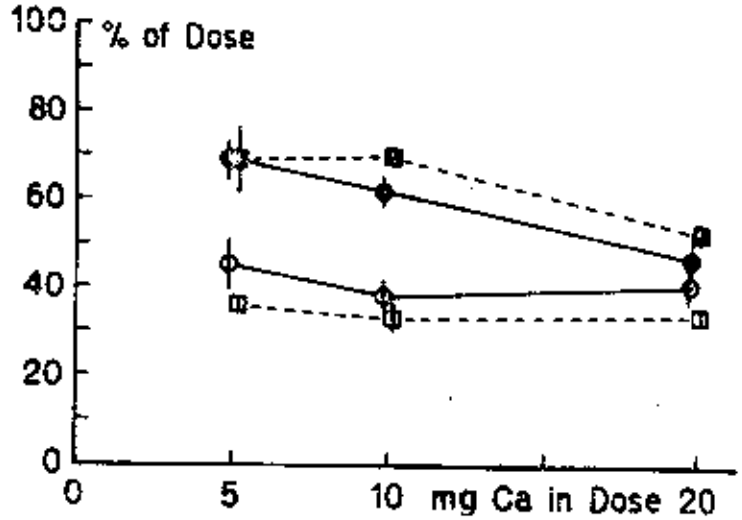

Fig. 2. The Absorption of Ca^{45} and P^{32} at Varying Dosage of Ca in Rats Kept on a Ca "Free" Diet.
Squares: Ca^{45}. Circles: P^{32}. Open symbols: vitamin D deficient animals. Filled in symbols: vitamin D treated animals.
Each symbol indicates mean ± standard error of the mean.

Vitamin D	Nr of rats given the Ca dose of 5 mg	10 mg	20 mg
—	5	11	9
+	5	10	10

amount of Ca in the intestine. This will be apparent from Figure 3, where the amount of non-absorbed P is plotted against the amount of non-absorbed Ca. Vitamin D treatment resulted in a lower amount of non-absorbed P even when the difference in non-absorbed Ca was accounted for. The data seem to be in fairly good agreement with those of NICOLAYSEN given in Figure 1.

Table I gives the results of the second series, in which the animals were kept on a diet with a high Ca/P ratio. The test dose was the same in all the animals and contained 5 mg Ca and 2.5 mg P. In part of the animals the parathyroids had been removed 16 days earlier, which had no detectable influence on the absorption of Ca and P. The absorption of Ca was markedly increased by vitamin D, but there was no effect on the absorption of P.

Discussion.

The influence of vitamin D on the absorption of Ca was appreciable in both experimental series, but an effect on the absorption

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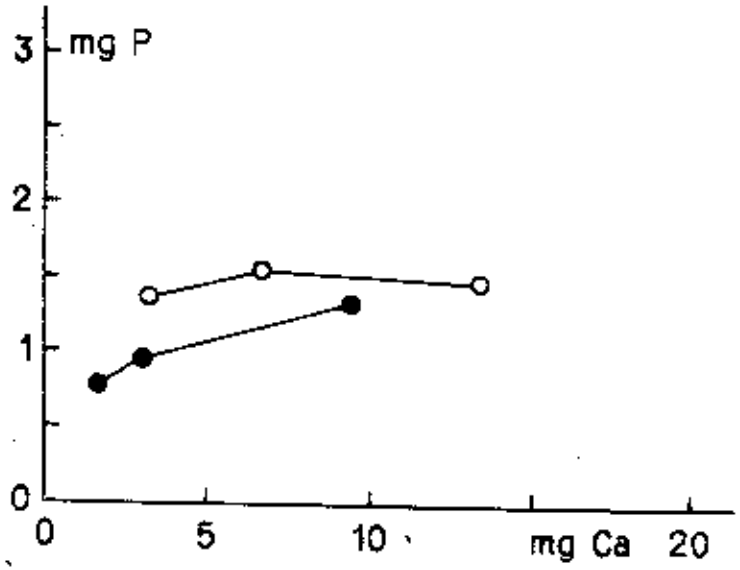

Fig. 3. Non-Absorbed P in Relation to Non-Absorbed Ca in Vitamin D Deficient Rats (Circles) and Vitamin D Treated Rats (Dots).
The values were calculated from the data in Figure 2.

Table 1.

The Effect of Vitamin D on the Absorption of Ca^{45} and P^{32} in Rats Kept on a Diet with a High Ca/P Ratio.

The values are expressed in per cent of the administered dose.

Vitamin D	Parathyroids present			Parathyroids removed		
	Nr. of rats	Ca^{45}	P^{32}	Nr. of rats	Ca^{45}	P^{32}
—	7	25	51	9	18	46
+	8	38	49	8	43	52
Difference		13	−2		25	6
± standard error		4.0	7.1		3.8	6.3
P %		< 1	> 50		< 0.1	> 25

of P was detectable only in the first series. Under the conditions of the first experimental series, in which the animals were deficient in Ca, vitamin D exerts a marked effect on the growth and general well-being of the animals, presumably by restoring the lowered serum Ca to normal (CARLSSON 1952). We have observed no similar action in animals kept on the diet with a high Ca/P

ratio used in the second series. It therefore seems probable that the effect on the absorption of P seen in the first series is indirect and related in some way to the action on the growth of the animals.

The observations of NICOLAYSEN (l. c.) seem to be in agreement with this interpretation, as far as can be judged by the available data. The rats used in his experiments gained in body weight about 1 gram daily. This gain was regular in the rats given vitamin D, whereas the vitamin D deficient rats stopped growing after 4 to 7 weeks. In the early stage of vitamin D deficiency the fecal excretion of P was not always affected by vitamin D, while in the late stage the effect was apparent in all the experiments. Thus even here the influence of the vitamin on the absorption of P seemed to be related to the growth of the animals. As in the present experiments the absorption of Ca was invariably improved by the vitamin.

The experiments so far discussed do not exclude the possibility that the concentration of Ca ions in the intestinal tract plays an important rôle for the absorption of the phosphate of an ester such as phytin, the Ca salt of which has an extremely low solubility. Indeed this is indicated by the work of DAY (1940), in which the intestinal concentration of Ca ions was varied by adding citrate to the diet. In accordance with the present findings citrate addition was without influence on the absorption of P, when the diet contained no phytin, whereas the effect was marked, when phytin-containing diets were used. It therefore seems probable that the effect of vitamin D on the absorption of P, as observed in ordinary balance experiments by *e. g.* ALBRIGHT and SULKOWITCH (1938), may be at least partly referable to a reduced precipitation of Ca phytate resulting from an increased absorption of Ca.

Summary.

The absorption of P^{32} given orally as inorganic phosphate together with varying amounts of Ca was studied in vitamin D deficient rats and in rats treated with this vitamin.

In Ca deficient animals vitamin D increased the absorption of phosphate. The effect was probably indirect and related to an improvement of the general condition of the animals. In rats kept on a diet with a high Ca/P ratio vitamin D had no influence on

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the absorption of phosphate. In both instances the absorption of Ca was increased by the vitamin.

The absorption of inorganic phosphate was found to be fairly independent of the amount of Ca in the intestinal tract.

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34 EXOGENOUS VITAMIN D AND CALCIUM BINDING PROTEIN IN THE GUINEA PIG. M. W. Chapman*, W. C. Pond, A. H. Taylor, E. H. Wasserman, F. H. Craig* and L. Krook. N. Y. State Col. of Agric. & Life Sciences and N. Y. State Veterinary Col., Cornell Univ., Ithaca, N. Y. 14850.

The objectives were to determine if the guinea pig needs exogenous vitamin D, and if calcium binding protein (CaBP) is vitamin D-dependent. Two to 4-day old, male, English short hair guinea pigs were placed in the dark. They were conditioned for one week on a basal, semi-purified diet complete except for vitamin D. Groups of guinea pigs were then placed on one of the following diets for periods up to 16 weeks: I) basal; II) basal + 220 I.U. vitamin D_3/100 g diet (Control); III) basal with the Ca reduced by 2/3. In Diet III, all supplemental Ca was removed for the last week. Results: ^{47}Ca absorption from the intestine and deposition in the femur were not affected by lack of vitamin D in Diet I. Total serum Ca decreased in Ca-deficient, vitamin D-deficient animals in Diet III. No significant change was found in CaBP concentration on any treatment. No histological signs of rickets were found in the growth plate of the humerus for any treatment. Changes which we previously interpreted as the start of rickets were shown by serial sections of the growth plate to be only normal variation. Parathyroid activity, as judged histologically, was increased by vitamin D-deprivation and by reduction of dietary Ca. Conclusions: Under these experimental conditions it appears that (1) with a normal dietary Ca/P ratio the guinea pig does not need exogenous vitamin D to prevent rickets during the major part of growth and that (2) intestinal CaBP is not dependent on exogenous vitamin D. If exogenous vitamin D is required, then it is needed in extremely minute quantities, undetectable in the diet by an independent biological assay. (Supported in part by NIH Nutrition Training Grant No. GM01221).

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On the Subcellular Location of Vitamin D Metabolites in Intestine*

(Received for publication, February 2, 1970)

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SUMMARY

Following the intravenous injection of radioactive vitamin D_3 no radioactivity appeared in intestinal chromatin isolated according to the procedure of Marushige and Bonner, *J. Mol. Biol.*, 15, 160 (1966). Instead the radioactivity of intestinal nuclei remained with a membrane fraction which is separated from chromatin at the initial step in the preparation. When "chromatin" was prepared by the method of Haussler, Myrtle, and Norman, *J. Biol. Chem.*, 243, 4055 (1968) as much as 40% of the intestinal radioactivity appeared in the chromatin fraction in the case of chicks and 20% in the case of rats. However, the chromatin prepared by this method was heavily contaminated with membranes and cell organelles whereas the Marushige and Bonner preparations were composed of homogeneous chromatin as revealed by electron microscopy. Chemical analysis and ultraviolet spectra confirmed the electron microscopic data. Thus the previous contention that intestinal chromatin is a major site of vitamin D metabolite location is without experimental basis.

In 1965, it was shown that actinomycin D prevents both the intestinal transport of calcium and bone mineral mobilization responses to vitamin D (1-3). Subsequent experiments conclusively established that the expression of vitamin D action must involve transcription of DNA and protein synthesis (4). A more direct demonstration of the effect of vitamin D on transcription of DNA resulted from experiments on pulse labeling of intestinal RNA by ^{3}H orotic acid or ^{3}H-uridine (5-7).

It therefore appeared that vitamin D must be involved in some way with intestinal nuclei. With the synthesis of ^{3}H-labeled vitamin D_3 of high specific activity (8), an examination of the subcellular location of vitamin D and its metabolites was possible. Stohs and DeLuca (9) and Haussler and Norman (10) found that the cytoplasm and nuclei contained most of the ^{3}H in intestine from a dose of 10 i.u. ^{3}H-labeled vitamin D_3. The ^{3}H of the nuclear fraction was found primarily as a polar metabolite or metabolites of vitamin D (9), although Haussler and Norman (10) had concluded it to be in the form of unchanged vitamin D_3. Little ^{3}H from ^{3}H-labeled vitamin D_3 could be found in intestinal nuclei prepared in 1% citric acid, in nuclei isolated from sucrose containing Triton X-100, or in deoxyribonucleoprotein. Because citric acid and Triton X-100 (11, 12) treatment removes the outer nuclear membrane, it was concluded that a major site of ^{3}H from ^{3}H-labeled vitamin D_3 is the nuclear membrane (9). Haussler, Myrtle, and Norman (13) and Haussler and Norman (14), on the other hand, have concluded that chromatin is the major location of the ^{3}H from ^{3}H-labeled vitamin D_3 in intestinal mucosa. Despite repeated examination using high specific activity ^{3}H-labeled vitamin D_3, we were unable to confirm their findings. Instead, the present results not only support the previous report of Stohs and DeLuca (9), but also provide an explanation for the discrepancy between the conflicting observations from the two laboratories. Clearly, no ^{3}H from either vitamin D_3 or [26,27]^{3}H-25-hydroxycholecalciferol can be found when pure chromatin is prepared from intestine by the procedure of Marushige and Bonner (15).

MATERIALS AND METHODS

Preparation of Rats—Male weanling rats (Holtzman Company, Madison, Wisconsin), weighing 50 to 60 g, were housed in individual hanging wire cages and given food and distilled water *ad libitum*. They were fed a diet adequate in calcium (0.47%) and phosphorus (0.3%) as previously described (16). After 5 weeks the rats were deficient in vitamin D as evidenced by lowered serum calcium and reduced growth and were then used for experiments.

Preparation of Chicks—One-day-old white Leghorn cockerels were obtained from Sunnyside Hatcheries, Oregon, Wisconsin. They were fed a purified rachitogenic diet as previously described (17) for 3 to 4 weeks before they were used for the experiments.

Radioactive Compounds—[1,2]^{3}H-labeled vitamin D_3 (8) (specific activity of 70,000 dpm per i.u. and 108,000 dpm per i.u.); [22,23]^{3}H-labeled vitamin D_3 (24) (specific activity of 155,000 dpm per i.u.); and [26,27]^{3}H-25-hydroxycholecalciferol[1] (220,000 dpm per i.u.) were synthesized in this laboratory. Vitamin D-deficient rats or rachitic chicks were given 0.20 to 0.75 μg of ^{3}H-labeled vitamin D_3, ^{3}H-labeled vitamin D_4, or ^{3}H-25-hydroxycholecalciferol intraingularly in 0.04 ml of ethanol for rats or in

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[1] T. Suda and H. F. DeLuca, in preparation.

TABLE I

Radioactivity of intestinal chromatin isolated by method of Marushige and Bonner (15) from rats given ^{3}H-labeled vitamin D compounds intrajugularly

Vitamin D-deficient rats were given the indicated dose in 0.04 ml of ethanol, intravenously.

Compound administered	Dose	Time after dose	Percent of total radioactivity in homogenate found in chromatin
	μ*Ci/rat*	*hours*	%
[1,2]^{3}H-labeled vitamin D_3	0.25	4	1
[1,2]^{3}H-labeled vitamin D_3	0.25	8	0
[1,2]^{3}H-labeled vitamin D_3	0.75	15	0
[22,23]^{3}H-labeled vitamin D_4	0.25	4	0
[22,23]^{3}H-labeled vitamin D_4	0.50	15	0
[26,27]^{3}H-25-hydroxycholecalciferol	0.20	1½	2

TABLE II

Radioactivity in chromatin isolated by Marushige and Bonner procedure (15) from chicks given [1,2]^{3}H-labeled vitamin D_3, intrajugularly

Experiment	Radioactivity			Percent of total radioactivity of homogenate in chromatin
	Homogenate	Precipitate from EDTA step	Chromatin	
	dpm/μg DNA			%
1	0.95	0.3	0.05	5.5
2	0.75	0.23	0.05	6.6

TABLE III

^{3}H content of intestinal chromatin isolated by Haussler et al. (13) procedure from rats or chicks given [1,2]^{3}H-labeled vitamin D_3

Vitamin D-deficient rats and vitamin D-deficient chicks were given 0.75 μg and 0.25 μg [1,2]^{3}H-labeled vitamin D_3, respectively, intrajugularly 15 hours before killing.

Sample	Radioactivity	Percent of total radioactivity of homogenate
	dpm/μg DNA	%
Rat homogenate	0.19	100
Rat chromatin	0.04	21
Chick homogenate	0.51	100
Chick chromatin	0.21	41

TABLE IV

Radioactivity during isolation of chromatin from rat intestinal nuclei after intrajugular [1,2]^{3}H-labeled vitamin D_3

Vitamin D-deficient rats were given 0.25 μg [1,2]^{3}H-labeled vitamin D_3 intravenously 12 hours prior to killing. There were four rats in a group and each value represents the average of four experiments.

Procedure	Total radioactivity	Percent of total radioactivity of homogenate
	dpm	%
Crude nuclei	1600	65
EDTA supernatant	1150	50
EDTA precipitate (crude chromatin)	514	15
Tris buffer wash precipitate	50	2
Sediment in 1.7 M sucrose (chromatin)	0	0
EDTA supernatant centrifuged at 50,000 rpm 2 hours (membrane fraction)	1030	45

0.1 ml of vitamin D-deficient chick serum for chicks. All animals were fasted 12 to 15 hours before they were killed by decapitation.

Chromatin Preparation—Approximately 40 to 50 cm of the small intestine from the duodenal end was quickly removed and chilled in ice-cold 0.25 M sucrose, 0.05 M Tris-chloride (pH 7.5), 0.005 M $MgCl_2$, and 0.025 M KCl solution. The intestinal segment was removed, slit open, washed with the solution, blotted on filter paper, and laid on a stainless steel plate held at 0°. The mucosa was removed by scraping with a microscope slide. Chromatin was prepared from the pooled mucosa of two to four animals by the method of Marushige and Bonner (15) and the method of Haussler *et al.* (13).

Purified Nuclei—Nuclei with the double membrane intact were prepared by a modification of the Chauveau technique (18). Crude homogenates (50%) were prepared in 2.2 M sucrose solution containing 4 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2 mM $NaHCO_3$ (pH 6.5). The homogenates were centrifuged for 3 hours at 7,000 × *g* in a No. SW 25.1 rotor using a Spinco model L2 ultracentrifuge. The nuclei sediment were then disrupted with a Branson Sonifier model LS 75 at a setting of 6 for 30 sec.

Chemical Analysis—Protein determinations were made by the diphenylamine method (10) with bovine serum albumin as the standard.

DNA and RNA were determined according to the procedure of Schneider (20). DNA content of chromatin was determined without trichloroacetate precipitation. Calf thymus DNA and yeast RNA were used as standards.

Phospholipids were extracted from the samples by the procedure of Bligh and Dyer (21). The chloroform phase was dried under an air stream and digested with concentrated H_2SO_4 and 46% perchloric acid. The phosphorus content was then measured by the method of Chen (22).

Tritium was measured with a Packard Tri-Carb liquid scintillation counter, model 3003, employing an external standard for determination of counting efficiency. A toluene-based scintillation solution was used (8).

Electron Microscopy—Chromatin preparations were fixed in Karnovsky (23) fixative for 1 hour and washed three times 5 to 5 min each in 0.1 M cacodylate buffer adjusted to pH 7.2. The pellets were postfixed for 1 hour in 2% OsO_4 buffered with 0.1 M cacodylate adjusted to pH 7.2. They were dehydrated in graded ethanol solutions prior to embedding in Epon (Fisher Scientific Company). After polymerization, Epon embedded sections were stained with Reynolds lead citrate (24) and examined with a Philips EM 200 electron microscope.

RESULTS

In the first series of investigations, repeated attempts were made to demonstrate the presence of ^{3}H in the chromatin isolated from rats given either [1,2]^{3}H-labeled vitamin D_3 or [22,23]^{3}H-labeled vitamin D_4 intrajugularly (Table I). When the pro-

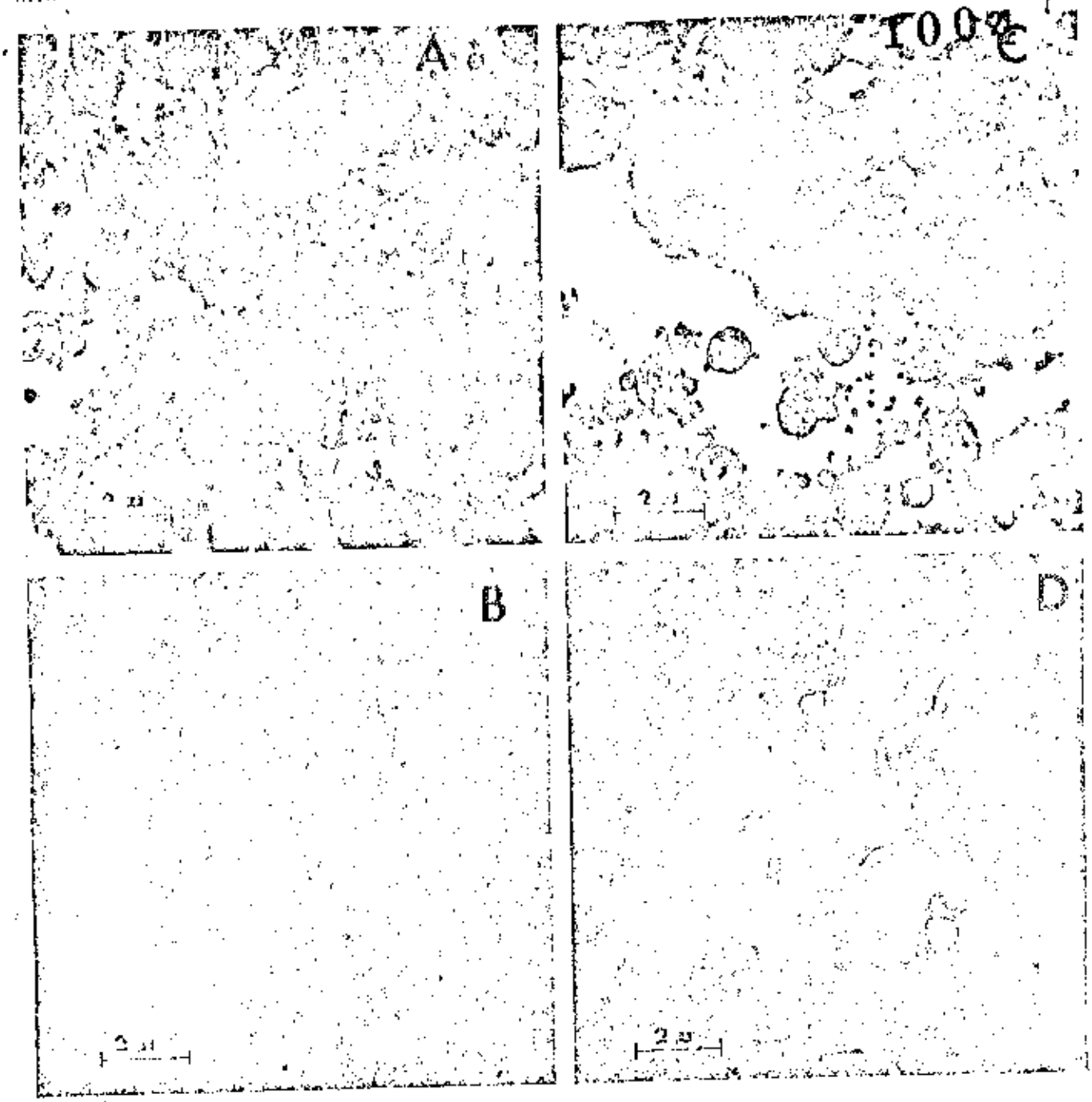

FIG. 1. Electron micrographs of chromatin preparations from intestinal mucosa. × 8000. *A*, rat preparation, Haussler *et al.* procedure (13); *B*, rat preparation, Marushige and Bonner procedure (15); *C*, chick preparation, Haussler *et al.* procedure (13); *D*, chick preparation, Marushige and Bonner procedure (15).

cedure of Marushige and Bonner (15) was used for the isolation, no ^{3}H could be found in the intestinal chromatin at 4, 8, or 15 hours after the administration of the radioactive vitamins. These results are clearly at variance with those of Haussler *et al.* (13) and Haussler and Norman (14).

Rachitic chicks were used next because the results of Haussler *et al.* (13) were obtained with chicks. The data shown in Table II demonstrate that no more than 0.6% of the total ^{3}H in the homogenate could be found in the chick intestinal chromatin 15 hours after administration of [1,2-^{3}H] labeled vitamin D, when the procedure of Marushige and Bonner (15) was used for the isolation. Again these results are at variance with those of Haussler *et al.* (13).

Because Haussler *et al.* (13) and Haussler and Norman (14) significantly modified the procedure of Marushige and Bonner, it appeared that this might account for the discrepancy. When chromatin was isolated by this procedure, as much as 10% of the total radioactivity in the homogenate was found in this fraction in the case of chicks, but only 20% of the total radioactivity was found in the chromatin fraction in the case of rats (Table III).

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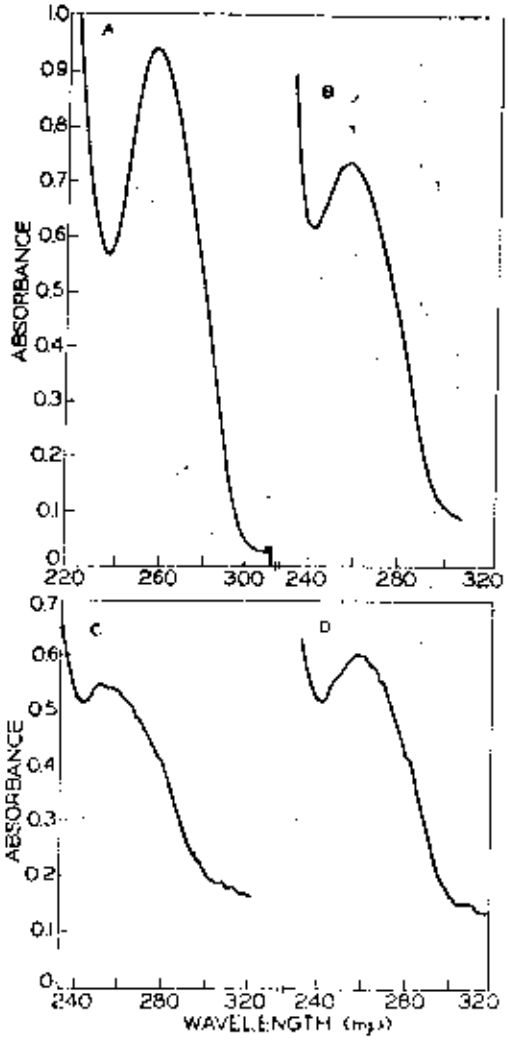

FIG. 2. Ultraviolet spectra of various intestinal chromatin preparations. *A*, rat chromatin prepared by the Marushige and Bonner procedure (15); *B*, chick chromatin prepared by the Marushige and Bonner procedure (15); *C*, rat chromatin prepared by the Haussler *et al.* procedure (13); *D*, chick chromatin prepared by the Haussler *et al.* procedure (13).

Thus the results of Haussler *et al.* (13) could be repeated, but the question remained as to whether the interpretation of the location of radioactivity in chromatin was valid.

When the fate of ^{3}H in the homogenate during the chromatin isolation by the Marushige and Bonner procedure from intestines of rats given [1,2-^{3}H]-labeled vitamin D$_3$ is examined, it is evident that the major portion of radioactivity is removed during the EDTA treatment (Table IV). When the supernatant fluid after EDTA treatment is sedimented for 2 hours at 50,000 rpm in a Spinco L-2 ultracentrifuge using a No. 50 rotor, the radioactivity is located in a particulate fraction not associated with chromatin, while the pure chromatin contains no radioactivity.

Chromatin prepared by the two methods, from both chicks and rats, was therefore observed in thin sections by electron microscopy (Fig. 1). The electron microscopic appearance of chromatin prepared by the procedure of Marushige and Bonner resembled the normal granular and filamentous nucleoplasm. This nuclear ground substance and chromatin did not stain well with osmium or lead, as does membranous material. There was only the slightest trace of membranous material in the Marushige and Bonner preparations. The preparation from rat intestine was the most nearly homogeneous.

TABLE V

Chemical composition of chromatin, pure nuclei, and homogenates from rats or chicks

Source	Ratio of RNA to DNA	Ratio of protein to DNA	Ratio of phospholipid phosphate to DNA
Literature values			
Rat liver nuclei (26)	0.22	4.8	
Rat liver homogenate (26)	3.7	65.6	
Chick liver chromatin (13)	0.04	4.3	
Rat liver chromatin (15)	0.013	1.67	
Chick intestinal chromatin (13)	0.12	3.7	
Experimental values			
Rat intestinal chromatin (Marushige and Bonner procedure)	0.037	0.94	0.002
Chick intestinal chromatin (Marushige and Bonner procedure)	0.09	0.66	0.006
Chick intestinal chromatin (Haussler *et al.* procedure)	0.12	1.64	0.010
Chick intestinal nuclei	0.33	3.28	0.012
Rat intestinal homogenate	4.9	73	0.39
Chick intestinal homogenate	1.6	84	0.65

The preparations made by the method of Haussler *et al.* (13) were heavily contaminated with membranous components and cellular organelles (Fig. 1, *A* and *C*), chiefly mitochondria and vesicles. These vesicles and membrane fragments could be formed during preparation from any of the cellular membranes. Although the nuclear membrane is not seen encircling the condensed nucleoplasm, the presence of nuclear membrane fragments certainly is not eliminated (Fig. 1, *A* and *C*).

The ultraviolet absorption spectra (Fig. 2) provide additional evidence of purity for the preparations obtained by the Marushige and Bonner method, especially those from rat intestine. On the other hand, the other preparations were impure by this criterion as well.

Chemical analyses of chromatin preparations as well as pure intestinal nuclei and homogenate were carried out. The results shown in Table V leave little doubt that the chromatin obtained by the Haussler *et al.* procedure (13) is quite different from that obtained by the Marushige and Bonner procedure (15). The phospholipid content of the Haussler *et al.* (13) chromatin was almost exactly that of pure nuclei from chick intestine. These results clearly accord with the electron microscopic findings and raise questions in regard to the conclusions of Haussler *et al.* (13) and of Lawson, Wilson, and Kodicek (25).

DISCUSSION

In considering mechanisms of vitamin D action from the standpoint of now well established involvement of vitamin D with transcription of a specific gene or genes of intestinal mucosa, the subcellular location of the active form of the vitamin is of central importance. Stohs and DeLuca (9) concluded that the nuclei contained some 50% of the ^{3}H of intestinal homogenates from rats given 0.25 μg of [1,2,^{3}H]-labeled vitamin D$_3$. Because

the two different agents, citric acid and Triton X-100, which remove the outer nuclear membrane, also remove about 80% of the ^{3}H from the nuclei, it was concluded that the nuclear membrane is the principal location of ^{3}H in this organelle. Hausler *et al.* (13), on the other hand, concluded that the ^{3}H in a ^{3}H-labeled vitamin D_3 is found principally in chromatin rather than in the nuclear membrane. Unfortunately, these investigators modified the procedure of Marushige and Dounce for recovering chromatin without providing adequate evidence of purity of the preparations. In fact, their results which show some 60% of the total deoxyribose of the homogenate in their chromatin preparation is suggestive of membrane contamination. Because we could not confirm their results in either rats or chicks when the Marushige and Dounce procedure was used, we undertook a combined chemical and electron microscopic examination of the various chromatin preparations. There is little doubt from electron microscopy, ultraviolet absorption spectra, and chemical analysis that the Hausler *et al.* preparation is far from pure chromatin, whereas the Marushige and Dounce preparation is quite homogeneous especially in the case of rats. Clearly the conclusion that the ^{3}H from [1,2-^{3}H]-labeled vitamin D_3 in intestinal nuclei is in the chromatin is not warranted. From our experience, it is evident that care should be exercised by investigators to characterize thoroughly any cell fraction before conclusions on subcellular location of a substance are made. Unfortunately, the Hausler *et al.* method of chromatin preparation has already been applied by Lawson *et al.* (25).

The question then remains: where in the intestinal nuclei is the active form of vitamin D_3 located? The present results still are consistent with location in the nuclear membrane. Certainly the nuclear radioactivity remains with a particulate fraction during the isolation of chromatin from nuclei but is not bound to pure chromatin. Unfortunately, all cell fractionation methods are subject to the same uncertainties that prohibit absolute conclusions. It is possible that autoradiography will provide additional evidence and may succeed in establishing the location of the active form of vitamin D in the intestinal cell. Recently [26,27]-^{3}H-25-hydroxycholecalciferol of extremely high specific activity has been synthesized and shows promise of providing the required autoradiographic information. Until that time, it can only be stated that nuclei of the intestine contain much of the active form of vitamin D (either 25-hydroxycholecalciferol or further metabolites), probably in the nuclear membrane.

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R.C. Clause

The Margin of Safety.—Because of differences in the potency of the products used, it is difficult to make any exact statement concerning the size of dose necessary to produce toxic effects. In short time experiments, however, disastrous results are not apparent on normal diets until the dose administered reaches at least from 25,000 to 50,000 times the minimum antirachitic dose.

In experiments continued for three or four generations, harmful effects which may not be apparent in the first generation may appear in the third or fourth. For example, Light, Miller and Frey of the Fleischmann Laboratories have found that in rats fed the Steenbock diet 2965, doses of 40 units daily, continued throughout three or four generations, have no effect on the mineral metabolism. No pathologic calcification of the tissues occurred, and the ash of the bones was normal. The fourth generation of these rats, however, was much more sensitive to overdosage (6,000 units daily) than were young, normal rats whose ancestors had never received any excessive amounts of vitamin D.

The harmful effects of considerably larger doses of 2,500 units daily were just noticeable in the first generation but were marked in the fourth. The fourth generation rats were very lame in the hind legs, apparently because of spontaneous fractures of the tibia, and the long bones were very low in ash. The head of the long bone was hypercalcified, but the shaft was so soft that it could be bent with the fingers.

Evidently disastrous results are produced in the first generation only when enormous doses are fed. Smaller doses, just on the verge of toxicity in the first generation, may produce a cumulative effect in the third or fourth. The fact should be emphasized, however, that even these doses are several thousand times the minimum dose required to prevent or cure rickets.

THEORIES CONCERNING THE CAUSE OF TOXICITY

Thus far in this discussion it has been assumed that the toxic symptoms produced by enormous doses of irradiated ergosterol are caused by excessive amounts of vitamin D. Opinion on this point is, however, divided, and certain investigators, notably Windaus and his associates at the University of Göttingen, believe that the toxic effects may be produced not by vitamin D but by other substances produced simultaneously with it by the action of ultraviolet rays. Their reasons for this belief will be discussed in the following paragraphs.

VITAMIN D INTAKE AND THE HYPERCALCEMIC SYNDROME

The Committee on Nutrition of the American Academy of Pediatrics in 1963 published a memorandum entitled, "The Prophylactic Requirement and the Toxicity of Vitamin D," (Pediatrics, 31:512, 1963). The Committee believed at that time that there was circumstantial evidence relating vitamin D intake to the pathogenesis of the mild form of infantile hypercalcemia, and probably also of the severe form, which, in addition to hypercalcemia, manifests typical facies, supravalvular aortic stenosis, and a mental defect.

Since the appearance of this memorandum, evidence has appeared which suggests that the severe form of the disease may arise *in utero* (Lancet, 2:745, 1963; New Engl. J. Med., 271: 117-120, 1964). Although it is not established whether maternal vitamin D intake or metabolism play a role in the pathogenesis of the fetal condition, the possibility must be considered. Under the circumstances, and despite the indication that the disease is rare and the population at risk very small, it seems reasonable to recommend public health measures which may reduce the incidence of one form of mental retardation and one form of congenital heart disease. However, such recommendations are justified only in so far as a much larger normal population is not simultaneously placed in jeopardy from vitamin D deficiency.

It is generally considered that a total intake of 400 units of vitamin D/day will meet the needs of normal infants and children and that a similar intake during the last half of pregnancy and during lactation amply meets the requirements of both mother and fetus. In view of the possibility that maternal factors may play a role in the causation of the severe form of infantile hypercalcemia, the Committee believes that there is reason to reiterate and amplify its previous recommendations with respect to the use of vitamin D, and pending further evidence, to emphasize the wisdom of applying these recommendations to pregnant women as well as to the infants and children of North America.

The Committee is fully cognizant of the nutritional requirements for vitamin D and the importance of maintaining a daily intake which will protect the infant and the growing child from the development of rickets. In addition, it is aware that the judicious addition of vitamin D to milk has virtually eradicated vitamin D deficiency rickets in the United States. The recommendations which follow should be read in the context of what is known about tolerance of normal humans of all ages to high doses of vitamin D and the remarkable rarity of the hypercalcemic syndrome of infancy. They reflect not only the best judgment of the Committee with respect to vitamin D requirements, but also take cognizance of the risk which may be incurred by a certain small group of infants when pregnant women or infants consume amounts of vitamin D which are little more than the recommended daily intake.

1. The Committee wishes to reiterate the statement made in its published memorandum with respect to vitamin D fortification of foods other than milk or infant formula products: ". . . the present practice of enriching foods other than milk and infant formula products is not justified, and discontinuation of this practice is to be recommended."

2. The addition of vitamin D to fluid milk, evaporated milk, and to special milk products and milk substitutes designed for consumption by infants is desirable and should continue. However, the vitamin D fortification should be carefully controlled so that in normal usage the total daily consumption will not be more than 400 units (i.e., 400 units/quart of fluid milk, or 400 units per reconstituted quart of formula products), as is the present practice in the United States. The addition of vitamin D to other foods may serve only to increase the intake by infants, children, and pregnant women to levels beyond requirement.

3. Special purpose foods, designed to serve as the total daily source of nutrient or calories of infants, children, pregnant or lactating women, may contain vitamin D. The vitamin should be added in an amount that does not exceed 400 units in the total daily intake.

4. At the present time, various vitamin

preparations provide different amounts of vitamin D, some considerably in excess of 400 units in the recommended daily dosage. The Committee suggests that no preparations sold over the counter should provide more than 400 units of vitamin D in the amount of the product designated on the label as the recommended daily intake. Since it is practice to dispense vitamin preparations designed for use by infants in liquid form, adjustment by the physician of intake from supplements to levels below 400 units/day remains feasible if consideration of total vitamin D available to the infant makes such reduction desirable. The Committee suggests that products containing amounts of vitamin D in excess of 400 units in the recommended daily dosage be restricted to sale on prescription.

5. If in the future as unambiguous causal relation between vitamin D intake of the pregnant woman or the infant and the severe form of the hypercalcemic syndrome can be proved, the Committee would favor labeling of all vitamin products containing vitamin D so as to indicate potential toxicity if taken in excess of the recommended daily dosage. In addition, the user should be advised to seek medical advice before administering greater amounts of the product.

6. Before prescribing vitamin D supplements for any patient, the physician should assess the total intake of vitamin D from other sources, and unless high doses of vitamin D are required to treat specific disturbances of calcium and phosphorus metabolism, he should attempt to establish an intake of vitamin D of approximately 400 units per day from all sources. This applies for pregnant and lactating women as well as for infants and children. Normal adults living under ordinary conditions do not require supplementary vitamin D.

A copy of this letter has been forwarded to the Food and Drug Administration for consideration.

Errata acknowledged subsequently in Pediatrics have been corrected in this reprint.

Committee on Specifications. 1972

Food Chemicals Codex, Ed. 2

Committee on Food Protection, National Academy of Sciences
National Research Council
Washington, D.C.

J Appl Physiol 10, 98-102, 1957

Effects of Massive Doses of Vitamin D on Physiological Behavior of Calcium in Cattle[1, 2]

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ABSTRACT

CONRAD, H. R. AND SAM L. HANSARD. (U. of Tennessee-AEC., Agric. Res. Program, Oak Ridge.) *Effects of massive doses of vitamin D on physiological behavior of calcium in cattle.* J. Appl. Physiol. 10(1): 98-102. 1957.—Tracer doses of calcium-45 were used as markers in conjunction with balance trials, blood and tissue distribution studies with paired Hereford calves to assess changes in calcium metabolism when 5 million units of vitamin D were fed for 5-day period. The true digestibility of calcium was increased, denoting increased absorption and confirming the findings reported by others. Increased absorption due to vitamin D feeding was accompanied by several related effects, including decreased endogenous fecal losses, a transitory increase in absorbed radiocalcium in the blood, more rapid movement of radiocalcium through the plasma, a threefold increase in net calcium retention and increased deposition of radiocalcium in areas of new bone growth. Extended feeding of vitamin D for two additional 5-day periods produced no apparent deleterious effect on calcium metabolism. Increased calcium concentration in the kidney and esophagus was observed but had disappeared after 8 days of vitamin D feeding. Autoradiographs of femur bone sections indicated that deposited radiocalcium in the areas of new bone growth was exchanged more rapidly under conditions of extended vitamin D feeding. Analytical data supported these observations.

INCREASED absorption of calcium is considered the major physiological action of vitamin D on calcium metabolism (1-3). Serum calcium levels are raised as a result of increased calcium absorption when calcemic doses of vitamin D are administered (1). In experiments with dairy cows, short-time prepartum feeding of massive doses of vitamin D raised serum calcium above the normal level and prevented milk fever (parturient paresis) (4), which is a metabolic disturbance characterized by a precipitous drop in serum calcium (3, 5). Later results showed that massive oral doses of vitamin D increased calcium and phosphorus absorption from the digestive tract and retention in the body tissue of mature cattle (6). In general, the increased retention is attributed to an accentuation of normal calcification of the skeleton brought on by increased absorption (2), but some reports in the literature (7) suggest that vitamin D may have a direct effect on mineralization of the bone. In this study radiocalcium was used to obtain further information on the physiological effects in the young growing bovine of short-time feeding of massive doses of vitamin D.

METHODS

Ten 6-month-old Hereford calves, weighing an average of 400 pounds, were used in this study. After allowing a period for physiological adjustment to a ration of previously determined composition, the animals were placed in metabolism units (8, 9) for 7-day calcium and phosphorus balances.

A single dose of 1 mc of radiocalcium ($Ca^{45}Cl$) was administered orally and intravenously, respectively, to the pair-mates on the initial day of the balance trial. Radioisotope procedures used have been described previously (8, 10). Six of the calves were dosed orally with 5 million U.S.P. units of vitamin D (viosterol) daily for 5 days and the remaining four calves served as

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[3] On leave of absence from Ohio Agricultural Experiment Station, Wooster, Ohio.

controls. Periodically, samples of jugular blood were taken throughout the experimental period and the animals were killed following a 30 day period during which time four calves received vitamin D for two additional 5-day periods. Table 1 shows the procedure followed.

Selected tissue samples were taken for radioassay and calcium determinations by methods as described previously by Comar *et al.* (11). The right femur from each animal was removed and prepared for autoradiographs. Total calcium and phosphorus were determined by standard procedures.

RESULTS

Calcium Excretion and Retention. Average daily excretion of fecal calcium and percentage intake absorbed expressed as true digestibility are shown in table 2 for six vitamin D treated calves and for the four control calves receiving no extra vitamin D. The percentage of orally and intravenously administered radiocalcium appearing in the feces is shown graphically in figure 1 as the cumulative percentage of Ca^{45} excreted in relation to time after dosing. As with mature cattle (12), a higher average true digestibility and a lower daily rate of excretion of total calcium was observed when massive doses of vitamin D were fed daily. The excretion curves in figure 1 reflect the increased absorption by calves receiving supplemental vitamin D as less Ca^{45} is excreted. Similar patterns of excretion in relation to time after dosing for both groups indicate that the time of passage through the alimentary tract was approximately the same.

TABLE 1. EXPERIMENTAL PLAN USED FOR ADMINISTRATION OF VITAMIN D AND RADIOCALCIUM

Vitamin D Feeding*	Method of Ca^{45} Administration (No. of Calves)		Remarks
	Oral	I.V.	
None	2	2	Killed after 32 days
25,000,000 U	1	1	Killed after 32 days
75,000,000 U	1	1	Vitamin D given on 1-5, 16-21 and 26-30 days after Ca^{45} administration; killed after 32 days
75,000,000 U	1	1	Vitamin D given on 1-5, 11-15 and 21-25 days after Ca^{45} administration; killed after 32 days

* Daily dose of vitamin D was 5,000,000 U.S.P. units, viosterol in oil.

† 1 mc of Ca^{45} chloride administered as a single dose on 1st day of vitamin D feeding.

TABLE 2. EFFECT OF FEEDING MASSIVE ORAL DOSES OF VITAMIN D* ON THE DIGESTIBILITY, EXCRETION AND RETENTION OF CALCIUM AND CALCIUM-45 IN HEREFORD CALVES 6 MONTHS OLD

	Vitamin D	No Vitamin D
No. of animals	6	4
Vitamin D, U/day	5,000,000	None
Ca intake, gm/day	10.08	8.92
Fecal Ca, gm/day	4.90	7.10
Urinary Ca, gm/day	.15	.08
Ca retained, gm/day	4.96	1.74
Ca; apparent digestibility %	52.2	20.6
Fecal endogenous Ca, gm/day†	1.64	2.27
Ca; true digestibility, %‡	69.8	47.7
Ca^{45} excreted in feces, % dose		
I.V. dose	10.9	9.9
Oral dose	39.1	54.7
P; apparent digestibility	58.2	40.3

* Viosterol; administered orally once daily in capsules.

† Fecal endogenous calcium determined concurrently by isotope dilution and comparative procedures (9).

‡ True digestibility calculated as $100 - \frac{(\text{fecal Ca} - \text{endog. Ca})100}{\text{Ca intake}}$.

A distinct effect from vitamin D feeding on endogenous calcium losses was observed. The daily fecal endogenous output was decreased while calcium excreted in the urine, although a relatively small proportion of the total excretion, was increased when vitamin D was fed. As a result of increased absorption and a net decrease in endogenous losses, calcium retention was increased accordingly as shown in table 2.

The fecal excretion curves for intravenously dosed calves were parallel and essentially the same. Since the intravenous dose of Ca^{45} and the vitamin D were administered simultaneously excretion of a large proportion of the radiocalcium had occurred before the vitamin D reached the site of either absorption or excretion.

Total Calcium and Ca^{45} in the Blood. The means and standard deviations of the average daily plasma calcium values were 12.1 ± 1.3 and 12.2 ± 1.0 mg/100 ml for the vitamin D and control groups, respectively. Thus the calcemic effect observed in mature parturient cattle when massive doses of vitamin D were fed (4) was not produced in the 6-month-old

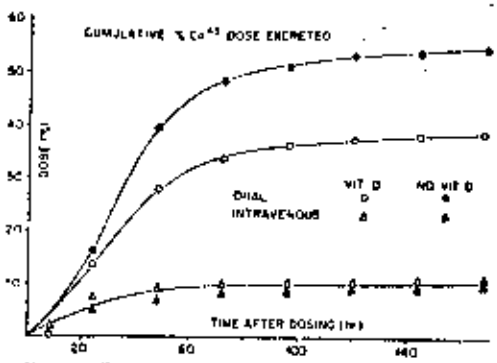

FIG. 1. Cumulative fecal excretion of orally and intravenously administered calcium-45 by calves with and without massive oral doses of vitamin D.

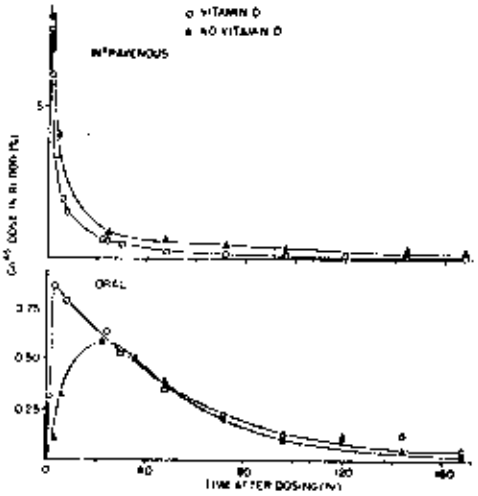

FIG. 2. Calcium 45 concentration in total blood as function of time in calves with and without massive doses of vitamin D following intravenous and oral administration.

calves used in this experiment. This difference may be associated with the comparatively higher blood disappearance rates and the higher maximum exchangeability of tissue calcium known to occur in young calves (9, 13).

Intravenously dosed calcium-45 disappeared from the blood with greater rapidity in the vitamin D group (fig. 2). Data for the 24- to 144-hour period were analyzed graphically using procedures previously outlined (13, 14). The following exponential equations were found to represent radiocalcium disappearance from the blood when the 24-hour values were adjusted to 100%.

Vitamin D treated:

$$C(t) = 62e^{-0.054t} + 38e^{-0.012t} \quad (1)$$

Untreated calves:

$$C(t) = 100e^{-0.0128t} \quad (2)$$

The terms of such equations have been correlated with specific physiological processes (13–15). Thus it is possible that the first term of the equation is a mathematical expression of the increased movement of radiocalcium from the plasma pool caused by the feeding of massive doses of vitamin D. Calculation of turnover rate for radiocalcium by the formula used by Thomas, Litovitz, Rubin and Gieschickter (14) showed that the blood radiocalcium in vitamin D treated calves was disappearing nearly four times faster than that in their untreated controls during this period (5.1% hr. vs. 1.3% hr.).

The line graphs in figure 2 also show comparisons of average total blood radiocalcium when calcium-45 was administered orally. The Ca^{45} in the total blood (13) increased more sharply and rose to a higher maximum in the vitamin D treated group reflecting the increased absorption of calcium previously noted in table 2.

Tissue Distribution of Total Calcium and Ca^{45}. A comparison of calcium concentration in selected soft tissues and bones of calves fed 5 million units of vitamin D daily with that of an untreated group is presented in table 3. The data are classified according to total amount of vitamin D received by the calves and number of days elapsing between date of the last vitamin D fed and killing of the animal. Two main points of interest are illustrated. First, in calves receiving a total of 75 million U.S.P. units of vitamin D and killed 2 days after the date of last vitamin D feeding, a marked increase in calcium was found in the kidney and a slight increase was noted in esophageal tissue. However, these tissues were of normal calcium concentration in similarly treated calves killed 8 days after vitamin D feeding was terminated, suggesting that the calcium accumulation in these soft tissues was of a temporary and migratory nature when vitamin D was fed for the 15-day period. It is generally known that prolonged feeding of massive doses of vitamin D will cause calcification of soft tissue including the kidney and various other soft tissues (16).

Second, the calcium concentrations of samples obtained from the femur, rib and mandible

TABLE 3. CALCIUM CONCENTRATION* IN SELECTED TISSUES OF HEREFORD CALVES FED 5 MILLION UNITS OF VITAMIN D DAILY FOR DIFFERENT PERIODS OF TIME

Group	1	2	3	4
No. of animals	2	3	2	3
Vitamin D, total, units in million	None	15	75	75
Days after last vitamin D		30	1	8
Blood plasma	.177	.120	.151	.127
Kidney	.161	.185	.269	.136
Gastrocnemius	.072	.051	.044	.058
Esophagus	.096	.106	.119	.108
Aorta	.120	.108	.120	.157
Femur shaft	275.5	200.1	218.5	231.5
Femur epiphysis	85.4	83.1	86.0	86.1
Rib shaft	130.5	140.8	154.3	134.3
Rib epiphysis	38.1	60.1	38.6	31.7
Mandible	160.4	168.5	145.3	150.0

* Calcium expressed as milligrams of Ca per gram of fresh tissue

TABLE 4. EFFECT OF MASSIVE ORAL DOSES OF VITAMIN D ON CONCENTRATION OF INTRAVENOUSLY AND ORALLY ADMINISTERED CALCIUM-45 IN THE BONE.* RELATIVE CALCIUM-45 CONCENTRATION EXPRESSED AS SPECIFIC ACTIVITY ($Ca^{45} \times 10^8/Ca^{40}$)

	Intravenous		Oral		Oral	
	Vit. D	No Vit. D	Vit. D	No Vit. D	Vit. D	No Vit. D
No. of animals	3	2	3	2	3	2
Vit. D, millions of u/day	5	None	5	None	5	None
Femur shaft	3.33	2.83	2.48	1.40	4.20	4.29
Femur epiphysis	6.00	6.03	5.58	3.25	9.01	9.55
Rib shaft	7.30	7.73	4.90	3.80	8.20	11.17
Rib epiphysis	6.57	6.05	9.14	3.11	15.63	9.14
Mandible	9.58	11.80	8.24	4.04	13.40	11.88

* Corrected to percentage of Ca^{45} absorbed.

are remarkably constant. This observation would seem to minimize the possibility that extended feeding of massive doses of vitamin D at the rate of 5 million units per day resulted in demineralization of the bone as has been observed when toxic doses of vitamin D were administered to rats (17).

Results of analysis of bone ash from femurs, ribs and mandibles for radiocalcium activity are presented in table 4. The data are expressed in terms of specific activity of Ca^{45}. Higher concentration of orally administered radiocalcium was found in bone ash from calves treated with vitamin D, whereas no notable differences were observed when the method of administering radiocalcium was through the jugular vein. When considered in conjunction with the absorption and excretion data (table 2), this is in agreement with the accepted theory (3) that vitamin D functions primarily to increase intestinal absorption of calcium which in turn results in increased calcium lay-down. This conclusion is further borne out by data presented in table 4 showing that, after tissue-specific activity was adjusted for the amount of administered dose absorbed (13), major differences were eliminated.

Autoradiographs made from sagittal sections of femoral bones obtained from the calves given vitamin D for various periods of time reflected the pattern of Ca^{45} deposition and verified the radiocalcium findings. Extended feeding of vitamin D appeared to affect Ca^{45} deposition both in the periosteum and in the new trabecular bone of the metaphysis. This was seen as irregular and broken lines of calcium-45 deposition in the epiphyseal region in calves which received 5 million units of vitamin D for three 5-day feeding periods. Specific activities of femur epiphyseal samples were 6.03, 5.34 and 6.94, respectively, for calves receiving no vitamin D, 75 million units in 15 days and 5 million units in 5 days. Thus the quantitative chemical data were in agreement with the observed autoradiographic picture. These observations further indicated increased exchange and movement of calcium in the areas of new bone growth as a result of feeding massive doses of vitamin D for extended periods.

DISCUSSION

Massive oral doses of vitamin D produced a sequence of effects in the calcium metabolism of calves which arose primarily from increased absorption, although endogenous fecal calcium appeared to be decreased simultaneously. The data presented provide no information on functional processes in the gastrointestinal tract which caused increased absorption. However, in rat experiments using Ca^{45} Harrison and Harrison (1) found that the increase in absorption efficiency produced by vitamin D resulted from an extension of conditions favoring calcium absorption beyond the proximal two-thirds of the small intestine. This would permit increased reabsorption from the intestine of endogenous calcium (9) as well and thereby reduce the net quantity excreted in the feces of these young growing calves.

The increased absorption of Ca^{45} produced a transitory increase in blood radiocalcium but total blood calcium remained essentially constant during the vitamin D feeding period. In prepartum cows it has been possible to increase total blood calcium with this level of vitamin D feeding (4), but this does not hold for calves. This can be explained by the marked differences observed in calcium metabolism between young and old cattle (13, 18).

The increased disappearance rate from the blood of vitamin D treated calves of intravenously administered Ca^{45} may further be considered a dilution effect by increased absorption and resorption of stable calcium ions. Since total blood calcium was maintained at normal levels, the rate of movement of calcium through the plasma would necessarily be increased, as shown in the results.

Calcification and accretion of calcium in tissue appeared to be increased by vitamin D. Based on the retention values presented in table 2, the increased rate of calcification was approximately threefold. Epiphyseal and periosteal regions of the growing bone were the principal areas of increased radiocalcium deposition. However, radiochemical assays of soft tissue indicated increased calcium deposition of a transitory nature in the kidney and esophagus after an extended period of vitamin D feeding. It has been pointed out, however, that with young calves and with mature cows (5, 6) the increased deposition of Ca^{45} in the bone may be attributed primarily to increased absorption. Thus the increased deposition could be explained simply by increased availability. The extent to which deposition is possible, however, is limited by bone growth. Therefore, it may be postulated that the additional calcium available in the tissue of vitamin D treated animals is deposited first in areas of new bone growth where calcification occurs readily but, after these regions acquire their physiological maximum and total blood calcium is raised over a period of time, calcification may occur in the soft tissues.

Urinary excretion of calcium, although increased slightly by vitamin D, was of insufficient quantity to influence the total calcium metabolism.

The technical assistance of Tom Clark, William Lyke, Ruth Bell, Everett Kincaid and Robert Sellers is gratefully acknowledged.

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STUDIES ON MILK FEVER IN DAIRY COWS. V. THE EFFECT OF MASSIVE ORAL DOSES OF VITAMIN D ON ABSORPTION, EXCRETION, RETENTION AND BLOOD LEVELS OF CALCIUM AND PHOSPHORUS[1]

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The efficiency of calcium absorption and mobilization is involved as a major contributory factor in the onset of milk fever. A study concerned with changes in the physiological behavior of calcium and phosphorus when massive doses of vitamin D are fed to dairy cattle assumes significant importance. Recent development of radioisotope procedures for the separate estimation of endogenous and exogenous fecal calcium and phosphorus provided the means for further study of this important problem. *Editor.*

This study is concerned with changes in the physiological behavior of calcium and phosphorus when massive oral doses of vitamin D are fed to cattle.

Recently Hibbs and Pounden *(13)* have shown that 30 million units per day of vitamin D fed 3-8 days prepartum prevented milk fever in mature cows with previous milk fever histories. The protection afforded by vitamin D was attributed to its calcemic effect, which was indicated by the relatively high blood calcium level maintained during the critical postpartum period. Other studies *(21)* have suggested that the increased blood calcium level was not due to parathyroid stimulation, because parathyroid activity was suppressed after 7 days of vitamin D feeding at thirty million units per day. In support of this, various workers *(1, 15)* using other species have found that calcemic doses of vitamin D increased absorption of calcium from the intestine, but parathyroid hormone secretion actually declined *(1)* and parathyroid size decreased *(16)*. The fact has been established experimentally in a number of species that vitamin D increases the absorption of calcium and phosphorus when added to a diet low in vitamin D *(6, 10, 17, 19)*. These findings suggest that the calcemic effect of vitamin D is elicited chiefly through an increased intestinal absorption of calcium and phosphorus.

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The marked diminution of calcium absorption with increased age in cattle *(8, 9)* and the characteristic occurrence of milk fever in aged cows as opposed to young cows *(11)* suggest that the efficiency of calcium absorption and mobilization is involved as a major contributory factor in the onset of milk fever. Ward *et al.* *(25)* found by conventional balance methods that aged cows which developed milk fever were in severe negative calcium balance for 15 days prepartum whereas others maintained their calcium stores, and that primiparous Jerseys appeared to store calcium, prepartum. The relation of age to calcium metabolism was indicated also by Moodie *et al.* *(18)*, who showed that the decline in serum calcium and rise in serum magnesium increased with age up to the fourth calving.

With the development of radioisotope procedures for the separate estimation of endogenous and exogenous fecal calcium and phosphorus *(9, 14, 22)*, an opportunity was presented for further study of the specific effects of vitamin D when fed prepartum to cattle in amounts sufficient to prevent milk fever.

EXPERIMENTAL PROCEDURE

This study involved 24 concurrent chemical and radiocalcium and phosphorus-32 balances with four lactating, two nonlactating cows (aged purebred or grade Jerseys), and two mature Hereford steers. All animals were oriented in metabolism units *(7)* following pre-experimental adjustment to rations of known composition.

In general three consecutive balances with each animal were carried out, including periods before, during, and after the feeding of 20 to 30 million units of vitamin D by capsule daily for five consecutive days. A viosterol-oil solution containing one million U.S.P. units per gram was used as a source of vitamin D. A single oral or intravenous tracer dose of radioactivity was administered to the animals by methods previously described *(9)* on the first day of the balance period. In the first phase of this study tracer doses of radiocalcium chloride were administered orally and intravenously, respectively, to paired animals on adequate rations, and periodic blood samples were taken throughout the 5- to 7-day balance trials. The procedure was then repeated several weeks later with phosphorus-32 in similar trials with two of the animals.

Feed, feces, urine, and milk samples were obtained routinely, dried overnight at 100° C., and ashed at 625° C. in muffle furnaces for 18 hours. The dry ash was then dissolved in 6 *N* HCl, an aliquot was taken, and the calcium was precipitated as the oxalate after adjusting the pH. Phosphorus was determined colorimetrically by a modification of the method of Fiske and Subba-Row *(4)*. Analyses for radiocalcium were carried out as described previously *(2)* and for radiophosphorus by direct counting of the wet ashed samples. Total calcium, phosphorus, and radioactivity of the periodic blood samples were determined by conventional procedure *(9)*.

Endogenous fecal losses were determined by using concurrently the comparative method described by Hansard *et al.* *(9)* and the isotope dilution *(11, 22)* procedure modified to include a single tracer dose of radioactivity *(9)*.

RESULTS

Results of the calcium and phosphorus balances before, during, and after feeding massive oral doses of vitamin D are summarized in Tables 1 and 2. A pronounced rise in calcium absorption was observed during vitamin D feeding. The average true digestibility coefficients for calcium increased from 33.1 to 50.3 for nonlactating cows (Table 1). True digestibility of the total phosphorus was found also to increase during the 5-day feeding period when 20 million units of vitamin D were fed daily to mature steers (Table 2). This increased true digestibility was accompanied by a marked decrease in endogenous calcium losses and an increase in net calcium retention (Table 1). A further increase in per cent true digestibility of both calcium and phosphorus occurred during the 5-day period immediately following vitamin D feeding.

The effects of vitamin D feeding upon fecal endogenous losses and retained calcium in the lactating cow were small with only a slight effect on net calcium retention. Thus it appeared that the vitamin D effect on calcium storage was most pronounced in dry cows. The lactating cows absorbed a greater percentage of their calcium intake than the nonlactating animals (Table 1).

TABLE 1

The effect of feeding massive oral doses of vitamin D[a] to mature cattle on the digestibility, excretion, and retention of total calcium and calcium[b]

	Nonlactating cattle[b]			Lactating cattle[c]		
	Before vitamin D feeding	During vitamin D feeding	After vitamin D feeding	Before vitamin D feeding	During vitamin D feeding	After vitamin D feeding
No. of animals	2	4	4	3	4	3
Vitamin D (*units/d.*)	none	30,000,000	none	none	30,000,000	none
Calcium intake (*g.*)	26.9	30.3	27.7	64.8	59.4	54.1
Fecal calcium (*g.*)	27.9	20.8	20.0	46.3	39.5	29.9
Urine calcium (*g.*)	0.1	0.0	0.5	0.2	0.0	0.3
Milk calcium (*g.*)	—	—	—	10.4	10.2	8.8
Calcium retained (*g.*)	-1.1	8.8	7.3	5.8	8.5	11.0
Ca. apparent digestibility (%)	-9.9	31.8	27.9	28.6	33.5	45.1
Fecal endogenous calcium (*g.*)	11.3	5.7	7.2	9.8	8.8	9.3
Ca. true digestibility (%)[d]	33.1	50.8	54.3	44.3	49.6	60.8
Ca^{45} excreted in feces (% *dose*)						
I.V. dose	19.6	18.8	21.7	—	17.7	—
Oral dose	80.7	78.2	53.9	—	53.7	—
Phos. apparent Digestibility (%)	9.9	44.1	41.9	27.4	45.5	48.8

[a] Viosterol. Administered daily by capsule.
[b] Two mature steers and three dry dairy cows used in this group.
[c] Four dairy cows in 5th-8th month of lactation used in this group.
[d] Differences between means due to vitamin D feeding significant at the 1% level of probability.

TABLE 2

The effect of feeding massive oral doses of vitamin D[a] to mature steers on the digestibility, excretion, and retention of total phosphorus

	Before vitamin D feeding	During vitamin D feeding	Directly after vitamin D feeding
No. of animals	2	4	2
Vitamin D (*units/d.*)	none	20,000,000	none
Phosphorus intake (*g/d.*)	30.3	27.9	26.0
Fecal phosphorus (*g/d.*)	29.2	21.9	14.8
Urine phosphorus (*g/d.*)	1.22	3.09	4.73
Phosphorus retained (*g/d.*)	-0.3	3.5	6.5
Phos. apparent digest. (%)	3.5	21.5	43.0
Fecal endogenous phos. (*g/d.*)	13.5	9.4	10.4
Phos. true digest. (%)	47.6	54.5	63.5
Total endogenous phos. loss (*g/d.*)	14.7	13.1	15.1

[a] Viosterol. Administered twice daily in capsules.

Although fecal endogenous phosphorus losses were decreased during the period of vitamin D feeding, a concomitant rise in total urine phosphorus approximately equaled this decrease; thus the net endogenous phosphorus losses for all animals were about the same for three feeding periods (Table 2).

The daily changes in calcium and phosphorus absorption and in blood levels are shown graphically in Figure 1. From the first day of vitamin D feeding there was an immediate rise in per cent of calcium and phosphorus intake absorbed, which persisted throughout the next 10 days of the test. On the other hand, total plasma calcium and inorganic phosphorus were not markedly affected until the fifth day of vitamin D feeding, at which time

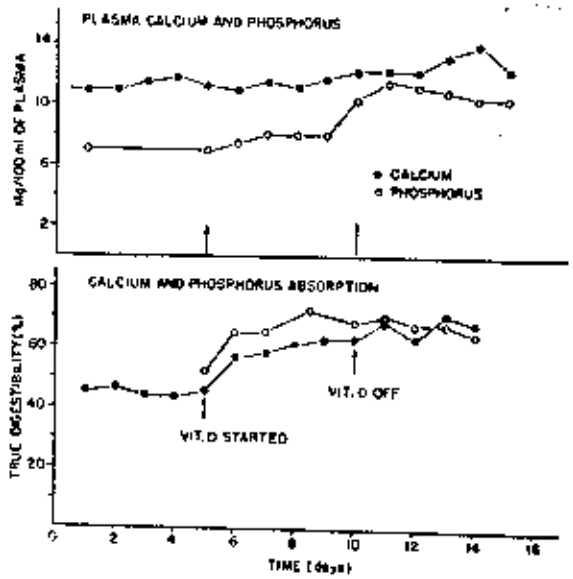

FIG. 1. Changes in true digestibility and blood levels of total calcium and phosphorus as a result of feeding mature steers 20 million units of vitamin D for 5 days.

the plasma calcium level began to increase slightly. This was accompanied by a marked increase in plasma inorganic phosphorus (Figure 1). It is noteworthy that both the per cent intake absorbed and the blood calcium and phosphorus levels remained at a maximum for the 5-day period following vitamin D feeding.

The increase in true digestibility of calcium was accompanied by an increase in radiocalcium concentration in the blood of nonlactating cattle dosed orally with Ca-45, whereas blood radiocalcium was approximately the same in the lactating cows receiving vitamin D as that of the control group (Figure 2).

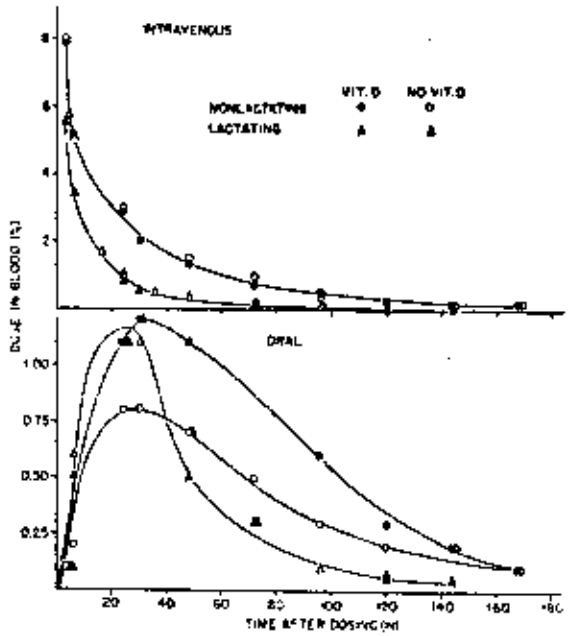

FIG. 2. The per cent of orally and intravenously administered radiocalcium in the blood as a function of time after dosing. Two lactating and two nonlactating cows included in each group with and without vitamin D.

Blood radiocalcium concentration was not affected by vitamin D feeding in the animals receiving Ca-45 intravenously. However, radiocalcium disappearance rate from the blood appeared to be more rapid in the lactating than nonlactating cows, even though their average age was approximately the same (Figure 2).

Analysis of these curves in Figure 2 by the methods of Flexner *et al.* (5) and Thomas *et al.* (21), as outlined by Hansard *et al.* (8) for determining the rate of exchange of calcium between the plasma and other metabolic pools, showed that 39% of the calcium disappeared from the blood per minute in the nonlactating cows whereas in lactating cows this rate was calculated to be 54% per minute.

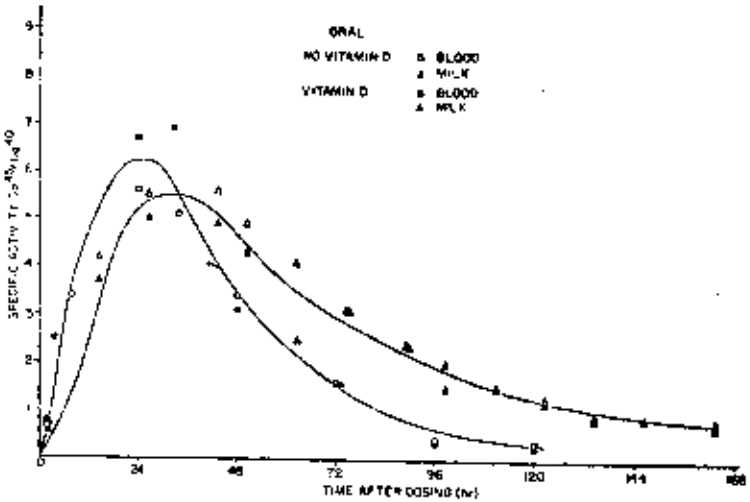

FIG. 3. The specific activity $\left(\frac{Ca^{45} \times 10^4}{Ca^{40}}\right)$ of blood and milk in relation to time after administering radiocalcium orally in a single pair of cows with and without vitamin D.

Observations on the average specific activity of the blood and milk from two cows receiving vitamin D, corrected for average time of secretion, are plotted graphically in Figure 3 with similar results from other cows. These limited data indicate that vitamin D did not affect the proportion of Ca-45 to Ca-40 secreted in the milk. A lag of approximately 12 hours was observed in the peak specific activities between the blood and milk curves, and the specific activity of the milk calcium remained higher than that of the blood throughout the remainder of the collection period. The rates of decline of both blood and milk values are exponential and they are essentially parallel.

DISCUSSION

The principal fundamental action of feeding massive oral doses of vitamin D appears to be an increase in the rate and quantity of calcium and phosphorus absorbed from the digestive tract. This effect was accompanied by a decreased excretion of these minerals into the intestinal tract and thus necessarily an increased net retention of both calcium and phosphorus. In subsequent experiments with 6-month-old calves sacrificed 7 days after dose administration tissue distribution studies showed Ca-45 concentration to be higher in the trabecular bone of the vitamin D fed calves than in that of their controls (3). That the decreased endogenous loss is a secondary effect, perhaps caused by the increased movement of calcium and phosphorus from the gastrointestinal tract into the blood, is suggested by the fact that total blood calcium increased slightly during vitamin D feeding (Figure 1). This increase has been found to be due primarily to an increase in ionic calcium (12).

Vitamin D feeding caused only a slight increase in plasma calcium levels, whereas the increase in net retention of calcium resulting from increased absorption was marked. Thus, it is apparent that the homeostatic mechanism regulating blood calcium in cattle was not seriously overwhelmed by the feeding of twenty to thirty million units of vitamin D for a 5-day period. These results suggest further that the massive doses of vitamin D employed by Hibbs and Pounden *(13)* for prevention of milk fever had no adverse physiological effects upon the bone storage mechanisms but rather augmented calcium reserves which could be drawn upon during the critical postpartum period. Evidence that prepartum enhancement of calcium reserves is a sound approach to prevention of milk fever is found in the work of Ward *et al. (25)*. They showed that cows which had milk fever were in greater negative calcium balance for the 15 days preceding parturition than normally freshening cows.

The per cent true digestibility of calcium and phosphorus increased during the 5-day period immediately following vitamin D feeding. This may have been due in part to a time lag in passage through the digestive tract and/or to the residual effects of absorbed vitamin D. Although increased absorption was maintained throughout this period, it is known from previous experience that preventive action of vitamin D for milk fever is limited to about 2 days or less after feeding is terminated. This suggests that vitamin D in preventing milk fever may have an additional effect other than increased mineral absorption from the gastrointestinal tract. The increased calcium retention observed in these experiments indicated that mineralization of the bone was taking place, possibly associated with low parathyroid hormone activity during vitamin D feeding.

Early studies with massive doses of vitamin D have demonstrated an increase in the urinary excretion of phosphates *(1)*. This is considered to be a separate action independent of increased gastrointestinal absorption. It has been shown by these studies that feeding 20 to 30 million units of vitamin D per day for a 5-day period markedly increased urinary phosphate excretion in mature cattle. The next 5-day period following the termination of the vitamin D feeding was characterized by *(a)* high plasma inorganic phosphate levels, *(b)* high plasma calcium, *(c)* further increase in urinary phosphates, and *(d)* a tendency for the endogenous fecal losses of calcium and phosphorus to return toward the pre-vitamin D level. These observations suggest that following vitamin D feeding, mobilization of the calcium was occurring. This may indicate return of parathyroid activity.

The increased rate for the disappearance of radiocalcium (Figure 2) from the blood of the lactating cows (54%) compared to nonlactating cows (39%) is interpreted as being indicative of the increased rate of calcium movement through the plasma caused by secretion into the milk. In this experiment the lactating cows were secreting in the milk an average of 10 g per day; therefore, a greater rate of calcium movement would be expected (Table 1). The lag in the peak specific activity of the milk calcium and its higher specific

activity during subsequent sampling periods is in agreement with similar results reported and discussed by Visek *et al. (23)* and Swanson *et al. (20)*. This observation is indicative of a variable calcium reserve in the udder or a variation in the exchangeability of stored calcium with plasma calcium.

SUMMARY AND CONCLUSIONS

Tracer doses of Ca-45 and P-32 were used as markers in balance trials and blood studies with mature lactating and nonlactating cattle to evaluate the changes in absorption, excretion, and retention of these minerals when massive doses of vitamin D were given.

These studies show that massive oral doses of vitamin D increased calcium and phosphorus absorption from the digestive tract. There was a decrease in endogenous losses accompanied by an increase in net retention, which extended throughout a 5-day period following vitamin D feeding. These changes in the absorption pattern for calcium and phosphorus were accompanied by a delayed increase in blood levels of calcium and phosphorus. A rise in blood phosphorus was observed to precede the rise in blood calcium. The increase in net absorption of total calcium by nonlactating cattle was accompanied by an increase in radiocalcium concentration in the blood. Radiocalcium disappeared from the blood of lactating cows at a faster rate than from nonlactating cows. Vitamin D was without effect on specific activity of milk calcium.

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Infantile Hypercalcemia and Vitamin D

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Often subtle, perhaps manifested only by a "failure to thrive," infantile hypercalcemia may well prove to be a significant cause of mental retardation and cardiovascular anomalies. The author discusses steps that may permit early detection of the disease, as well as a public health program that could prevent the development of hypercalcemia in the unborn infant.

According to present evidence, the incidence of recognized infantile hypercalcemia is something like one case 20,000 live births; its severe form, characterized by mental retardation and cardiovascular abnormalities, is thought to affect about one infant in 200,000.

Present evidence, however, may be inadequate. The condition is usually both elusive and transient, and it thus may often go undiagnosed. Quite possibly infantile hyper- may prove to be something more than the rare that current statistics suggest. It may, for example, be implicated in cases of "minimal cerebral dysfunction," now suspected of constituting a public health problem of small dimensions ("A 'Traveling' Neurologic Service," HOSPITAL PRACTICE, July).

There are indications, moreover, that infantile hypercalcemia, whatever its incidence, may be a largely preventable disease. For that reason alone it should merit attention. Its etiology, still imperfectly understood, appears linked with maternal ingestion of vitamin D during or before pregnancy, and the disorder may thus represent yet another example of the dangers of overmedication.

The disease was first described in the early 1950's by British pediatricians, who named it "idiopathic hypercalcemia of infancy," and almost simultaneously by investigators in West Germany. Two more or less distinct forms, mild and severe, were recognized early. Most investigators, myself included, have since come to believe that these are simply extremes in the spectrum of a single disease process; a minority view, however, is that the two forms may represent two different etiologies.

The mild form of the disease generally presents as a subtle "failure to thrive," becoming clearly manifest between three and eight months of age. Serum tests usually reveal hypercalcemia and sometimes azotemia as well. On occasion, however, the serum is normal by the time the tests are made so that hypercalcemia at an earlier period remains conjectural. This is probably the major source of ambiguity in estimating the incidence of the disease.

The most satisfactory treatment in mild hypercalcemia is marked restriction of calcium intake (calcium-free milk), temporary elimination of vitamin D, and avoidance of sunlight. In some instances cortisone has also been used. With this regimen the mild form of hypercalcemia can usually be cleared up without permanent sequelae, provided it is caught promptly enough. Even with adequate therapy, however, some children develop nephrocalcinosis. Whether this occurs probably depends on the amount of calcium in storage within the body. Another possible sequela is mild retardation. Here, too, the critical factors seem to be how early the condition is recognized and how soon therapy is instituted. The relationship between retardation and hypercalcemia is obscure, since these children do not usually have abnormal mineral deposits in the brain.

Severe hypercalcemia is much more easily diagnosed than the mild variety. The failure to thrive is obvious, and the serum abnormality is more severe. Moreover, most of the infants have the "elfin" facies characteristic of the disease. Mental retardation is almost invariably present, often with strabismus, as is nephrocalcinosis or some other kidney disorder. Skeletal abnormalities include dense mineralization at the base of the skull and in the metaphyses of the long bones. Abnormalities in dentition are also sometimes present.

This pattern of abnormalities was established quite early in the process of defining the disease. More recently it has become apparent that a cardiovascular component is nearly always present also — usually supravalvular aortic stenosis, evidenced by a systolic murmur. This usually arises from a thickening of the subendothelial tissue of the aorta. The

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Characteristic elfin facies – high, prominent forehead, epicanthal folds, underdeveloped bridge of nose, overhanging upper lip – apparent in nine-month-old child with infantile hypercalcemia. Associated supravalvular aortic stenosis can be seen on x-ray as narrowing above aortic valve. Pulmonary valvular and pulmonary valvular stenosis were also present.

few cases without aortic stenosis usually show other cardiovascular abnormalities including coarctation of the aorta, hypoplasia of the thoracic and abdominal aorta, stenoses of the celiac and superior mesenteric arteries, or peripheral pulmonary stenosis.

Therapy for severe hypercalcemia is much the same as for the mild form of the disease, with the possible addition of surgical correction of certain cardiovascular anomalies. But even with the best therapy, the prognosis is poor. The mental defect, usually severe, is permanent. Renal dysfunction may lead in turn to frequent infection; death, from the combination of ailments, sometimes supervenes.

As with the mild form, the interrelationships of the various features are by no means clear. The nephrocalcinosis may be a direct consequence of the hypercalcemia, and some investigators believe that the same is true of the aortic stenosis. However, the spectrum of pathological features may equally well prove to have some other, still unidentified, cause.

It should be noted that there seems to be no sharp line between the two forms of the disease. Until recently, for example, it had been accepted that patients with a normal facies (hence, presumably, only mildly affected) constituted a distinct clinical group free of mental retardation. However, at Johns Hopkins a retrospective study of patients with supravalvular aortic stenosis has undermined this assumption. In some of these patients, hypercalcemia was no longer present, but they showed other of its sequelae, such as dental abnormalities and intellectual impairment in discrete areas. Yet a significant number had normal facies. This same study, incidentally, suggested that certain cardiovascular abnormalities or mild mental abnormalities, often not diagnosed until well after infancy, may represent delayed effects of an undetected episode of the disease (see table on page 92).

Even when infantile hypercalcemia was first recognized as a pathologic entity, it was apparent that there are striking similarities between its manifestations and those of vitamin D intoxication. In animal experiments it has been shown that high doses of the vitamin given during gestation produce cardiovascular and dental abnormalities, and these abnormalities strongly resemble those of severe hypercalcemia. On the other hand, there are important differences: Hypervitaminosis D can at times produce bone rarefaction, in notable contrast to the osteosclerosis seen in hypercalcemia.

The suspicion that vitamin D may somehow be implicated was strengthened by the appearance of several hundred cases of hypercalcemia in Great Britain in the early 1950's – shortly after an increase in vitamin D fortification of the powdered milk formula supplied by the National Health Service. Studies showed that some infants had been receiving up to 4,000 units of vitamin D over long periods – 10 times the presently recommended prophylactic dose, though still below the so-called toxic level.

The formula was changed, and vitamin D fortification of most other foods was stopped; at this point it was estimated that the average infant's intake of the vitamin had been halved. A study in 1959 showed that the incidence of infantile hypercalcemia had

apparently fallen off, but only slightly. My own feeling is that the actual drop may have been minimized by a higher of suspicion, and therefore bet- se-finding, in the 1959 study. A second study, in 1960-61, showed a further, this time quite marked, drop to less than half the "original" (1953-55) level -- but no estimates were made at that time of whether there had been a further decrease in vitamin D intake among infants. Additional confusion stemmed from the fact that whatever the actual level of vitamin consumption, the overwhelming majority of infants had shown no ill effects at any time.

The obvious conclusion was that if vitamin D was implicated it could only operate via some special metabolic condition in the affected infants. Perhaps they were themselves synthesizing too much of the vitamin, or perhaps their metabolic mechanisms for degrading or excreting it were impaired. But no evidence was found to support these theories.

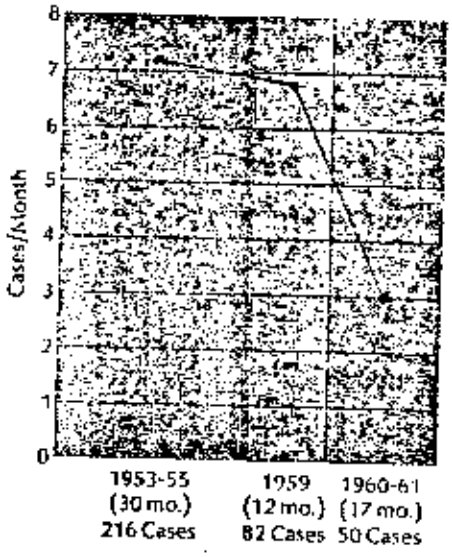
The next possibility was hypersensitivity, that is, not an increased quantity of the vitamin but an exaggerated se to it. Consistent with this were experiments showing that rats could be sensitized to the vitamin. If they were given large quantities of vitamin D and calcium, allowed to recover, and then challenged with vitamin D they showed more marked hypercalcemia than was evident in the nonsensitized controls.

Meantime, it was also determined that neither infantile hypercalcemia nor supravalvular aortic stenosis was genetically caused.

If, then, the disease involved a disturbance in calcium metabolism that was innate yet not inherited, an obvious possibility was that events in utero were responsible. This hypothesis was strengthened by the finding that in more severe cases the facies, as well as murmurs and other evidence of cardiovascular involvement, could be detected as early as the first or second week of life.

There was also circumstantial evidence pointing to a prenatal etiology. A number of cases of infantile hypercalcemia had been reported from West Germany, where the "stoss" approach to rickets prophylaxis had some popularity and physicians often gave huge doses (single injections of up to 600,000 I.U.) of vitamin D to pregnant women as well as to infants. Moreover, the epidemiological evidence from Great Britain could apply almost as well to the mothers as to their offspring. Pregnant women also consumed milk in which the level of vitamin D supplementation was then considerably higher than in either the U.S. or Canada; and British physicians prescribed supplemental vitamin D for women as well as for children — to say nothing of the probability of additional, self-prescribed vitamins.

But if excessive vitamin D intake during pregnancy did indeed produce



Incidence of infantile hypercalcemia was surveyed at three different periods by British Paediatric Association, which felt that the decline was genuine but that data were insufficient to prove relationship to reduced vitamin D intake.

some cases of infantile hypercalcemia in Great Britain and Germany — and the evidence is far from watertight — it does not appear to be a necessary condition for the disease. At least our own retrospective studies at Johns Hopkins, though they turned up a probable association between supravalvular aortic stenosis and (earlier) hypercalcemia in infants, adduced only the most tenuous evidence of excess intake of the vitamin by the

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In Johns Hopkins study of hypercalcemic children with supravalvular aortic stenosis (SAS), aortic hypoplasia was found to extend to the abdominal region in at least nine cases. In x-ray at left, mesenteric artery stenosis can be noted (arrow). Pulmonary artery stenosis (in x-ray at right) was also frequently associated with aortic hypoplasia and with SAS.

mothers. Thus we were once again forced to go back to hypersensitivity, though now in the mother rather than, or in addition to, the child.

Vitamin D sensitivity is intrinsically difficult to investigate. The main criterion at present is a rise in serum calcium in response to a normally ineffective dose of the vitamin. But we also know that in any individual the serum calcium can fluctuate between fairly wide limits, as can the serum response to a given vitamin dose.

Nonetheless, the evidence we have certainly suggests, if it does not prove, that some mothers of hypercalcemic infants are at least transiently hypersensitive to vitamin D. Perhaps significantly, the sensitivity is also associated with symptoms in the mother similar to those of hyperparathyroidism, so it is conceivable that undetected or subclinical cases of that condition may play a role in developing sensitivity. Thyroid disturbances may also play a part, either alternative or supplementary, since we now know that that gland elaborates calcitonin, a hormone that lowers plasma calcium levels.

We do know quite definitely that the fetus can be affected by maternal intake of vitamin D. A study of 58 normal pregnancies showed that administration of the vitamin in the usually recommended daily supplemental dose of 250 I. U., plus supplemental calcium at the usual level of 1.44 gm/day, brought about increased bone density in the fetus. And experiments with rats and other animals clearly demonstrate that maternal vitamin D has a dose-related effect on the fetus, producing bone abnormalities at low toxic levels, death at higher ones. But it is still uncertain how these fetal changes come about, whether indirectly, through changes in the mother's calcium metabolism (calcium is known to pass the placenta), or directly, through passage of the vitamin into the fetal circulation (there is as yet no clear evidence on whether this can occur, although it seems probable).

At any rate, there seems enough evidence of various sorts to warrant several conclusions about the nature of infantile hypercalcemia:

1. The more severe form of the disease almost certainly has its genesis in utero, probably through temporary maternal overreactivity to vitamin D.

2. The less severe, borderline forms probably but not certainly stem from intrauterine events. They may result from postnatal sensitization but more likely stem from either fetal sensitization through the placenta or from maternal sensitization of a milder type or at a later stage of pregnancy than in the severe form. This sensitization is not allergic but rather may result from excessive skeletal mineralization following long exposure to large doses of vitamin D and calcium.

It should be stressed that although these conclusions are tentative there is at present no plausible alternative to the maternal sensitivity hypothesis,

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Complications of Infantile Hypercalcemia in Children with Supravalvular Aortic Stenosis

	Case	Sex	Age	Age Murmur Detected	Infant Milestones (months)			I.Q. Range	Ocular Abnormalities	Dentition Affected	Skeletal Abnormalities	Abnormal Kidney Function	Multiple Cardiovascular Abnormalities
					Sat	Walked	Talked						
Typical Elfin Facies	1	F	11	3 months	Late	17	30	31-42		•			•
	2	M	2	2 months	20	No	No	50-58	•			•	•
	3	F	11	2 months	6	24	24+	40-50		•			•
	4	M	8	5 weeks	6	12	12	63-109		•			•
	5	F	6	6 weeks	7	30	42	50-60		•	•		•
Unhyphical Facies	6	M	8	1 month	8	15	24	53-77		•		•	•
	7	M	6	9 months	17	36	36	30-32	•	•	• • •	•	•
	8	F	10	6 years	8	22	Late	40-57		•			•
	9	M	3	9 months	9	18	24+	42-57		•	• •		•
Normal Facies	10	M	16	Birth	9	24+	36	42-92		•		•	•
	11	M	15	4 years	9	13	12	52-102					•
	12	F	13	7 years	5	12	12+	58-94	–			•	•
	13	M	13	4 years	5	14	9	70-119		•			•
	14	M	11	1 year	3	12	11	81-118					•
	15	M	12	Birth	4	16	24						

11 patients with supravalvular aortic stenosis seen over a five-year period were studied retrospectively at Johns Hopkins. Typical hypercalcemic sequelae were found in mild, intermediate, and severe cases. The intermediate group had facies unlike the severe elfin prototype but markedly different from those of siblings and parents. All children were normocalcemic when studied; in three, some confirmation of a hypercalcemic state during infancy was available. The differences among the three groups were mainly of degree, but the nature and site of complications were essentially similar for all. The findings support an association between hypercalcemia and supravalvular aortic stenosis. This need not be cause and effect; it could equally well reflect separate responses to disturbed maternal metabolism, and maternal hypervitaminosis D could explain anomalies.

at least as regards the severe disease.

If we accept this hypothesis, what level of vitamin D supplementation constitutes a danger level? Obviously, will depend on the mother's level reactivity, and there is as yet no reliable way of measuring this. There is an attested case of a severely hypercalcemic infant born to a mother whose vitamin D intake was no more than 300 or 400 I.U. per day. In another instance, a mother who had previously produced a severely hypercalcemic child bore a normal infant despite a daily intake of 600 to 700 I.U. during the last seven months of the second pregnancy. Given data of this sort, there seems no possibility of completely preventing infantile hypercalcemia short of putting all pregnant women on a vitamin D–free diet. And this would undoubtedly produce far more sickness, in the form of rickets, than it would prevent.

On the other hand, the British epidemiological evidence certainly suggests that the higher the vitamin D intake (among mothers and/or infants), the higher the incidence of hypercalcemia. And if one assumes that what is true of nearly all drugs is also true vitamin D — that sensitivity or hypersensitivity varies from one individual to another over a more or less continuous spectrum, so that the higher the dosage the larger the proportion of the population suffering ill effects — this suggestion is clearly supported.

Thus the simplest preventive step for limiting, if not eliminating, infantile hypercalcemia would be to ensure as far as possible that no pregnant woman receive more than the recommended amounts of either vitamin D or calcium in her daily diet. With so many commercially prepared foods now supplemented with the vitamin, this is not an easy task. The problem could be greatly simplified if a number of public health proposals, several of them suggested a few years ago by the Committee on Nutrition of the American Academy of Pediatrics, were put into effect:

1. Allow the marketing of vitamin D–free milk, both canned and fresh, while continuing vitamin supplementation of most milk supplies as an antirachitic measure.

Discontinue vitamin D supplementation of all other foodstuffs. Failing this, the amount of the vitamin should be indicated more prominently and explicitly on labels.

3. All proprietary preparations containing vitamin D should contain no more than 400 I.U. per recommended daily dose. Product information in such works as *Physicians' Desk Reference* should be revised to reflect this recommended daily maximum.

4. The dietary intake of all patients, but especially of infants and pregnant women, should be carefully evaluated before supplemental vitamin D is prescribed for them.

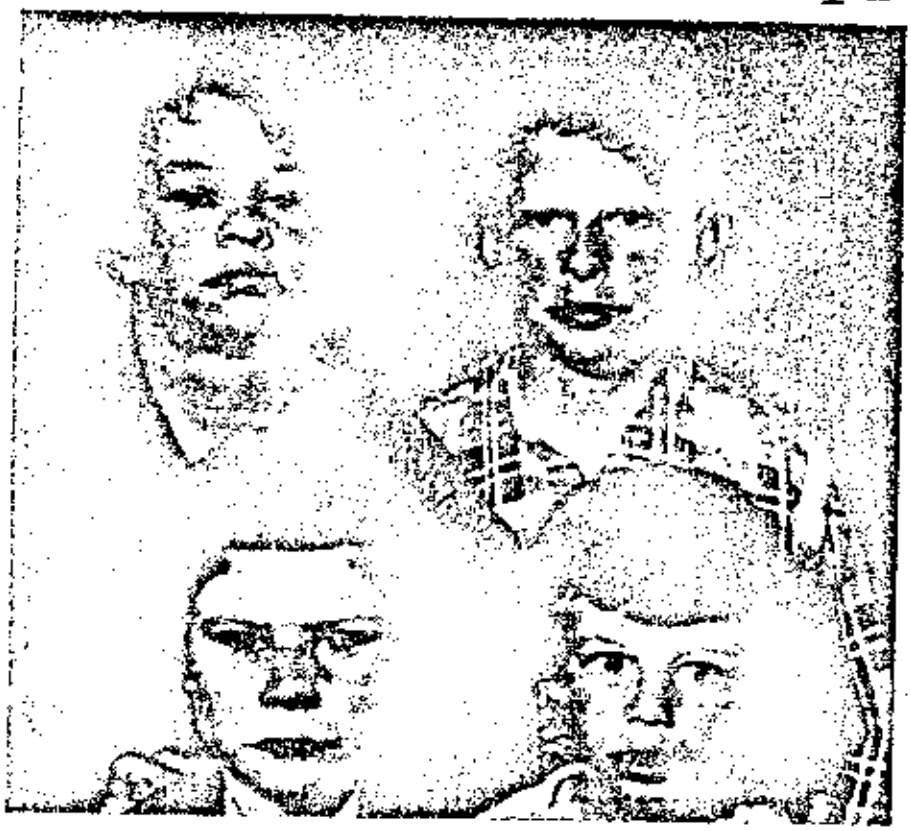
5. Vitamin D intake during pregnancy should be continuously and carefully supervised.

Aside from these preventive measures, early detection is probably the most important element in lessening the damage caused by infantile hypercalcemia. But here again, unfortunately, there are no simple and foolproof diagnostic rules.

Obviously the mother who has already borne a hypercalcemic infant, whether definitely diagnosed or suspected in retrospect, should be closely watched. Calcium levels and calcium and vitamin D intake of mother and child should be checked.

In regard to the infant, severely affected cases present few problems of detection. As already noted, they almost always show the typical elfin facies, mental retardation, and cardiovascular defects. But children without the obvious stigmata of severe involvement are hard to spot. Certain symptoms, however, should raise the physician's index of suspicion: failure to thrive, irritability, hypotonia, poor appetite, vomiting. In such cases, especially if there is no ready explanation for the symptoms, a serum calcium determination may be desirable.

It is obvious that the above recommendations are not much more satisfactory to the conscientious physician than are the etiological explanations to the conscientious physiologist. Improved prevention and detection will probably have to wait for better understanding of its relationship to vitamin D. And this may well require years of investigation, as did the demonstration of a relationship between vitamin D deficiency and rickets. □



Early link between unusual facies of infantile hypercalcemia and supravalvular aortic stenosis came from Dr. J. C. P. Williams and colleagues in Auckland, New Zealand. In 1961 they reported that facial resemblance of three of these children to the fourth, in whom SAS had been discovered at operation, led to repair of the anomaly in all.

Metabolism and Subcellular Location of 25-Hydroxycholecalciferol in Intestinal Mucosa*

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ABSTRACT: The metabolism and subcellular distribution of 25-hydroxycholecalciferol, a biologically active metabolite of cholecalciferol (vitamin D_3) was investigated in the intestinal mucosa of vitamin D deficient rats. The mucosal nuclear fraction accumulates approximately 50 and 25% of the total cellular ^{3}H after administration of 0.025 and 0.25 μg of [26,27-^{3}H]-25-hydroxycholecalciferol, respectively. Both 1,2-^{3}H- and 26,27-^{3}H-labeled 25-hydroxycholecalciferol are converted *in vivo* by the intestinal mucosa into polar metabolites, referred to as peak V and peak VI. Within 30 min after a 0.025-μg dose of [26,27-^{3}H]-25-hydroxycholecalciferol, 72% of the nuclear radioactivity is peak VI, the more polar of the two metabolites. Two hours after the dose the relative amount of peak VI steadily decreases and after 8 hr peak V has increased to 58% of the total tritium-containing metabolites in the nuclear fraction. The time course of appearance of these metabolites indicates peak VI is the probable precursor of peak V. The paucity of these polar metabolites in the cytoplasmic fractions suggest they are of nuclear origin and hence may be of significance in understanding at the nuclear level the mode of action of vitamin D on the calcium transport mechanism in the small intestine.

The currently accepted model for vitamin D action in the small intestine proposes that specific genetic information is unmasked in response to the vitamin, leading ultimately to the synthesis of specific functional protein(s) involved in a calcium transport system (DeLuca, 1967). A response in calcium absorption is known to lag several hours after administration of vitamin D and recent evidence partially explains the reason for this lag period. First it has been conclusively established that vitamin D_3 (cholecalciferol) must be converted enzymatically by the liver into an "active form" (Ponchon and DeLuca, 1969a; Horsting and DeLuca, 1969) which has been identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a).[1] The next important event in vitamin D action occurs at or in the nucleus of the intestinal mucosal cell where the vitamin and its metabolites accumulate (Stohs and DeLuca, 1967). Then by an unknown mechanism, a metabolite of vitamin D stimulates the synthesis of nuclear RNA, as demonstrated by the increased incorporation of [^{3}H]orotic acid into nuclear RNA (Stohs *et al.*, 1967) as well as the increased DNA template activity of these cells (Halloran and DeLuca, 1969). Presumably this nuclear RNA includes specific mRNA which codes for the protein components of the calcium transport system. Both of these nuclear responses occur well before any detectable response in the calcium transport mechanism (Blunt *et al.*, 1968b; Wasserman and Taylor, 1968), but undoubtedly RNA synthesis adds substantially to the lag in vitamin D action.

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[1] This compound may also be referred to as 25-OH vitamin D_3, 25-HCC, or peak IV.

Recent studies on the metabolism of [^{3}H]vitamin D_3 have demonstrated that other metabolites, more polar chromatographically than either cholecalciferol or 25-hydroxycholecalciferol, are present in certain tissues and blood following administration of physiological doses of the radioactive vitamin (Ponchon and DeLuca, 1969a,b; Haussler *et al.*, 1968; Lawson *et al.*, 1969). However it has not been established if these metabolites are produced early enough to be of importance in understanding the lag in vitamin D response. Hence in order to evaluate the biological significance of these polar metabolites a new approach had to be taken. The production of the "active form" of vitamin D is undoubtedly closely regulated *in vivo* to facilitate calcium homeostasis, therefore it is obvious that meaningful studies of vitamin D metabolism in target tissues must be carried out at the level of the active form rather than the dietary form of the vitamin. The synthesis of high specific activity [^{3}H]-25-hydroxycholecalciferol has provided the tool necessary to follow this new approach.

The studies described in this paper were undertaken to establish that the polar metabolites of vitamin D_3 actually originate *in vivo* from the active form, to identify the subcellular location and origin of these metabolites as well as to analyze the sequence of their appearance in the intestinal mucosa following intravenous administration of physiological doses of [^{3}H]-25-hydroxycholecalciferol.

Experimental Procedure

Animals. Male rats (Holtzman Co., Madison, Wis.) obtained at 20 days of age were maintained individually in hanging wire cages and given food and distilled water *ad libitum*. The purified diet (Guroff *et al.*, 1963) fed throughout these experiments contained 0.47% calcium and 0.3% phosphorus. Vitamins A, E, and K in Wesson oil were placed on top of fresh diet three times a week. After 4 weeks on this diet the rats were vitamin D deficient by the criteria of lowered serum calcium levels and retarded growth.

Isotopes. [26,27-^{3}H]-25-Hydroxycholecalciferol with a specific activity of 1.3 Ci/mmole (T. Suda and H. F. DeLuca, in preparation) was synthesized in this laboratory. [1,2-^{3}H]-25-Hydroxycholecalciferol with a specific activity of 650 mCi/mmole was prepared from the plasma of chicks previously administered [1,2-^{3}H]cholecalciferol (Neville and DeLuca, 1966). Radiochemical purity of these preparations was routinely checked by silicic acid chromatography (Ponchon and DeLuca, 1969b).

The [5-^{3}H]orotic acid (New England Nuclear Corp., Boston, Mass.) used in the RNA pulse-labeling experiments had a specific activity of either 4.1 or 940 mCi per mmole.

Isotope Administration and Mucosa Preparation. All animals were fasted for 12 to 15 hr prior to sacrifice. After placing the animals under light ether anesthesia the specific isotopically labeled compound was administered intrajugularly in 0.02 ml of 95% ethanol, at one of the time intervals before sacrifice. The rats were killed by a sharp blow on the head, the peritoneal cavity was quickly opened, and 48 cm of the duodenal end of the small intestine was removed and placed in ice-cold 0.25 M sucrose-TKM buffer (pH 7.4).[1] The entire small intestine was removed for extraction in the whole tissue experiments. All intestinal segments were freed of fat and mesentery, then slit open, washed in fresh buffer, blotted on absorbant paper, and placed mucosal side up on an ice cold stainless steel plate. The mucosa was then scraped from the serosa and submucosal muscle layers with a glass slide (Crane and Mandelstam, 1960).

Tissue Fractionation. Mucosal scrapings from six rats were weighed and a 10% homogenate was prepared in 0.25 M sucrose-TKM buffer utilizing fifteen strokes of a motor-driven glass-Teflon Potter–Elvehjem homogenizer (A. H. Thomas Co., Philadelphia, Pa.) operated at 800 rpm. The subcellular fractionation closely followed the procedure of Schneider and Hogeboom (1950). The crude nuclear fraction was obtained by centrifugation at 600*g* for 10 min in a refrigerated Lourdes centrifuge equipped with a 9-RA rotor. After washing the nuclear pellet twice, the postnuclear supernatant was centrifuged at 7500*g* for 10 min to obtain the mitochondrial fraction. The mitochondrial pellet was washed once. The 7500*g* supernatant was then centrifuged at 105,000*g* for 60 min in a Spinco Model L-2 ultracentrifuge employing a No. 50 titanium rotor to obtain the microsomal (pellet) and cytoplasmic (supernatant) fractions. All fractionation and centrifugation steps were conducted at 0–4°. Each subcellular fraction was subsequently resuspended in an appropriate amount of 0.25 M sucrose-TKM buffer. Subcellular suspensions not immediately extracted were held at −20°. These operations were carried out individually in the experiments involving the 0.25-μg doses and on pooled mucosa in the experiments involving the 0.025-μg doses. In the 0.25-μg experiments after appropriate aliquots of the six nuclear suspensions and supernatants were removed the remainder of each respective fraction was combined and subsequently extracted as described below.

Extraction and Chromatography of Vitamin D Metabolites. The radioactive nuclear suspensions and supernatants were extracted by the chloroform–methanol procedure described by Lund and DeLuca (1966). Two parts of methanol and one part of chloroform were added to eight-tenths part of either a nuclear suspension or 105,000*g* supernatant. This one-phase system was shaken vigorously for 2 min and then allowed to stand for 30 min at room temperature. A two-phase system was produced by addition of one part chloroform and then allowing the mixture to stand overnight in a cold room. The chloroform layer was then removed and evaporated to dryness on a flash evaporator. Residual water in this phase was removed as an azeotrope by repeated additions of 100% ethanol at the end of the drying step. The final lipid extract was dissolved in Skellysolve B (predominately *n*-hexane; bp 67–68°) for chromatography.

Silicic acid chromatography of the lipid extracts was carried out according to the procedure described in detail by Ponchon and DeLuca (1969b). Columns (1 × 55 cm) of activated silicic acid were prepared in Skellysolve B. The ^{3}H-containing samples were eluted from the columns by sequential additions of 250 ml of 75% (v/v) diethyl ether in Skellysolve B, 400 ml of 100% diethyl ether, 300 ml of 5% (v/v) methanol in diethyl ether, and 200 ml of 50% (v/v) methanol in diethyl ether to the holding chamber of an exponential gradient generating apparatus. The constant volume mixing chamber initially contained 230 ml of Skellysolve B. The columns were stripped of metabolites with 200

[1] Abbreviation used is: TKM, 0.05 M Tris-HCl–5 mM $MgCl_2$–0.025 M KCl.

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TABLE I: Effect of 0.25 μg of 25-Hydroxycholecalciferol on the Incorporation of [^{3}H]Orotic Acid into Nuclear RNA of Rat Intestinal Mucosa.[a]

Experiment	Time (hr)	Specific Activity of [^{3}H]RNA (dpm/OD) 25-HCC	Control
1	0.5	149 ± 32[b]	150 ± 45[b]
2	1	157 ± 30	119 ± 28
3	2	242 ± 17	140 ± 4
4	4	244 ± 11	204 ± 16

[a] Vitamin D deficient rats were administered 0.25 μg of 25-hydroxycholecalciferol intravenously and sacrificed 0.5, 1, 2, or 4 hr later. Each rat received 50 μCi of [5-^{3}H]orotic acid intravenously 30 min prior to sacrifice. [b] Standard deviation. *Each experiment was replicated at least four times.*

TABLE II: Percentage Distribution of ^{3}H-Containing Metabolites in Serum after a 0.25-μg Dose of [26,27-^{3}H]-25-Hydroxycholecalciferol.[a]

Metabolite (Peak)	Minutes after Administration of Dose 15	30	60	120	180
IV	69.8	74.7	91.5	88.6	88.9
V	7.9	3.1	3.8	7.1	7.7
VI	10.3	1.8	3.0	4.3	1.9
VII	12.0	20.4	1.7	0	1.5

[a] Six vitamin D deficient rats were each injected, intrajugularly, with 0.25 μg of [26,27-^{3}H] 25-OH vitamin D_3 and each group was sacrificed at the indicated times after dosage. Data are expressed as percentage of total ^{3}H eluted from the silicic acid column.

ml of methanol applied directly to the column at the end of the 50% methanol gradient. The gradients produced by this system are referred to as A, B, C, D, and E, respectively, in the figures below. Fractions (10 ml) were collected from all columns.

RNA Pulse Labeling. Vitamin D deficient rats were fasted 12-15 hr and then 0.25 μg of 25-HCC was administered intrajugularly at the desired time.[a] Before sacrifice (0.5 hr) each rat received 50 μCi of [^{3}H]orotic acid intrajugularly. Both injections were given during light ether anesthesia. Crude nuclei from the pooled mucosa of 2-3 rats in each treatment group were obtained by centrifugation as described above, but utilizing 0.25 M sucrose 0.05 M Tris-HCl buffer (pH 7.4). The RNA was isolated by the hot phenol method as described by Stobs *et al.* (1967). The absorbancy and spectra of isolated RNA samples dissolved in deionized water were obtained with a Beckman Model DB-G recording spectrophotometer.

Radioactivity Determination. Aliquots of homogenates, subcellular fractions, and nuclear RNA were prepared for tritium determination by drying each sample in a counting vial under an infrared lamp. Deionized water (1 drop) and 0.5-2.0 ml of NCS solubilizer (Amersham/Searle Corp., Des Plaines, Ill.) was added to each vial. After incubation at 37° for 24-hr solubilization was complete and 15 ml of toluene counting solution (Herberg, 1960) was added.

Tritium content was measured with a Packard Tri-Carb liquid scintillation spectrometer, Model No. 3000 (Packard Instrument Corp., LaGrange, Ill.) equipped with an external standardization system. Counting efficiencies were approximately 35% for chromatographic samples and 20-25% for samples containing NCS reagent.

Results

The results of a time course study (Table I) reveal that a stimulation of nuclear RNA synthesis occurs in response to a 0.25-μg intrajugular dose of 25-hydroxycholecalciferol. Since this response is maximal by 2 hr after administration it is evident that the dose-response time course of 25-HCC, at the nuclear level, is markedly shorter than with a comparable dose of vitamin D_3.

The distribution of ^{3}H-containing metabolites in the lipid extracts of blood serum from rats intravenously dosed with 0.25 μg of [26,27-^{3}H]-25-hydroxycholecalciferol is shown in Table II. At each time period reported the major component appears to be unchanged 25-HCC (peak IV). The small amounts of peak V (a metabolite(s) eluted with 5% methanol in ether) and peak VI (a metabolite(s) eluted with 50% methanol in ether) in the serum extracts are not artifactual however, since the injected 25-HCC was radiochemically pure. At the earliest time periods, 15 and 30 min, substantial amounts of a peak VII metabolite are eluted from the columns by the 100% methanol gradient. The significance of the rapid appearance and disappearance of peak VII is not known.

TABLE III: Percentage Distribution of ^{3}H-Containing Metabolites in Intestinal Mucosa after an Intrajugular Dose of [^{3}H]-25-Hydroxycholecalciferol.[a]

Label Position	Metabolite (Peak)	Minutes after Administration of Dose 3	15	30	60	180
26,27-^{3}H	IV	82.2	56.7	44.4	57.6	52.7
	V	9.7	9.6	8.2	10.2	25.7
	VI	8.1	33.7	47.4	33.2	21.6
1,2-^{3}H	IV			57.8		
	V			17.6		
	VI			24.6		

[a] Six vitamin D deficient rats were each injected, intrajugularly, with either 0.25 μg of [26,27-^{3}H]-25-OH vitamin D_3 or 0.1 μg of [1,2-^{3}H]-25-OH vitamin D_3 and each group was sacrificed at the indicated times. Data are expressed as percentage of total ^{3}H eluted from the column.

[a] The nonradioactive 25-HCC was kindly supplied in part by the Upjohn Co., Kalamazoo, Mich., and the Philips-Duphar Co., Weesp, The Netherlands.

TABLE IV: Subcellular Distribution of ^{3}H in Intestinal Mucosa after a 0.25-μg Dose of [^{3}H]-25-Hydroxycholecalciferol.[a]

Fraction	Minutes after Administration of Dose			
	15	30	60	120
Crude nuclei	26.9 ± 2.2[b]	31.5 ± 4.3	32.2 ± 5.2	21.7 ± 3.5
Mitochondria	6.3 ± 0.8	6.3 ± 1.2	5.5 ± 1.8	5.6 ± 1.5
Microsomes	10.9 ± 1.3	9.7 ± 2.3	9.4 ± 1.8	9.9 ± 2.2
Supernatant	52.5 ± 3.7	51.0 ± 7.8	52.3 ± 5.5	58.9 ± 2.7
Average total mucosal dpm recovered per animal	12,853	19,074	19,474	19,883

[a] Six vitamin D deficient rats were each injected, intrajugularly, with 0.25 μg of [26,27-^{3}H]-25-OH vitamin D_3 and each group was sacrificed at the indicated times. Data are expressed as percentage of total ^{3}H recovered. [b] Standard deviation.

The results of silicic acid chromatography of lipid extracts from intestinal mucosal homogenates prepared from vitamin D deficient rats given 0.25 μg of [26,27-^{3}H]-25-HCC are summarized in Table III. As early as 15 min after the injection, a marked conversion of peak IV (25-HCC) into peak VI has occurred and by 30 min 47% of the chromatographed chloroform-soluble radioactivity is present as peak VI. The relative abundance of peak V rises steadily at 1 hr after the dose concomitant with a decrease in the relative amount of peak VI.

It is apparent that 25-HCC is rapidly converted *in vivo* into polar metabolites. Therefore, to verify these results a 30-min *in vivo* study was carried out with 0.05 μg of [1,2-^{3}H]-25-HCC. While the design of this experiment was not strictly comparable with the experiments using 26,27-^{3}H-labeled 25-HCC, the data shown in the lower section of Table III establish that peak V and peak VI metabolites are produced *in vivo* from [1,2-^{3}H]-25-HCC as well.

The distribution of ^{3}H within the components of intestinal mucosal cells from vitamin D deficient rats that had received 0.25 μg of [26,27-^{3}H]-25-HCC is shown in Table IV. The crude nuclear fraction contains 22–32% of the total cellular ^{3}H while the cytoplasmic fraction (105,000*g* supernatant) represents at least 51% of the total ^{3}H. There is a slight decrease in the ^{3}H content of the nuclei at 120 min and a corresponding ^{3}H increase in the cytoplasm. The reason for this is not clear at present. Nevertheless, the ^{3}H content of the nuclei and cytoplasm remain relatively constant throughout the 2-hr period after injection.

The chromatographic profiles of lipid extracts of both the nuclear and cytoplasmic fractions of mucosal cells from vitamin D deficient rats sacrificed 60 min after receiving 0.25 μg of [26,27-^{3}H]-25-HCC are presented in Figure 1. The relative proportions of peaks IV, V, and VI are 27, 15, and 58% of the total ^{3}H, respectively, in the nuclei and 87, 5, and 8%, respectively, in the cytoplasm.

A diagrammatic representation of the metabolite distribution in the intestinal mucosa from chromatographic profiles of nuclear and cytoplasmic lipid extracts at 15, 30, 60, and 120 min after administration of 0.25 μg of [26,27-^{3}H]-25-HCC (peak IV) is shown in Figure 2. Substantial metabolism of 25-HCC is clearly evident in the nuclear fractions as early as 15 min after the dose. Peak VI reaches a maximum level 60 min after administration. The increase in peak VI closely

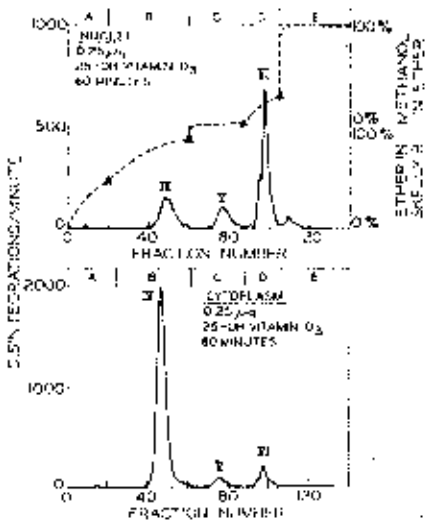

FIGURE 1: Silicic acid chromatography of lipid extracts of intestinal mucosal nuclei and cytoplasm 60 min after 0.25 μg of [26,27-^{3}H]-25-OH vitamin D_3. ( ) Radioactivity; ( ) gradient.

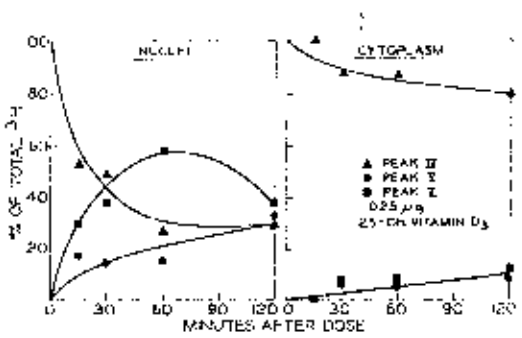

FIGURE 2: Conversion of [26,27-^{3}H]-25-hydroxycholecalciferol into peak V and peak VI in the nuclei and cytoplasm of intestinal mucosa after a 0.25-μg dose.

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TABLE V: Subcellular Distribution of ^{3}H in Intestinal Mucosa after a 0.025-μg Dose of [^{3}H]-25-Hydroxycholecalciferol.[a]

Fraction	Minutes after Administration of Dose					
	7.5	15	30	60	120	480
Crude nuclei	56.7	41.8	48.4	41.8	52.6	48.3
Mitochondria	5.4	6.3	6.3	3.8	2.5	2.3
Microsomes	15.5	13.3	14.0	14.6	7.6	6.9
Supernatant	22.4	38.6	31.3	39.8	37.3	42.5
Total dpm recovered	8,504	16,545	17,806	31,960	20,246	11,135

[a] Six vitamin D deficient rats were each injected, intrajugularly, with 0.025 μg of [26,27-^{3}H]-25-OH vitamin D_3 and each group was sacrificed at the indicated times. Data are expressed as percentage of total ^{3}H recovered.

coincides with the disappearance of the precursor metabolite, 25-HCC. The relative amount of peak V in the nuclear fraction rises steadily during the time course and since the 25-HCC content remains relatively constant after 60 min it appears plausible that peak V could be derived metabolically from peak VI rather than from 25-HCC. The major metabolite in the cytoplasmic fraction is unchanged 25-HCC. The paucity of metabolites more polar than 25-HCC in the cytoplasm strongly suggest that peaks V and VI are of nuclear origin and not the product of extraintestinal metabolism.

As we have stated above, the circulating level of 25-HCC under normal steady-state conditions is low and the concentration of this metabolite present at intestinal sites at any given time is probably not equivalent to the concentration available to these sites after a 0.25-μg intrajugular dose of this form of vitamin D. Hence to confirm the results obtained with 0.25-μg dose levels, the above subcellular experiments were repeated and expanded employing 0.025-μg intrajugular injections of [^{3}H]-25-HCC.

In contrast to the 0.25-μg experiments the nuclear and cytoplasmic fractions of mucosal cells accumulate 42–57 and 22–42% of the total cellular ^{3}H, respectively, at 7.5, 15, and 30 min, and 1, 2, and 8 hr, following 0.025 μg of [26,27-^{3}H]-25-HCC (Table V).

Profiles of the silicic acid chromatography of lipid extracts from the mucosal cell nuclei of vitamin D deficient rats 0.25, 1, and 8 hr after administration of 0.025 μg of [^{3}H]-25-HCC are shown in Figure 3. Within 15 min following the injection, 50% of the nuclear radioactivity is in the form of peak VI and 7% as peak V. One hour following the dose, 65% of the total radioactivity is in peak VI, however, by 8 hr after the dose peak V represents the major ^{3}H-containing metabolite. In addition, substantial amounts of a metabolite or more likely a conjugate of a metabolite, of lower polarity than 25-HCC, has formed by 8 hr after dose administration.

The distribution of ^{3}H-containing metabolites in the nuclear fraction from the mucosa 7.5, 15, and 30 min, and 1-, 2-, 5-, and 8-hr postadministration of 0.025 μg of 25-HCC is presented diagrammatically in Figure 4. The disappearance of 25-HCC (peak IV) is rapid as peak V and VI accumulate. The marked hyperbolic shape of the peak VI curve helps support the earlier suggestion that peak VI is the precursor of peak V. The cytoplasm contains substantial amounts of the polar metabolites by 8-hr postinjection but not at 2 hr (Table VI).

Discussion

The stimulatory effect of 25-hydroxycholecalciferol on nuclear RNA synthesis (Table I) provides additional support for the hypothesis that the 25-hydroxy metabolite is the active form of vitamin D at the organ level and furthermore demonstrates that it has an effect on the nucleus of intestinal mucosal cells. We are assuming the intestinal mucosa of the vitamin D deficient rat is a fully differentiated tissue and the RNA stimulation observed in the present experiments is largely a reflection of the rapid transcription of that section of the genome which carries the code for the vitamin D sensitive components of the calirum transport system. Since the administration of 25-HCC bypasses the activation mechanism in the liver, the response in RNA synthesis is considerably shorter than that noted by Stohs and DeLuca (1967b) with 10 (0.25 μg) or 2000 (50 μg) IU of vitamin D_3. Blunt *et al.* (1968b) demonstrated that 25-HCC given intravenously initiates calcium transport by everted sacs of rat small intestine 2–3 hr after injection while an equivalent dose of vitamin D_3 requires 8–10 hr to elicit a detectable response. Within this 2–3-hr period, mRNA and protein synthesis must have occurred. Therefore our evidence that a stimulation in RNA occurs within 1 hr and is maximal by 2 hr after a 0.25-μg dose of 25-HCC is consistent with the 3-hr effect on calcium transport in gut sacs.

The present report provides clear evidence that the intestinal

TABLE VI: Distribution of ^{3}H Metabolites in Intestinal Mucosal Cytoplasmic Fraction after Administration of 0.025 μg of [^{3}H]-25-Hydroxycholecalciferol.[a]

Metabolite	Hours after Administration	
	2	8
IV	92.0	44.6
V	3.4	37.9
VI	4.6	17.5

[a] Six vitamin D deficient rats were administered 0.025 μg of [26,27-^{3}H]-25-OH vitamin D_3, intrajugularly, and each group was sacrificed 2 and 8 hr later. Data are expressed as percentage of total radioactivity eluted from the column.

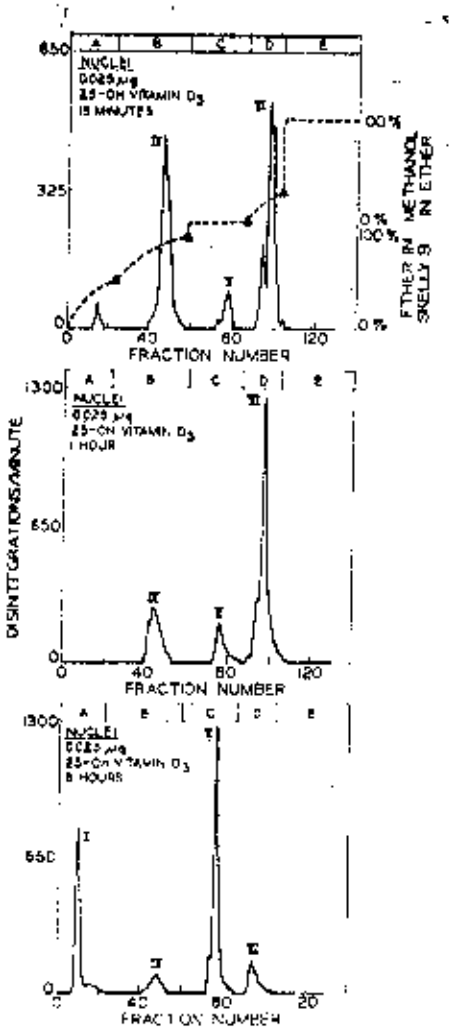

FIGURE 3: Silicic acid chromatography of lipid extract of intestinal mucosal nuclei after 0.025 μg of [26,27-^{3}H]-25-OH vitamin D_3. (— · —) Radioactivity; (- - -) gradient.

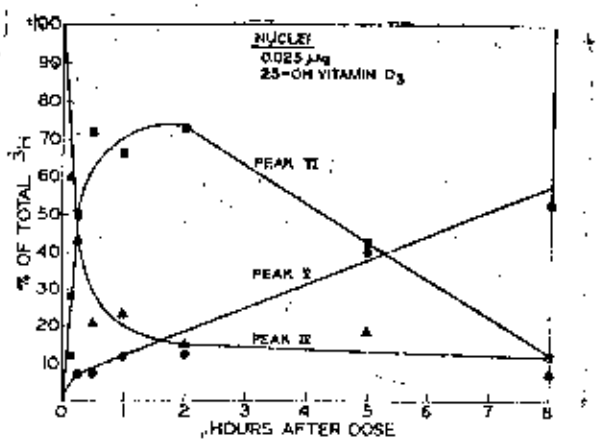

FIGURE 4: Production of metabolites in intestinal mucosal nuclei following intravenous administration of 0.025 μg of [26,27-^{3}H]-25-hydroxycholecalciferol.

mucosal cell nucleus is a probable site of further metabolism of 25-HCC (Figures 2 and 4). The presence of substantial quantities of peaks V and VI in the nuclear fractions and small quantities in the blood and cytoplasmic fractions support this suggestion. It should also be kept in mind that 25-HCC has a direct *in vitro* effect on both bone mobilization (Trummel *et al.*, 1969) and calcium transport (Olson and DeLuca, 1969); therefore meaningful metabolism would have to be at the target tissue level.

From the data at two dose levels this report establishes that [^{3}H]-25-HCC is rapidly converted into peak VI which probably is subsequently converted into peak V. The simultaneous production of peak V and peak VI *via* separate pathways cannot be completely ruled out from our data however. The time course of appearance of these metabolites suggests that peak VI is more likely to be of biological significance, but only future experiments could clarify this point. Stohs and DeLuca (1967) found 80% of the radioactivity from [^{3}H]vitamin D_3 in chick mucosal nuclei in the form of polar metabolites. Similarly Haussler *et al.* (1968) and Lawson *et al.* (1969) have reported the existence of polar metabolites in chick mucosal chromatin and nuclei, respectively. The comparison of our data with data of these investigators must be approached with some reservation. The chick has been shown to rapidly metabolize physiological intravenous doses of vitamin D_3 while in the rat the rate of 25-HCC formation and hence further metabolism is much slower (DeLuca, 1967; G. Ponchon and H. F. DeLuca, 1969, in preparation). Moreover, our experiments were conducted with the active form (25-HCC) administered directly, allowing for the first time evaluation of rapid metabolic conversions otherwise evident only at long periods after vitamin D administration. Lawson *et al.* (1969) report the presence of a tritium deficient polar metabolite (peak P) in the nuclear debris fraction of chick mucosa after 20 IU (0.5 μg) of [1-^{3}H,4-^{14}C]vitamin D_3 were administered. While the chromatographic system employed was not described in detail, peak P was eluted from a silicic acid column with a steep methanol in ether gradient. Therefore this fraction appears to be equivalent to both peak V and peak VI in our chromatographic profiles since a subtle stepwise methanol gradient is necessary to separate these two metabolites. Our data with [1,2-^{3}H]-25-HCC and [26,27-^{3}H]-25-HCC of high specific activity, however, unequivocally establish that peaks V and VI are produced *in vivo* from both specifically labeled compounds.

Recently Kodicek (1969) reported that the radioactivity from 0.375 μg of [1-^{3}H, 4-^{14}C]-25-HCC is largely present as peak P in the crude nuclear fraction by 8 hr after the dose. While the design of the experiment is not strictly comparable with ours and tritium loss in peak P was again observed, it should be noted that if peak V plus peak VI and peak P are essentially equivalent the data are complementary. Kodicek hypothesized on the basis of the tritium loss and the apparent polarity of the fraction that peak P is a ketone derivative of 25-HCC formed *via* an undetected hydroxylated intermediate. It is indeed possible that peak VI is the hydroxylated intermediate and peak V is the ketone derivative postulated by Kodicek. This sequence of reactions would explain the apparent conversion of peak VI into peak V observed in our experiments.

The significance of the possible metabolic conversions of 25-HCC within the intestinal mucosa is not evident at present.

Further conversion may be necessary to establish specificity in target tissue. With such a mechanism, 25-HCC would constitute a "circulating active" form while additional metabolites (peaks V and VI) would constitute "tissue active" forms. This mechanism would explain the specificity and differences in the physiological expression of vitamin D action in bone and intestinal tissues. Bruchovsky and Wilson (1968) reported testosterone is converted by target tissues to additional biologically active metabolites. They have suggested that enzymes which catalyze these further metabolic steps are present only in testosterone sensitive tissues.

An alternative suggestion to explain the significance of further 25-HCC metabolism would be that a regulatory mechanism is necessary within the intestine to limit or shut down the synthesis of calcium transport components prior to mucosal cell destruction within the intestinal lumen. Further work is clearly necessary to establish the validity of the above suggestions or other possible mechanisms before the significance of the intracellular metabolism of 25-hydroxycholecalciferol is fully understood.

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IDIOPATHIC HYPERCALCÆMIA IN INFANTS WITH FAILURE TO THRIVE

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With illustrations on plate

MUCH interest has been aroused by the success of biochemical methods in the investigation of the wasted infant. This interest has been further stimulated by recognition of the syndrome of "idiopathic hypercalcæmia, with failure to thrive" (Lightwood 1952a), in which anorexia, vomiting, and constipation are associated with wasting, hypotonia, and elevation of the serum-calcium and blood-urea levels. Clinically the condition simulates infantile renal acidosis; the aetiology is unknown; and spontaneous recovery is the rule.

We here describe 16 cases seen in the Royal Belfast Hospital for Sick Children and the Ava Hospital during the period April, 1952 to June, 1954. 1 case previously reported (Creery 1953) of idiopathic hypercalcæmia with renal impairment, osteosclerosis, craniostenosis, hypertension, and general retardation (Fanconi and Girardet 1952, Schlesinger, Butler, and Black 1952) is not included, because it is at present uncertain what relation this type of case bears to the syndrome under review.

Observations

ÆTIOLOGY

Sex.—There were 11 females and 5 males in the series.

Pregnancy and Delivery.—Pregnancy was normal in 10 cases; in the remainder it had been complicated by considerable vomiting. There were 3 twin pregnancies, one twin from each pair (1 identical, 2 dissimilar) being affected. 5 of the infants were first-born and 2 were premature. Delivery was normal in 10 cases and of unknown type in 1. There were 3 forceps deliveries, 1 breech, and 1 caesarean section. 1 infant suffered neonatal asphyxia.

Feeding Before Onset of Symptoms.—5 infants were never breast-fed, 3 were breast-fed for less than a week, and 7 for less than four weeks. 1 infant was breast-fed for two months. Subsequent feeding in 15 cases was with dried milk fortified with vitamin D (7 were fed on full-cream dried milk, 6 on half-cream followed by full-cream milk, and 2 on half-cream milk alone). 1 infant was fed on liquid cow's milk. In several cases mixed feeding had started.

Vitamin D Before Onset.—2 infants did not have vitamin-D supplements. The remainder had supplements varying from 400 to 1600 international units (I.U.) daily; the majority had a daily dose in the region of 800 I.U. An attempt has been made to assess the approximate total vitamin-D intake from a consideration of the known diets and vitamin additions. From this it would appear that 2 infants had about 700 I.U. daily, 6 had 1100–1600, 7 had 1800–2400, and 1 had 3200.

Preceding Alkali Therapy.—7 infants had varying but regular dosage with alkali preparations: 3 had magnesia in some form and 4 had alkaline carminative mixtures.

Preceding Illness and Therapy.—A preceding gastroenteritis was noted in 2 cases. In 1 of these infants this was associated with pyelonephritis and otitis media. 1 infant was said to have had a severe common cold, 1 a sore throat, and 1 otitis media. In only 1 case was there a history of sulphonamide medication.

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SUMMARY OF THE CASES

Case no.	Age at onset (mos.)	Age at examination (mos.)	Duration to clinical recovery (mos.)	Highest serum-calcium (mg. per 100 ml.)	Highest blood-urea (mg. per 100 ml.)	Follow-up: Duration (mos.)	Follow-up: Last known serum-calcium (mg. per 100 ml.)	Follow-up: Last known blood-urea (mg. per 100 ml.)	Stature	Other remarks
1	1½	17	18	16	35	18	10·3	30	Average	Mentally retarded (if not connected with illness)
2	3	13	13	15·8	124	18	12·8	49·7	Below average	Secondary rise in serum-calcium age 27 months
3	9	12	4	13	33	13	8·7	33	Below average	..
4	1	7	11	16·8	92·0	9	12	45	Average	..
5	Birth	17	22	12·3	39	6	11·2	30·3	Average	..
6	2	13	16	14·2	61	8	11·7	14	Average height. Underweight	Urinary infection at follow-up
7	6	12	8	13·8	43	5	10·6	46·4	Slightly below average	Anæmic at follow-up (62%)
8	7	14	11	12·5	46	1	11	28·5	Average height. Underweight	Frequent tonsillar sepsis
9	3½	7	11	14·5	96	6	11·7	43	Average height. Underweight	Anæmic at follow-up (58%)
10	1½	6	7	13·1	52	6½	11·7	37·5	Below average	..
11	5	11	..	14·5	80	..	..	..	..	Lost sight of
12	7½	7½	1	14·7	78	3	12·3	32	Average	..
13	5	9½	..	14·4	98·5	..	..	..	..	Remains in active stage
14	6	11	6	14·1	40·4	1	11·4	36·1	Average height. Underweight	..
15	8	10	..	17·3	100	..	..	..	..	Remains in active stage
16	6	8	..	17·5	105	..	..	..	..	Remains in active stage

THE CLINICAL PICTURE

These infants appeared to thrive at first and the onset of definite symptoms was usually clear-cut, at ages from six weeks to nine months (see table). In 1 infant, symptoms were present from birth. Vomiting—often unspectacular but occasionally severe and projectile—was the initial symptom in 8 cases, and it occurred in all but 1 of the 16. In 4 cases the illness began with constipation and in 3 with refusal to feed. In 1 case vomiting and constipation were noticed more or less simultaneously.

Vomiting tended to be most severe during the early part of the illness and gradually subsided. Periodic exacerbations occurred, but it ceased with the child's recovery. Constipation was obstinate in most cases, the stools being infrequent, small, and hard, and it resisted the household remedies which were given with remarkable perseverance. Proprietary alkali preparations were commonly used regularly (twice or thrice weekly) or daily for periods of weeks or months with a singular lack of success. 12 infants were given magnesia preparations, with or without additional alkaline carminatives or citrate. 1 received an occasional dose of carminative and 1 an occasional dose of magnesia; 2 had no alkali therapy. Other laxatives were frequently given without effect.

All the infants failed to gain weight normally, and some were 1–5 lb. below their expected weights. Definite anorexia was noted in 12 infants and distaste for solids was conspicuous. 11 of the mothers thought that their infants passed an excessive amount of urine. The mothers mentioned thirst in 6 cases, pallor in 5, and "muscular weakness" in 4.

Physical examination revealed little apart from wasting of varying degree. Growth in length appeared to be retarded in 4 of the 11 infants who were measured; head circumference was within normal limits and craniostenosis was not noted.

Despite their poor physical condition most of these infants were alert and contented, though some showed curious apathy. This pleasant nature, associated with a diminutive appearance, sunken eyes, and a slender neck, has sometimes suggested the diagnosis.

Clinical evidence of dehydration was relatively uncommon (6 cases) and was only seen where vomiting had been severe or long-continued. Though 8 infants were regarded as pale, anæmia was not a feature of the illness; haemoglobin values were commonly within normal limits for age and in only 4 cases were they below 75% (50%, 60%, 65%, and 70%). Only 1 infant was regarded as mentally retarded. Hypotonia—involving particularly the legs and sometimes extreme—was usual (14 cases). Other less constant findings were unexplained episodes of pyrexia (8 cases), faecal masses in the colon (6 cases), soft apical systolic murmurs (3 cases), and hypertension (2 cases out of 13 in which blood-pressure readings were made). Intercurrent infections (colds, bronchitis, tonsillitis, otitis) were seen in 6 cases. After periods ranging from one to twenty-two months (see table) vomiting finally stopped, appetite slowly improved, constipation lessened or disappeared and the infants began to gain weight and strength.

THE BIOCHEMICAL PICTURE

In all but 2 cases the initial examination of the serum showed a calcium level above 12 mg. per 100 ml., and in some cases it was as high as 16–17 mg. The blood-urea level was almost always raised, the mean being 44 mg. per 100 ml. The highest values obtained for calcium and urea in each case are shown in the table. It should be noted that some of these values were probably in part attributable to dehydration, as shown by rises in the specific gravity of the plasma. Inorganic-phosphorus levels were within the normal range for children of this age, the mean value being 5·6 mg. per 100 ml. Values for

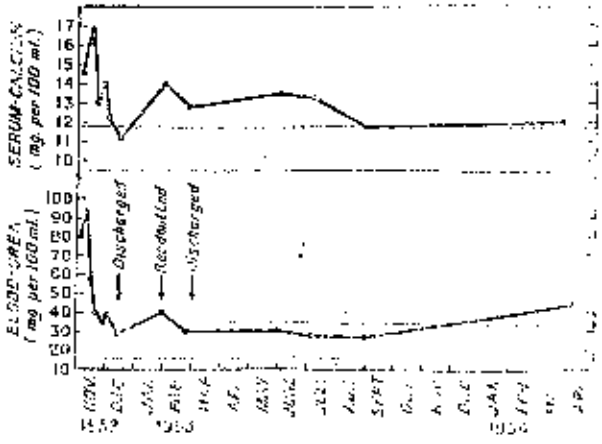

Fig. 4—Serum-calcium and blood-urea levels during treatment and follow-up.

sodium, chloride, and potassium were normal. The alkali reserve was generally within normal limits, but occasionally a little high. Estimation of plasma-proteins by salt fractionation gave normal readings (with an occasional high total value) the albumin/globulin ratio being normal. Electrophoresis on paper (13 cases) showed in 7 cases an increase in the α_2-globulin at the expense of the α-globulin; in 2 cases the γ-globulin was also increased. Alkaline phosphatase occasionally showed a slight rise, and in 3 children the serum-cholesterol was raised. Serum-magnesium (7 cases) was within normal limits of 1·3 mg. per 100 ml. The changes in the serum-calcium and blood-urea during the illness are illustrated in the chart of a typical case (fig. 4). The raised calcium, paralleled by a raised urea, characterises the active stage of the condition; reversion to rather high normal values takes place after clinical recovery. In some cases the blood-urea showed a much slower return to normal.

The urine was examined in 15 cases. The reaction was varied; only 1 infant passed a persistently alkaline urine. Proteinuria was not a feature, and on microscopy a few pus cells were seen. 1 infant, however, had a pyuria with a heavy growth of *Staphylococcus aureus* and proteus. Scanty growth of *Escherichia coli* was found in 6 cases and scanty proteus in 4; in 4 cases the urine was sterile. In 7 cases chromatography of the urine was normal; 1 case showed excretion of phenylalanine in unusual quantity, and 1 of proline.

OTHER INVESTIGATIONS

On radiography, calcification in the kidneys was not detected in any case. As excess of intestinal gas commonly obscured the renal tracts, straight radiography and pyelography (2 cases) gave unsatisfactory results. The density of the skull, pelvis, vertebræ, and thoracic cage always appeared to be within normal limits. Radiographs of the wrists were taken in 15 cases; these were normal in 3 cases but 12 showed transverse bands of increased density at the metaphyseal margins of the lower ends of the radius and ulna with a similar appearance in the metacarpals in some cases (figs. 1 and 2). In 8 cases a similar appearance was noted in the femur and tibia (fig. 3). These bands were single, narrow, and well defined, quite unlike "growth lines" (which were present in addition in 4 cases); they were reminiscent of the dense lines seen in lead poisoning. In 1 child the bands were seen to develop as the illness progressed. In 4 children followed to recovery, the bands have disappeared. In 7 cases an additional feature has been a zone of slight rarefaction in the metaphysis, proximal to which the normal density of the diaphysis has been resumed.

Electroencephalography (3 cases) and electrocardiography (2) showed nothing abnormal.

THE DIAGNOSIS

In the absence of any of the common causes of wasting in infancy, a biochemical examination of the serum may be of value in suggesting the diagnosis. The pyuria of pyelonephritis is generally more severe than that of idiopathic hypercalcæmia, and the latter can be distinguished from pink disease by the absence of irritability, photophobia, rashes, and tachycardia. But idiopathic renal acidosis cannot be satisfactorily diagnosed on clinical grounds alone.

A study of these 16 cases, and of 13 cases of renal acidosis occurring during the same period, displays certain points which may help in the differential diagnosis. The infants with renal acidosis had less often had vitamin D added to their feeds, and their illness had begun at a rather earlier age. The cardinal symptoms of anorexia, vomiting, constipation, and failure to thrive were common to both syndromes. Irritability and sighing respirations were additional but inconstant features of renal acidosis. Wasting was present in both groups of cases. Fæcal masses, clinical dehydration, and unexplained

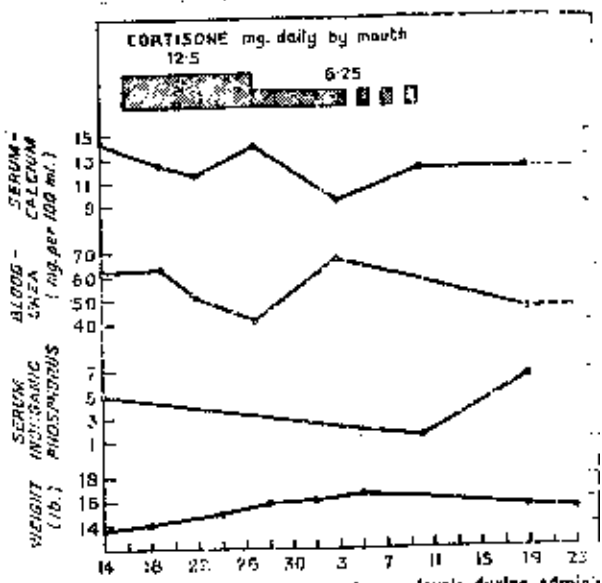

Fig. 5—Blood calcium, phosphorus, and urea levels during administration of cortisone.

pyrexia were more often seen in renal acidosis, while hypotonia, uncommon in renal acidosis, was characteristic of the hypercalcæmic syndrome. Urine containing a trace of protein, and alkaline or neutral in reaction, was characteristic of renal acidosis; in the hypercalcæmic cases the reaction was variable and proteinuria was absent. Minor pyuria was found in both. Radiological sclerosis of the metaphyseal margin, similar to that noted in idiopathic hypercalcæmia, has been observed in several cases of renal acidosis. The final distinction was made on the serum chemistry. The essential difference was the finding of a persistently lowered alkali reserve in the cases of renal acidosis. This acidæmia was not a feature of idiopathic hypercalcæmia, the alkali reserve being generally within normal limits or in some cases slightly raised.

A transient elevation of the serum-calcium was found in 9 of the 13 cases of renal acidosis. In 3 cases this was noted on the original biochemical examination but disappeared when alkali therapy commenced. In 1 case hypercalcæmia was found on the first examination and once again during the course of therapy. 2 other cases showed raised serum-calcium levels as isolated incidents during the course of treatment and while acidosis was still present. In 1 case the hypercalcæmia persisted after return of the alkali reserve to normal. In 2 cases a high serum-calcium was found on one occasion after apparent recovery from the renal acidosis. In all other respects these cases were typical of renal acidosis.

TREATMENT

Of the 16 children, 14 were admitted to hospital and the other two were observed as outpatients. 13 received no treatment apart from symptomatic measures to relieve constipation and (when present) dehydration and infection. These infants were given their usual diets with vitamin-D supplements. So as to reduce the vitamin-D intake further, a change was usually made from vitamin-D-fortified milks to liquid cow's milk. In several cases a synthetic diet of low calcium content and free of vitamin D was given for short periods: it was composed of soya flour, liquid glucose, peanut oil, and arrowroot made up with water and contained about 75 mg. calcium per pint. In 2 of these cases, in which the periods of trial were probably too short, the results were inconclusive; but in 1 case this diet produced a gratifying response both clinically and chemically.

1 infant a course of cortisone was given: the dose was 12·5 mg. daily by mouth, with gradual withdrawal: potassium chloride and oxytetracycline (terramycin) were given prophylactically, and a normal diet was continued. Clinical improvement was striking and the gain in weight was accelerated. The changes in the serum (fig. 5) comprised a fall in calcium, phosphorus, urea, and potassium levels with subsequent rise on cessation of therapy which was followed by a fall in weight. The fall in the phosphorus level was particularly marked. The urinary excretion of calcium was not estimated. It is possible that this child was already entering the recovery stage and that some of the changes were non-specific effects of cortisone. Nevertheless the biochemical response is of interest and merits further study. Similar findings were reported by Dent, Flynn, and Nabarro (1953) in cases of sarcoidosis with hypercalcæmia and impairment of renal function.

FOLLOW-UP (SEE TABLE)

3 infants remain in the active stage of the disease and 1 has been lost sight of. 12 have been followed from apparent clinical recovery for varying periods. 4 of these have apparently regained their normal rate of growth and reached their expected height and weight, but 8 still remain below their expected stature. 4 of these 8 still show blood-urea levels at or slightly above the upper limit of normal, in 1 case even after eighteen months. Urine at follow-up has shown minor pyuria ("occasional pus cell") in 5 cases and scanty infecting organisms in 7. 1 infant had a florid *Esch. coli* urinary infection. 2 infants had become anæmic.

Discussion

The clinical and chemical features of these cases are typical of the condition as described by Lightwood (1952a and b) and Payne (1952). In view of the known tendency to spontaneous recovery, and the lack of hypophosphatæmia, hyperparathyroidism appears an unlikely cause. It has been suggested (Creery 1953) that alkali medication might be a causal factor, but in the present series the syndrome has been seen to develop in the absence of such therapy. Similarly there is no direct evidence to implicate previous pyelonephritis as an ætiological agent, and the pyuria commonly found is probably secondary in nature (Lightwood 1952b).

Smith (1951) stated that ingestion of increasing amounts of calcium led to larger fæcal losses but also to surprising degrees of increased retention, and that the degree of retention depended mostly on the amount of calcium in the infant's diet, which is 4–6 times as great with cow's milk as with human milk. Stearns (1939) observed that retention of calcium was much greater when vitamin D was added, especially if the diet was alkaline in ash; that infants fed with ample vitamin D tended to have serum-calcium levels at the upper limit of normal; and that the urinary excretion of calcium (which she said was normally under 20 mg. in twenty-four hours) was higher in those infants who were fed on cow's milk. It is known that increased calcium excretion through the kidneys may initiate tubular calcification (Albright and Reifenstein 1948, Dufault and Tobias 1954) and further that chronic hypercalcæmia may give rise to hyalinisation of the glomeruli (Dent, Flynn, and Nabarro 1953) with subsequent renal impairment and azotæmia.

Thatcher (1936), in his report of a case which presented a clinical picture similar to the syndrome under discussion, attributed the illness to vitamin-D intoxication, although the intake had not been markedly excessive by accepted standards. Variation of personal sensitiveness to vitamin D was suggested by Bicknell and Prescott (1942). Of interest in this connection is the observation of Jeans (1950) that vitamin D in a daily dosage of about 1800 I.U. may give rise to anorexia, loss of weight, and retardation of linear growth. He regarded these as mild manifestations of hypervitaminosis D during therap of an order usually considered reasonable and harmles He also pointed out that an additional feature of vita min-D intoxication in infants was a dense deposition c mineral in the zone of provisional calcification in th metaphyses of long bones, this deposition being mad at the expense of the diaphysis. Similar findings ha previously been reported by Ross and Williams (1939 and further elaborated by Ross (1952), who noted i some cases a zone of rarefaction proximal to the dens line, and periosteal changes. Lightwood (1953), i seeking an explanation for the hypercalcæmia, has pu forward the hypothesis that there may be an abnorma sensitivity to vitamin D in these children. Unequivoca evidence of such a sensitivity is difficult to obtain especially as it may be only temporary.

In one of our children in whom the serum-calcium ha returned to normal without specific treatment and who wa receiving a high calcium intake, the addition of 2000 I.U vitamin D daily was accompanied by a rise in the serum calcium from 11 mg. per 100 ml. to 12·4 mg.; in a period o two weeks the blood-urea rose from 36 mg. per 100 ml. t 46 mg. Yet on continuing the vitamin D for a further te days the serum-calcium fell to 11·4 mg. and the blood-urea t 36·4 mg. Throughout these changes the child continued t improve clinically.

If, however, this postulate of abnormal sensitivity i correct, the radiological changes in the long bones o our cases, similar in nature to those described in hypervitaminosis D, are probably of significance.

In our series there is clear evidence for high calcium intake from early infancy. The low rate and short duration of breast-feeding is notable, and no example of the syndrome has so far been seen in a fully breast-fed infant. Evidence for hypercalciuria is less definite. Reliable figures for twenty-four-hour urinary excretion of calcium were obtained in 7 cases, the figures being 67·1, 54, 48·2, 40, 50·5, 60·7 and 47·75 mg.; but none was obtained in the early stages of the disease. If one accepts Stearns's figure of 20 mg. as normal, these figures probably represent increased levels of urinary calcium. On the other hand Knapp (1947) gave normal figures of 1–82 mg. daily for infants under six months old fed on cow's milk and added vitamin D; so there seems to be considerable variation from infant to infant. In all but 2 of our cases vitamin-D intake had been more than adequate by accepted standards, and in 5 cases it was over 2000 I.U. daily. Calcium and vitamin-D intakes of this order make it probable that there was alimentary over-absorption of calcium, which could well be the basis of the syndrome. In artificially fed infants, 30–50% of ingested calcium is said to be retained (Eliot and Park 1950). Several four-day calcium balance experiments in this series have had to be abandoned or the results discarded because of vomiting, and satisfactory balances have been carried out on 3 infants. 2, fed on diets high in calcium (such as they were accustomed to receive before admission) and without added vitamin D, showed retention of 57% and 56·7%. A further infant, fed on vitamin-D-fortified milk with a high total calcium content had a retention of 41·5%. These experiments, again, were not made in the early stages of the disease. We think that they indicate retention of calcium at the upper limit of normal.

If hypercalcæmia can be caused by such overabsorption, most if not all of the other findings can be satisfactorily explained. Anorexia, vomiting, constipation, muscular weakness, and hypotonicity are all probably related in some part to the hypercalcæmia per se. Loss of weight and failure of linear growth could result from insufficient nutrition. Impairment of renal function will follow hypercalcæmia and hypercalciuria if present. Tubular calcification may be sufficient to give rise to pyuria and may predispose to the mild urinary infection so commonly found. Polyuria and thirst are presumably

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of renal origin. Harris (1954) has pointed out that sulphadimidine can adversely influence renal function in these cases, and such therapy is rightly condemned. Changes in the plasma proteins may be compensatory and directed towards the carriage of the increased serum-calcium. It is tempting to regard the electrophoretic evidence of an increased α_2-globulin as analogous to the increase in the copper-carrying protein caeruloplasmin in hepatolenticular degeneration (Holmberg and Laurell 1947, 1948). Changes in γ-globulin concentration may be the result of intercurrent infection. The relatively minor changes which we have noted in the long-bones of our cases seem likely to be related to interference with growth in combination with hypercalcaemia and increase in the "calcium-phosphorus product" (Creery 1953, Harris 1954). The hypercalcaemia sometimes found in idiopathic renal acidosis is at present unexplained: it may arise in a way similar to that suggested above, or it may be related to changes in the acid-base balance of the blood.

To prevent this condition it would seem that we must constantly encourage breast-feeding and discourage unnecessarily large doses of vitamin D especially when vitamin-fortified dried milks and foods are being used. A rational therapy should include a diet low in calcium and free from vitamin D, such as has recently been suggested by Ferguson and McGowan (1954). From our experience we cannot say definitely how long this treatment should be continued, but we think that it is of most value to tide the infant over the acute stage of the illness and should be followed by a gradual return to normal feeding without excessive milk and with no added vitamin D. The results of such treatment may perhaps be regarded with some scepticism in the light of the spontaneous recovery which can confidently be anticipated.

The immediate prognosis is good, and all our infants have so far survived. A long follow-up will be needed to determine whether renal function remains unimpaired in those cases in which the blood-urea is still raised, and to see whether normal stature is always attained.

Summary

The clinical, chemical, and radiological features of 16 cases of idiopathic hypercalcaemia of infants, with failure to thrive, are described.

The only constant aetiological factor is a high calcium intake from early infancy, usually associated with a more than adequate amount of vitamin D in the diet.

The possible mechanism of production of the syndrome, based on alimentary over-absorption of calcium, is discussed. The prophylaxis, treatment, and prognosis are outlined.

We are indebted to Prof. F. M. B. Allen and Dr. J. A. Smyth for their help at all stages of this study. We wish to thank Dr. M. J. L. Fraser, Dr. J. B. T. Logan, Dr. V. K. St. G. Breakey, Dr. W. A. B. Campbell, and Dr. A. A. H. Gailey for permission to include cases in the series; Dr. N. A. J. Carson for much help with the biochemical investigation; Dr. D. Boyd and Dr. H. B. McDowell for radiological assistance; Dr. O. D. Fisher and Dr. C. M. B. Field for clinical summaries of some of the cases; and Dr. P. E. Dent for advice. Our thanks are also due to those members of the resident medical, nursing, laboratory, radiographic, and secretarial staffs whose cooperation has been so greatly appreciated.

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Effect of Hypervitaminosis D Upon the Phospholipids of Metaphyseal And Diaphyseal Bone.* (32353)

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Previous work has shown that the hypervitaminotic D state affects the quantity of lipids which can be extracted from the matrix of rat bone(1,2). Hypervitaminosis D increased the total lipids, phospholipids, triglycerides, cholesterol, cholesterol esters, and total fatty acids of the diaphyseal and metaphyseal long bones. The greatest change was in the phospholipid fraction. There is no information available in the literature on the nature of the phospholipids of long bones or on the mechanism of action of vitamin D in lipid metabolism. Consequently, the following experiments were carried out to determine which phospholipids were present, whether the effect of vitamin D on phospholipid metabolism is confined to a single phospholipid or concerns all major phospholipid components, and to ascertain whether the changes are the result of increased synthesis or decreased break-down.

Materials and methods. Male hooded rats of the R.V.H. strain averaging 110 g were maintained on a complete synthetic laboratory diet (Purina Labina). The hy[illegible]inotic D group received 15,000 international units of vitamin D_2† dissolved in sesame oil per 100 g of body weight daily. The control animals were dosed with an equal volume of sesame oil. Between days 14 and 18, 11 control

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† Vitamin D_2 (calciferol) kindly supplied by N. V. Philips-Roxane Co. The vitamin D was dissolved in a small amount of absolute alcohol and diluted with sesame oil so that the concentration of vitamin D in sesame oil was 15,000 units/ml.

and 12 vitamin D treated animals were decapitated and the humeri, femora and tibiae removed and cleaned of soft tissue. The epiphyses were pried off and the marrow of the long bones was removed by means of a syringe. The bones were then prepared and incubated in Krebs-Ringer-Bicarbonate (pH 7.4) according to the method of Deiss, Holmes, and Johnston(3). Ten microcuries of P^{32} as $NaHPO_4$ were added to each flask. After 6 hours of incubation, the bone fragments were removed, washed immediately with cold saline, and lyophilized. They were ground in a Spex Grinder in the cold for 2 minutes and the lipids were extracted and washed according to the method of Folch *et al*(4). This procedure was found to remove all but .05% of added inorganic P^{32}. The bones of the right extremities were kept separate from those of the left and prior to incubation the latter bones were placed in a steam bath for 5 minutes. The uptake of P^{32} into the phospholipids of the steamed bones never exceeded 1% of that of the non-steamed ones. Ash weight was determined by ashing an aliquot of dried fat-free bone in a muffle furnace at 680° C for 48 hours. The fraction of organic matter was calculated by subtracting the per cent ash from the total dry, fat-free weight. Lipid phosphorus was determined on an aliquot of the chloroform-methanol extract by the method of Marinetti(5) and phospholipid calculated by multiplying the phosphorus content of the lipid extract by a factor of 25(6). An aliquot of the chloroform-methanol extract was counted in a Packard Tri-Carb liquid scintillation counter and suitable corrections were made for background, decay, and quenching.

An additional group of 6 control and 6 hypervitaminotic D animals was maintained for 24 days, at which time they were sacrificed and their bones were removed, prepared and the lipids extracted as described above. Aliquots of the extracts were applied to thin-layer chromatograms using activated silica gel-G and the solvent system of Wagner *et al*(7), was used for chromatographic separation. Identification and quantification of the individual phospholipid classes were carried out by the method of Blecher and Abramson(8). Total phospholipid, ash, and per cent of organic content were determined as outlined above. The use of 2-dimensional thin-layer chromatography according to the method of Skidmore and Entenman(9) failed to reveal any significant additional phospholipids.

Results. As can be seen in Tables I and II, the rats which had been receiving high doses of vitamin D failed to gain weight at the same rate as the controls and there was a statistically significant increase in the organic fraction of their bones, as has been shown before(10). Table I shows that there is an increase in the amount of phospholipid of these bones whether this value is expressed as mg/g of bone or mg/g of the organic fraction. The P^{32} uptake was significantly increased whether it was expressed as counts per g bone or counts per mg of phospholipid. Table II shows that lysolecithin, sphingomyelin, lecithin, and phosphatidylethanolamine constitute the major phospholipids of the matrix of diaphyseal and metaphyseal bone. One unidentified phospholipid making

TABLE I. P^{32} Uptake by Bones of Hypervitaminotic D Animals 11-18 Days on Treatment.

	Control (11 animals)	Hypervitaminosis D (12 animals)
Final wt (g)	170 ± 4.2	146 ± 6.3***
Bone—Ash (% dry, fat-free wt)	65.3 ± 1.47	58.5 ± 1.34***
Bone—Organic fraction (% dry, fat-free wt)	34.7 ± 1.45	41.5 ± 1.34***
Phospholipid—Bone (mg/g)	1.97 ± .22	3.21 ± .37***
Organic fraction (mg/g)	5.64 ± .35	7.93 ± .58**
P^{32} uptake—Counts/min/g bone	7629 ± 1502	15,701 ± 2041 **
Counts/min/mg phospholipid	3441 ± 764	6,418 ± 1400 **

*** Significance at .1% level ($p < .001$).
** " " 1% " ($p < .01$).
* " " 5% " ($p < .05$).

± Standard error

TABLE 11. Phospholipids of Bones by Hypervitaminotic D Animals 24 Days on Treatment.

	Control (6 animals)		Hypervitaminosis D (6 animals)	
Final wt	182 ± 5.2		121 ± 5.8 ***	
Bone – Ash (% dry, fat-free wt)	65.0 ± 1.37		58.1 ± 1.46 ***	
Bone – Organic fraction (% dry, fat-free wt)	35.0 ± 1.35		41.6 ± 1.17 ***	
	mg/g organic fraction	% of total phospholipid	mg/g organic fraction	% of total phospholipid
Total phospholipid	7.03 ± .42		11.60 ± .64 ***	
Lecithin	3.63 ± .21	57.6	5.99 ± .28 ***	51.1
Phosphatidylethanolamine	2.22 ± .18	31.5	3.01 ± .20 ***	26.5
Sphingomyelin	.53 ± .09	7.6	1.19 ± .11 ***	10.3
Lysolecithin	.30 ± .07	4.3	.60 ± .09	5.1
"X"	.16 ± .04	2.2	.46 ± .06	4.1

*** Significance at .1% level ($p < .001$).
** " " 1% " ($p < .01$).
* " " 5% " ($p < .05$).

± Standard error

up 2-4% of the total also was found. It travelled between phosphatidylethanolamine and the solvent front and contained neither amino acids nor sugars. It can be seen that the increase in phospholipids resulting from excess vitamin D is the result of a rise in all phospholipids, rather than a selective increase in any one and that it is in all probability due to an increased synthesis based on the P^{32} incorporation.

Discussion. The recovery of lipid phosphorus applied to thin-layer chromatograms was between 85 and 90% and was quite constant. All extracts were compared with known standards whose recoveries also were within this range. Two faint additional spots were found on the 2-dimensional thin-layer chromatograms and these corresponded to very small amounts of phosphatidic acid and cardiolipin. No other unidentified fractions were found and it is therefore felt that the major phospholipids of the matrix of metaphyseal and diaphyseal rat bone as extracted by the Folch procedure(4) have been described.

An interrelation between calcification and lipids has been observed by Irving(11). He found a sudanophilic material at the zone of calcification of the epiphyseal line of calves and suggested that lipids and particularly phospholipids may be involved in bone formation. Johnson(12) also has implicated lipids in the series of events which lead to calcification of bone matrix in many tissues. Using histochemical techniques, he has shown that neutral lipids are present between the osteoblast and the calcifying front. After the affinity for neutral lipid stain disappears, the tissues will stain for phospholipid and this affinity disappears as calcification occurs. These histologic observations are strongly suggestive that phospholipids may play a role in osteogenesis. It is well established that an excess of vitamin D has profound effects on the chemical composition and morphology of skeletal tissue(10,13,14). Recently it has been shown that the lipids of bone are increased in hypervitaminosis D and that the most marked increase occurs in the phospholipid fraction(1,2). It is possible that the involvement of vitamin D in skeletal metabolism is through its role in phospholipid metabolism. It is known that in hypervitaminosis D there is a significant increase in the organic fraction of bone and that also the collagen and mucopolysaccharide content of this fraction is increased(15). Therefore it is not too surprising that the lipid content of bone also is elevated since it too is associated with the organic fraction. Further, there is accelerated bone production in the medullary cavities of long bones with large quantities of uncalcified osteoid occurring about the original trabeculae(13,14). It is unlikely that calcification cannot keep up with the increased rate of osteogenesis because there is,

indeed, a hypercalcemia in this condition. The present findings of an increased rate of phospholipid formation as measured by the uptake of P^{32} are consistent with the increased rate of bone production. Thus, in hypervitaminosis D, since the rate of matrix formation is greater than normal, it may be that the rate of calcification is inhibited by the presence of excess lipids and in particular phospholipids. It may be that calcification cannot occur until the phospholipids are removed. This could account for the decreased mineral content of the skeleton in hypervitaminosis D. It seems unlikely that any particular phospholipid is specifically involved since the present study showed that all phospholipids were increased proportionately.

Thompson and DeLuca (15) and Hoyosha (16) have shown that the vitamin D-deficient state leads to a diminished incorporation of P^{32} into the phospholipids of rat mitochondria from intestine and kidney but not from liver. The present work clearly indicates an increased incorporation of P^{32} into the phospholipids of bone in the presence of excess vitamin D. Thus, vitamin D status of the animal has significant effects on the phospholipid metabolism of tissues which are influenced by this vitamin, *i.e.*, kidney, intestine and skeleton.

Summary. The phospholipids of the diaphyseal and metaphyseal portion of the long bones of rats have been analyzed. Lecithin, phosphatidylethanolamine, sphingomyelin and lecithin constitute the major phospholipids with small amounts of cardiolipin and phosphatidic acid also being present. Hypervitaminosis D was found to cause a significant increase in all phospholipids without a selective action on any one component. The incorporation of P^{32} into the phospholipids was found to be increased indicating an increased synthesis rather than a decreased break-down. It is suggested that the accumulation of lipid material may be related to the failure of the osteoid in hypervitaminosis D to calcify properly.

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THE ANTAGONISM BETWEEN CORTISONE AND VITAMIN D: EXPERIMENTS ON HYPER-VITAMINOSIS D IN RATS

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SUMMARY

Hypervitaminosis was produced in young growing rats and the effect of daily injections of cortisone acetate (CA) was studied on growth, metastatic calcification, urinary phosphorus excretion and bone ash.

Cortisone treatment did not ameliorate the weight-loss, clinical appearance, histological lesions or the increased urinary phosphorus excretion of the hypervitaminotic rats. Control rats, treated with CA alone, showed no untoward effects at the dose levels employed.

It is concluded that cortisone does not act as a direct antimetabolite towards vitamin D. The antagonistic effect of the hormone observed in man may be due to reversal of the toxic action of vitamin D by an indirect mechanism, affecting the metabolic sites, such as intestinal tissue, kidney tubules and/or skeletal tissue, on which both vitamin D and cortisone appear to act.

Certain pathological conditions in man associated with hypercalcaemia have been attributed to the toxic effects of vitamin D, either as the result of overdosage or of hypersensitivity to the vitamin. In a small proportion of patients with Boeck's sarcoidosis, hypercalcaemia occurs, accompanied by some of the other manifestations of vitamin D intoxication. Cortisone has a dramatic effect on the plasma-calcium levels, hypercalcaemic symptoms and renal function [Lovelock & Stone, 1951; Shulman, Schoenrich & Harvey, 1952; Dent, Flynn & Nabarro, 1953; Anderson, Dent, Harper & Philpot, 1954; Henneman, Dempsey, Carroll & Albright, 1956]. The hypercalcaemic symptoms and the reaction to cortisone are those observed also in typical hypervitaminosis D [Albright & Reifenstein, 1948; Fanconi, 1956; Winberg & Zetterström, 1956]. This led Dent and his co-workers [1953, 1954] to suggest that, in Boeck's sarcoidosis, the hypercalcaemic syndrome might be due to a hypersensitivity towards vitamin D. The occurrence of idiopathic hypercalcaemia of infants also has been attributed to an overdosage and/or hypersensitivity to vitamin D, with varying emphasis on one or other of these aetiological factors, and in this condition, too, cortisone has a beneficial effect [Creery & Neill, 1954; Lowe, Henderson, Park & McGreal, 1954; Bonham Carter, Dent, Fowler & Harper, 1955; Fanconi & Spahr, 1955; Macdonald & Stapleton, 1955; Stapleton & Evans, 1955; Forfar, Balf, Maxwell & Tompsett, 1956; Morgan, Mitchell, Stowers & Thomson, 1956].

Anderson *et al.* [1954] postulated that cortisone and vitamin D act antagonistically by virtue of a substrate competition mechanism. It is evident from the beneficial effect of cortisone on the pathological conditions previously mentioned, that there is some interrelationship between vitamin D and corticosteroids. Additional support is

provided by the observation of Bazzi & Nicolosi [1951] that administration of large doses of vitamin D_2 or D_3 decreased appreciably the urinary excretion of 11-hydroxycorticosteroids in man. The finding of two abnormal Δ^1-3-ketosteroids in urine of patients suffering from vitamin D-resistant osteomalacia [Kodicek, 1956] would indicate a disturbance of ketosteroid turnover, in a disease associated with a disordered vitamin D metabolism. The negative calcium balance induced in humans by cortisone can be counteracted by administration of vitamin D and calcium [Fischer & Hastrup, 1954].

Relatively few experiments on animals have been reported, and the results are not in accordance with the observations in man. Wilson, Care & Anderson [1957] have found that nephrocalcinosis, induced in rats by moderate doses of vitamin D, was not prevented by cortisone. Polemann & Froitzheim [1953] reported that cortisone did not mitigate the toxic symptoms of vitamin D_2 over-dosage in mice, though admittedly the amount of cortisone used was small relative to the vitamin D dose. A synergistic rather than an antagonistic action is implied in the findings of Selye & Bois [1957] that calcium deposits in the gastric mucosa of rats were only produced by vitamin D if cortisone acetate (CA) was given simultaneously. The observations of Hanssler [1956] also suggest a synergistic effect, since cortisone, administered to rachitic rats on a low calcium–high phosphorus diet, appeared to simulate the effect of vitamin D in producing ossification.

Though the evidence previously referred to indicates that there is an interaction between vitamin D and corticosteroids, at least in some species, it is not clear whether this interrelation exists at the site of action of the vitamin, or whether a direct antimetabolite antagonism is involved, as suggested by Anderson *et al.* [1954]. In the latter event, administration of cortisone should mitigate, in rats as well as in man, the characteristic toxic effects of vitamin D excess observed in rats [Kreitmar & Moll, 1928; Harris & Moore, 1929]. At the suggestion of Prof. Dent, the following experiments were therefore carried out, a short report of which has already been presented [Cruickshank & Kodicek, 1957].

EXPERIMENTAL METHODS

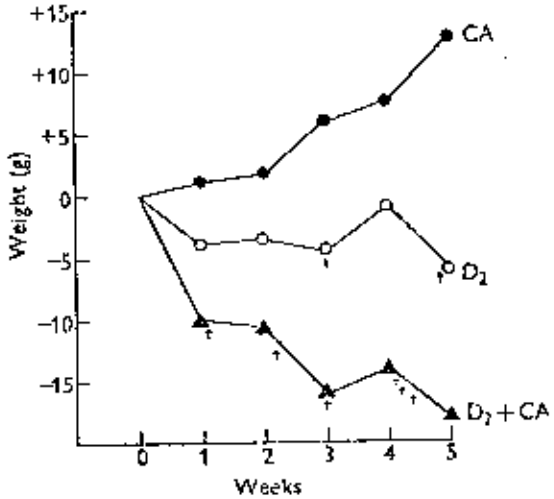
Twenty-four rats, weighing about 70 g, were divided into three groups of eight each and given the rachitogenic diet 2965 of Steenbock for 5 weeks. In group 1 each rat received *per os* 1 mg vitamin D_2 in arachis oil daily. The animals in group 2 were similarly treated with vitamin D_2 and, in addition, injected i.m. with 1 mg CA (Merck and Co. Inc.)/rat/day. The rats in group 3, which served as controls, were each injected daily with 1 mg CA and given a maintenance dose of vitamin D_2 (approx. 1·25 μg/rat/week).

The rats were weighed weekly. Urinary phosphorus was estimated by the method of Fiske & SubbaRow [1925] at the beginning and end of the experiment. The ash content of the dry, fat-free bone was estimated. All the animals were examined roentgenologically, and histological examination of tissues from representative rats in each group was carried out.

RESULTS

Growth

Text-fig. 1 shows the average growth curves of the groups of rats during the experimental period of 5 weeks, at the end of which the surviving animals were killed. Of the animals receiving a massive dose of vitamin D_2 alone, two died during the experimental period, the remaining six decreased in weight and were in very poor condition. In the rats given CA in addition to vitamin D_2, the loss in weight was appreciably greater. After the 1st week their general condition deteriorated rapidly, the fur became dank and moist, the appetite poor, and six of the eight rats died. All the control rats receiving CA only, appeared in normal health and continued to increase in weight throughout the experiment.



Text-fig. 1. Effect of cortisone acetate (CA) on average growth of hypervitaminotic (D_2) rats.
† Death of animal.

Urinary excretion of phosphorus

Initially only traces of phosphorus (< 0·06 mg/rat/day) were found in the urine in all groups. At the end of the experiment it was found that the administration of high doses of vitamin D_2 had increased the urinary excretion of phosphorus to 2·7–3 mg/rat/day, irrespective of whether CA was given or not. In the control rats, receiving CA only, the excretion of phosphorus in the urine was low, 0·14 mg (Table 1).

Bone ash

The average amount of ash in the bones of rats given vitamin D_2, with or without CA, was 43 and 41 % respectively, while in the control group the content was somewhat higher, 48 % (Table 1).

X-ray examination (Plate, figs. 1–6)

At the end of the experiment, the bones in the control group, receiving CA only, appeared normal, and no signs of osteoporosis were present; the rats receiving high doses of vitamin D_2, with or without CA, showed swelling of the distal parts of the radius and ulna, and varying degrees of circumscribed osteoporosis, particularly in

the proximal parts of the diaphysial bone of the tibiae, indicating a disturbance of mineralization. No roentgenological signs of gross calcification in tissues were observed.

Table 1. *Effect of cortisone acetate (CA) on toxicity of vitamin D_2 in rats*

Daily dose (mg)	No. of rats	Deaths	Aver. weight gain or loss (g)*	Urinary P† (mg/rat/day)	Bone ash (%)	Calcification in organs
I. Vitamin D_2, 1	8	2	− 6	3·0	41	++
II. Vitamin D_2, 1+CA, 1	8	6	−18	2·7	43	++
III. CA, 1	8	0	+13	0·14	48	0

* Change in weight during experimental period of 5 weeks.
† Determined at end of experiment.

Autopsy

In the control group nothing abnormal was noted, except that the adrenals were small. In the rats receiving vitamin D_2 only, haemorrhagic cystitis and occasional stones in the renal pelvis or ureters were observed. The kidneys were mottled. Von Kossa stain revealed, macroscopically, calcium deposits in the arch and descending part of the aorta. The bones appeared soft, friable and occasionally haemorrhagic. The same post-mortem picture was noted in animals given both vitamin D_2 and CA, except that the adrenals were small.

Histological examination (Plate, figs. 7–10)

Sections of tibiae, ribs, heart, muscles, liver, kidneys and aorta were stained with haematoxylin and eosin, and sections of the latter two tissues by the Von Kossa stain [Pearse, 1953] for calcium deposits. It was found that rats receiving the high doses of vitamin D_2, irrespective of whether they were treated with CA or not, had microscopically-visible deposits of calcium salts in the endothelial layers of the aorta, particularly in the region of the arch and descending portion. The kidneys of these rats also showed mineral deposits, particularly in the glomeruli, occasionally accompanied by atrophic changes. Sections of tissues of rats receiving CA alone, showed no abnormalities. No lesions were found histologically in liver, heart or muscles of the rats in any of the groups. In the hypervitaminotic rats that died during the course of the experiment, no metastatic calcification was noted macroscopically or histologically, but in every case haemorrhagic cystitis of the bladder was present.

Only a few sections of bones were examined. An abundance of osteoid tissue in and around the old trabeculae, without any pathological change in epiphysial cartilage, occurred in the bones of all rats which received high doses of vitamin D_2, with or without CA. The findings resembled those previously described by Harris & Innes [1931], Follis [1955] and Gebauer [1956]. No such changes were observed in bone sections of the control rats receiving CA only.

DISCUSSION

The results showed that treatment of hypervitaminotic rats with cortisone did not ameliorate the weight loss, clinical appearance, histological lesions or the increased urinary phosphorus excretion, in contrast to the positive findings in man, where

cortisone has a definite beneficial effect on the syndrome of vitamin D toxicity. These negative findings are confirmed by the recent observations of Wilson *et al.* [1957] that cortisone did not prevent nephrocalcinosis induced in rats by moderate doses of vitamin D. As previously stated, a direct antimetabolite competition between cortisone and vitamin D presupposed a positive effect of cortisone in rats as well as in man. Since we did not observe such an effect, we conclude that a substrate competition mechanism does not appear to be involved. The beneficial effect of cortisone in human conditions, confirmed by many workers, can thus be explained only by postulating another alternative, that cortisone counteracts indirectly the toxic action of vitamin D by influencing the metabolism of certain tissues, on which the vitamin also appears to act. It has been shown that cortisone in high doses affects the bones of growing animals [Follis, 1951*a*, *b*; Sissons & Hadfield, 1955], and that it induces osteoporosis in human beings [Albright & Reifenstein, 1948; Shorr, 1953; Eliel & Heaney, 1954]. Its effect may be mediated either through a lowered rate of formation of bone matrix [Albright & Reifenstein, 1948; Carlsson & Rosengren, 1955], or through a decrease in the absorption of calcium by the intestinal mucosa [Anderson *et al.* 1954]. However, cortisone may also exert its influence by altering the renal mechanism of electrolyte excretion, by decreasing the tubular phosphate reabsorption and by increasing the reabsorption of bicarbonate [Roberts & Randall, 1955; Giebisch, Macleod & Pitts, 1955]. In the present state of our knowledge it is impossible to tell which of these metabolic sites, viz. intestinal tissue, kidney tubules and/or skeletal tissue, are principally involved.

The ineffectiveness of CA in rats might be explained by species differences. According to Follis [1951*b*] who has studied the changes in skeletal structure resulting from massive doses of cortisone, the response to the hormone appears to vary from species to species. In studies on wound healing also, species differences in the response of connective tissue to cortisone have been demonstrated [Bangham, 1951; Clayton, 1954].

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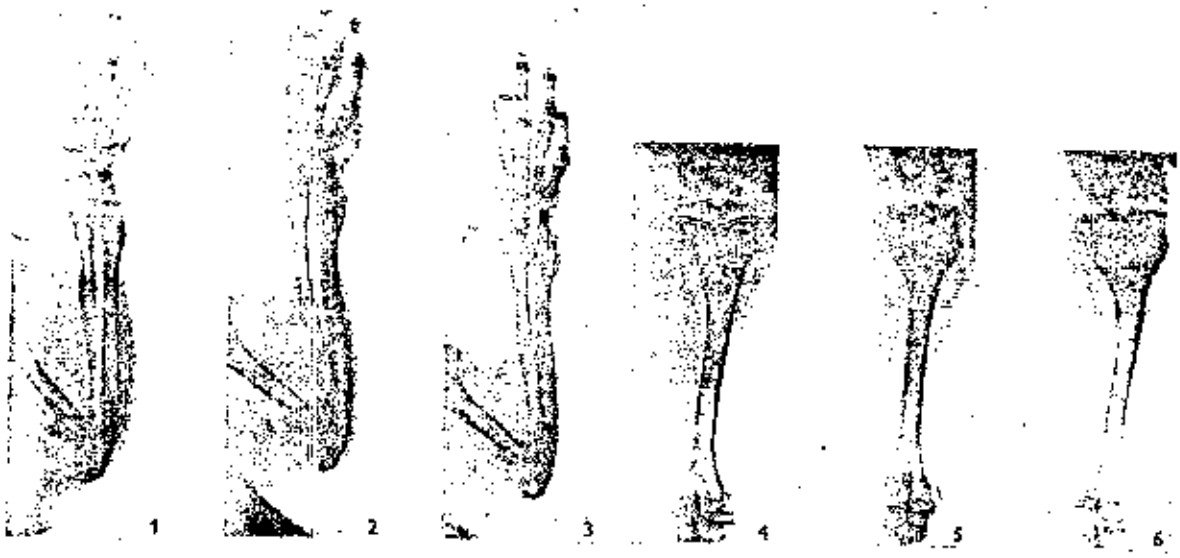
DESCRIPTION OF PLATE

Fig. 1. Control rat receiving CA only. No signs of osteoporosis.
Fig. 2. Rat receiving high doses of vitamin D_2. Swelling of distal parts of radius and ulna.
Fig. 3. Rat receiving high doses of vitamin D_2 and CA. Swelling of distal parts of radius and ulna and small degree of circumscribed osteoporosis.
Fig. 4. Control rat receiving CA only. No signs of osteoporosis.
Fig. 5. Rat receiving high doses of vitamin D_2. Swelling of proximal part of tibia, and circumscribed osteoporosis.
Fig. 6. Rat receiving high doses of vitamin D_2 and CA. Swelling of proximal part of tibia and uneven density of bone.
Fig. 7. Control rat. Tibia, normal. Haematoxylin and eosin, × 185.
Fig. 8. Rat receiving high doses of vitamin D_2. Tibia, abundance of new osteoid tissue in and around old trabeculae. Haematoxylin and eosin, × 185.
Fig. 9. Rat receiving high doses of vitamin D_2 and CA. Kidney, showing calcium deposits in glomerulus. Von Kossa, × 1250.
Fig. 10. Rat receiving high doses of vitamin D_2 and CA. Descending aorta. Calcium deposits in endothelial and sub-endothelial layer. Von Kossa, × 1500.



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Vitamin D activity of plasma in idiopathic hypercalcaemia

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Idiopathic hypercalcaemia is a condition noted in young children, from a few months to a few years old, and is so called because of the absence of any obvious cause for the typical elevated levels of serum calcium. The signs and symptoms of the disease have been described by Lightwood (1952*a*,*b*), Payne (1952), Fanconi, Girardet, Schlesinger, Butler & Black (1952), Lightwood & Stapleton (1953) and Creery (1953). The condition is characterized by hypercalcuria, hypercalcaemia, azotaemia and renal dysfunction. There is also an increased absorption of Ca from the intestine, elevation of serum citrate level, increased renal phosphate reabsorption and growth retardation; all these effects are closely similar to those noted after administration of excess vitamin D (Fellers, 1959), except that in idiopathic hypercalcaemia there is usually no evidence that abnormally large amounts of vitamin D have been administered.

Lightwood & Stapleton (1953) observed that the disease could occur in one or other of two forms, the benign or mild, and the severe. The signs listed above are noticeable in both types of patient, but those with the severe form typically display several further features, physical and mental retardation, osteosclerosis and cardiac systolic murmurs, and they frequently demonstrate a typical facies known as 'elfin' face; the severe condition may be chronic and so further developed than the benign or mild form, which may be a more acute and transient manifestation of the disease.

The toxic manifestations, the renal and cardiac damage, the osteosclerosis and the mental and physical retardation are probably the result of the elevated serum Ca levels, because the disease can be controlled by measures that control serum Ca, e.g. cortisone administration, induction of a negative Ca balance by administration of a diet low in Ca or low in Ca and rich in phytic acid, or administration of ethylenediaminetetra-acetate (EDTA) (Creery & Neill, 1954; Ferguson & McGowan, 1954; Bonham-Carter, Dent, Fowler & Harper, 1955; Stapleton, McDonald & Lightwood, 1956; Morgan, Mitchell, Stowers & Thomson, 1956).

Investigators have frequently remarked on the close correspondence between the condition noted in vitamin D intoxication and idiopathic hypercalcaemia (e.g. Lowe, Henderson, Park & McGreal, 1954; Creery, 1953; Creery & Neill, 1954; Schlesinger, Butler & Black, 1956; Lightwood & Stapleton, 1953; Bonham-Carter *et al.* 1955; Fellers, 1959; Morgan *et al.* 1956; Fellers & Schwartz, 1958*a*; Rhaney & Mitchell, 1956) and it has indeed been suggested that this condition may be in fact vitamin D intoxication in children especially susceptible to the toxic action of this vitamin (Morgan *et al.* 1956).

The observation that idiopathic hypercalcaemia is much rarer in the USA, Canada and Sweden than in the UK has also been put forward as evidence that vitamin D may be implicated (Morgan *et al.* 1956), because, in 1956, the vitamin D

content of infant milk foods was lower in those countries (about 40 i.u. 100 ml in the USA) than in the UK (about 130 i.u./100 ml). Since 1957 the vitamin D content of infant dried milks in the UK has been reduced (now about 40 i.u. 100 ml), and the vitamin D content of infant cereal foods has also been similarly decreased (from about 600–1000 i.u./oz to about 200 i.u./oz) thus markedly decreasing the average vitamin D intake of children in this country. It is not yet possible to state whether these changes have been associated with a significant reduction in the incidence of idiopathic hypercalcaemia, which, over the past few years, has amounted to about seventy to eighty cases yearly.

Idiopathic hypercalcaemia could easily be explained if there had been an excessive intake of vitamin D (and no evidence for it could be discovered in most instances in spite of detailed inquiries). An abnormal sensitivity to the vitamin, an abnormally high absorption and retention, a decrease in breakdown of the vitamin or its elimination from the body or an abnormality in Ca metabolism might also each offer an explanation for this disease.

Vitamin D activity can normally be demonstrated in a number of tissues of the body, since these tissues, or suitable extracts thereof, when orally administered to rachitic rats may cure rickets to some degree. Only in a very few instances has the compound responsible for this vitamin D activity been identified, by, for instance, radiochemical methods combined with chromatographic and chemical procedures (Kodicek, 1956; Kodicek & Ashby, 1960; Kodicek, Cruickshank & Ashby, 1960). Thomas, Morgan, Connor, Haddock, Bills & Howard (1959) have shown that the vitamin D activity of serum is non-dialysable, that this activity is located, after electrophoresis, between the α_1- and α_2-globulins and that it is so whether the vitamin D (ergocalciferol) is administered to the patient before examination of the serum or added to the serum sample in vitro. Assay of pooled human serum fractions demonstrated vitamin D activity in the α-globulin-containing fractions (IV-1, IV-4), with traces in fraction IV but none in fractions I, II or III. Fellers (1959) reported that the vitamin D activity of the serum of a child receiving 60 000 i.u. day was distributed about equally between the α-lipoprotein and albumin fraction and the high-density β-lipoprotein fraction. From these observations it may be concluded that the vitamin D activity of serum is probably due to the presence of a compound having vitamin D activity and associated with a macromolecule.

Little is known of the fate of vitamin D when administered in physiological doses because of the extreme difficulty of measuring the minute quantities involved. Cruickshank & Kodicek (1953), Kodicek (1956) and Fellers (1959) followed the fate of ergocalciferol by radiochemical procedures, using ^{14}C-marked ergocalciferol, or by biological methods after administering large doses (40 000 i.u.) to the rat. These workers have shown that, under these conditions, the concentrations in the liver 24 h after dosing were much higher than in the blood and other tissues, but that, within the next few days, the concentrations fell more rapidly in the liver than in the blood. Cruickshank & Kodicek (1953) also showed that only a small proportion of the administered vitamin D could be recovered from the test rats (6% after 1 day and less than 2% after 4 days). This poor recovery was shown, by tests with ^{14}C-

marked ergocalciferol (Kodicek, 1956; Kodicek & Ashby, 1960) to be due partly to poor absorption and partly to degradation of the vitamin within the body. The percentage absorption and degradation were also found to be similar for doses of 40 000 i.u. or 400 i.u./rat, and, at least at 24 h after administration, the vitamin concentrations were higher in the liver than in the other organs of rats given either of these doses. Vollmer (1939) demonstrated vitamin D activity (about 20 i.u./g) in skin and liver samples obtained at post-mortem from a child who had been given, within the 3½ days before death, $1{\cdot}6 \times 10^6$ i.u. vitamin D.

Eaton, Spielman, Loosli, Thomas, Norton & Turk (1947) showed that liver concentrations in the young calf were initially higher than plasma concentrations of the vitamin after administration of 50 000 i.u. vitamin D, but that the plasma concentration fell only slowly during the subsequent 42-day period. Heymann (1937) noted in the rabbit that 1 week after administration of 200 000 United States Pharmacopoeia units of vitamin D the vitamin activity was higher in blood plasma than in the other tissues and that plasma levels remained higher than those in other tissues for at least the next 12 weeks. In a series of tests on dogs and rabbits, Warkany (1936, 1937) showed, like Heymann (1937), that the serum vitamin D activity, once elevated by administration of a single large dose (100 000 United States Pharmacopoeia units of vitamin D), only slowly returned to previous levels (4–6 weeks), even though the initial rise in vitamin D activity was rapid, occurring within the first 1 or 2 days after dosing.

There is little evidence as to the relative concentrations of the vitamin in liver and blood under physiological conditions, but Devaney & Munsell (1935) reported the vitamin D activity of liver from pigs, sheep and cattle as ranging from about 0·1 to 0·5 i.u./g, and Warkany (1937) has shown the vitamin D activity of serum samples from these species to be between about 0·5 and 1·5 i.u./ml. Thus, under physiological conditions the liver reserves may be small compared with the quantity circulating in the blood.

In the pig, Quarterman, Dalgarno & Adam (1963) have shown that, after treatment for 1 month with daily doses of 250 000 i.u. vitamin D_3/day, the vitamin D activities of the liver and blood were both elevated to about the same extent (to 27 and 23 i.u./g respectively), whereas at a much lower dose level (350 i.u./day) the liver had an activity only about half that of the blood (0·21 and 0·52 i.u./g respectively). Within a week of withdrawal of the vitamin D_3 doses, it was noted that the vitamin D activity of the liver in both groups had fallen to about half that of the blood. Blood vitamin D activities steadily fell over the next 6 months, to 0·15 and 0·08 i.u./g in those groups that had been given the high and low doses of vitamin respectively. Liver vitamin activities were found to be about half those of the blood in both groups throughout this period.

Most of the blood vitamin D activity has been shown to be in the plasma rather than the corpuscles (Hess, Light, Frey & Gross, 1932). The foregoing observations would suggest that, at least when large doses of the vitamin, whether vitamin D_2 or vitamin D_3, are administered, some of it may be preferentially deposited in the liver, though within a relatively short period the liver concentration may fall and

that in the blood may then be higher. At lower, presumably physiological, doses, the blood levels are higher than those of the liver and all other organs examined.

In man the vitamin D activity of blood serum was found, in Cincinnati, to be about 1 i.u./ml (Warkany, 1936; Warkany & Mabon, 1940), whereas in Baltimore (Thomas *et al.* 1959) and in the UK (Cuthbertson, 1963) a similar but slightly higher mean value (about 2 i.u./ml serum or plasma) was reported. The liver vitamin D activity has been determined in two subjects (Cuthbertson, 1963), both aged 10–12 months. The control, not suffering from any disease of Ca metabolism, showed an activity of about 3 i.u./g liver, but the hypercalcaemic patient's liver contained less, about 1 i.u./g liver. Serum vitamin D assays were not carried out on either subject, but it can be seen that the liver activity was not greatly different from what would have been expected in the plasma of these patients.

The serum vitamin D activity may quickly be raised in man by administering the vitamin; with daily doses the rise may continue from a few hours after the first dose to reach a maximum level after several weeks (Thomas *et al.* 1959; Warkany, Guest & Grabill, 1942). As in animals, the serum activity, once elevated, is slow to subside, several months at least being required for return to normal levels (Warkany *et al.* 1942).

Table 1. *Serum vitamin D activity (i.u./ml) of patients given daily doses of ergocalciferol for periods of more than 1 month*

Daily dose (i.u.)	Serum vitamin D activity: Arthritic patients (Warkany *et al.* 1942)	Serum vitamin D activity: Hypoparathyroid patients (Thomas *et al.* 1959)
50 000	16·5	40, 40
100 000	—	30, 48, 50
150 000	66	
180 000	33	
200 000	44	18, 8
240 000	33	
400 000	130, 90	
500 000	90, 130	—

Table 1 shows the maximum serum vitamin D activities attained in two groups of patients who had been prescribed known high levels of the vitamin for periods of time never less than 1 month and mostly considerably longer. One group (Warkany *et al.* 1942) suffered from chronic proliferative arthritis and the other (Thomas *et al.* 1959) from hypoparathyroidism. The results in Table 1 clearly show that the serum vitamin D activity cannot be precisely predicted from the dose given. Even the level attained with a constant dosage may vary unpredictably (Warkany *et al.* 1942), but in at least one patient, and perhaps in others, part of this variation may have been due to failure to take the dose prescribed. Thomas *et al.* (1959) have shown that the relation between serum Ca level and serum vitamin D activity is also variable. Thus it appears that there are from one person to another large quantitative differences in response to a given dose of vitamin D.

A number of determinations of vitamin D activity have been made on serum (or plasma) samples from patients with idiopathic hypercalcaemia and are summarized in Table 2 (Lang & Eiardt, 1957; Fellers & Schwartz, 1958*a,b*; Fellers, 1959; Thomas *et al.* 1959; Smith, Blizzard & Harrison, 1959; Cuthbertson, 1963).

Table 2. *Vitamin D activity of plasma from patients with idiopathic hypercalcaemia*

Reference and country of origin of samples	No. of patients and type* of idiopathic hypercalcaemia	Serum (or plasma) vitamin D activity (i.u./ml)	Time taken for vitamin D activity to decline to normal level (months)
Lang & Eiardt (1957); W. Germany	1, severe	4	~8
Fellers & Schwartz (1958*a*); USA		20–30	>18
Fellers & Schwartz (1958*b*); USA	3, severe	20–60	>17 <26
Fellers (1959); USA		20	?
Thomas *et al.*† (1959); UK	2, mild	Normal (1–3)	—
Smith *et al.* (1959); USA	1, severe	23	>6
Cuthbertson (1963); UK	6, mild 5, severe	Normal (1–3) Normal (1–3)	—

*Mild or severe according to the criteria of Lightwood & Stapleton (1953).
†Patients suffering from mild form of disease (R. G. Mitchell, 1962, personal communication).

The plasma and serum vitamin D activities recorded in samples from two patients with the benign form of the disease (R. G. Mitchell, 1962, personal communication) by Thomas *et al.* (1959) and by Cuthbertson (1963) on other similar patients, were not significantly different from normal values or from values determined on control patients, i.e. 1–3 i.u./ml. Similarly, in serum from a patient suffering from the severe form of the disease, Lang & Eiardt (1957) found a vitamin D activity of about 4 i.u./ml, i.e. a little above normal, as might perhaps have been expected in this patient, who had received u.v. light treatment and daily doses of 1000–10 000 i.u. vitamin D, apart from the administration of a total of 1 600 000 i.u. vitamin D in three separate doses during the previous 2 years!

The patients with the severe condition, reported by Fellers & Schwartz, 1958*a,b*) and by Smith *et al.* (1959), present a different situation, in that the high serum activities (Table 2) found were such as would only be expected to result from the administration of large amounts (more than 20 000 i.u.) of the vitamin daily. The vitamin D intake of these patients, as reported by Fellers & Schwartz (1958*a*) and by Smith *et al.* (1959), was in no way abnormal, ranging from about 1000 to 5000 i.u./day and would certainly not be expected to have induced such high serum levels in normal patients. These results differ markedly from those found

by Cuthbertson (1963) (Table 2) in a series of five patients with the severe type of disease, i.e. suffering from mental retardation, systolic murmurs, osteosclerosis and the typical 'elfin' facies. Plasma samples were not obtained from three of these patients until they had been on a diet free from added vitamin D for a period of from 3 weeks to 6½ months; it is thus possible that the vitamin D activity may have fallen during this period. Even so, had these patients resembled those of Fellers & Schwartz (1958*a*) or of Smith *et al.* (1959), the vitamin D activity would still have been elevated even after this lapse of time, for these authors noted elevated serum activity from 6 months to 2 years after stopping the administration of vitamin D. Plasma samples were taken from the other two severely affected patients, in one instance before transfer to a diet free from vitamin D; the sample from the other patient was taken 10 days after removal of all sources of vitamin D from the diet. These two samples were both shown to have vitamin D activities within the normal range and not to differ in this respect from control samples assayed at the same time.

The condition noted in the severely affected patients studied by Cuthbertson (1963) cannot therefore be attributed to unusually large doses of vitamin D (because these infants had received average amounts, from 1000 to 3000 i.u. vitamin D/day) or to unusually high levels of circulating vitamin D (which could have been brought about by abnormally effective absorption, by decreased elimination of vitamin D or by other means); they would seem best to be explained by an unusual sensitivity to doses of the vitamin normally quite harmless or to some other derangement in Ca metabolism. The severely affected patients observed in the USA by Fellers & Schwartz (1958*a*) and by Smith *et al.* (1959) all had exceptionally high serum vitamin D activities, sufficient easily to account for the observed state of these patients, not unlike that to be expected from chronic vitamin D intoxication; it is thus not necessary to postulate any abnormal sensitivity to vitamin D in these patients. The high level of circulating vitamin D is, however, more difficult to explain; it could have arisen from undisclosed or unknowing administration of large doses of vitamin D before or during treatment, though it is unlikely that it would have escaped notice. Even if it had occurred, the serum vitamin D activity would not, to judge from the work of Warkany *et al.* (1942), have been expected to remain elevated for so long after removal of vitamin D from the diet. It may therefore be that certain instances of the severe form of idiopathic hypercalcaemia are brought about by a derangement of vitamin D metabolism that permits high levels of circulating vitamin D activity even when the subjects have received only average amounts from the diet (1000–5000 i.u./day). In patients with the benign form of the disease, all studies on serum and plasma vitamin D activity (Thomas *et al.* 1959; Cuthbertson, 1963) have shown the levels to lie within the normal range, so that the condition may be due to an unusual sensitivity to the vitamin. It is also possible, in both the severe and benign forms of the disease, that a derangement of Ca metabolism is primarily responsible, in that the condition is associated with an unusually efficient absorption of Ca (Creery & Neill, 1954; Bonham-Carter *et al.* 1955; Morgan *et al.* 1956), and that improvement follows reduction of the serum Ca levels, whether it

is brought about by treatment with cortisone, EDTA or phytic acid, or simply by maintenance on a diet low in Ca (Morgan *et al.* 1956). The single observation on liver stores, in a patient suffering from the benign form of the disease (Cuthbertson, 1963) and a control patient, again supports the suggestion that vitamin D metabolism is not deranged. Both the hypercalcaemic patient and the control had been receiving 3000 i.u. vitamin D/day, the control up till the time of the patient's death, though the vitamin had been withdrawn from the diet of the hypercalcaemic patient about 1 month before death. The fact that rather less vitamin D activity was found in the patient's liver than in that of the control is thus not surprising in view of the small amounts of vitamin D found in the livers of animals not given large doses of the vitamin (Devaney & Munsell, 1935) and the known rapid rate of disappearance of vitamin D from the livers of animals given the vitamin (Heymann, 1957; Warkany, 1936, 1937; Quarterman *et al.* 1963). These observations on liver vitamin D activity, as on plasma and serum activities, have failed to demonstrate anything abnormal about vitamin D activities encountered in at least the benign form of idiopathic hypercalcaemia. Rat biological assays, which have been used in all the studies quoted, can throw no light on the suggestion by Thomas *et al.* (1959) that the abnormality may reside in the accumulation within the body, and the failure to eliminate it, of a vitamin D derivative, such as, for instance, dehydrotachysterol, which may have a potent effect on Ca metabolism in man and yet have little or no antirachitic effect in the rat. Further investigation is required to determine whether the disease may be due to an abnormal sensitivity to vitamin D, since these patients show toxic responses to doses closely similar to those commonly needed to ensure normal utilization of Ca, or whether the condition may be due to an error in Ca metabolism and so to a large extent independent of vitamin D intake, at least within the range of vitamin D intakes commonly encountered in this country.

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The vitamin D activity of plasma of children with idiopathic hypercalcaemia

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Similarities between idiopathic hypercalcaemia of infants and vitamin D intoxication have led to the suspicion that the former may be caused by giving needlessly large doses of vitamin D to children who are more than usually sensitive to its effects or who absorb it with unusual efficiency. This view has been strengthened by the rarity of idiopathic hypercalcaemia in the USA and in Sweden, countries in which the vitamin D intake of children used to be much less than in the UK (Morgan, Mitchell, Stowers & Thomson, 1956).

In the USA Smith, Blizzard & Harrison (1959) reported a serum vitamin D activity of 23 i.u./ml in one patient, and Fellers & Schwartz (1958*a*, *b*) noted high activity, between 20 and 60 i.u./ml, in three patients. These findings support the view that idiopathic hypercalcaemia may be caused by overdosage with vitamin D. On the other hand, Thomas, Morgan, Connor, Haddock, Bills & Howard (1959) reported essentially normal vitamin D activities (1–3 i.u./ml) in samples of serums sent to the USA from patients in the UK. The work to be reported here was carried out further to investigate the condition in the UK.

EXPERIMENTAL

Samples. Samples of heparinized plasma, 5–10 ml, from patients suffering from idiopathic hypercalcaemia were tested, together with similar samples obtained at approximately the same time from control patients of about the same age and sex but not suffering from any abnormality of calcium or vitamin D metabolism. All diagnoses were made by the physicians and pathologists named in the acknowledgements, to whom I am deeply grateful for providing the plasma samples.

In one instance, serum samples from the hypercalcaemic patient and from the control were provided but this mistake is not thought to have affected the results, because in other experiments no significant differences could be detected between the vitamin D activity of serum and plasma from the same specimen of blood. All samples were frozen quickly (to prevent protein separation) and stored in the deep freeze until assayed. Tests not reported here have shown that storage in a deep freeze for a period of several months, with melting and immediate refreezing at monthly intervals, or storage for up to 14 days in the refrigerator (4°) does not lead to loss of vitamin D activity.

Livers from a child who died in idiopathic hypercalcaemia and from a control child dying from another condition were stored in the deep freeze until required. The livers were saponified by the method of E. Kodicek (personal communication). To each 50 g

Table 1. *Plasma vitamin D activity and serum calcium level of children suffering from mild idiopathic hypercalcaemia and of control children*

(Numbers in parentheses denote days before (−) or after (+) transfer to diet low in Ca and vitamin D. NR, not recorded)

Patient and sex ...	M.B., ♂	E.F., ♀	M.P., ♂	H.K., ♀	G.S., ♂	S.W., ♀
Date of birth	14. iii. 62	9. ii. 59	27. iv. 61	13. xi. 61	17. iv. 61	9. ii. 59
Date vitamin D withdrawn	1. x. 62	14. x. 59	16. xi. 60	1. x. 62	25. x. 61	11. i. 60
Date of introduction of diet low in vitamin D and Ca	1. x. 62	30. x. 59	16. xi. 60	1. x. 62	1. xii. 61	8. ii. 60
Approximate daily vitamin D intake before treatment (i.u.)	1350	2000	NR, probably 1000	2500	1000	2000–3000
Serum Ca at or near date of diagnosis (mg/100 ml)	12·9 (−14) 10·8 (−52)	13·0 (0)	11·4 (−5)	13·2 (−4)	11·5 (+36)	13 (−80) 9·8 (0)
Serum Ca at or near date of vitamin D assay (mg/100 ml)	12·9 (−14) 10·8 (+15)	13·0 (0)	10·4 (+9)	9·4 (+15) 10·7 (+29)	12·9 (+41) 12·4 (+43)	—
Plasma vitamin D activity (i.u./ml)	2·9 (−2)	3·0 (+3)	2·0 (+7)	2·1 (+25)	1·5 (+42)	1·4 (+140)
Plasma vitamin D activity of control patient (i.u./ml)	2·3	—	3·0	3·6	1·7	1·1

Mean plasma vitamin D activity of patients 2·1 i.u./ml and of controls 2·3 i.u./ml.

Table 2. *Plasma vitamin D activity and serum calcium level of children suffering from severe idiopathic hypercalcaemia and of control children*

(Numbers in parentheses denote days before (−) or after (+) transfer to diet low in Ca and vitamin D)

Patient and sex ...	A.C., ♀	P.B., ♂	A.B., ♂	S.T., ♂	S.D., ♀
Date of birth	15. v. 61	12. v. 59	17. iv. 61	26. ii. 61	26. iv. 59
Date vitamin D withdrawn	25. xi. 61	12. xi. 60	7. i. 62	11. xi. 61	17. x. 59
Date of introduction of diet low in vitamin D and Ca	25. xi. 61	9. xii. 60	7. i. 62	11. xi. 61	17. x. 59
Approximate daily vitamin D intake before treatment (i.u.)	1500	1000	1000	3000	1700
Serum calcium at or near date of diagnosis (mg/100 ml)	15·3 (−15) 16·1 (−11)	11·7 (+3)	11·4 (−1)	14·9 (+3)	14·7 (0)
Serum calcium at or near date of vitamin D assay (mg/100 ml)	16·1 (−11) 11·7 (+9)	11·8 (+11)	9·5 (+17)	13·4 (+16)	8·3 (+190) 9·2 (+210)
Plasma vitamin D activity (i.u./ml)	2·0 (0) 2·0 (+12)	* (+10)	1·7 (+18)	2·0 (+25)	1·0 (+200)
Plasma vitamin D activity of control patient (i.u./ml)	2·0, 2·0	*	1·7	2·0	1·0

Mean plasma vitamin D activity of patients 1·7 i.u./ml and of controls 1·7 i.u./ml.

* Statistical examination of the values from this assay showed that the vitamin D activity of the control and hypercalcaemic plasma did not differ significantly though the values were not sufficiently precise to permit a definitive statement of vitamin D activity.

of [illegible] acid 30 ml absolute alcohol and 4 ml saturated aqueous KOH. The mixture was then heated for 30 min on a steam-bath and then allowed to cool. The digest was extracted four times with equal volumes of peroxide-free diethyl ether. The extracts were washed with water to eliminate alkali, dried over anhydrous sodium sulphate, evaporated and taken up in arachis oil. The extracts were diluted further with arachis oil and assayed by the routine line-test procedure as used for oily vitamin D preparations in these laboratories (International Union of Pure and Applied Chemistry, 1959) on forty-eight rats in each assay. The vitamin D activity of the plasma samples was also determined by the line-test rat assay method. Litters of four or eight rats were placed on the rachitogenic diet at 21 days of age. After 14 days they were divided into groups, each of at least six rats, matched for litter origin but otherwise randomly selected. The animals in two groups were each given, daily, either 0·1 or 0·2 ml of a standard arachis oil solution containing 2 i.u. vitamin D/ml, those in the other groups being all given, daily, 0·1 ml of arachis oil with either 0·05 or 0·1 ml of the plasma sample. All doses were given orally by syringe for a 10-day period, at the end of which the animals were killed, the tibias were dissected, and the degree of rickets was assessed by the line-test procedure. The vitamin D activity of the test sample was then calculated in terms of the standard.

Table 3. *Vitamin D content of liver from a child with mild idiopathic hypercalcaemia and from a child not suffering from any known derangement of calcium or vitamin D metabolism*

	Hypercalcaemic child*	Control
Weight of liver (g) ...	175	136
Total vitamin D activity and fiducial range ($P.=0·95$) (i.u./liver)	120 (50–150%)	435 (43–1300%)
Vitamin D intake (i.u./day)	3000 until 1 month before death	3000

* First case described by Rhaney & Mitchell (1956).

Table 4. *Plasma vitamin D activity of children with different vitamin D (ergocalciferol) intakes*

Child and sex ...	K.B., ♀	P.H., ♂	M.P., ♀
Age	15 months	8 years	9 years
Condition	Aminoaciduria	Cystinosis	Vitamin D intoxication
Ergocalciferol dose (i.u./day)	1000	20000	150000
Period of dosing before test	8 months	6 years	20 months
Plasma vitamin D (i.u./ml)	3	≃10	> 20*

* The activity of this sample could have been very much higher than indicated.

RESULTS

The results obtained on examination of samples from six patients with the mild and five with the severe form of idiopathic hypercalcaemia are summarized in Tables 1 and 2 respectively, together with other relevant facts and the vitamin D activity of the samples from the control patients.

Table 3 provides information on the liver samples, and Table 4 on the [illegible]ts obtained on samples from three children with precisely defined vitamin D_2 (ergocalciferol) intakes.

DISCUSSION

The results given in Tables 1 and 2 show that the vitamin D activity of all the samples examined fell within the range 1·0–3·6 i.u./ml, which is in close agreement with results of determinations made by Thomas *et al.* (1959) on normal subjects. Further, they show that the values for patients with the severe or the mild form of the disease do not differ from each other or from those for the controls. One of the patients, H.K., was a twin, and her twin sister was used to provide a control plasma sample. Both children were brought up and fed in the same way and given the same vitamin supplements until admission of the affected twin to hospital, except that for some time before entering hospital the affected twin had a poorer appetite.

In only five instances were samples obtained when the patient had been on a diet free from vitamin D for less than 10 days. It could therefore be argued that plasma vitamin D activity might originally have been high but that this activity had fallen in a number of the patients during the time they had been kept on the vitamin D-free regimen before assay. Had it been so, then the difference between the activities of samples from patients and controls should have been much greater for patients M.B., A.C., E.F., P.B. and M.P. than for the others who had been under treatment longer before examination. In fact, the values for these patients did not differ from those for their controls any more than did the others in this series.

Smith *et al.* (1959) and Fellers & Schwartz (1958*a*, *b*) showed that the high serum vitamin D activity noted in their patients with idiopathic hypercalcaemia fell only slowly after they had been transferred to a vitamin D-free diet. Warkany, Guest & Grabill (1942) and Thomas *et al.* (1959) have shown that serum vitamin D activity, once elevated by administration of vitamin D, may take several months to return to normal. Thus if any of the patients in this series had had significantly elevated plasma vitamin D activity before withdrawal of vitamin D, then this value would be expected still to have been higher than the control value for several months afterwards. In fact, significantly higher levels were not noted in any of the patients in this series, so that it is unlikely that the condition in these patients could have been associated with a high plasma vitamin D activity.

This conclusion is supported by the figures in Table 3, which show that the liver stores of a child who died in idiopathic hypercalcaemia were in fact smaller than those of a child of the same age who had been receiving about the same amount of vitamin D. The hypercalcaemic child had not been given any vitamin D for 1 month before death. This fact could explain the lower vitamin D content of the liver, but the fact that the child was certainly suffering from idiopathic hypercalcaemia at death shows that a high vitamin D status is not necessary for demonstration of the condition. Unfortunately the vitamin D activity of the plasma could not be determined, but the liver vitamin D activity, about 1–2 i.u./g, was not much different from that expected in plasma from children of this type. Devaney & Munsell (1935) showed the vitamin D

content of livers of sheep, cattle and pigs to be about 0·1–0·5 i.u./g, which is somewhat lower than the activity of serum (0·5–1 i.u./ml) found in the same species by Warkany (1937). More recently Quarterman, Dalgarno & Adam (1963) have shown in the pig that blood vitamin D activity is higher than that of the liver. Our findings may therefore indicate that in man, as in some other species, liver vitamin D stores are small, at least in individuals receiving moderate amounts of the vitamin.

Table 4 shows that 1000 i.u. vitamin D/day can maintain a plasma vitamin D activity of about 3 i.u./ml, doses of 20000 i.u./day can cause a marked rise in the plasma vitamin D activity, and administration of 150000 i.u./day causes a still further increase in plasma vitamin D activity. It appears that the plasma vitamin D activities found both in the idiopathic hypercalcaemic patients and the controls of this series are what would be expected from the vitamin D intakes of the children as calculated from the available information on the foods and vitamin supplements given to them. The results obtained agree with those reported by Thomas *et al.* (1959) for two mildly affected patients (R. G. Mitchell, 1962, personal communication) but differ markedly from those of Fellers & Schwartz (1958*a*, *b*) and Smith *et al.* (1959). The last two groups of workers reported high serum vitamin D activities in their hypercalcaemic patients (all of the severe type). My own observations do not support their hypothesis that an abnormality of vitamin D metabolism associated with an increase in serum vitamin D activity is a necessary prerequisite for the disease, though it may sometimes be so. A more likely explanation is an abnormal sensitivity in these patients to the toxic effects of vitamin D or an abnormality in calcium metabolism not caused by vitamin D. However this may be, the results show that in this series of patients the disease could not be attributed either to an unusually high intake of vitamin D or to an unusually high plasma vitamin D activity.

SUMMARY

1. The plasma vitamin D activities of a series of eleven children suffering from idiopathic hypercalcaemia (five with the severe and six with the mild form of the disease) were found not to be significantly elevated in comparison with values determined at the same time on plasma samples from healthy children of like sex and age.

2. The vitamin D content of the liver of a patient dying with idiopathic hypercalcaemia was found to be lower than that of the liver from a control patient not suffering from any derangement of calcium or vitamin D metabolism.

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 70(6); 952, 1967 1267

Consumption of vitamin D in fortified and natural foods and in vitamin preparations

Data were obtained from seven-day records of the daily intake of vitamin D in measured amounts of fortified and natural foods and in vitamin preparations. The 150 subjects ranged in age from newly born to 17 years. The daily average varied from 21 to 1,800 I.U. Of the total group, 9.3 per cent consistently had intakes above 1,000 I.U.; 31.3 per cent, below 400 I.U.; 3.3 per cent, less than 100 I.U.; and 50 per cent, from 400 to 800 I.U., optimal range. Milk was found to be the most uniform source of vitamin D for all age groups; other vitamin D fortified foods were not needed to bring consumption to the desired level.

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BECAUSE OF THE widespread fortification of foodstuffs and the increasing awareness and understanding of hypervitaminosis D,[1-4] it was deemed important to study the actual daily intakes of vitamin D from various sources as ingested by a group of children and adolescents.

METHOD

Subjects. The subjects, chosen within the Seattle and Everett area and ranging in age from newly born to 17 years, were in good health at the time of the study. Six groups of 25 subjects each were studied. Infants from birth through 12 months were randomly selected from the Well-Child Clinic of the Division of Child Health, the Department of Pediatrics, University of Washington. All of the subjects in the other five age groups were randomly selected within the Everett and Edmonds public school districts. They were grouped as follows.

Preschool	(2 to 5 years)
Early elementary	(6 to 8 years)
Late elementary	(9 to 11 years)
Early adolescence	(12 to 14 years)
Later adolescence	(15 to 17 years)

Data gathering. A tabulated form consisting of two sections was designed for recording the intakes of vitamin D: one from fortified foods and the other from natural food sources. The list of fortified foods was

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compiled by examining the labels of all food items in five local supermarkets. The fortified foods, which varied from cereal products to diet foods, were listed on the data sheet. Sixty-five different kinds of ready-to-eat cereals were checked: seven were fortified with vitamin D, varying in amounts from 40 to 400 I.U. per one-half cup serving. Margarine, milk, chocolate flavorings, diet foods, and Tiger Shake had varying amounts of vitamin D added. The second part of the data sheet consisted of foods which contain in their natural state 150 I.U. or more of vitamin D per half cup: tuna, shrimp, sardines, salmon, and herring. A place to record the kind, amount, and regularity of intake of a vitamin preparation was provided at the bottom of the sheet.*

Procedure. Careful instructions were given to the subjects, according to their ability to participate, and to the mothers who would need to do the recording. The recording procedure was explained by identifying the foods listed on the data sheet; techniques for measuring the amounts of foods eaten were demonstrated, and an example of the recording technique was given in detail. Those participating were instructed to return the data sheet after seven consecutive days of recording. The same data sheet was used for all groups with each sheet keyed to make it anonymous. The records were collected for 150 subjects during the period from November 1, 1965, to December 17, 1965.

RESULTS

The data revealed widespread average daily intakes (a.d.i.) for the total group (Fig. 1), as well as variability in the daily intakes of the individual subjects. The range of the preschool group, with the highest a.d.i. (660 I.U.), was 21 to 1,137 I.U.; in the infancy group, with the lowest a.d.i. (462 I. U.), it was 130 to 914 I.U.; in the 150 subjects the a.d.i. was 547 I.U., including all sources, and the range was 21 to 1,800 I.U. Table I records the amount contributed by each source of vitamin D to the total a.d.i. for each age group. Milk is shown as the most uniform and dependable source of vitamin D for all age groups.

The a.d.i.'s in this study were not particularly high. Two, or 1.3 per cent, had an a.d.i. greater than 1,500 I.U. for the seven days; 12, or 8 per cent, averaged 1,001 to 1,500 I.U.; and 14, or 9.3 per cent, averaged 801 to 1,000 I.U. There were high individual intakes not reflected in the a.d.i. Twice during the week, three consumed above 2,000 (2,910, 2,300, 2,140), and three others, 1,500; seven, over 1,000 on two or three days; and four, over 1,000 I.U. at least once in the seven days.

The optimal range of intake for growth and for calcium and phosphorus retention has been stated as 400 to 800 I.U.[5,6] with 340 to 600 I.U.[5,7] giving maximum increases in linear growth. In this study, 75 subjects, or 50 per cent, had an a.d.i. from 400 to 800; 62, or 41.3 per cent, had between 340 to 600; 47, or 31.3 per cent, had less than 400; and 5, or 3.3 per cent, had less than 100 I.U. For six individuals no vitamin D intake was recorded for five out of the seven days.

COMMENTS

The source of vitamin D which made the greatest contribution to the total intakes varied with the age group and with eating patterns as shown in Table II. As the child grows older, he consumes a wider variety of foodstuffs and, therefore, includes many of the fortified foods. This can result in irregular and unpredictable consumption of vitamin D.

Vitamin preparations were important sources of vitamin D for the infancy and preschool groups. The consumption of fortified foods increased with age. The group aged 9 through 14 years consumed consistently more fortified foods such as cereals, chocolate flavorings, margarines, and diet foods, than did other age groups. This is also the group in which there were individuals with high intakes between 1,500 and 2,000 I.U. An analysis of an individual case shows an

*The natural prophylactic agent, toddy, was not included in the computation of vitamin D intake.

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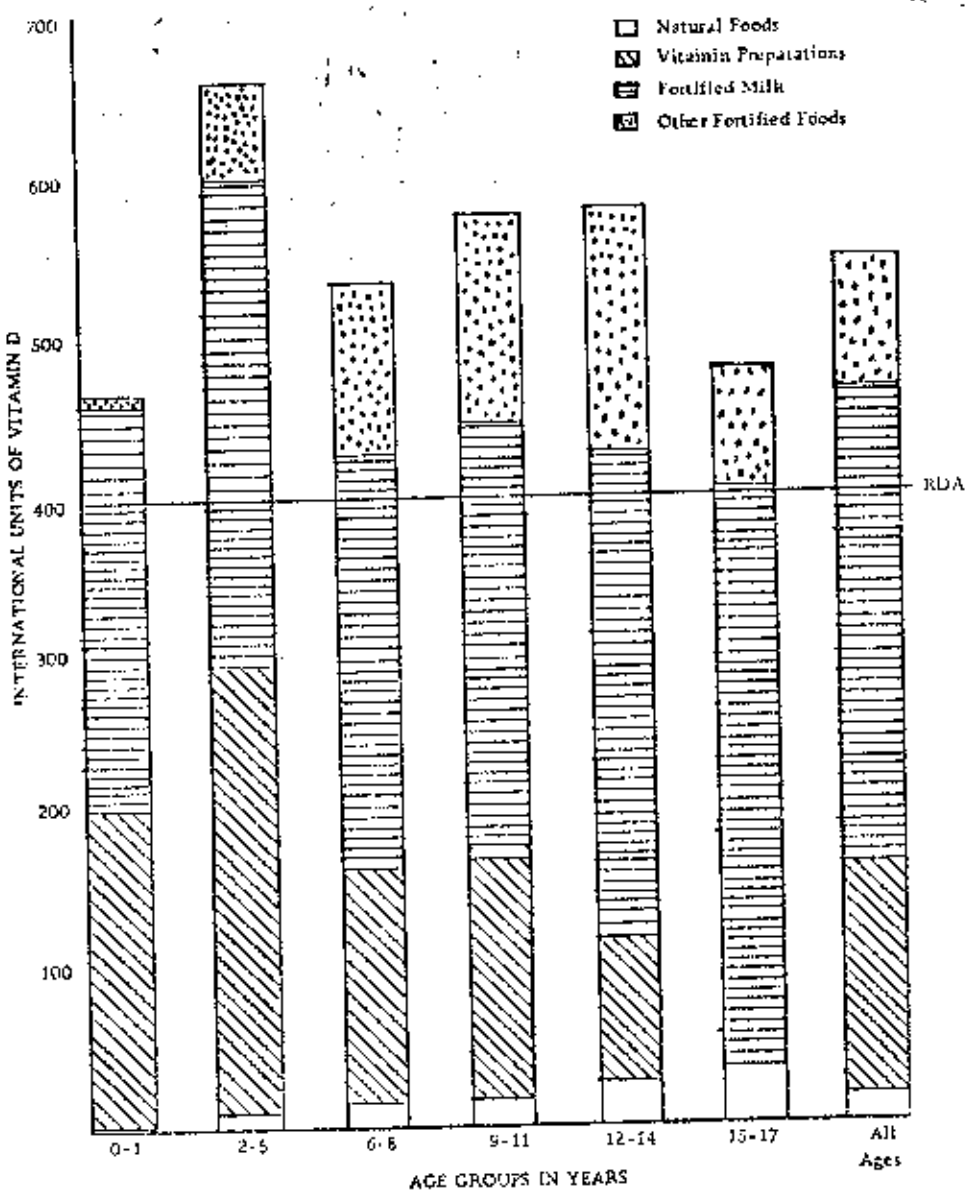

Fig. 1. Average daily intakes of vitamin D by age groups and sources.

Table I. Average daily intakes and contributing sources of vitamin D for each age group (I.U.)

Group	Total a.d.i.	Vitamin prep.	Milk	Food sources*		Total
				Fortified	Natural	
Infants	462	201	257	4	Trace	261
Preschool	660	283	309	59	9	377
Early elementary	532	146	280	91	15	386
Older elementary	578	151	270	135	46	427
Early adolescents	579	92	308	153	26	487
Older adolescents	477	0	367	74	36	477
All groups	517	145	300	84	18	402

*Other than milk.

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Table II. Range (high and low) of the average daily intakes of vitamin D by sources and age-groups*

Age groups	Sources						
	Cereals	Dairy	Diet foods	Margarines	Chocolate flavorings	Natural foods	Vitamin prep.
0–12 mo.	0–16	248–262	0	0.6–1.9	0–4	0–2	201
2–5 yr.	6–32	238–360	0	13–22	18–42	2–30	272–292
6–8 yr.	0–72	247–313	0	23–35	20–41	8–28	136–148
9–11 yr.	35–121	262–288	0–16	16–31	8–37	0–47	128–168
12–14 yr.	0–79	287–336	0–16	32–42	31–64	2–73	92
15–17 yr.	0–35	325–387	0–20	26–47	8–38	20–66	0

*Expressed in international units.

eleven-year-old boy with a day's intake of 2,910 I.U. from two cups of fortified hot chocolate (made with fortified milk), three cups of fortified cereal, two cups of fortified milk, and some fortified margarine. In most instances, intakes above the recommended level of vitamin D were the result of consumption of vitamin D-fortified foods in addition to taking vitamin preparations, as shown in Fig. 1.

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ITALIAN TRANSLATION

Vitamin D Overdosage Syndrome

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Vitamin D, when administered in excess (both in a single "stoss" dose and in moderate, long-term dosage), can give rise to a characteristic hypervitaminic syndrome.

In 1949, R. Debré reported the first large-scale case study, covering 250 subjects with hypervitaminosis D, among which there were 11 fatal cases; in 1953, Frontali, on the basis of 300 cases reported in the literature (including 17 fatal cases) and while reporting 5 personal cases, described the symptoms caused by an excess dosage of vitamin D and defined them as being characterized by anorexia, vomiting, stypsis, intermittent fever, hepatomegalia, hypercalcemia, decrease of serum phosphatases, hypercalciuria, hyposthenuria and polydipsia.

In fact, we are dealing here with a syndrome, the external pathology, physiopathology and symptoms of which have already been the object of extensive discussions, but which, nevertheless, is still of special interest today in view of the variable nature and frequency of the cases observed.

It is noted that the doses capable of provoking the syndrome of vitamin D overdosage vary according to the constitutional state of every single subject (Willi); not all subjects to which high doses of vitamin D are administered are subject to phenomena of intolerance and overdosage. Generally recognized

factors, which play a considerably important role in determining whether subjects can be affected by vitamin D, include the age, the state of mineralization, and according to Illig and Prater, solar irradiation, and, according to Willemin-Clog and G. Menut, reduced growth, and in some cases the coincidence (Goormaghtigh 1938, Hooft 1948, Fanconi 1952, Johnson 1952, Waylor 1955, Royer 1958, Willemin-Clog and G. Menut 1968) of particular conditions, such as thyroidal insufficiency and sarcoidoses of Besnier-Baeck-Schaumann.

We must point out, however, that in the majority of cases it is difficult to understand the mechanism underlying and responsible for hypervitaminosis D.

Acute and chronic cases have been described, and light transitory forms, with spontaneous recovery and only slight signs of generic intoxication (nausea, vomiting, anorexia, polyuria, dehydration and signs of hypoadrenalism), as well as severe forms with more marked functional signs and primarily with irreversible anatomic complications (with calcareous deposits in the epiphyses, in newly formed bones, in the entire vascular stystem, in the heart and kidneys).

In regard to age, as was stated above, it plays a prominent role in determining the syndrome of hypervitaminosis D, which occurs most frequently in the early (primary stage) of infancy, particularly between 6 and 24 months (Willemin-Clog and Menut), and more rarely during the second and third stage of infancy (Hollinger and Pattee, Durand, Faccini, Serafini and Svikolos).

The present case study was prompted by the relatively high incidence of vitamin D overdosage syndromes encountered within a short period of time (8 cases in about 60 days); this fact has stimulated us to analyze our case records during the period of 1960 to June 1963, in order to obtain therefrom inferences (deductions) of a clinical and practical order.

The analysis of our cases was based primarily on studies of the renal function, and in particular we have tried to study in depth the tubular functional capacity (a residue of the harmful effect of vitamin overdosage), with repeated control of 24-hour diuresis, of the number of ions excreted with urine within 24 hours, and of the acidification capacity of the kidneys with measurements of the urinary pH.

We have also performed studies of the blood chemistry, by controlling (checking) electrophoresis, alkaline reserves, (blood electrolytes) azotemia, and cholesterolemia.

In regard to the present knowledge of the effect exerted by plasma electrolytes on cellular functions in general and on cells of the nervous system and of the myocardium in particular, we have performed a study of electroencephalographic and electrocardiographic charts. In addition, we have tried to exclude eventual diseases of the endocrine glands (concerning the hypophysis, parathyroid glands, adrenal glands and gonads), and the eventual influence of mineral active hormones (desoxycorticosterone) and of glucoactive hormones (insulin and adrenalin), exerting multiple and various electrolytic effects.

Case Study

Our case study covers 12 infants affected by intoxication due to Vitamin D overdosage, of varying severity, between 7 and 30 months old, undergoing treatment in the Pediatrics Division of the Udine Civil Hospital.

The clinical diagnosis, based on objective and case history data, was confirmed in all cases by laboratory data. Each case is fully summarized in the attached Table I.

TABLE 1

Observation Osservazione ① N. Nome, sesso, età	② Data del ricovero	3 Motivo della prescrizione	4 Dose di vit. D somministr. Totale mgr	per day per die mgr	5 Sintomatologia che ha condotto il paziente alla nostra osservazione 6 Anoressia	7 Stipsi	8 Vomito	9 Perdita di peso	10 Anemizzazione	11 Poliuria	12 Polidipsia	13 Ipertermia	14 Convulsioni	15 Stato tossico (disidratazione cutanea)	16 Astenia	17 Mutamento del carattere
1) Z. E. ♀, 13 m. (months)	1-9-1959	a) Ricostituente	120	1,2	+	+	+	+	++	+	+		+			
2) B. M. ♂, 10 m. (year)	1-7-1959	a) Ricostituente	120	4	+	+		+	++	+	+	+		+	+	
3) C. D. ♀, 1 a., 1 m. (year)	20-8-1959	b) Profilassi antirachitogena	240	1,5	+	+	+	+	+	+	+	+		+	+	a) Sonnolenza
4) P. B. ♂, 9 m.	15-10-1960	c) Profilassi della tetania rachitogena	30	2,5	+	+		+	++	+	+		+	+	+	Instabilità
5) Z. D. ♀, 2 a. (years)	16-7-1951	c) Profilassi della tetania rachitogena	105	2	+	+		+	++	+	+			+	+	Instabilità b)
6) C. G. ♂, 10 m.	1-3-1963	b) Profilassi antirachitogena	150	1,6	+	+	+	+	++	+	+			+	+	Instabilità
7) M. G. ♀, 1 (6) (year)	27-3-1963	a) Ricostituente	50	1,7	+	+		+	+	+	+	+		+	+	Irrequiet. c)
8) C. A. ♀, 7 m.	17-4-1963	a) Ricostituente	50	1,6	+	+		+	+	+	+			+	+	Irrequiet. c)
9) N. V. ♀, 1 (2), 1 m. (year)	5-6-1963	a) Ricostituente	25	1,5	+	+		+	++	+	+			+	+	Irrequiet. instabilità d)
10) C. N. ♀, 2 (6)	1-7-1963	a) + b) Ricostituente profilassi antirach.	60	1,5	+	+	+	+	++	+	+	+		+	+	Irrequiet. c)
11) C. M. ♀, 1 (6) (years)	10-7-1963	a) Ricostituente	60	1,5	+	+	+	+	++	+	+	+		+		Irrequiet. c)
12) M. L. ♂, 2 (6) a., 4 m.	26-7-1963	a) Ricostituente	26,5 + derab.	1,7	+	+	+	+	++	+	+	+				Apatia e)

Clinical Data Referring to Each Case Are Reported

1-Name, sex, age
2-Date of admission to hospital
3-Purpose of prescription
4-Doses of Vitamin D administered
5-Symptoms which led patient to our observation
6-Anorexia
7-Stypsis
8-Vomiting
9-Weight loss
10-Degree of anemia
11-Polyuria
12-Polydipsia
13-Hyperthermia
14-Convulsions
15-Toxic state (skin dehydration)
16-Asthenia
17-Personality changes

(Under (3) - from top to bottom:)

a-Tonic
b-Antirachitogenic prophylaxis (prevention of rickets)
c-Prevention of rachitogenic (rickets) tetany

(Under (17) - from top to bottom:)

a-Sleepiness
b-Instability
c-Restlessness
d-Restlessness and instability
e-Apathy

TABLE TABLE 1 (cont.)

18 Diagnosi di ricovero	19 Dati anamnestici	20 Classificazione anxologica secondo de Toni Gobessi (medie genovesi)	P.A. ARTERIAL PRESSURE	E.C.G.	E.E.G.	21 Prova pitressina	22 Prova di alcalinizzazione	23 Segni radiologici	Terapia Therapy	Evolution Evoluzione
a) Cistite; accessi convulsivi	Allattamento materno		105/75		+	Neg.				Favorable
Cistite	a) Allattamento materno		105/75	+		Neg.				Favor.
Cistite b)	b) Genitori consanguinei, allattamento materno	Pachisomia a)	125/75			Neg.		+	a) Reidratante alcalinizzante	Favor.
b) Crisi convulsiva	a) Allattamento materno	Tiposomia b)	95/55		+	Neg.		—	a) Reidratante alcalinizzante	Favor.
c) Crisi convulsiva	a) Allattamento materno	Microsomia c)	80/55	+	+	Neg.		+	a) Reidratante alcalinizzante	Favor.
b) Cistite	a) Allattamento materno	Ipozomia d)	90/55	+	+	Neg.		+	a) Reidratante alcalinizzante	Favor.
d) Turbe del carattere	a) Allattamento materno	Nanismo, leptosomia e)	80/50	+	+	Neg.	a) Esito patologico	—	a) Reidratante alcalinizzante + desametaz. b)	Favor.
b) Cistite	a) Allattamento materno	Tiposomia b)	85/55	+	+	Neg.	a) Esito patologico	+	Reidratante alcalinizzante + desametaz.	Favor.
b) Cistite	a) Allattamento materno	Macroleptosomia f)	95/60	+	+	Neg.		—	a) Reidratante alcalinizzante b)	Favor.
e) Tossicosi	a) Allattamento materno	Ipersomia g)	105/70	+	—	Neg.	a) Esito patologico	—	a) Reidratante alcalinizzante + desametaz.	Favor.
1) Turbe del carattere	a) Allattamento materno	Tiposomia b)	90/65	+	—	Neg.		+	a) Reidratante alcalinizzante + desametaz.	Favor.
Turbe del carattere	a) Allattamento artif.	Tiposomia b)	65/50	+	—	Neg.	b) Nei limiti della norma	—	a) Reidratante alcalinizzante	Favor.

19-Case history data
20-Embryological classification of Toni-Gobessi (Genoa media)
21-Pitressin test
22-Alkalinization test
23-Radiological signs

(Under (18) - from top to bottom:)

a-Cystitis; convulsive fits
b-Cystitis
c-Convulsive crises
d-Personality changes
e-Toxicoses

(Under (19) - from top to bottom:)

a-Maternal feeding
b-Parents related by blood, maternal feeding

(Under (20) - from top to bottom:)

a-Pachisomia
b-Typosomia
c-Microsomia
d-Hyposomia
e-Nanism, leptosomia
f-Macroleptosomia
g-Hypersomia

(Under (22) - from top to bottom:)

a-Pathological result
b-Within normal limits

(Under "Therapy", from top to bottom:)

a-Alkalizing rehydration agent
b-Desametazone treatment (?)

Cases 1 and 2 were the objects of a communication made by Prof. 1. Sala to the Friuli Medical Society in 1960.

A more extensive description is given of case 12, in view of the special features of the clinical pictures due to the association of vitamin D_2 and norandrostenolone phenyl propionate (durabolin).

Case #12. M. L., 2 years and 3 months old, male, born at Pagnacco (Udine) on 13 April 1961, admitted to hospital on 23 July 1963. Nothing special in the ancestry, parents living and in good health, not related by blood. First-born child. Born at the end of pregnancy with a normal physiological course, easy delivery. Regular psycho-physical development.

The parents, on medical advice, started to give the child, on 9 June 1963, as a tonic, an oral dose of 400,000 international units (I.U.) of vitamin D_2 every eight days, and in addition, two teaspoonfuls daily (10 cc/day) every 15 days parenterally of a syrup of a colloidal calcium base containing 1500 I.U. vitamin D_2 per cc together with 5 mg norandrostenolone phenyl propionate.

Altogether, the child received, during 45 days, a total of 3,075,000 I.U. of vitamin D_2, equal to 76.5 mg vitamin (1.7 mg/day) and 15 mg norandrostenolone phenyl propionate (durabolin).

At the start of treatment, the child exhibited symptoms of anorexia, progressive pallor and restlessness. After the second vial of durabolin, the child showed crises of psychomotor agitation and priapism. Since the condition of the child deteriorated as a result of a significant weight loss and the appearance of polydipsia and polyuria, he was admitted to the hospital for a check-up. On objective examination, the child exhibited a poor general condition, intense pallor, a threadlike pulse of 140, an

anelastic superdehydrated skin, a low-intensity protomesosystolic breathing on foci of the base and on the mesocardium, and anosteotendinous hyperreflexia. Laboratory tests (Tables I, II, III) confirmed the clinical signs of vitamin D intoxication. The evolution of the disease showed a rapidly favorable course, although only a rehydrating and alkalizing treatment was carried out.

TABLE 2

Case no.	Ca	P	K	Na	Cl	ALKALINE RESERVES	Fosfatasi alcaline UKA (1)	Azotemia (2)	Colesterolemia (3)	Glicemia (4)	A	Elettroforesi (5)					(6)
												α_1	α_2	β	γ	QA/G	Prot. tot.
1)	15,4	4,5	—	—	—	—	—	32	—	—	—	—	—	—	—	—	—
2)	17	4,9	13,5	340	371	50	13	25	139	1	—	—	—	—	—	—	6,5
3)	11,5	4,5	—	—	510	—	6	25	—	—	—	—	—	—	—	—	—
4)	11,9	5,1	—	—	—	38	6	26	—	—	—	—	—	—	—	—	—
5)	11,5	4,8	—	—	—	38	8	24	—	—	—	—	—	—	—	—	—
6)	10,7	4,2	—	—	—	41	3	30	—	—	—	—	—	—	—	—	—
7)	12,15	3,2	14,5	—	635	33	4	26	235	0,85	46	7	16	15	14	0,85	5,60
8)	10,85	4,7	15	305	381	35	8	32	270	0,90	46	7	17	12	18	0,85	6,90
9)	11,15	5,3	—	—	—	42	15	28	—	—	—	—	—	—	—	—	—
10)	13,05	3,2	17	285	549	30	6	36	195	1,15	47	7	17	16	13	0,83	7,20
11)	12,4	4,5	—	—	—	41	7	25	229	—	46	7	19	15	13	0,85	7,90
12)	20,4	2,8	—	—	—	43	7	26	131	—	59	5	12	12	21	1	6,45

Blood Chemical Data Referring to Each Case Are Reported

1-Alkaline phosphatases UKA
2-Azotemia
3-Cholesterolemia
4-Glycemia
5-Electrophoresis
6-Total proteins

Blood chemical data referring to each case are listed in Table II, and urinary data are listed in Table III.

Results of alkalinization tests are given in Table IV; unfortunately, for contingent reasons, the ability to maintain a correct acid-base equilibrium could be investigated only in four cases out of 12.

TABLE 3

Caserta (1)	Diuresi 24 h cc	P H urine Sd (2)	P H urine 24 h (3)	Albuminuria 24 h (4)	Sedimento urine 24 h (5)	Ca urine 24 h	P urine 24 h	Na urine 24 h	K urine 24 h	Cl urine 24 h
1)		1014	6			+++				
2)		1008	8	—	++	162				2600
3)	1300 *	1006	7	+	+	250				
4)	1300 *	1004	8	—	—					
5)	1700	1006	8	—	—	231				
6)	1900	1004	8	—	—	380	340	2710	610	
7)	1950	1004	8	—	—	368	1310	1820	810	1809
8)	1000 *	1011	8	—	+	240	1630	1270	680	1914
9)		1006	7	—	—	261				
10)	1100 *	1007	8	+	—	215	1267	1590	390	2810
11)	1750	1005	8	+	+	321	614	700	342	655
12)	1200		7	—	—	190				

(6) *) I dati segnati sono parziali in quanto data l'età dei pazienti non è stato possibile una raccolta completa.

Data Referring to Urinalysis Are Reported

1-Diuresis, cc/24 hour
2-Urine density, 24 hour
3-pH of urine, 24 hour
4-Albuminuria, 24 hour
5-Urine sediment, 24 hour
*6-The data listed in the table are partial figures, due to the fact that a complete collection of urine was not possible in view of the age of the patients.

TABLE 4

(2) Prima del carico di bicarbonato (3) Controlli eseguiti (4) Dopo il carico di bicarbonato

	I	II	III	IV	I	II	III	IV
Case # 7 case pH urine	6,9	7,1	7,3	7	7,5	8	7,8	8
R.A.	33	37	32	35	44	41	36	44
Case # 8 case pH urine	7,5	7,4	7,8	7,8	8	8	8	8
R.A.	39	41	35	33	41	31	38	37
Case # 10 case pH urine	8	7,3	7,3	7,5	8	7,8	7,9	8
R.A.	30	33	35	32	39	37	34	34
Case # 12 case pH urine	7	7,1	6,5	7	7,5	8	7,8	8
R.A.	43	46	48	45	50	52	48	50

ALKALINE RESERVES

1-Alkalinization test (administration of 6 g Na bicarbonate within 24 hrs., i.e. 0.25 g/hr). Determinations of urinary pH and alkaline reserves in blood were carried out every 6 hrs. within the 24-hr. period immediately preceding and following bicarbonate administration; during this period, the patients were kept on a diet and on alkalizing solutions.
2-Prior to bicarbonate administration
3-Controls performed
4-After bicarbonate administration

Discussion

For all the cases presented here, both the case history, the objective examination, and the clinical data have made it possible to exclude, first of all, whether the syndrome encountered in each case does or does not represent a basic morbid condition (neoplasms of the skeleton, multiple myeloma, bone atrophy, etc., as a hyperparathyroidism, milk-alkaline syndromes, and anidiopathic hypercalcemia).

The vitamin D doses administered to our patients must be considered today as definitely excessive (too high) for children of their age, even though the noticeable individual variation in their sensitivity makes it difficult to determine what is the effectively excessive dose of this vitamin.

In summary, we have reported in Table 1, 12 cases of intoxication due to vitamin D_2 overdosage, and in 10 of these cases it was possible to establish a clearcut picture of renal tubular acidosis. The group involving a hypercalcemia exhibits common typical urinary symptoms, such as polyuria, hyposthenuria, pollakiuria, etc. This takes place in primary hyperparathyroidism, in sarcoidoses, in the hypercalcemia of tetraplegics with the sudden arrest of osseous growth (poliomyelitis), inidiopathic hypercalcemia, and finally in hypervitaminosis D. The disturbances of the renal function, common to these syndromes, reflect the struggle of the organism against hypercalcemia, thus giving rise to the picture of renal tubular acidoses, as described by Pines and Mudges in 1951 (Amer. J. Med. 11, 302, 1951), determined by a tubular pathology well defined by now, although its pathogenesis has not yet been clarified.

The clinical picture has been given the name of the syndrome of Lightwood (1953), who was the first one to describe it. In 1936, Butter, Walson, and Faber described a case of acidosis with diffuse nephrocolcinosis in a male child. In 1946, Albright discussed these cases as states of renal tubular insufficiency without a glomerular insufficiency.

In order to understand this syndrome, we must remember the important role of renal control in the acid-base equilibrium, and that a hypotonic glomerular filtrate (pre-urine) becomes isotonic under the effect of the proximal tubule: it reabsorbs 85% of the water and sodium; 80% of chlorides and bicarbonates; the major part of K, Ca, Mg, P, S, glucose, proteins, and amino acids, and thus the filtrate becomes hypertonic due to the effect of the distal tubule, which reabsorbs the remaining portion (20%) of bicarbonates, of water, chloride, phosphates and separates from them K and ammonia. The activity of the tubule cannot be explained only on the basis of enzymic processes promoted and inhibited by various hormones and other substances. Vitamin D exerts a substantial effect on the activity of the tubule: it intervenes in the regulation of the acid-base equilibrium, in the elimination of amino acids, proteins and citric acid, and above all in the elimination of Ca and P. A slight intoxication by vitamin D causes acute and subacute disruptions of these various tubular functions and, in the final analysis is the cause of renal tubular acidosis, that is of a renal tubular insufficiency without glomerular insufficiency and thus without a hyperazotemia.

A good demonstration of this fact are the eight cases out of 12, in which, as far as it was possible, we have studied the tubular activity (Tables 1, 2, 3).

In Table 1, other data of interest can be noted:

1) First, out of 12 cases, 7 are females and 5 males, and this fact agrees with what is generally admitted; namely, that sex does not play any role whatsoever in the determination of the clinical picture.

2) We are dealing here with subjects which are all very young, 11 of which are in their early infancy, and one is 28 months old.

3) In regard to the date of hospital admission of each patient, we find that none of the cases were admitted in winter or in late fall. All authors are in agreement that solar radiation is a predisposing factor for hypervitaminoses (Illig and Prater, Royer, Fanconi). There is no doubt that the seasonal factor has played a considerable role in our cases. In fact, three cases were admitted in the spring, while eight were admitted during the summer, and one in early autumn.

4) In all cases, the motive (purpose) of prescribing vitamin D was the treatment of rickets, and in two cases for the treatment of an assumed latent spasmophilia.

5) The doses administered in each case were always high, both if we consider the daily doses, which vary in our case study (the minimum daily doses were 48,000 I.U., and the maximum daily doses were 200,000 I.U.), and the total dosage (between 1,200,000 and 10,000,000 I.U.). In our data, it does not seem that the clinical picture was influenced as much by the total dose, as rather by the daily dose (cases 4, 7, 8, and 9); in fact, cases 7, 8, and 9 had received high daily doses during a brief period of time.

6) We shall not dwell a long time on the symptoms of overdosage, since these are well recognized elements; we shall merely call attention to the neurological and psychic symptoms, which are often present in such cases. Cases 2 and 4 had exhibited convulsions, while the history of 10 cases discloses character disturbances.

7) Six children were admitted for reasons of cystitis, 4 for character disturbances, and 2 for convulsions.

8) All infants, except one, were breast-fed with maternal milk, a condition which is far from favoring the appearance of rickets.

9) The embryological classification includes 3 typosomics, 4 physically overdeveloped infants, and 3 physically underdeveloped infants. It is known that a normal child tolerates vitamin D to a lesser extent than a rachitic child. There is no doubt that an error in prescription for the child, whatever the the child is, either of deficient or excessive development or typosomic, is not modified in any way by its constitutional aspect.

10) Nine electrocardiographic charts showed clear signs of hypercalcemia, and seven out of ten electro-encephalographic charts were already altered.

11) The pitressin load test was negative in all patients.

12) In six cases, therapy consisted simply in the administration of rehydrating and alkalizing agents, due to a greater state of injury, treatment with desametazone was also used. In every case, progress was favorable, even in distance controls.

From the data given in Tables 2, 3, and 4, we can see that the plasma and urinary data are trulycharacteristic for hypercalcemic states.

Among the blood chemical data, we shall dwell on the following:

1) The greatly lowered alkaline reserves in 8 cases out of 9. The reduction of the CO_2 percentage present in blood is due to the decreased renal acid genesis (formation), a cause of loss of bicarbonates, and thus of a minor CO_2 reabsorption.

In 4 cases, the tubular genetic acid power was studied by carrying out the alkalinization test with repeated administrations of Na bicarbonate; the result of this test was quite significant and probationary for the reduction of this power. An increase of the urinary alkalinity was noted, and a slight increase the alkaline reserves of, which nevertheless always remained low and never reached normal values.

As was noted in a normal subject, administration of bicarbonate is followed by a very small increase, or no increase at all, of urinary alkalinity and, on the contrary, by an increase of the alkaline reserve in blood.

The data obtained by us with this test in the four subjects examined (Table 4) clearly demonstrate the existence of a tubular

damage, that is the inability of the renal tubule to split bicarbonate by means of carbonic anhydrase and to reabsorb CO_2.

The data reported by us in Table 4 appear to us to be quite important, since even today we do not have a perfect knowledge not only about the action of vitamin D at the renal level, but mainly about the more immediate damage caused by the vitamin at this point.

In 1958, de Luca et al. were the first to demonstrate experimentally in vitro, the harmful effect of vitamin D on renal mitochondria.

On the basis of the experiments of de Luca et al., Harman, Scarpelli et al. have studied and demonstrated in the rat, by means of histological and histochemical techniques, the alterations caused at the renal level by the administration of a vitamin dose of 50,000 units. On the basis of his own experiments, Scarpelli concludes that the renal pathology caused by hypervitaminosis D is the consequence of an initial hydropic degeneration of mitochondria, which in turn is the cause of a serious reduction of the diaphorase activity of DPN and TPN and of the isocitric dehydrogenase complex, and; therefore, indirectly a cause of the accumulation of citric acid at the renal level.

The hydropic degeneration of mitochondria is followed by the appearance of positive PAS granule, at first in the cells of the high tracts of the nephron, and then also in the distal tubule. Such granules, according to Eisenstein, Gropp, and Scarpelli, consist of mucroproteins mobilized from the fundamental substance of the bone by massive doses of vitamin D, and, in addition, the high concentration of mucoproteins favors the abnormal deposition of calcium in the form of Ca citrate.

But among the first manifestations of the altered renal function, following vitamin D overdosage, is a disruption of the mechanism

regulating the acid-base equilibrium. In the urine, Na_2HPO_4, by substitution of a sodium ion by a hydrogen ion, is converted into NaH_2PO_4, and thus the elimination of acid valencies takes place; the hydrogen ions required for this conversion are provided by carbonic acid, formed at the level of tubular cells by hydration of CO_2 catalyzed by carbonic anhydrase.

If this enzyme is blocked by an inhibitor, the amount (number) of potential hydrogen ions is insufficient, and the urine becomes alkaline due to elimination of Na_2HPO_4.

Closely connected with the above mechanism of acid elimination is the tubular process of bicarbonate reabsorption, as a result of which a sodium ion is exchanged for a hydrogen ion, thus leading to the formation in the tubular urine of carbonic acid, which breaks down into $CO_2 + H_2O$. The CO_2 is then diffused through the cells of tubules and returns into the cycle. Thus, carboanhydrase inhibitors interrupt the production route of hydrogen ions (i.e. the hydration of CO_2 and the formation of $H_2 - CO_3$, donor of H^+), and the bicarbonate therefore can no longer be reabsorbed and appears in the final urine.

In summary, the alteration of the tubular function is marked by a lowered carboanhydrase activity and therefore a lowered production of H_2^+ and CO_2^-, and by an abnormal elimination of sodium diphosphate and bicarbonate.

The lowered production of H^+, which does not permit the transformation of sodium diphosphate into Na acid phosphate, reduces the elimination of acid valencies with the urine, and the urine itself is alkaline.

The absence of a cleavage of Na^+ bicarbonate into H_2CO_3 and Na^+ and the consequent lack of cleavage of H_2CO_3 into CO_2 and H_2O causes an increased elimination of bicarbonate and a lack of reabsorption of CO_2, which, in the ultimate analysis, means that the urine is alkaline and that the values of alkaline reserves are low, and therefore a metabolic acidosis is present.

All the facts reported above are confirmed in all our cases, and particularly in cases 5, 6, 8, and 10, when an alkalinization test was carried out (Table 4); prolonged administration of bicarbonate has increased the alkalinity of the urine but did not significantly modify the alkaline reserve of the blood.

The lowering of the alkaline reserve, according to what is commonly admitted and is referred by the formula of Rhona - Takahaschi - Gyorgy, and the state of acidosis are followed by a state of hypercalcemia, and the increase of Ca^{++} leads to hypokalemia (excess K content) and hyponatremia (excess Na content) (Loeb's formula). This has been verified in two of our cases.

The hypervitaminosic hypercalcemia, following the increased intestinal absorption of Ca^{++} and the reduced intestinal elimination of Ca^{++}, leads to a state of tubular acidosis; this acidosis aggravates the hypercalcemia.

2) <u>The calcemia is not high in all our cases</u>:
In regard to the calcemic data, the recent study of Mathieu should be noted, in which the author has controlled (checked) the toxic action of Vitamin D independently of the hypercalcemic state.

The fraction of hematic Ca^{++}, bound to proteins, is equal to about 33% of the total content, and increases or decreases parallel to the concentration of the serum proteins and perhaps above all to that of albumins; it increases further with a lowering of the pH.

In regard to our data on calcium, R.A. and albumins, it should be noted that the fraction of Ca^{++}, although high, is lower than that of Ca^{++} bound to proteins: therefore, even at normal concentrations, relatively high rates of ionized calcium, which is the notoriously active fraction, are present.

3) The lower content of phosphatases: is not only a pathological sign of cellular enzymatic activity (Franconi, de Luca, Paupe), but also a sign of antagonism between vitamin D and the parathyroid gland: vitamin D, by increasing calcemia, slows down the action of the parathryoid, an inhibition which is made evident by the increase of phosphatemia, phosphaturia and hypophosphatasemia.

4) The lowered proteinemia in some cases: we must consider the ease with which patients suffering from idiopathic hypoproteinemia are resistant to attacks of hypocalcemic tetany (Debre et al., Homburger and Peterman, Bonud and Hackett). Hypocalcemia, which occurs particularly frequently in the infantile age, and primarily in early infancy, is due both to a reduction of Ca^{++} in relation to the decrease of Ca^{++} proteinate, according to the known formula $\frac{Ca^{++} + \text{protein}}{\text{Ca proteinate}} = K$, and to its defective intestinal absorption notoriously present in small children (connected with secondary abnormalities of the pancreatic and intestinal functions with excessive elimination of calcium soaps in the stool).

This reduction of calcium ions, even though modest in scope, is sufficient to instigate a tetanic crisis, even without being followed by ionic modifications of other electrolytes and by variations of the blood pH.

Therefore, returning to our cases, hypoproteinemia, in which a marked hypoalbuminemia exists, would bring to our mind a hypocalcemic predisposition. This situation, however, cannot be confirmed; the hypoalbuminemia, secondary to the hepatic pathology, is itself the cause of the Ca^{++} reduction connected with it; but it is noted that the Ca^{++} bound to proteins does not have a functional importance, except as a deposit, while the increase or decrease of diffusible or ionized calcium are responsible for the symptoms of hypercalcemia or hypocalcemia, respectively.

We must point out that there is no direct pathogenetic relation between hypoproteinemia and relative hypercalcemia, but only between hypoproteinemia and the toxic action of vitamin D at the hepatic level.

Our first case, in which there are clear electrocardiographic signs of hypercalcemia, while laboratory calcemic values are normal, demonstrates the harmful effect on the heart of the increase in the diffusible Ca^{++} fraction.

Another fact which must be considered is the increase of bound α_2-globulins with the increase of serum mucopolysaccharides. This is a constant sign of hypervitaminosis D (Fanconi, de Luca, Gillman, Paupe). On the other hand, we know that every alteration of ossification processes is accompanied by an increase of serum mucoproteins, which is connected, as can be clearly seen in our cases, with an increase of α_2-globulins. For such cases, Fanconi in reality speaks about a true and actual "pathology (disease) of osteoblasts" following an overdosage of vitamin D.

6) Cholesterolemia has not been studied in all of our cases, although we have obtained some interesting data: 4 out of 6 cases in which it was investigated exhibited a hypercholesterolemia. The latter is an index of hypervitaminoses (Fanconi, Willi).

7) Azotemia was normal in all of our cases. It is noted that, according to the severity of the intoxication, glomerular alterations may occur, and consequently a renal insufficiency.

8) Polyuria was present in all of our cases. It is noted that it is related to the consequent increase of the glomerular filtrate (amounting to 38%, according to Paupe) and to the lower tubular absorption of water.

From Table 3, we can see that polyuria, hyposthenuria and alkaline urines were noted in all cases, a pathological sediment was observed in 5 cases, and albuminuria in 3 cases.

In all cases, a hypercalciuria and a noticeable hyperphosphaturia were present; in the urine also the concentrations of Na, K and Cl are much higher than the norm, and therefore clear signs of a disruption of the tubular function are present. The power (capacity) of concentration of the tubule is lacking or is reduced, as well as the power (capacity) of acidification and reabsorption of the most important ions.

The pitressin load test (Table 1) gave negative results and shows that polyuria does not depend on the hypophyseal state.

An examination of Table 3 leads us to believe that these subjects are affected by a true and actual renal saline diabetes, due to the abnormal elimination of ions. In fact, a marked beneficial effect was noted in these cases immediately after administration of synthetic steroids. Low doses of such steroids, in fact, caused a rapid reduction of the diuresis, due to increased tubular reabsorption of water and sodium on the part of the proximal tubule.

With regard to hypercalciuria, it is a consequence not only of hypercalcemia, but also of the toxic state with tubular damage; it is the cause, in the final analysis, of hypocalcemia, sometimes with a consecutive increase of the parathyroidal activity, and consequently of a successive compensatory reduction of the tubular reabsorption of phosphorus.

Our data clearly indicate the presence of conditions which are sufficient to provoke serious derangements.

In the syndrome of vitamin D overdosage, the tubular function is altered in a different way in various cases, and, in each case, at various times during the disease. In our present report, we have attempted to

study the tubular function of each patient, and we were confronted by problems of great practical and scientific interest, and we have presented, in a very generic form, data which unfortunately present difficulties in interpretation, and which, therefore while justifying only in a partial way the various pathogenic interpretations, emphasize the need for carrying out further studies.

Among the radiographic results, the one relating to the pelvis in case 4 is remarkably characteristic, since the compact (thick) and homogeneous aspect of the iliac blades can be noted in this case. The metaphysis region of the femurs (thigh bones) is homogeneously (uniformly) opaque, the cortical portion of the femoral hypophyses is thickened, and has more compact and homogeneous appearance. The visible ossification nuclei are more compact. The metacarpus bones also exhibit a more compact cortical portion, and the first two carpal nuclei of the capitate bone and the uncinate have a compact, net and hypercalcified appearance.

The electrocardiograms performed on our patients have yielded truly characteristic results; the cardiologist has twice made a precise diagnosis of a disionic state caused by a probable hypercalcemia, without any prior knowledge of biochemical data.

We believe that it is necessary here to restate point by point the clinical data referring to our cases which have studied to a greater extent. We only want to underline the fact that, out of the 12 cases cited by us, 10 occurred during the spring and summer, when we believe that, due to the increased physiological cutaneous solar irradiation and, therefore, due to the greater production of ergosterol and calciferol, there is a minor (lesser) requirement for exogenous (external) vitamin D.

We shall now mention quite briefly the therapy to which one must have recourse to favor the evolution (progress), usually spontaneous, of healing the syndrome of vitamin D overdosage.

In less severe cases, a simple suspension of vitamin administration usually results in a rapid improvement of the clinical picture. In more severe cases, symptomatic rehydrating and alkalizing therapy is sometimes sufficient, and at other times, one must have recourse to substances which block the intestinal absorption of calcium (cortisone, sodium phytate, EDTA), and, whenever possible, to a calcium-deficient diet.

In the description of our cases, we have dwelt particularly on the description of case 12, in which simultaneous high doses of vitamin D and anabolic compound (durabolin) were used as a tonic.

From Table 1, we can see that the symptomatology of vitamin D overdosage was greatly minimized. In the history of the patient and in the objective examination, signs of an overdosage of the anabolic compound have been reported.

At this point, we want to report the studies performed by Bertolotti and Vignolo (1961) and Bertolotti and Giordano, which take a cue from the hypothesis of Selye (1958). The latter, in regard to the capacity, which was experimentally and clinically demonstrated, of some substances with a steroid structure (methyltestosterone and 17-ethyl-19-nortestosterone) to inhibit the catabolic effects of vitamin D, considers methyltestosterone as a specific inhibitor of the toxicity of vitamin D. Bertolotti et al. have confirmed the effect exerted by norandrostenolone phenyl propionate on rats subjected to toxic doses of vitamin D. They have shown that the association of noradrostenolone phenyl propionate has a prophylactic and therapeutic action in the experimental intoxication of vitamin D; the former, however, appears to be of lesser extent than the latter. Thus,

the above authors point out a method for the prophylaxis and treatment of the intoxication caused by vitamin D overdosage.

Our case appears to be significant in this respect: although the patient had received high doses of vitamin D for a brief time (2.5 mg/day), toxic symptoms were noted, yet with only slight biochemical signs of vitamin D overdosage.

It is difficult to establish to what extent the action of the anabolic compound had weakened (minimized) the toxic picture; however, a comparison of this case with those in which more than 2 mg per day of vitamin D was administered reveals considerable differences from a biochemical standpoint: cases 2, 4, 5, and 11, as compared to case 12, exhibited a severe picture (pattern) of vitamin D overdosage with a hypercalcemia associated with a disionic state and renal, electrophoretic, electronencephalographic and electrocardiographic disturbances.

In case 12, in particular, only moderate clinical signs of a vitamin D intoxication were noted, and, as was already stated above, the data obtained from a biochemical study were within a normal range.

On the basis of these data, it does not seem correct to attribute the greater tolerance to vitamin D, observed in case 12, only to the constitution of the patient, and we consider it proper to attribute a certain value to the antivitaminic action of norandrostenolone phenyl propionate in this case.

We have already indicated the biological conditions which favor the establishment of a hypervitaminosis D syndrome. In reality, however, the actually more frequent causes in determining an overdosage include--the prescription of vitamin D by doctors for strictly tonic (restorative)

purposes, the prevalent custom of parents to treat their own children with vitamin preparations, even without the doctor's advice, and finally, the existence of commercial preparations containing high doses of vitamin D, such that actual dosages used in shock therapy are achieved as a result of low-dose administrations (Segni and Bruni).

Jeans and Stearns (1938) and Berry et. al. (1955) consider that daily doses of 2,000 units of vitamin D_2 are harmful for a subject in early infancy. Tumulty (1942), Sartori (1956) and Faccini et. al. (1959) believe that daily doses of 50,000 I.U. of vitamin D for 45 days constitute a high, but not excessive dosage.

Hollinger and Pattee (1956) make a distinction of toxic doses of vitamin D in relation to age and consider that a daily dose of 30,000 units is toxic for a suckling (unweaned) child, whereas, a dose of 50,000 I.U. is toxic for a child in the second and third stage of infancy and a daily dose of 150,000 I.U. is toxic for an adult.

In 1959, the Council on Foods and Nutrition established in a categorical manner that the vitamin D requirement for an immature or older child is equal to 400 units per day that such a dose is the one that determines the maximum retention of Ca^{++} and P, and that daily vitamin D doses superior to 1800-2000 units are already toxic for some children.

In the same year, H. Willi (Acta Helv. Paed. 14, 351, 1959), in regard to the prevention of rickets in immature infants, suggested the combined use of 500-1000 units of vitamin D and 400-800 mg/day of calcium diphosphate, starting on the second day of life, and later (1960) specified that the optimum antirachitic prevention is achieved in the infant artificially fed on cow's milk with a daily dose of only 500

units of vitamin D, whereas, dibasic calcium phosphate must be added to the above dose in the breast-fed infant, due to the known deficiency of calcium in maternal milk.

In the 1962 edition of his "Treatise on Children's Welfare", Bulgarelli suggests the use, in the prophylactic (prevention)field, of 400 units per day of vitamin D for suckling infants and for children in the second stage of infancy, and 800-1000 units per day for immature infants, during a period of about 3 weeks; as a therapeutic dose, the same author recommends 6000-10,000 units of vitamin De Toni, Debré, and Fanconi consider that 5 mg of vitamin D, equal to 200,000 units, should be able to cure a case of severe rickets and believe that rickets that cannot be cured with a dose of 200,000 units represents a vitamin D-resistant type of rickets.

Conclusions

The above cases, 8 of which have come under observation within a short period of time, force us to draw the following conclusions:

1) Attention should be drawn once more to the necessity of a cautious use of the vitamin, carried out in a scientific and conscious manner, and always after a thorough examination of the condition of each subject, especially when an antirachitic preventive treatment with vitamin D is intended.

2) In view of the assurance that the standard of living of young children is good and that rickets by now is a rare disease, we must presume that the biological conditions of young children in general (barring some exceptions) are perfectly balanced. Therefore, it is the duty of the doctor, whenever he must use drugs which are already

known to be toxic, to always exercise great caution, and especially today, in view of the great concern expressed both in medical and Paymens circles for the increased frequency of the so-called "iatrogenic" diseases (de Toni, Jr.).

3) The doctor should select at the proper time a suitable preparation, and should indicate its exact dosage (posology) and warn the parents about the dangers of the indiscriminate use of a drug considered moreover as totally harmless.

Summary

Vitamin D overdosage syndrome - 12 cases of vitamin D overdosage observed at the Department of Paediatrics of the Udine City Hospital are described. The pathogenesis and clinical picture are described, especially as regards the renal function in these patients, and the recognized preventive and therapeutic doses of vitamin D are mentioned. The fact that vitamin D should always be used with caution is stressed.

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IPERVITAMINOSI

Ospedale Civile di Udine - Divisione Pediatrica
Primario Prof. P. Schiaparelli

La sindrome da iperdosaggio di vitamina D

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La vitamina D può dar luogo, qualora venga somministrata in eccedenza (sia in dosi uniche urto, sia in dosi più modeste, ma per lungo tempo), ad una sindrome ipervitaminosica, caratteristica.

R. Debré nel 1949 segnalò la prima ampia casistica, concernente 250 soggetti con ipervitaminosi D_2, di cui 11 mortali; nel 1953 il Frontali sulla base di 300 casi della letteratura, di cui 17 mortali, trattando 5 propri casi, delinea la sintomatologia dovuta al superdosaggio di vitamina D_2 e la definisce caratterizzata da anoressia, vomito, stipsi, febbre irregolare, epatomegalia, ipercalcemia, riduzione delle fosfatasi seriche, ipercalciuria, iposteniuria, polidipsia.

Si tratta in realtà di una sindrome, la cui eziopatogenesi, fisiopatologia e sintomatologia sono state già ampiamente oggetto di trattazione, ma che, tuttavia, per la variabilità e la frequenza dei casi osservati, ancor oggi ha particolare interesse.

E' noto che le dosi capaci di determinare la sindrome da iperdosaggio D sono diverse in rapporto allo stato costituzionale di ogni singolo soggetto (Willi); non tutti quelli, ai quali vengono somministrate elevate dosi di vitamina D, vanno incontro a fenomeni di intolleranza o di sovradosaggio. Fattori, che hanno notevole importanza nel loro determinismo, sono, universalmente riconosciuti, l'età, lo stato di mineralizzazione e secondo Illig e Prater l'irradiazione solare e, secondo Willemin-Clog e G. Menut, la crescita ridotta e in alcuni casi la coincidenza (Goormaghtigh, 1938; Houft, 1948; Fanconi, 1952; Johnson, 1952; Waylor, 1955; Royer, 1958; Willemin-Clog e G. Menut 1963) di particolari condizioni, quali la insufficienza tiroidea o la sarcoidosi di Besnier-Baeck-Schaumann.

Si deve precisare, però, che nella maggior parte dei casi risulta difficile comprendere il meccanismo su cui si basa e per cui si attua l'ipervitaminosi D.

Sono stati descritti quadri acuti e cronici, forme lievi (transitorie, a guarigione spontanea) con solamente segni lievi di intossicazione generica (nausea, vomito, anoressia, poliuria, disidratazione e segni di iposurrenalismo), e forme gravi con più marcati segni funzionali e soprattutto con complicanze anatomiche irreversibili (con deposizioni calcaree nelle epifisi, nelle ossa neoformate, in tutto l'albero vascolare, nel cuore e nei reni).

Per quanto riguarda l'età, come già detto, essa ha un ruolo non indifferente nel determinismo della sindrome da ipervitaminosi D: è più frequente nella I infanzia, ed in particolare tra il 6° ed il 24° mese (Willemin-Clog e Menut), e più rara nella II e III infanzia (Hollinger e Pattee, Durand, Faccini, Serafini e Svikolos).

Spunto alla presente esposizione casistica è stata la relativamente elevata incidenza di sindromi da iperdosaggio di vitamina D, riscontrata in un tempo ristretto (otto casi in 60 giorni circa); tale fatto ci ha stimolato ad analizzare la nostra casistica dal 1960 al giugno 1963 al fine di trarne illazioni di ordine clinico e pratico.

Lo studio dei nostri casi è stato basato soprattutto sull'indagine della funzionalità renale, ed in particolare abbiamo cercato di approfondire le capacità funzionali tubulari (residue all'azione dannosa dell'iperdosaggio vitaminico), con il ripetuto controllo della diuresi nelle 24 ore, della quantità di ioni eliminati con le urine, nelle 24 ore, e della capacità di acidificazione del rene con il rilievo del pH urinario.

Abbiamo inoltre eseguito indagini ematochimiche: abbiamo controllato l'elettroforesi, la R.A., la elettrolitemia, l'azotemia e la colesterolemia.

In rapporto alle attuali conoscenze sull'influenza che gli elettroliti plasmatici hanno sulla funzionalità cellulare in genere e delle cellule del sistema nervoso centrale e del miocardio in particolare, abbiamo eseguito lo studio dei tracciati elettroencefalografici ed elettrocardiografici.

Abbiamo cercato inoltre di escludere eventuali endocrinopatie (riguardanti l'ipofisi, le paratiroidi, il surrene, le gonadi) e gli eventuali influssi di ormoni minerato-attivi (desossicorticosterone) e di quelli glicoattivi (insulina e adrenalina), a molteplice e varia azione elettrolitica.

Casistica

La nostra casistica riguarda dodici bambini, affetti da intossicazione da iperdosaggio di vitamina D, di varia gravità, di età compresa tra i 7 e i 30 mesi, ricoverati presso la Divisione Pediatrica dell'Ospedale Civile di Udine.

La diagnosi clinica basata sul dato anamnestico e obbiettivo è stata confermata in tutti i casi dai dati di laboratorio. Ciascun caso è sinteticamente riassunto nella allegata tabella I.

I casi 1 e 2 furono oggetto di una comunicazione del dott. prof. I. Sala alla Società Medica del Friuli nel 1960.

Il caso 12 lo presentiamo più ampiamente descritto, data la particolarità del quadro clinico dovuto all'associazione di vitamina D_2 e di fenilpropionato di norandrostenolone (durabolin).

Caso 12. — M. L., 2 anni e 3 mesi, ♂, nato a Pagnacco (Udine) il 12-IV-1961 ricoverato il 23-VII-1963.

Nulla al gentilizio; genitori, viventi e sani, non consanguinei. Primogenito. Nato a termine da gravidanza decorsa in modo fisiologico, da parto eutocico. Sviluppo psicofisico regolare.

A scopo ricostituente i genitori, su consiglio medico, hanno praticato dal 9-VI-1963 una fiala orale di 400.000 U I, di vitamina D_2 ogni otto giorni, più due cucchiaini pro die (10 cc al dì) di sciroppo a base di calcio colloidale e contenente 1500 U I di vitamina D_2 per cc, associato a 5 mgr di fenilpropionato di norandrostenolone ogni 15 giorni per via parenterale.

Tutto sommato il bambino in 45 giorni assunse 3.075.000 U I di vitamina D_2, pari a 76,5 mgr di vitamina (mgr 1,7 pro die) e 15 mgr di fenilpropionato di norandrostenolone (durabolin).

Dall'inizio della cura il bambino ha presentato anoressia, progressivo pallore e irrequietezza. Dopo la seconda fiala di durabolin ha avuto crisi di agitazione psicomotoria e priapismo. Essendo le condizioni del bambino peggiorate per un notevole calo ponderale, e per comparsa di polidipsia e poliuria, viene ricoverato per accertamenti. All'E.O.: condizioni generali scadenti, pallore intenso, polso 140 filiforme, cute ipoidratata anelastica, soffio protomesosistolico di lieve intensità sui focolai della base e al mesocardio, iperreflessia osteotendinea. Gli esami di laboratorio (tabelle I, II e III) hanno confermato i segni clinici da intossicazione di vitamina D. L'evoluzione della malattia è stata rapidamente favorevole, sebbene sia stata eseguita solo terapia reidratante e alcalinizzante.

Nella tabella II sono esposti i dati ematochimici riguardanti ciascun caso, mentre nella tabella III i dati urinari.

Nella tabella IV sono esposti i risultati delle prove di alcalinizzazione; purtroppo per ragioni contingenti ci è stato possibile indagare la capacità di mantenere il giusto equilibrio acidobasico solo in 4 casi su 12.

Discussione

Per tutti i casi presentati, sia l'anamnesi, sia l'esame obbiettivo, sia i dati clinici, hanno fatto innanzitutto escludere che la sindrome, in ciascuno di essi riscontrata, rappresenti il quadro collaterale e facoltativo o no di una condizione morbosa di base (neoplasie dello scheletro, mieloma multiplo, atrofia ossea ac., ecc.; iperparatiroidismo, sindrome-latte-alcali, ipercalcemia idiopatica).

Le dosi di vitamina D, assunte dai nostri pazienti si devono pensare oggi senz'altro eccessive per bambini della loro età, anche se la notevole variabilità individuale alla sensibilità rende difficile fissare quale sia la dose effettivamente eccessiva di vitamina.

In sintesi abbiamo citato nella tab. I 12 casi di intossicazione da iperdosaggio di vitamina D_2 in 10 dei quali è stato possibile accertare un quadro chiaro di acidosi tubulare renale. Il gruppo delle ipercalcemie possiede in comune dei sintomi urinari tipici: poliuria, isostenuria, pollachiuria, ecc. Ciò avviene nell'iperparatiroidismo primario, nella sarcoidosi, nella ipercalcemia dei tetraplegici con arresto brusco dell'accrescimento osseo (poliomielite), nell'ipercalcemia idiopatica e infine nell'ipervitaminosi D. Le turbe della funzione renale, comuni a queste sindromi, riflettono la lotta dell'organismo contro l'ipercalcemia: si ha il quadro di acidosi tubulare renale, così come descritto da Pines e Mudges nel 1951 (« Am. J. Med. », 11, 302, 1951), determinato da una tubulopatia ormai ben definita, sebbene a patogenesi non ancora chiarita.

Il quadro clinico prese il nome di sindrome di Lightwood (1953), che la descrisse per primo. Butler, Walson e Faber nel 1936 descrissero un caso di acidosi con nefrocalcinosi diffusa in un bambino. Albright nel 1946 parlava di questi casi come stati di insufficienza renale tubulare senza insufficienza glomerulare.

Per comprendere la sindrome, bisogna ricordare l'importanza del controllo renale sull'equilibrio acido-base e che il filtrato glomerulare ipotonico (preurina) diviene isotonico per opera del tubulo prossimale: esso riassorbe l'85 % dell'acqua e del sodio, l'80 % dei cloruri e dei bicarbonati, la maggior parte del K, Ca, Mg, P, S, glucosio, proteine e aminoacidi, e quindi il filtrato diviene ipertonico per opera del tubulo distale che riassorbe ulteriore parte di bicarbonati (restante 20%), dell'acqua, dei cloruri, dei fosfati, e secerne K e ammoniaca. L'attività del tubulo non si esplica solo per processi enzimatici sollecitati e inibiti da vari ormoni e da varie sostanze. La vitamina D ha notevole influenza sull'attività del tubulo: interviene sulla regolazione dell'equilibrio acido-base, sulla eliminazione degli amino-acidi, delle proteine, dell'acido citrico e soprattutto sull'eliminazione del Ca e P. L'intossicazione lieve da vitamina D, determina acuti e subacuti perturbamenti di queste varie funzioni tubulari ed è in ultima analisi causa di acidosi tubulare renale, cioè di insufficienza renale tubulare senza insufficienza glomerulare e quindi senza iperazotemia.

Di questo sono bene dimostrativi gli 8 casi su 12 di cui, per quanto ci è stato possibile, abbiamo indagato l'attività tubulare (Tabelle I-II-III).

Nella tabella I si rilevano altri dati interessanti:

1) innanzi tutto su 12 casi, 7 sono femmine e 5 maschi, e ciò concorda con quanto comunemente ammesso, cioè il sesso non incide per nulla nella determinazione del quadro clinico;

2) si tratta di soggetti tutti molto giovani, 11 della prima infanzia, 1 solo di 28 mesi di età;

3) per quanto riguarda la data di ricovero di ciascun paziente si riscontra che nessun caso è avvenuto in inverno o nel tardo autunno. Tutti gli AA. sono d'accordo che l'irradiazione solare sia un fattore predisponente alla ipervitaminosi (Illig e Prader, Royer, Fanconi). E' indubbio che nei nostri casi il fattore stagionale abbia avuto notevole influenza. Tre casi infatti sono stati ricoverati in primavera, ben 8 durante l'estate ed uno all'inizio dell'autunno;

4) in tutti i casi il motivo della prescrizione è stato di profilassi del rachitismo, e in due casi di una supposta spasmofilia latente;

5) le dosi assunte da ciascun caso sono state sempre elevate, sia se si considera la dose pro die che nella nostra casistica è varia (le dosi minime sono 48.000 U pro die, le massime sono 200.000 U pro die),

ABELLA I.
Sono esposti i dati clinici riferentisi a ciascun caso.

Osservazione N. Nome, sesso, età	Osservazione Data del ricovero	Motivo della prescrizione	Dose di vit. D somministrata Totale mgr	Dose di vit. D somministrata pro die mgr	Sintomatologia che ha condotto il paziente alla nostra osservazione: Anoressia	Stipsi	Vomito	Perdita di peso	Anemizzazione	Poliuria	Polidipsia	Ipertermia	Convulsioni	Stato tossico (disidratazione cutanea)	Astenia	Mutamento del carattere	Diagnosi di ricovero
1) Z. E. ♀, 13 m.	1-9-1959	Ricostituente	120	1,2	+	+	+	+	++	+	+		+				Cistite; accessi convulsivi
2) B. M. ♀, 10 m.	1-7-1959	Ricostituente	170	4	+	+		+	++	+	+	+		+	+		Cistite
3) C. D. ♀, 1 a., 1 m.	20-8-1960	Profilassi antirachitogena	250	1,5	+	+	+	+	+	+	+	+		+	+	Sonnolenza	Cistite
4) P. B. ♂, 9 m.	15-10-1960	Profilassi della tetania rachitogena	30	2,5	+	+		+	++	+	+		+	+	+	Instabilità	Crisi convulsive
5) Z. P. ♂, 2 a	16-7-1961	Profilassi della tetania rachitogena	105	2	+	+		+	++	+	+			+	+	Instabilità	Crisi convulsive
6) C. G. ♂, 10 m.	1-3-1963	Profilassi anticachitogena	150	1,6	+	+	+	+	++	+	+			+	+	Instabilità	Cistite
7) M. G ♀, 1 a.	27-3-1963	Ricostituente	50	1,7	+	+		+	+	+	+	+		+	+	Irrequiet.	Turbe del carattere
8) C. A. M. ♀, 7 m.	17-4-1963	Ricostituente	50	1,6	+	+	+	+	+	+	+			+	+	Irrequiet.	Cistite
9) N. A. ♀, 1 a., 1 m.	5-6-1963	Ricostituente	25	1,5	+	+		+	++	+	+			+	+	Irrequiet., instabilità	Cistite
10) C. N. ♀, 2 a	1-7-1963	Ricostituente pro filassi antirach.	60	1,5	+	+	+	+	++	+	+	+		+	+	Irrequiet.	Tossicosi
11) C. M. ♀, 1 a	16-7-1963	Ricostituente	60	1,5	+	+	+	+	++	+	+	+		+		Irrequiet.	Turbe del carattere
12) M. L. ♂, 2 a., 4 m.	26-7-1963	Ricostituente	76,5 + durab.	1,7	+	+	+	+	++	+	+	+				Apatia	Turbe del carattere

e sia la dose totale (compresa tra 1.200.000 ÷ 10.000.000). Nella determinazione del quadro clinico dei nostri dati non sembra che abbia influito tanto la dose totale, quanto quella giornaliera (caso 4, casi 7, 8 e 9); infatti i casi 7, 8 e 9 hanno avuto dosi giornaliere elevate per breve tempo;

6) non ci soffermiamo molto a parlare della sintomatologia da iperdosaggio, dato che questi sono elementi molto noti, vogliamo solo richiamare l'attenzione sulla sintomatologia neurologica e psichica, che è frequentemente presente in questi casi. I casi 2 e 4 hanno avuto convulsioni, conseguentemente alla somministrazione di vitamina, mentre la storia di 10 casi parla di turbe del carattere;

7) sei bambini furono ricoverati per cistite, 4 per turbe del carattere, 2 per convulsioni;

8) tutti, eccetto uno, furono allattati con latte materno, condizione questa che è molto lontana dal favorire il rachitismo;

9) la classificazione auxologica ha rilevato 4 tiposomici, 4 eccedenti e 3 deficienti nello sviluppo fisico. E' noto che il bambino normale sopporta meno bene del rachitico la vitamina D. E' indubbio che l'errore di prescrizione nel bambino, comunque sia, se deficiente di sviluppo o eccedente o tiposomico, non viene per nulla modificato dall'abito costituzionale;

10) nove tracciati elettrocardiografici presentano netti segni di ipercalcemia, e sette tracciati elettroencefalografici su dieci erano nettamente alterati;

11) la prova da carico di pirressina è stata negativa in tutti i pazienti;

12) la terapia è stata per sei casi semplicemente reidratante e alcalinizzante data la lievità dei sintomi. A quattro casi per il maggior stato di compromissione si è aggiunta anche terapia desametazonica. In ogni caso l'evoluzione è stata favorevole, anche a controlli a distanza.

Dalle tabelle II, III e IV si rileva che i dati plasmatici e urinari sono stati veramente caratteristici degli stati ipercalcemici.

Tra i dati ematochimici vogliamo soffermarci su:

1) *La R.A. fortemente abbassata in 8 casi su 9:* il ridursi della percentuale di CO_2 presente nel sangue è dovuta alla diminuita acidogenesi renale, causa di perdita di bicarbonati, e quindi di minore riassorbimento di CO_2.

In quattro casi è stato indagato il potere acidogenetico tubulare eseguendo la prova di alcalinizzazione con somministrazioni ripetute di bicarbonato di Na; l'esito è stato molto significativo e probativo per la diminuzione di tale potere. Vi è stato un aumento della alcalinità urinaria, un lieve aumento della R.A. la quale tuttavia è restata sempre bassa e non ha raggiunto mai i valori normali.

Come è noto nel soggetto normale al carico di bicarbonati segue un modicissimo, o dal tutto nullo, aumento della alcalinità urinaria ed invece un aumento della riserva alcalina nel sangue.

I dati da noi ottenuti con questa prova nei quattro soggetti esaminati (tabella IV) sono chiaramente dimostrativi per l'esistenza di un danno tubulare, cioè della incapacità del tubulo renale di scindere tramite l'anidrasi carbonica il bicarbonato e di riassorbire il CO_2.

Quanto da noi esposto nella tab. IV ci sembra molto importante, poichè ancor oggi non sono perfettamente conosciuti, non solo l'azione della vitamina D

Diagnosi di ricovero	Dati anamnestici	Classificazione auxologica secondo de Toni-Gobessi (medie genovesi)	P.A.	E.C.G.	E.E.G.	Prova pitressina	Prova di alcalinizzazione	Segni radiologici	Terapia	Evoluzione
Cistite; accessi convulsivi	Allattamento materno		105/75		+	Neg.				Favor.
Cistite	Allattamento materno		105/75	+		Neg.				Favor.
Cistite	Genitori consanguinei, allattamento materno	Pachisomia	125/75			Neg.		+	Reidratante alcalinizzante	Favor.
Crisi convulsiva	Allattamento materno	Tiposomia	95/55		+	Neg.		—	Reidratante alcalinizzante	Favor.
Crisi convulsiva	Allattamento materno	Microsomia	80/55	+	+	Neg.		+	Reidratante alcalinizzante	Favor.
Cistite	Allattamento materno	Ipozomia	90/55	+	+	Neg		+	Reidratante alcalinizzante	Favor.
Turbe del carattere	Allattamento materno	Nanismo, leptosomia	80/50	+	+	Neg.	Esito patologico	—	Reidratante alcalinizzante + desametaz.	Favor.
Cistite	Allattamento materno	Tiposomia	85/55	+	+	Neg.	Esito patologico	+	Reidratante alcalinizzante + desametaz.	Favor.
Cistite	Allattamento materno	Macrolepiosomia	95/60	+	+	Neg.		—	Reidratante alcalinizzante	Favor.
Tossicosi	Allattamento materno	Ipersomia	105/70	+	—	Neg.	Esito patologico	—	Reidratante alcalinizzante + desametaz.	Favor.
Turbe del carattere	Allattamento materno	Tiposomia	90/65	+	—	Neg.		+	Reidratante alcalinizzante + desametaz.	Favor.
Turbe del carattere	Allattamento artif.	Tiposomia	85/50	+	—	Neg.	Nei limiti della norma	—	Reidratante alcalinizzante	Favor.

« ...ello renale, ma soprattutto i danni più immediati che a tal punto essa provoca.

Per primi nel 1958 de Luca e coll. dimostrarono sperimentalmente l'azione lesiva « in vitro » della vitamina D sui mitocondri renali.

In base all'esperienza del de Luca e coll., Harman e Scarpelli e coll. ricercarono, e dimostrarono nel ratto, con tecniche istologiche e istochimiche le alterazioni provocate a livello renale dalla somministrazione di una dose di 50.000 U di vitamina. Su scorta delle proprie esperienze lo Scarpelli concluse che la nefropatia da ipervitaminosi D è conseguente ad una iniziale degenerazione idropica dei mitocondri, che è a sua volta causa di una riduzione grave delle attività diaforasiche del DPN e del TPN e del blocco della isocitrico-deidrogenasi e quindi indirettamente dell'accumulo di acido citrico a livello renale.

Alla degenerazione idropica dei mitocondri segue la comparsa di granuli PAS positivi, prima nelle cellule dei tratti alti del nefrone, poi anche nel tubulo distale. Tali granuli, secondo Eisenstein, Gropp e lo stesso Scarpelli, sono di mucoproteine mobilizzate dalla sostanza fondamentale dell'osso dalle dosi massive di vitamina D, e inoltre l'alta concentrazione di mucoproteine favorisce la deposizione abnorme di calcio sotto forma di citrato di Ca.

Ma tra le prime manifestazioni della alterata funzionalità renale, conseguente al sovradosaggio di vitamina D, è il perturbamento del meccanismo di regolazione dell'equilibrio acido-base. Nell'urina il Na_2HPO_4 per sostituzione di un sodioione con un idrogenione si trasforma in NaH_2PO_4 e si attua così l'eliminazione di valenze acide; gli idrogenioni necessari a questa trasformazione provengono dall'acido carbonico, che si forma a livello delle cellule tubulari dall'idratazione del CO_2 catalizzata dall'anidrasi carbonica.

Se questo enzima viene bloccato da un inibitore, la quantità di idrogenioni possibili è insufficiente e l'urina diventa alcalina per eliminazione di Na_2HPO_4.

Strettamente connesso al suddetto meccanismo di eliminazione degli acidi è il processo tubulare di riassorbimento dei bicarbonati, per il quale avviene che un sodioione viene scambiato con un idrogenione e si ha formazione pertanto nell'urina tubulare di acido carbonico che si dissocia in $CO_2 + H_2O$. Il CO_2 si diffonde quindi attraverso le cellule dei tubuli e ritorna in circolo. Gli inibitori della carboanidrasi interrompono quindi la via di produzione di idrogenioni (cioè l'idratazione del CO_2 e la formazione di H_2CO_3 donatore di H^+) ed il bicarbonato perciò non può venire riassorbito e compare nella urina definitiva.

In sintesi, per l'alterazione della funzionalità tubulare si ha diminuita attività carboanidrasica e quindi diminuita produzione di H_2^+ e di CO_2^-, eliminazione abnorme di bifosfato di sodio e di bicarbonato.

La diminuita produzione di H^+, che non permetta la trasformazione del bifosfato di sodio in fosfato acido di Na, riduce la eliminazione di valenze acide con le urine, e le urine stesse sono alcaline.

La mancata scissione del bicarbonato di Na^+ in H_2CO_3 e Na^+ e la conseguente mancata scissione dell'H_2CO_3 in CO_2 e H_2O è causa di aumentata eliminazione di bicarbonato e di mancanza di riassorbimento di CO_2, che in ultima analisi vuol dire urine alcaline, valore basso della riserva alcalina e quindi acidosi metabolica.

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TABELLA II.

Sono esposti i dati ematochimici riferentisi a ciascun caso.

Caso n.	Ca	P	K	Na	Cl	R.A	Fosfatasi alcalina UKA	Azotemia	Colesterolemia	Glicemia	A	Elettroforesi α_1	α_2	β	γ	QA/G	Prot. tot.
1)	13,4	4,5	—	—	—	—	—	32	—	—	—	—	—	—	—	—	—
2)	17	4,9	13,5	340	331	50	13	25	130	1	—	—	—	—	—	—	6,5
3)	11,8	4,5	—	—	510	—	6	28	—	—	—	—	—	—	—	—	—
4)	11,9	5,1	—	—	—	32	6	26	—	—	—	—	—	—	—	—	—
5)	11,5	4,8	—	—	—	38	8	24	—	—	—	—	—	—	—	—	—
6)	10,7	4,2	—	—	—	41	5	30	—	—	—	—	—	—	—	—	—
7)	12,15	3,2	14,5	—	655	33	4	26	235	0,85	46	7	18	15	14	0,85	5,80
8)	10,85	4,7	15	305	381	35	8	32	270	0,90	46	7	17	12	18	0,85	6,0
9)	11,15	5,3	—	—	—	42	15	28	—	—	—	—	—	—	—	—	—
10)	13,05	3,2	12	285	549	30	6	26	196	1,15	47	7	17	16	13	0,88	7,20
11)	12,4	4,5	—	—	—	41	7	24	220	—	46	7	19	15	13	0,85	7,90
12)	10,4	2,8	—	—	—	43	7	26	143	—	50	5	12	12	21	1	6,85

TABELLA III.

Sono esposti i dati che si riferiscono all'esame delle urine

	Diuresi 24 h cc	U+Z alutti Sd	P H urine 24 h	Albuminuria 24 h	Sedimento urine 24 h	Ca urine 24 h	P urine 24 h	Na urine 24 h	K urine 24 h	Cl urine 24 h
1)		1014	6			+++				
2)		1008	8	—	+	162				2600
3)	1300 *	1006	7	+	+	420				
4)	1300 *	1004	8	—	—					
5)	1700	1006	8	—	—	231				
6)	1900	1004	8	—	—	380	340	2710	610	
7)	1950	1004	8	—	—	368	1310	1820	810	1800
8)	1000 *	1011	8	—	+	240	1630	1220	680	1914
9)		1006	7	—	—	261				
10)	1100 *	1007	8	—	+	215	1267	1590	390	2810
11)	1750	1005	8	+	+	321	614	700	342	855
12)	1260		7	—	—	190				

*) I dati segnati sono parziali in quanto data l'età dei pazienti non è stato possibile una raccolta completa.

TABELLA IV.

Prova della alcalinizzazione (somministrazione di gr 6 di bicarbonato nelle 24 ore, cioè gr 0,25/h; i dosaggi del pH dell'urina e della riserva alcalina del sangue sono stati eseguiti ogni 6 ore nelle 24 ore immediatamente precedenti e seguenti il carico di bicarbonato, durante tale periodo i pazienti sono stati tenuti a dieta e a soluzioni alcalinizzanti).

	Controlli eseguiti: Prima del carico di bicarbonato I	II	III	IV	Dopo il carico di bicarbonato I	II	III	IV
7 caso: pH urine	6,9	7,1	7,3	7	7,5	8	7,8	8
R.A.	33	37	32	35	44	41	36	44
8 caso: pH urine	7,5	7,4	7,8	7,8	8	8	8	8
R.A.	39	41	35	33	41	34	38	39
10 caso: pH urine	8	7,3	7,3	7,5	8	7,8	7,9	8
R.A.	30	33	35	32	39	37	34	34
12 caso: pH urine	7	7,1	6,5	7	7,5	8	7,8	8
R.A.	43	46	48	45	50	52	46	50

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Quanto finora esposto trova conferma in tutti i nostri casi, e in particolar modo in quei casi 5, 6, 8 e 10 in cui si è eseguita la prova di alcalinizzazione (tabella IV); la somministrazione prolungata di bicarbonato ha aumentato l'acalinità delle urine, ma non ha modificato sensibilmente la riserva alcalina del sangue.

All'abbassamento della riserva alcalina, secondo quanto comunemente ammesso, così come riferisce la formula di Rhona-Takahashi-Gyorgy, ed agli stati di acidosi conseguono stati ipercalcemici, ed un aumento del Ca^{++} conduce ad ipokalemia ed iponatremia (formula di Loeb). Ciò si è verificato in due dei nostri casi.

L'ipercalcemia ipervitaminosica, conseguente ad aumentato assorbimento intestinale di Ca^{++} ed alla diminuita eliminazione instinale di Ca^{++}, conduce a uno stato di acidosi tubulare; l'acidosi aggrava l'ipercalcemia.

2) *La calcemia non in tutti i nostri casi è elevata*: per quanto riguarda i dati calcemici è noto il lavoro recente del Mathieu, in cui l'A. ha controllato l'azione tossica della vitamina D indipendentemente dallo stato ipercalcemico.

La frazione del Ca^{++} ematico, legato alle proteine, è intorno al 33 % del totale; aumenta o diminuisce parallelamente alla concentrazione delle sieroproteine e forse soprattutto delle albumine; esso aumenta ancora con l'abbassamento del pH.

In rapporto ai nostri dati di calcio, di R.A., di albumine, è da ritenersi che la frazione di Ca^{++} sia elevata, e minima sia quella del Ca^{++} legata alle proteine: quindi anche a concentrazioni normali, si hanno tassi relativamente elevati di calcio ionizzato, che è la frazione notoriamente attiva.

3) *Le fosfatasi abbassate*, non solo sono segno di patia delle attività enzimatiche cellulari (Franconi, de Luca, Paupe), ma anche dell'antagonismo fra vitamina D e paratiroide: la vitamina D, aumentando la calcemia, frena l'azione delle paratiroidi, inibizione che si mette in evidenza con l'aumento della fosfatemia, della fosfaturia e con la ipofosfatasemia.

4) *La proteinemia in alcuni casi abbassata*: da tener presente la facilità con cui gli ammalati di ipoproteinemia idiopatica vanno incontro ad accessi di tetania ipocalcemica (Debré e coll., Homburger e Peterman, Bonud e Hackett). L'ipocalcemia, che insorge particolarmente frequente nell'età infantile, e soprattutto nella prima infanzia, è dovuta sia alla riduzione del Ca^{++} in rapporto alla diminuzione del proteinato di Ca^{++} secondo la nota formula:

$$\frac{Ca^{++} + \text{proteina}}{\text{Proteinato di Ca}} = K$$

e sia al suo difettoso assorbimento intestinale notoriamente presente nel bambino (legato alle anomalie secondarie della funzionalità pancreatica e intestinale con eliminazione esagerata di saponi calcarei con le feci).

Questa riduzione del calcio ione, anche se spesso modesta, è sufficiente a scatenare la crisi di tetania pur non essendo seguita da modificazioni joniche degli altri elettroliti e da variazioni del pH del sangue.

Quindi, per tornare ai nostri casi, l'ipoproteinemia, in cui esiste una marcata ipoalbuminemia, farebbe pensare ad una predisposizione ipocalcemica. Questa situazione tuttavia non si avvera; l'ipoalbuminemia secondaria alla patia epatica, e si causa di una diminuzione del Ca^{++} ad essa legato, ma è noto che il Ca^{++} legato alle proteine non ha importanza funzionale se non di deposito, mentre l'aumento o la diminuzione del calcio, diffusibile o ionizzato, sono rispettivamente responsabili dei sintomi di ipercalcemia o di ipocalcemia.

Riteniamo che non vi sia un rapporto patogenetico diretto tra ipoproteinemia e ipercalcemia relativa, ma solo tra ipoproteinemia e azione tossica della vitamina D a livello epatico.

Il nostro primo caso, in cui vi sono netti segni elettrocardiografici da ipercalcemia mentre i valori calcemici rilevati in laboratorio sono normali, dimostra l'azione dannosa dell'aumento della frazione del Ca^{++} diffusibile sul cuore.

Altro dato da considerare è l'aumento delle alfadueglobuline legate all'aumento dei mucopolisaccaridi serici. Questo (Fanconi, de Luca, Gillman, Paupe) è segno costante di ipervitaminosi D. Si sa d'altra parte che ogni alterazione dei processi di ossificazione è accompagnata ad elevazione delle mucoproteine seriche, che si rileva, come nei nostri casi chiaramente, con l'aumento delle alfadueglobuline. Fanconi in realtà per questi casi parla di vera e propria « patia degli osteoblasti » conseguente all'iperdosaggio di vitamina D.

6) *La colesterolemia* non è stata indagata in tutti i nostri casi, tuttavia abbiamo dati interessanti: 4 casi su 6 in cui è stata indagata erano ipercolesterinemici. L'ipercolesterolemia è indice di ipervitaminosi (Fanconi, Willi).

7) *L'azotemia* in tutti i nostri casi è stata normale. E' noto che in rapporto alla gravità dell'intossicazione si possono avere alterazioni glomerulari e conseguente insufficienza renale.

8) *La poliuria* in tutti i nostri casi è stata presente. E' noto che è in rapporto all'aumento del filtrato glomerulare (del 38 % secondo Paupe) conseguente e al diminuito assorbimento tubulare dell'acqua.

Nella tabella III si rileva che in tutti i casi vi è stata poliuria, ipostenuria, urine alcaline; in 5 casi sedimento patologico e in tre albuminuria.

In tutti i casi si è avuta ipercalciuria e notevole iperfosfaturia; anche nelle urine, Na, K, Cl sono a concentrazione fortemente aumentata rispetto la norma; si hanno pertanto chiari segni di turbamento della funzione tubulare. Manca o è ridotto il potere di concentrazione del tubulo e quello di acidificazione e di riassorbimento dei più importanti ioni.

La prova da carico della pitressina (tabella I) ha dato esito di negatività e dimostra che la poliuria è indipendente dallo stato ipofisario.

L'esame della tabella III fa pensare alla esistenza in questi soggetti di un vero e proprio diabete salino

nale per l'abnorme eliminazione di ioni. Notevole stato infatti il beneficio che hanno avuto questi casi immediatamente dopo la somministrazione dello steroide di sintesi.

Infatti con dosi basse si è avuta una pronta riduzione della diuresi per aumentato riassorbimento tubulare di acqua e di sodio da parte del tubulo prossimale.

Per quanto riguarda l'ipercalciuria essa è conseguenza non solo della ipercalcemia, ma anche dello stato tossico con danno tubulare; è causa in ultima analisi di ipocalcemia talvolta con consecutivo aumento della attività paratiroidea e conseguentemente di una successiva compensatoria diminuzione del riassorbimento tubulare del fosforo.

I nostri dati parlano chiaro per condizioni, che sono sufficienti a provocare gravi squilibri.

La funzione tubulare nella sindrome da iperdosaggio di vitamina D è variamente alterata nei vari casi ed in ogni caso nei vari momenti della malattia. In questa nostra esposizione abbiamo tentato di indagare la funzionalità tubulare di ogni paziente, ci siamo trovati ad affrontare problemi di grande interesse pratico e scientifico, abbiamo presentato con una forma molto generica dei dati, che presentano purtroppo difficoltà interpretative e che perciò, mentre non giustificano che in modo parziale le varie interpretazioni patogenetiche, pongono in rilievo la necessità in proposito di ulteriori studi.

Dei reperti radiografici quello relativo al bacino del caso 4 è notevolmente caratteristico: vi si nota l'aspetto denso e omogeneo delle ali iliache. La regione metafisaria dei femori è in modo omogeneo opacizzata, la corticale delle ipofisi femorali è ispessita, più densa, più omogenea. I nuclei di ossificazione visibili sono più densi.

Anche le ossa del metacarpo presentano una corticale più densa, e i due primi nuclei carpali del grande osso e dell'uncinato si presentano densi, netti, ipercalcificati.

Gli elettrocardiogrammi eseguiti hanno dato esito veramente caratteristico; il cardiologo ha posto con precisione diagnosi, per due volte, di stato disionico da probabile ipercalcemia senza avere conoscenza dei dati biochimici.

Non pensiamo qui necessario risoffermarci punto per punto sui dati clinici dei nostri casi più ampiamente studiati. Vogliamo solo sottolineare che dei 12 casi, da noi citati, 10 sono avvenuti in primavera e in estate, allorchè è da pensare che per l'aumentata irradiazione solare fisiologica cutanea, e quindi per la maggior produzione dell'ergosterolo e di calciferolo, ci sia un minor fabbisogno di vitamina D esogeno.

Accenniamo molto in breve alla terapia a cui si ricorre per favorire l'evoluzione, di solito spontanea, verso la guarigione della sindrome da iperdosaggio di vitamina D.

Nei casi meno gravi la semplice sospensione della somministrazione della vitamina di solito conduce a rapido miglioramento del quadro clinico. Per i casi più gravi talvolta è sufficiente la terapia sintomatica reidratante e alcalinizzante, altre volte si deve ricorrere all'uso di sostanze, che bloccano l'assorbimento intestinale del calcio (cortisone, fitato di sodio, E.D.T.A.), e per quanto possibile a dieta carente di calcio.

Nella descrizione della casistica ci siamo soffermati particolarmente nella descrizione del caso 12, il quale ebbe contemporaneamente come ricostituenti dosi elevate di vitamina D e di anabolizzante (durabolin).

Dalla tabella 1 risulta che la sintomatologia da iperdosaggio di vitamina D fu molto attenuata.

Nella storia del paziente e all'E.O. sono segnalati i segni di iperdosaggio dell'anabolizzante.

A tal punto vogliamo ricordare le ricerche di Bertolotti e Vignolo (1961) e di Bertolotti e Giordano, i quali prendono spunto dalla ipotesi di Selye (1958). Quest'ultimo, a proposito della capacità dimostrata sperimentalmente e clinicamente da alcune sostanze a struttura steroidea (metiltestosterone e 17-etil-19-nortestosterone), di inibire gli effetti catabolici della vitamina D_2, ritiene il metiltestosterone quale specifico inibitore della tossicità della vitamina D. Bertolotti e Coll. hanno provato agli effetti del fenilpropionato di norandrostenolone su ratti sottoposti a introduzione di vitamina D a dosi tossiche.

Essi hanno dimostrato che l'associazione del fenilpropionato di norandrostenolone ha azione profilattica e terapeutica nell'intossicazione sperimentale da vitamina D; la prima tuttavia sembra minore della seconda.

Gli AA. indicavano, in tal modo, una via per la profilassi e la terapia dell'intossicazione da iperdosaggio di vitamina D.

Il nostro caso ci sembra significativo a proposito: pur avendo preso dosi elevate di vitamina D per breve tempo (mgr 2,5 pro die) ha presentato una sintomatologia tossica però con scarsi segni biochimici di iperdosaggio di vitamina D.

E' arduo stabilire quanto abbia agito l'anabolizzante nell'attenuare il quadro tossico, tuttavia il confronto di questo caso con quelli che hanno avuto somministrati più di due mgr di vitamina D pro die, palesa dal punto di vista biochimico notevoli diversità: i casi 2, 4, 5 e 11, rispetto al caso 12, presentano un grave quadro da iperdosaggio di vitamina D con ipercalcemia associata a stato disionico a turbe elettroforetiche, renali, elettroencefalografiche e elettrocardiografiche.

Nel caso 12 in particolare sono solo modici segni clinici da tossicosi - D, e, come già detto, l'indagine biochimica ha dato esito a dati nei limiti della norma.

Non ci sembra esatto, in base a questi dati, attribuire alla sola costituzione la maggiore tolleranza del caso 12 alla vitamina se riteniamo opportuno attribuire anche un certo valore all'azione antivitaminica del fenilpropionato del norandrostenolone.

Abbiamo già accennato alle condizioni biologiche, che favoriscono l'instaurarsi di una sindrome da ipervitaminosi D. In realtà però le cause realmente più frequenti nel determinismo dell'iperdosaggio sono la prescrizione di vitamina D da parte dei medici a scopo puramente ricostituente, l'uso invalso da parte

dei genitori di curare i propri figli con preparati vitaminici, anche senza il parere del medico, infine la esistenza di preparati commerciali contenenti dosi elevate di vitamina D, tali da raggiungere con piccole somministrazioni dosaggi veri e propri di terapia urto (Segni e Bruni).

Jeans e Stearns (1938) e Berry e coll. (1955) ritengono dannosa per un soggetto della prima infanzia la dose di 2.000 U di vitamina D_2 pro die.

Tumulty (1942), Sartori (1956) e Faccini e coll. (1959) ritengono che 50.000 U I di vitamina D al giorno per 45 giorni siano dosi elevate, ma non eccessive.

Hollinger e Pattee (1956) fanno la distinzione di dosi tossiche di vitamina D in rapporto all'età e ritengono che è tossica nel bambino lattante la dose giornaliera di 30.000 U, mentre nel bambino della seconda e terza infanzia lo è quella di 50.000 U I e nell'adulto quella di 150.000 U I quotidiane.

Nel 1959 il « Council on Foods and Nutrition » stabilì in modo categorico che il fabbisogno di vitamina D per un immaturo o per un bambino grandicello, è di 400 U pro die, che tale dose è quella che determina la massima ritenzione di Ca^{++} e di P, e che dosi superiori a 1800-2000 U di vitamina D pro die sono già tossiche per alcuni bambini.

Nello stesso anno H. Willi (« Acta Helv. Paed. », 14, 351, 1959), a proposito della profilassi rachitica nell'immaturo consigliava l'uso associato di 500 ÷ 1000 U di vitamina D, e 400 ÷ 800 mgr pro die di calcio bifosfato dal secondo giorno di vita, successivamente (1960) precisava che l'optimum d'una profilassi antirachitica si ottiene nell'allattato artificialmente con latte di mucca, con solo 500 U di vitamina D pro die, mentre nell'allattato al seno, per la nota carenza di calcio nel latte materno, è necessario aggiungere alla dose di vitamina sopraddetta il fosfato bifasico di calcio.

Il Bulgarelli, nell'edizione del 1962 del suo « Trattato di Puericultura », consiglia in campo profilattico 400 U pro die di vitamina D_2 per il lattante e per il bambino della seconda infanzia, e 800-1000 U pro die per l'immaturo, per circa tre settimane; lo stesso A., come dosi terapeutiche, indica 6000-10000 U pro die di vitamina. De Toni, Debré, Fanconi, considerano che 5 mgr di vitamina D, pari a 200.000 U, devono guarire il rachitismo grave e intendono che il rachitismo — che non guarisce con 200.000 U — è un rachitismo vitamina-D-resistente.

Conclusioni

Questi casi, otto dei quali sono giunti all'osservazione in breve lasso di tempo, fanno sentire il dovere di concludere:

1) con il richiamare ancora una volta l'attenzione sulla necessità di un'oculato uso della vitamina, fatto con scienza e coscienza sempre dopo approfondito esame delle condizioni di ciascun soggetto, specialmente allorchè si intenda praticare la profilassi antirachitica con vitamina D;

2) con l'affermazione che, siccome il tenore di vita della popolazione infantile è buono ed ormai il rachitismo è raro, si deve presumere che le condizioni biologiche dei bambini in genere (salvo eccezioni) siano perfettamente equilibrate. Pertanto dovere del medico, allorchè debba usare medicamenti già indicati come tossici, agire sempre con molta prudenza, e specialmente oggi in quanto « non soltanto il mondo medico, ma anche quello profano sono vivamente preoccupati per l'aumento di quelle malattie che sono denominate "jatrogene" (de Toni jr);

3) che sta al medico al momento opportuno scegliere il preparato adatto ed indicarne l'esatta posologia ed avvertire i genitori sulla pericolosità dell'uso indiscriminato di un farmaco ritenuto dai più totalmente innocuo.

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Ospedale Civile di Udine; Divisione Pediatrica (Primario: Prof. P. Schiaparelli).

G. De Luca e M. Cozzi: La sindrome da iperdosaggio di vitamina D. — Sono riportati in sintesi 12 casi di sindrome da iperdosaggio di Vitamina D, osservati presso la Divisione Pediatrica dell'Ospedale Civile di Udine. L'A. ne discute la patogenesi e il quadro clinico — soffermandosi in particolare sullo studio della funzionalità renale di questi pazienti — e conclude con il menzionare le dosi di vitamina D ritenute oggi le più appropriate, sia a scopo profilattico che terapeutico, e con il sottolineare la necessità che l'uso della vitamina D sia sempre oculato.

[« Min. Ped. », 16, 210-219, 1964]

Hôpital Civil de Udine; Service de Pédiatrie (Chef de Service: Prof. P. Schiaparelli).

G. De Luca et M. Cozzi: Le syndrome d'hyperdosage de vitamine D. — 12 cas de syndrome d'hyperdosage de vitamine D sont rapportés, observés au Service de Pédiatrie de l'Hôpital Civil de Udine. L'A. en discute la pathogénie et le tableau clinique; l'attention est attirée sur l'études de la fonction rénale de ces malades. Un rappel des doses les plus correctes de vitamine D est fait, au point de vue prophylactique et thérapeutique, ainsi que de la nécessité d'un emploi contrôlé de la vitamine D.

[« Min. Ped. », 16, 210-219, 1964]

From the Department of Paediatrics (Head: Prof. P. Schiaparelli), City Hospital, Udine.

G. De Luca and M. Cozzi: Vitamin D overdosage syndrome. — 12 cases of vitamin D overdosage observed at the Department of Paediatrics of the Udine City Hospital are described. The pathogenesis and clinical picture are described, especially as regards the renal function in these patients, and the recognised preventive and therapeutic doses of vitamin D are mentioned. The fact that vitamin D should always be used with caution is stressed.

[« Min. Ped. », 16, 210-219, 1964]

Aus dem Bürgerspital Udine, Kinderabteilung (Chefarzt: Prof. Dr. P. Schiaparelli).

G. De Luca und M. Cozzi: Über das Syndrom durch Hyperdosierung von Vitamin D. — Es werden 12 Fälle von Syndrom durch Hyperdosierung von Vitamin D zusammengefasst, die in der Kinderabteilung des Bürgerspitals Udine beobachtet wurden. Der Verf. bespricht deren Pathogenese, sowie auch das klinische Bild, indem

besonders auf dem Studium über die Nierenfunktion ser Kranken verweilt. Abschliessend erwähnt er die Dosen von Vitamin D, die heutzutage als am meisten passend betrachtet werden, und zwar sowohl zum prophylaktischem, wie auch therapeutischem Zweck. Zuletzt unterstreicht der Verf. die Notwendigkeit, dass die Anwendung von Vitamin D immer eine vorsichtige sein soll.

[« Min. Ped. », 16, 210-219, 1964]

Hospital Civil de Udine; Reparto Pediátrico (Jefe del Servicio: Prof. P. Schiaparelli).

G. De Luca y M. Cozzi: El síndrome por hiperdosificación de vitamina D. — Se exponen en síntesis 12 casos de síndrome por hiperdosificación de Vitamina D, observados en el Reparto Pediátrico del Hospital Civil de Udine. El autor discute la patogénesis y el cuadro clínico deteniéndose en particular en el estudio de la funcionalidad renal de estos pacientes, y concluye mencionando las dosis de vitamina D que hoy día se consideran como las más apropiadas, tanto para fines profilácticos como terapéuticos, y haciendo hincapié sobre la necesidad que el empleo de la vit. D sea siempre preciso.

[« Min. Ped. », 16, 219-219, 1964]

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LA PEDIATRIA ALL'ESTERO

PÉDIATRIE

(Lione - Vol. 18 - N. 1 - 1963)

P. Giraud e coll.: Il test del cromo radioattivo. Suo interesse in campo pediatrico. — Gli AA., in occasione di 20 osservazioni di bambini affetti da emopatie diverse, hanno esplorato la sopravvivenza eritrocitaria con la tecnica che utilizza il radiocromato di sodio. Espongono il principio del metodo ed in particolare i diversi fattori che concorrono a formare la curva di radioattività ematica, spiegando la scomparsa della radioattività fissata sulle emazie marcate. Trattano della tecnica per la fissazione del radionuclide e dei risultati ottenuti da questo tipo di ricerche su bambini affetti da anemie emolitiche congenite ed acquisite, da anemie ipocromiche insorte nel decorso del rachitismo grave e da anemie legate a varie affezioni viscerali. La diminuzione progressiva della radioattività eritrocitaria corrisponde alla regolare distruzione delle emazie marcate e permette pertanto di apprezzare la durata di vita della cellula. Nei casi in cui si sospetta una iperemolisi, studiando attraverso conteggi esterni la sede di questa iperemolisi con contatori a livello del fegato, della milza e dell'area precordiale, e valutando i rapporti epato-spleno-cardiaci ottenuti ad intervallo di tempo regolare, se il rapporto aumenta a favore della milza si può pensare ad una emolisi splenica; una curva di sequestrazione epatica è in favore di una emolisi periferica con accumulo di metaboliti dell'Hb a livello del fegato.

Nella malattia di Minkowski Chauffard la durata di vita delle emazie autotrasfuse è raccorciata.

Nella drepanocitosi vi è una diminuzione della durata di vita secondo questo metodo. Nella emoglobinosi C non vi sarebbero modificazioni. In tre casi di anemie emolitiche costituzionali atipiche i valori sono risultati di 25 giorni, 20 giorni e 6 giorni. Nelle anemie emolitiche acquisite, in due bambini in cui le prove furono praticate in stato di equilibrio ematologico, i valori erano normali.

Nelle anemie ipocromiche in corso di rachitismo grave, su 7 soggetti solo 3 presentavano normale tempo di supravvivenza eritrocitaria. Nelle anemie da cause diverse vi sarebbe una durata di vita normale del donatore sano al malato; variabile invece la durata di vita delle emazie autotrasfuse. Nelle anemie ipocromiche ferriprive l'emivita radioattiva è accorciata. Nelle nefropatie la durata di vita è considerata normale, mentre nelle emopatie maligne non è stata effettuata la determinazione per la continua necessità di trasfusioni.

NICOLAS ANDRY AWARD

Vitamin D: New Horizons*

HECTOR F. DELUCA†

Clin. Orthop. 73: 4-23 (1971)

The initial thrust toward understanding the mechanism of action of vitamin D was made in 1919 when Sir Edward Mellanby successfully produced experimentally the disease rickets in dogs.[44, 45] This disease could be prevented by means of cod liver oil or it could be cured by the same substance. Sir Edward Mellanby incorrectly concluded that the anti-rachitic activity was due to the newly discovered fat-soluble vitamin A of McCollum and coworkers. By means of stability studies McCollum quickly excluded this activity as being due to the vitamin A, demonstrating quite unequivocally that a new vitamin had been discovered, namely the anti-rachitic factor, which was called vitamin D.[54] It was on this basis that the assumption was made that vitamin D must be an accessory food substance or vitamin rather than the hormonal substance it really is. Huldshinsky,[32] for example, had shown quite clearly that rachitic children could be cured by ultraviolet rays from sunlight or from ultraviolet lamps. This apparent dichotomy was resolved at the hands of Steenbock and Black, who demonstrated that the ultraviolet light could produce the anti-rachitic substance not only in the skin of animals, but also in their diets.[71, 72] He then showed quite successfully that the anti-rachitic substance was found in the non-saponifiable fraction of the diet.[73] This work, which was confirmed by Hess and Weinstock,[29, 30] accomplished two major things. First, it eliminated rickets in the western world since mere irradiation of food could impart vitamin D activity in it. Second, it laid the ground work for the isolation and identification of vitamin D_2 which was accomplished in 1932 by Askew *et al.*[2] and by Windaus *et al.*[92] *Waddell*[83] first recognized that a new form of vitamin D must exist since the anti-rachitic activity of impure cholesterol fractions was very great in chickens and rats but the anti-rachitic activity imparted to plant-related sterols was very high in mammals and very low in birds. He correctly concluded that a new form of vitamin D must exist in the irradiated cholesterol fraction. It was Windaus *et al.* who successfully synthesized 7-dehydrocholesterol[91] and thus were able to isolate and identify the structure of vitamin D_3,[93] the normal form of the vitamin in all animals.

Because the vitamins were isolated and identified as accessory food factors, it has been assumed that vitamin D is in fact a vitamin. However, we now know that when man began to shield his body from ultraviolet light derived from the sun he prevented an important reaction from occurring in his skin, namely the conversion of 7-dehydrocholesterol in the epidermis to

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vitamin D_3. He, therefore, began to require vitamin D in his diet to replace that which was not being made in his skin. Vitamin D then became a vitamin as an artifact of civilization or, in the case of animals, as an artifact of domesticity. It is important to note, however, that the actual process of formation of vitamin D_3 in the skin from 7-dehydrocholesterol has not been extensively studied. We do know that 7-dehydrocholesterol exists in high concentrations in the epidermis and that ultraviolet light can reach the epidermis. We also know that ultraviolet light induces photoisomerization of 7-dehydrocholesterol to a number of related compounds, one of which is precholecalciferol which converts readily to cholecalciferol or vitamin D_3. It must be emphasized, however, that the actual process has not been studied from a chemical or biochemical point of view and remains as an uninvestigated biochemical system.

Following the identification of vitamin D_3 by Windhaus *et al.*[33] virtually no work was carried out on the metabolism of vitamin D and, in fact, work on the mechanism of vitamin D action remained at a standstill until approximately 1958. Even at that time it had been assumed that vitamin D_3 must be active *per se* and not be converted to metabolically active forms. This concept was reinforced further by the early work of Kodicek and his collaborators with radioactive vitamin D_3 of very low specific activity.[34] In recent years, however, there has been a gigantic rebirth of interest in the vitamin D mechanism and its metabolism. It will be the subject of this review to bring up-to-date, insofar as possible, the recently evolving concepts of vitamin D metabolism and action.

PHYSIOLOGY OF VITAMIN D ACTION

The first concept to be overturned in recent years is that vitamin D is actually involved in the mineralization process of bone.[14] Because the end-effect of the vitamin is to induce calcification of bone, it had been assumed by investigators over some 50 years that the vitamin must participate in the calcification process itself. In fact, numerous investigations have been directed at this point and the results have been uniformly disappointing. It now has become increasingly clear that the calcification process itself does not involve vitamin D. Perhaps the first blow dealt to the calcification concept was in 1924–25 when Shipley *et al.* demonstrated that in rickets the calcium-times-phosphorus product of the plasma is low.[69, 73] In fact, this has been a diagnostic test of vitamin D deficiency in man. This important and fundamental fact was ignored until 1956 when Neuman and Neuman[50] introduced the idea that blood is normally supersaturated with regard to bone mineral and that collagen merely serves to catalyze the crystallization of hydroxyapatite from a supersaturated solution. In a follow-up publication Neuman[49] showed that the plasma of rachitic animals is undersaturated with regard to bone mineral and hence calcification fails because of the failure of supply of calcium and phosphate to the calcification sites. It is, therefore, evident that the essence of vitamin D action is the elevation of plasma calcium and phosphorus to supersaturating levels which are required for normal mineralization of bone[15] (Fig. 1). There is the possibility that vitamin D may induce osteoblasts and other bone-forming

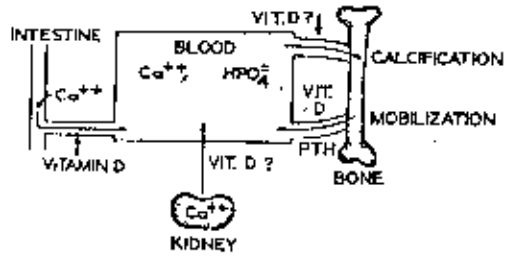

FIG. 1. Diagrammatic representation of physiologic actions of vitamin D.

cells to facilitate the transfer of calcium and phosphorus from the extracellular fluids to the calcification sites. The only evidence to support this concept at the present time is primarily clinical, in which certain cases of vitamin D-resistant rickets will show a calcification response to large doses of vitamin D in the absence of any appreciable change in plasma calcium and phosphate concentrations. However, direct evidence is lacking at the present time and represents an important area of investigation. There is no doubt that calcification does fail in vitamin D deficiency even in rats maintained on a normal calcium and phosphorus diet.[4] However, it is not clear whether there is a direct participation of vitamin D at the bone site to make available calcium and phosphorus from the extracellular fluid to the calcification sites in bone.

It is certain in any event that vitamin D functions to bring about the elevation of plasma calcium and phosphorus to supersaturation levels with regard to the hyroxyapatite of bone mineral (Fig. 1). The vitamin brings about the elevation of plasma calcium and phosphorus in at least two well-known mechanisms. The oldest known, and perhaps most firmly established, is that of increasing intestinal calcium absorption. This fundamental effect of vitamin D was first discovered by Orr *et al.* when they noted great losses of calcium in the feces of rachitic children.[39] They correctly concluded that in vitamin D deficiency there is a failure of calcium absorption. This important concept was unfairly discredited and largely ignored until Nicolaysen established on a very firm basis that vitamin D increases intestinal absorption of calcium.[53, 54, 55] He was able to demonstrate this fundamental action of the vitamin in intact animals in which he demonstrated quite conclusively that the loss of calcium in the feces in vitamin D deficiency is not due to an excessive loss of endogenous calcium. He was able to establish with isolated intestinal loops the conclusive participation of vitamin D in the calcium absorption process. This fundamental observation has been confirmed many times and in more recent years using more elegant techniques involving radiocalcium.[64] Nicolaysen was able to demonstrate that vitamin D does not increase phosphate absorption *per se*, but rather improves phosphate absorption secondarily to the improvement in calcium absorption.[33] In this manner the calcium-times-phosphate product of the plasma is elevated at the expense of calcium and phosphorus of intestinal lumen.

Of equally great importance to the overall vitamin D mechanism is the concept that the vitamin is involved in the mobilization of mineral from old or previously formed bone to the plasma. This phenomenon might appear paradoxical to the end-effect of vitamin D (namely, increased calcification), but in actual fact, by this mechanism, mineral can be utilized from old bone to bring about mineralization of newly formed organic matrix. This mechanism is also essential in the maintenance of plasma calcium concentration for normal bodily functions. This important function of vitamin D was not discovered until 1951 when Carlsson and coworkers[1, 9] established it firmly, not only at high doses of the vitamin but also at normal physiologic doses. As a follow-up to this, Harrison *et al.*[27] in 1958 noted that parathyroid hormone is unable to induce the mobilization of bone mineral in the vitamin D-deficient rat. This fundamental observation was confirmed and extended in the author's laboratory, and the suggestion was made that the parathyroid hormone-induced bone mineral mobilization requires the presence of at least small amounts of vitamin D whereas the phosphate diuretic action of the hormone is vitamin D-independent.[64] In any case, the dependency upon vitamin D of the bone mineral mobilization process has been clearly demonstrated. By this mechanism the vitamin also elevates plasma calcium

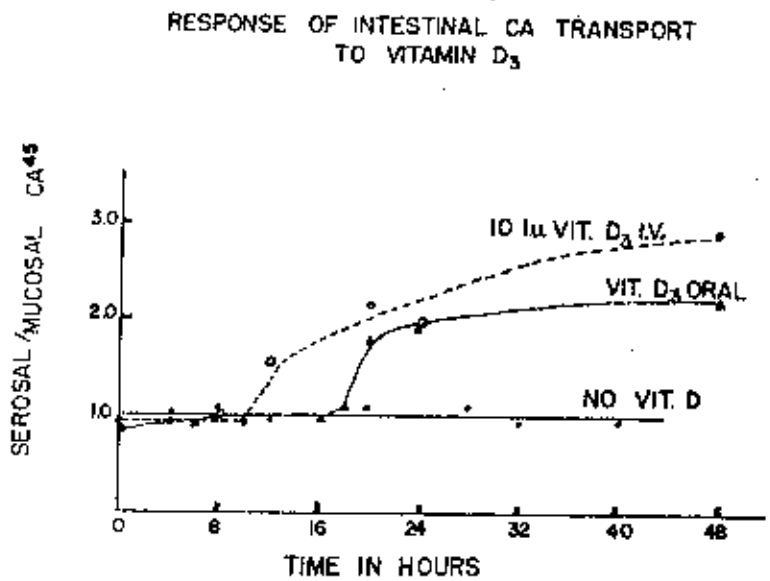

FIG. 2. Intestinal calcium transport response to 0.25 μg vitamin D_3 given intravenously or orally to vitamin D-deficient rats. The intestinal transport was measured by the everted sac technic.

and phosphate concentration. This effect, operating in concert with the increased intestinal absorption effect, brings about the elevated plasma calcium and phosphate concentration to supersaturation levels which are in turn necessary for normal mineralization of bone.

In 1941 Harrison and Harrison[25] suggested that vitamin D might be involved in the renal tubular reabsorption of phosphate. Although attractive at that time as an explanation of vitamin D action, it now is clear that the extensive phosphate loss in the vitamin D-deficient animal is probably due to a secondary hyperparathyroidism in which the excessive parathyroid hormone brings about a phosphate wasting in the renal tubules. This question has not been adequately investigated and the possibility remains that vitamin D may participate in phosphate handling by the kidney, but these phenomena must be separated from the parathyroid hormone action in any experimental plan.

In 1960 Grant[11] reported that vitamin D improves renal tubular reabsorption of calcium. Although this finding is of obvious interest, it perhaps plays a very small part in the overall calcification end-effect of vitamin D. In any case the role of vitamin D in the kidney has yet to be critically examined. It is indeed possible that improved tubular reabsorption of calcium and/or phosphate may take place in response to vitamin D and that this would also contribute to the overall calcification end-effect of the vitamin. Figure 1 illustrates in a diagrammatic fashion what is known concerning the physiologic action of vitamin D in the maintenance of plasma calcium and phosphate levels required for normal calcification.

METABOLISM OF VITAMIN D

As will become obvious from the ensuing discussion, it has become increasingly apparent over the past 10 years that the function of vitamin D cannot be intelligently discussed without considering its metabolism. It must first be recognized that there exists a large time lag between the time of administration of vitamin D and its first observable physiologic effect.[14] To illustrate this point, Figure 2 shows that the increase

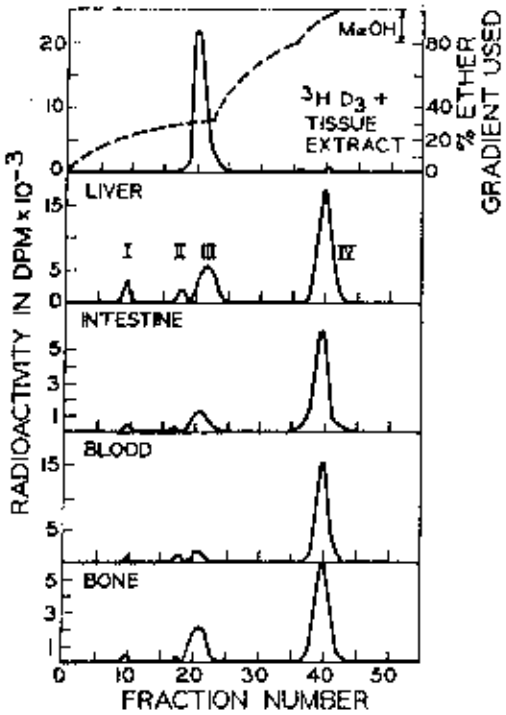

FIG. 3. Silicic acid column chromatographic profile of chloroform extracts of tissues from rats given 10 IU vitamin D_3 12 hours before sacrifice. . . . . radioactivity; — — — solvent gradient; III represents unchanged vitamin D_3.

in intestinal calcium transport as measured by the everted sac technic does not occur until some 10 to 12 hours following an intravenous injection of 0.25 μg of vitamin D_3. Obviously, the lag is considerably longer when the dose is given orally. This lag, which was first appreciated by Carlsson and Hollunger[13] and later by many investigators, has consumed much attention from our laboratory personnel for the past 10 years. Of obvious interest is whether the vitamin is transported rapidly enough to the target tissues, namely intestine and bone. Secondly, does the vitamin have to be altered or changed into a metabolically active form before it can function? Before these two fundamental questions could be answered, it was necessary to chemically synthesize radioactive vitamin D of sufficient specific activity so that experiments with truly physiologic doses could be carried out. It must be recalled that vitamin D is an extremely potent substance in which as little as 0.025 μg will produce a physiologic response in the experimental rat. Thus, three radioactive preparations of sufficient activity to permit experiments with 0.25 μg or less were successfully synthesized in the author's laboratory; vitamin D_3 labeled in the 1,2 position with tritium[51]; vitamin D_2 randomly labeled with carbon 14[33]; and vitamin D_4 labeled in the 22,23 position with tritium.[16] With these preparations the first question was successfully answered; that is, it became obvious that radioactivity from radioactive vitamins D_2, D_3, and D_4 following their intravenous injection appeared within minutes in the target tissues of vitamin D action. Clearly then, the time lag in vitamin D response was not due to a failure of transport of the radioactive vitamin to the target tissues.

The question of whether vitamin D must be converted to a metabolically active form, however, was to provide our first important insight into the functional metabolism of vitamin D. Previous workers had failed to detect biologically active metabolites of vitamin D.[14] This was undoubtedly due to the fact that the specific activity of the radioactive vitamin D used was so low that massive amounts of the vitamin were required before it could be detected in the animal, thus precluding the detection of small amounts of functional metabolites. Biologically active metabolites were then first successfully demonstrated in this laboratory in 1964.[57] It was not until 1966, however, that Lund and DeLuca[16] demonstrated the existence of a metabolite more polar than vitamin D_3 which had biological activity at least equal to, and perhaps greater than, the parent vitamin. This metabolite was detected by injecting radioactive vitamin D into vitamin D-deficient rats; after a certain

length of time, the rats were killed and their tissues extracted with methanol-chloroform. The chloroform extracts were chromatographed on silicic acid columns which at that time, using crude gradient technic, separated four radioactive components (Fig. 3). The first radioactive component was soon identified by Lund *et al.* as esters of vitamin D and long-chain fatty acids.[39] Fraser and Kodicek[18, 19] also detected and worked extensively on the esters of the vitamin and long-chain fatty acids. The second component remained unidentified, but both components I and II remained small regardless of dose or time after dose. Component III proved to be unchanged vitamin D_3, while component IV is the very polar metabolite fraction which contained the markedly biological active metabolite of the vitamin. Not only was this metabolite fraction more effective than the vitamin in the cure of rickets in rats, it improved intestinal absorption and bone mineral mobilization; and most important, it acted much more rapidly on these parameters than the parent vitamin.[36] With the successful improvement of silicic acid column chromatographic technics in our group[41] and the use of large experimental animals, namely the pig, sufficient quantities of this metabolite could be generated in their plasma to permit chemical isolation and identification. After an extensive effort in 1968, Blunt *et al.*[6, 7] successfully isolated the active principle from this fraction of the plasma of pigs and by means of nuclear magnetic resonance spectrometry, mass spectrometry, ultraviolet spectrophotometry and other physical means identified its structure as 25-hydroxycholecalciferol (25-HCC) (Fig. 4). To confirm the identification and to provide large amounts of the material for clinical testing in cases of vitamin D-resistant rickets and other related bone diseases, we were able to successfully synthesize this important substance by two chemical methods.[5] One of these methods, shown in Figure 5, has now

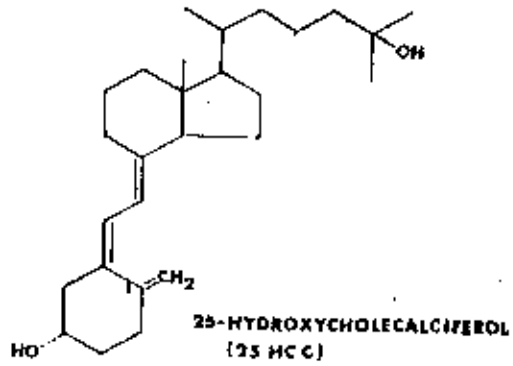

FIG. 4. 25-Hydroxycholecalciferol (25-HCC).

been utilized to synthesize radioactive 25-HCC. This is of great importance in our further understanding of the metabolism and functional aspects of vitamin D action. The biologically active metabolite from vitamin D_2 was also isolated in pure form and identified as 25-hydroxyergocalciferol (25-HEC) in this laboratory (Fig. 6).[76, 77]

The 25-HCC proved to be 1.5 times more effective than vitamin D_3 in the cure of rickets in rats and chicks.[8] Most important, however, is the fact that it stimulates intestinal calcium transport much more rapidly than an equal dose of vitamin D_3, as shown in Figure 7. It also acts much more rapidly than vitamin D_3 in inducing bone mineral mobilization *in vivo*. Although these two important findings suggest that the 25-HCC may be the circulating active form of the vitamin, it does not constitute conclusive proof. In this laboratory an isolated vascularly perfused intestinal system has been developed for the rat. Using this isolated intestine it has been possible to demonstrate the classic effect of pre-treatment of the animal with vitamin D_3 on improved rate of calcium transport. However, this system does not respond to vitamin D_3 infused in massive amounts in the vascular system. By way of contrast, this isolated, D-deficient perfused intestine will respond quite rapidly to 25-HCC in small amounts in the vascular

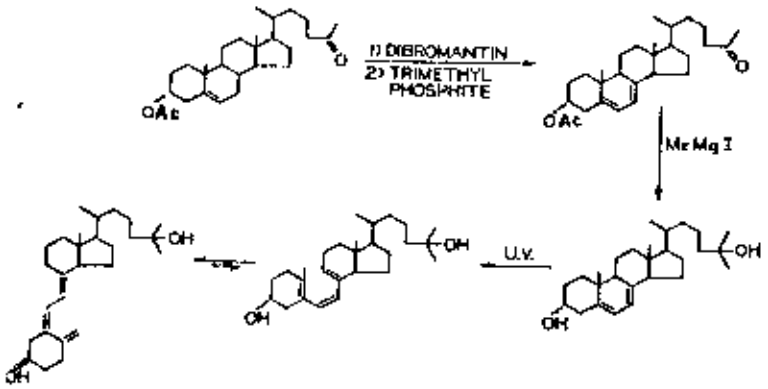

FIG. 5. Synthesis of 25-HCC starting with 3-acetoxy norcholesta-5-ene-25-one. (Reproduced with permission of the publisher from Blunt and DeLuca, The synthesis of 25-hydroxycholecalciferol. A biologically active metabolite of vitamin D_3, Biochemistry 8:671 1969.)

system.[58] Thus, in an isolated end-organ of vitamin D action 25-HCC is extremely effective in inducing the classical action of vitamin D whereas vitamin D_3 itself is without effect. In an analogous fashion, it had been observed by scientists carrying out tissue culture experiments with bone that parathyroid hormone readily induced mobilization of bone mineral from isolated pre-natal bone in culture. On the other hand, massive amounts of vitamin D produced little or no effect despite the fact that it is well known that vitamin D is involved in inducing bone mineral mobilization *in vivo*. This puzzling situation was solved when 25-HCC became available. This metabolite of vitamin D, and not the vitamin itself, was able in extremely small amounts to induce bone mineral mobilization in isolated cultures.[62] Thus in the two known end-organs of vitamin D action, vitamin D_3 itself is without effect whereas the 25-hydroxy derivative is extremely effective. This illustrates that 25-hydroxylation is essential for the action of vitamin D on its end-organs, and more specifically it shows that the 25-HCC is at least the hormonal form or circulating active form of the vitamin.

It is obvious that if the isolated end-organs of vitamin D cannot respond to vitamin D_3 but can respond to the 25-hydroxy derivative that the 25-hydroxylation must not take place in the end-organs. Of obvious importance is the site at which this hydroxylation must take place. It had been observed that following the injection of radioactive vitamin D the plasma radioactivity drops very rapidly; at the same time liver radioactivity rapidly increases until some 60 per cent of the dose can be found in the liver.[63] Shortly thereafter the radioactivity begins to leave the liver and appears once again in blood. However, it appears in the blood as the 25-hydroxy derivative. Thus it appears that the liver plays a major initial role in the metabolism of vitamin D and is probably the organ which carries out the 25-hydroxylation. This belief was clearly substantiated when "hepatectomized" rats were unable to synthesize 25-HCC from vitamin D_3.[63] In fact, these animals showed no evidence of vitamin D metabolism suggesting that the 25-hydroxylation is also the initial metabolic modification on the molecule. Unequivocal evidence of 25-hydroxylation in the liver was obtained when Horsting and DeLuca demonstrated conclusively that perfused liver is able to convert vitamin D_3 to 25-HCC.[31] More important, however, was the fact that they were able to demonstrate that homogenates of liver fortified with reduced pyridine nucleotide, magnesium ions and oxygen were able to hydroxylate vitamin D_3 to 25-HCC. This system has been extensively studied very recently and it has been conclusively shown that there

is an absolute requirement for molecular oxygen and a requirement for reduced pyridine nucleotide, although in whole homogenates it appears that both DPNH and TPNH can supply hydrogen for the hydroxylation.

The 25-hydroxylation of vitamin D_3 in the liver is an unusual one to begin with inasmuch as a tertiary hydrogen is involved. That it is in fact an unusual hydroxylation is shown in Table 1 which illustrates that respiratory inhibitors are unable to inhibit the hydroxylation but, even more important, carbon monoxide to oxygen at a ratio of 3:1 are unable to inhibit the hydroxylation of vitamin D_3. This is surprising in view of the fact that carbon monoxide is a very potent inhibitor of the standard steroid hydroxylation system of both liver and adrenal glands. Diphenyl-p-phenylenediamine, the lipid peroxidation inhibitor, also has no effect on the hydroxylation system, indicating that peroxidation is not involved in the reaction. Feeding of phenobarbital for as long as a week also had no effect on the level of the vitamin D 25-hydroxylase in liver. These results suggest that the standard hydroxylation mechanism normally observed in liver and adrenal glands is not involved in the vitamin D activation to its 25-hydroxy derivative.

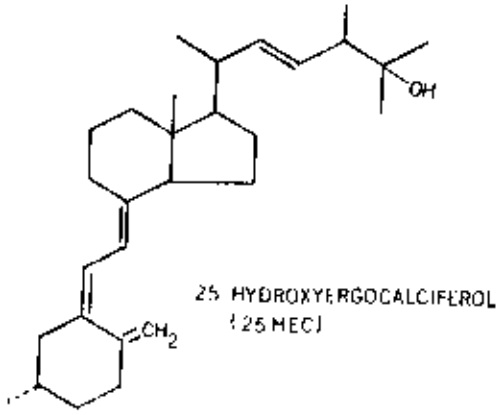

FIG. 6. 25-Hydroxyergocalciferol (25-HEC).

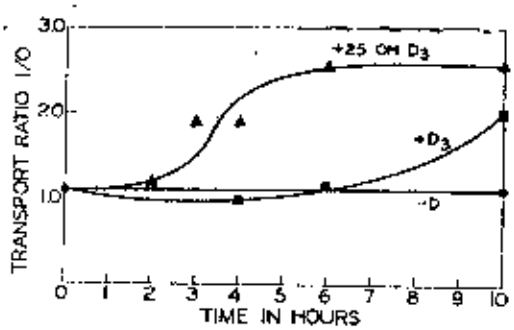

FIG. 7. Intestinal calcium transport response to either 0.25 μg 25-HCC or vitamin D_3 given intravenously to vitamin D-deficient rats.

Table 2 shows that the hydroxylation system resides in at least two cell fractions. Crude mitochondria, microsomes and nuclei are unable to carry out the hydroxylation by themselves. However, when they are fortified with cytoplasmic fraction, each of these crude preparations can carry out the hydroxylation. Pure nuclei prepared by the 2.3 molar sucrose method are unable to carry out the hydroxylation even in the presence of the cytoplasmic fraction, whereas pure mitochondria are the most effective. The microsomal fraction does have some activity in the presence of the cytoplasmic fraction but this is probably due to mitochondrial fragment contamination. This hydroxylation reaction is unique to liver inasmuch as whole homogenate of intestine and kidney is unable to catalyze the hydroxylation. It is also noteworthy that the cytoplasmic fraction of kidney or intestine is unable to satisfy the cytoplasmic requirement.

Of major interest to the physiologists is the fact that 25-HCC strongly inhibits hydroxylation of vitamin D to the 25-hydroxy derivative (Fig. 8). This potent product inhibition can be demonstrated both *in vivo* and *in vitro* and probably represents an important control mechanism for the secretion of active vitamin D into the plasma. Of even greater interest is the fact that 25-hydroxydihydrotachysterol is unable to exert

TABLE 1. Inhibitor Study of Vitamin D 25-Hydroxylase System of Rat Liver Mitochondria

Inhibitor	*25-HCC Produced*
	DPM
None	4,700
Antimycin A (0.2 nmoles/g protein)	6,500
Rotenone (30 nmoles/g protein)	9,600
NaCN (1.3 mM)	7,800
NaN_3 (1.3 mM)	5,100
Diphenyl- p-phenylene diamine (3.8×10^{-6}M)	5,100
$N_2:O_2$ — 3:1	4,200
$CO:O_2$ — 3:1	3,400

Incubations were carried out as described by Horsting and DeLuca,[31] but liver mitochondria plus liver supernatant served as the enzyme system.

the same inhibitory effect as the 25-HCC. This very important observation now makes possible an explanation of the well-known phenomenon that dihydrotachysterol at high doses is much more effective than vitamin D in the treatment of hypoparathyroidism and other diseases, whereas at very low doses vitamin D is much more effective than the dihydrotachysterol. It now appears that dihydrotachysterol is hydroxylated in the liver to form the 25-hydroxy derivative. This process is not product-inhibited, and thus the dihydrotachysterol in total is readily hydroxylated to its 25-hydroxy derivative. Vitamin D, on the other hand, is hyroxylated to a small extent before product inhibition begins to have a marked effect. This feedback control limits the total effectiveness of large amounts of vitamin D_3.

Not only do these phenomena explain why large amounts of dihydrotachysterol are more effective than large amounts of vitamin D_3 in the treatment of bone disease, but they also provide an explanation as to why vitamin D is stored and conserved for long periods of time. The product inhibition of vitamin D hydroxylase probably on the one hand prevents toxicity due to large doses of vitamin D_3 and conserves vitamin D_3 for use at times when the intake or production of vitamin D_3 is very low. It should be noted that the 25-HCC inhibition of the hydroxylase is probably competitive with the substrate. Thus, very large amounts of vitamin D can overcome the inhibition allowing the production of excess 25-HCC which in turn brings about toxicity. Thus the 25-hydroxylation step represents an important physiological control point which undoubtedly plays a major role in the regulation of 25-HCC or the hormonal form of vitamin D in the body.

The essentiality of the 25-hydroxylation raises a question as to whether some diseases, such as familial hypophosphatemia or pseudo-vitamin D-deficiency disease, may represent a defect in the 25-hydroxylation mechanism. Thus far in no disease, with the possible exception of certain types of hepatic disease, is there evidence of failure of hydroxylation. This may seem surprising but upon further consideration it is evident that if an essential reaction such as the 25-hydroxylation did not take place undoubtedly the animal would not have survived and hence such a mutation will have been undoubtedly lethal.

The fact that no disease has yet been found where there is a specific defect in the hydroxylation of vitamin D does not mean that 25-HCC is not a therapeutically effective compound. The 25-HCC has now been successfully used at relatively low doses to treat vitamin D-resistant hypoparathyroidism,[30] a number of cases of familial hypophosphatemia, pseudo-vitamin D-deficiency disease and Fanconi syndrome. It is now being tested in such diseases as renal osteodystrophy, vitamin D-resistant osteoporosis, and other forms of bone disease wherein large amounts of vitamin D have been used as treatment in the past. It is likely that 25-HCC will be more effective at lower doses.

Most important, however, is the fact that the 25-HCC is cleared from the body much more rapidly than is vitamin D_3 and hence represents a much safer compound to use therapeutically, since when toxicity appears, withdrawal of 25-HCC should be a sufficient and effective treatment whereas when a toxicity level of vitamin D_3 is achieved, the patient is in great danger for a long period of time following withdrawal of the vitamin and often requires cortisol treatment.

Following the synthesis of radioactive 25-HCC it was possible to investigate the metabolism of this active form of vitamin D in the target tissues to examine whether it is 25-HCC or an even further metabolite which might be considered the metabolically active form of the vitamin. Surprisingly, as shown in Figure 9, 25-HCC is metabolized very rapidly to more polar compounds in intestine, bone, kidney, and liver.[12, 13] Of great interest are the peak V metabolites derived from these tissues. These metabolites

TABLE 2. Subcellular Localization of Vitamin D 25-Hydroxylase of Liver

Fraction	*25-HCC Produced*
	DPM
Whole homogenate	9,200
Crude nuclei	0
Crude mitochondria	0
Crude microsomes	0
Cytoplasm	0
Pure nuclei (2.3 M sucrose method) + cytoplasm	0
Pure mitochondria (0.44 M sucrose) + cytoplasm	8,800
Microsomes + cytoplasm	4,400
Crude nuclei + cytoplasm	4,500
Mitochondria + kidney cytoplasm	0

The reaction was carried out as described by Horsting and DeLuca,[31] except cell fractions isolated from equivalent weights of tissue were used as the source of the enzyme.

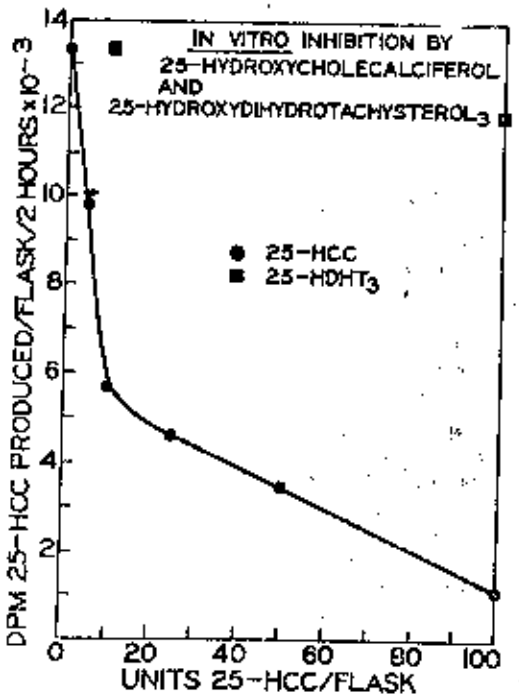

FIG. 8. Inhibition of 25-hydroxylation of vitamin D_3 by 25-HCC but not 25-hydroxydihydrotachysterol$_3$. The hydroxylation reaction was carried out with liver homogenates as described by Horsting and DeLuca.[31]

are chromatographically different from each other. The bone produces two peak V materials, one which is identical with the peak V of intestine and another which has been identified as 21,25-dihydroxycholecalciferol (Fig. 10).[78] This peak V material from bone has a highly specific action on the mobilization of bone mineral and it is attractive to put forth the hypothesis that 25-HCC represents the circulating or hormonal form of the vitamin and that it is converted to a specific tissue or to metabolically active forms. The peak V from intestine has not yet been identified nor has the peak V from the kidney. The 25,26-dihydroxycholecalciferol has been isolated and identified from hog blood and appears to originate in the liver.[79] Thus to summarize the current work on the metabolism of the vitamin (Fig. 11),

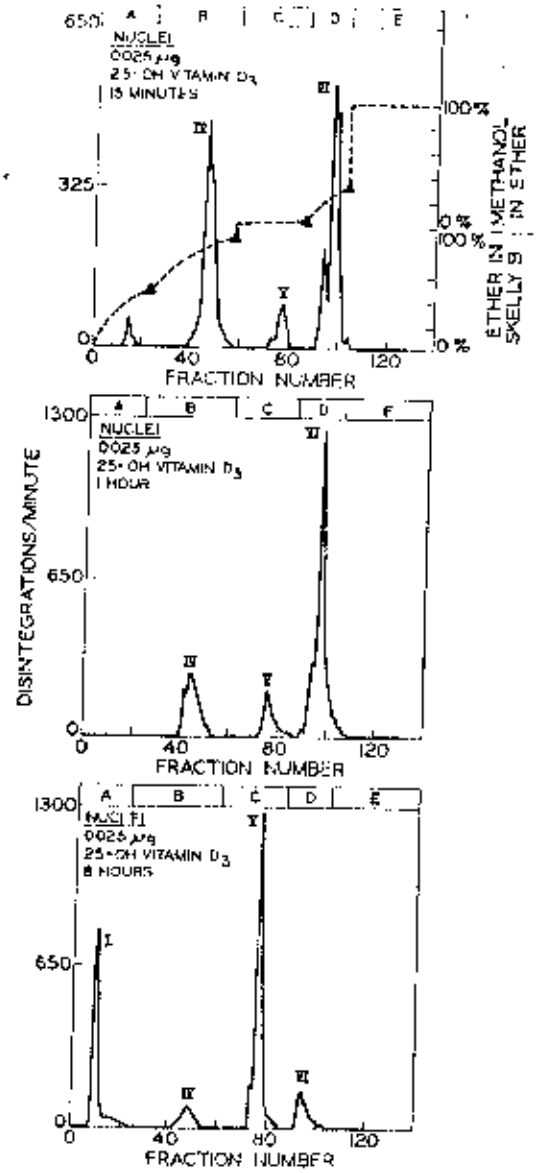

FIG. 9. Silicic acid column profiles of intestinal nuclear extracts from rats given 0.25 μg 26,27-^{3}H 25-HCC. (Reproduced with permission of the publisher from Cousins *et al.*: The metabolism and subcellular location of 25-hydroxycholecalciferol in intestinal mucosa, Biochemistry 9:1453, 1970.)

it appears that vitamin D_3 is the natural form of the vitamin generated in the skin and also represents a dietary form of the vitamin. It can be considered the storage form of the vitamin and it is converted in the liver by a tightly controlled reaction to the 25-HCC which represents a hormonal or circulating active form. In each tissue specific metabolically active forms appear. Recently the intestinal metabolically active form was isolated and identified as 1,25-dihydroxyvitamin D_3.[56]

MECHANISM OF VITAMIN D ACTION

There has been much recent progress in the understanding of the mechanism of action of vitamin D. In 1965 Zull *et al.* showed quite conclusively that all physiologic responses to vitamin D are prevented by the prior administration of actinomycin D.[94, 95] If the actinomycin D is administered after the vitamin has been given, it is no longer able to elicit the block of either bone mobilization or intestinal calcium transport. A number of other control experiments were run illustrating that actinomycin D was acting in the expected fashion. Furthermore, other protein and RNA synthesis inhibitors are able to partially block the action of vitamin D. Other workers established that actinomycin D inhibits the action of vitamin D on intestinal calcium absorption in the chick.[56] These results made it possible to conclude that the expression of vitamin D action must involve RNA and protein synthesis. This idea received additional support when it could be demonstrated that vitamin D stimulated the pulse-labeling or synthesis of intestinal nuclear ribonucleic acid.[75] In addition, Hallick and DeLuca very recently have shown that chromatin isolated from rats given vitamin D shows an increased template activity to pure *E. coli* RNA polymerase.[21] These results suggest that in some way vitamin D must bring about the unmasking of genetic information responsible for vitamin D action.

The exact site of actinomycin D sensitivity in the vitamin D mechanism has remained obscure until recently when Tanaka and DeLuca showed clearly that actinomy-

cin D prevents the metabolism of 25-HCC to its more polar peak V metabolites (Fig. 12).[80] If the actinomycin D is given after the radioactive 25-HCC, it is no longer able to block the conversion of 25-HCC to peak V. From these results it is evident that 25-HCC must itself interact with the nucleus whereupon it induces the formation of an enzyme or enzymes which are responsible for its conversion to its polar peak V metabolite. Its peak V metabolite then in some way brings about the increased intestinal calcium transport activity. At the present time it is not possible to say in what way the peak V metabolite brings about the function of vitamin D in the various target tissues. The existence of the very polar peak V metabolites from vitamin D_3 was first demonstrated by Haussler *et al.*,[28] and more recently they have ascribed considerable intestinal calcium transport activity to this metabolite.[47] Lawson *et al.* have also found this metabolite; they found further that in the conversion of vitamin D_3 to this metabolite, tritium is lost from the 1 position.[35] These results have been confirmed in our laboratory. At the present time an intensive effort is being made to isolate and identify this further metabolite of vitamin D in the intestine and to determine by which mechanism it is able

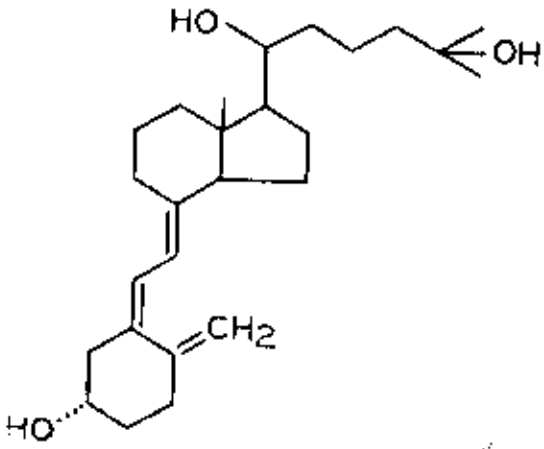

FIG. 10. 21,25 - Dihydroxycholecalciferol (25-DHCC).

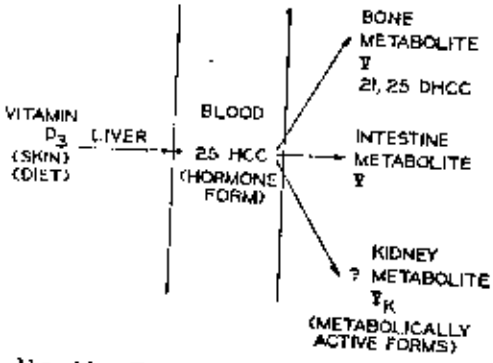

FIG. 11. Functional metabolism of vitamin D to metabolically active forms.

to bring about the increased intestinal calcium transport.

Of importance to the vitamin D-induced calcium transport mechanism is the subcellular location of the 25-HCC and its polar metabolites. There is general agreement that there are two cell fractions of intestine which contain the bulk of radioactivity derived from radioactive vitamin D_3 or 25-HCC.[11, 74] These fractions are the nuclear fraction and the cytoplasmic fraction. It has been suggested that in the nucleus the radioactivity exists in the nuclear membrane[74]; other suggestions are that it is located associated with the chromatin fraction.[28] Recent evidence[11] suggests that a conclusion that the radioactivity is found in the chromatin is premature from the present available data.

NATURE OF THE CALCIUM TRANSPORT COMPONENT FORMED IN RESPONSE TO VITAMIN D IN INTESTINE

Although the vitamin D-stimulated intestinal absorption of calcium phenomenon was studied many times and by many investigators following the work of Nicolaysen, little new was revealed regarding the mechanism of action of vitamin D. Schachter and Rosen,[67] utilizing the everted gut sac method of Wilson and Wiseman,[90] provided the first clear evidence of the nature of the calcium

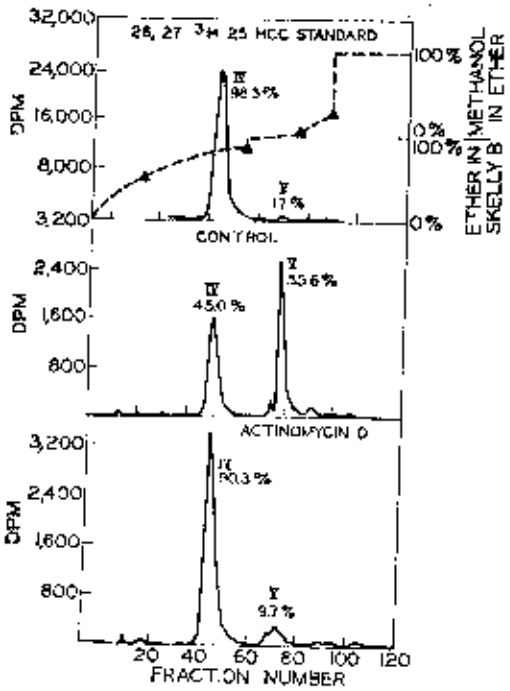

FIG. 12. Actinomycin D inhibition of 26, 27-^{3}H 25-HCC metabolism in intestine to peak V. Peak IV represents unchanged 25-HCC.

absorption process in response to vitamin D.

They were able to demonstrate that this process is an active one in which calcium is transported against a concentration gradient. This was quickly confirmed by Harrison and Harrison[24] who found in addition that vitamin D stimulates transfer of calcium even under conditions where little active transport is occurring.[26] They concluded then that vitamin D must be involved in the penetration of calcium into intestinal tissue where an active transport mechanism could operate to transfer calcium against an electrochemical potential gradient. Wasserman and his colleagues,[84] Schachter,[66] and Martin and DeLuca[19] provided clear evidence that calcium is transported against an electrochemical potential gradient in the small intestine of animals given vitamin D. Martin and DeLuca[19] and Wasserman and Kallfelz[85] have shown that vitamin D stimulates the rate of calcium flux in both the mucosal-to-serosal and serosal-to-mucosal directions. The dilemma nevertheless exists as to whether the vitamin D-induced mechanism is involved in the actual active transport mechanism or whether it is involved in the increased penetration of calcium into the mucosal cells. Schachter *et al.* have concluded, on the basis of experiments with intestinal slices, that vitamin D is involved in both the penetration of calcium across the brush border surfaces and the expulsion of calcium from the serosal surface.[68] Direct evidence that vitamin D is involved in the initial transfer of calcium across the mucosal surface has now been provided by experiments with short-term incubations and in which only the mucosal surface is exposed to ^{45}Ca.[39] Under these conditions, pre-treatment of animals with vitamin D results in intestines which possess a very marked increase in the ability to transfer calcium across the brush surface. Adams *et al.*,[1] using a polyene antibiotic to induce a leaky brush border surface, have provided additional evidence that the action of vitamin D is on the brush border surface. Whether or not vitamin D is involved in the expulsion of calcium from the serosal surface of intestine has not been critically examined at the present time. There seems, therefore, to be universal agreement that the vitamin D-induced mechanism involves an increased rate of calcium transfer across the brush border surface. Electron microscopic evidence for this has also been provided by Sampson *et al.*[63] in which granules of calcium can be shown to accumulate at the brush border surface in vitamin D-deficient intestine; immediately following vitamin D these granules disappear and are replaced by large numbers of calcium granules in the mitochondria. These results suggest that following the vitamin D-stimulated transfer of calcium across the brush border surface, the mitochondria scavenge the calcium keeping the intracellular calcium concentration

very low indeed. Despite an earlier report, recent work in this laboratory[19] and in that of Harrison and Harrison[25] has clearly established that the calcium transport system requires the presence of sodium ions. Sodium is required for the expulsion of calcium from the serosal surface.[40] To complete the picture, therefore, of the calcium transport mechanism it appears that a downhill sodium gradient at the serosal surface results in an additional energy push to expel calcium against an electrochemical potential gradient. A high sodium to potassium extracellular ratio is, of course, maintained by the well-known sodium potassium ATPase pump system on the serosal surface.

There is much disagreement regarding the nature of the vitamin D-stimulated component of the calcium transport system. Wasserman and coworkers[81, 86-88] have discovered, isolated in pure form and studied extensively a calcium-binding protein which appears in the intestine following vitamin D. The amount of calcium-binding protein is related inversely to the calcium concentration of the diet. Thus, chicks on a low calcium diet have high amounts of calcium-binding protein while chicks on a high calcium diet have very much less. In the absence of vitamin D, little or no calcium-binding protein make makes its appearance regardless of diet. There is little doubt that the calcium-binding protein is a likely candidate for the role of the vitamin D-stimulated calcium transport component. Certain discrepancies, however, still remain to be answered. For example, in the time following repletion of a vitamin D-deficient animal there is in fact a very poor correlation between the amount of calcium-binding protein and the amount of calcium absorption.[17] Ebel *et al.*[17] have shown with an immunoassay technic that calcium-binding protein is present at all times in small amounts following vitamin D when increased intestinal absorption of calcium is obvious. However, Harmeyer and DeLuca[27] have shown that there is no quantitative relationship between calcium-binding protein and amount of calcium absorption during this initial period. It is nevertheless possible that only a small amount of calcium-binding protein is needed to carry out the increased calcium absorption resulting from vitamin D.

The calcium-binding protein of the chick has a molecular weight of 28,000 and binds 1 mole of calcium per mole of protein. Surprisingly, the calcium-binding protein in rat intestine which appears following vitamin D has a molecular weight of somewhere around 13,000 (Drescher and DeLuca, unpublished results). Only additional investigation can provide the answer as to whether the calcium-binding protein is one of several components, or the only component, made in response to vitamin D which functions in the calcium absorption system.

In our laboratory it had been demonstrated that the initial transfer of calcium across the brush border surface of intestine is markedly inhibited by a nitrogen atmosphere, suggesting this process to be energy requiring.[39] This led to a search for a calcium-dependent adenosine triphosphatase of the brush border surface as a possible transport substance. Such an adenosine triphosphatase was clearly found and shown to appear immediately following vitamin D.[41, 43] The correlation of its appearance with increased intestinal absorption of calcium in response to vitamin D appears quite good. The amount of the ATPase however, seems to be somewhat variable depending upon the preparation. It must be realized that this system is visualized as a transport system which utilizes ATP to transfer calcium. The hydrolysis of ATP is merely a convenient method of measuring the transport system since it is not possible to measure transport once a membrane is ruptured. Nagode *et al.*[48] have provided evidence that the alkaline phosphatase of brush borders is identical with the calcium-

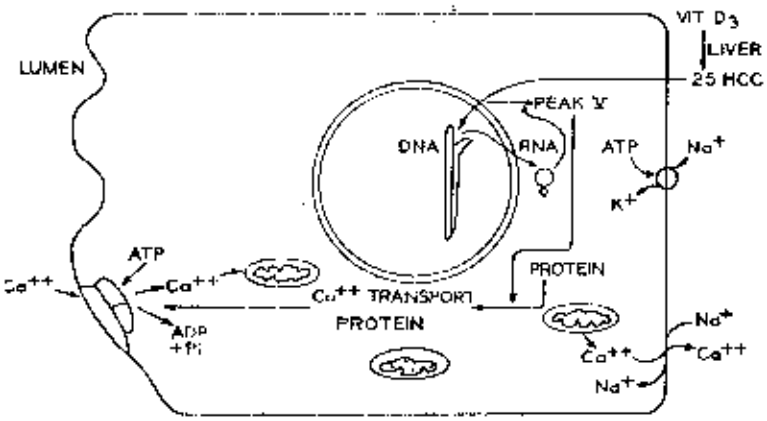

FIG. 13. Summary of a working hypothesis of mechanism of vitamin D action on intestinal mucosa.

dependent adenosine triphosphatase. Their results have substantiated the results in this laboratory regarding existence of the ATPase following vitamin D repletion of vitamin D-deficient rats and chicks.

It is evident that there is no uniform agreement as to the nature of the vitamin D-stimulated transport material in intestine. There is no doubt that the calcium-binding protein makes its appearance following vitamin D, and likewise there is no doubt that the calcium-dependent adenosine triphosphatase of brush borders also makes its appearance following vitamin D. The question is, how to resolve both concepts into a reasonable mechanism of vitamin D action in stimulating intestinal calcium transport. It is possible that the adenosine triphosphatase is a composite of an energy-dependent transfer system and the calcium-binding protein of Wasserman. If this proves to be the case, it may well be that the calcium-binding protein is the substance that appears following vitamin D and that this substance at the brush border becomes part of a transport system which can then be measured as a calcium-stimulated ATPase. Only additional investigation can provide evidence for this exciting concept.

Some evidence has been provided by MacGregor *et al.*[8] that vitamin D is involved in the de novo synthesis of the calcium-binding protein. However, the data that they present suggests that they are merely measuring turnover of the calcium-binding protein since chicks maintained on a borderline amount of vitamin D are used as controls and compared to other animals given massive amounts of vitamin D. In our laboratory, clear evidence (Drescher and DeLuca, unpublished results) has been provided in the rat that the calcium-binding protein is not made de novo following vitamin D treatment, but rather is formed from a preexisting protein by some unknown mechanism under the influence of the vitamin.

Finally it should be mentioned that little biochemical work has yet been carried out on the bone mineral mobilization system which responds to vitamin D. The reason for this is primarily technical inasmuch as biochemical studies are difficult to carry out with a heterogeneous and physically hard tissue such as bone. This represents another important area remaining for concerted biochemical investigation.

SUMMARY

Figure 13 illustrates the mechanism which can be visualized as explaining all present data regarding the action of vitamin D in intestine. The storage form of vitamin D is obtained by the animal either from his diet or by irradiation of his skin. This substance is stored throughout the body in either body

fat, liver, blood or other tissues and is converted by the liver to 25-HCC, the circulating or hormonal form of the vitamin. This process of conversion is very tightly feedback-controlled by 25-HCC itself. This represents an important control mechanism which conserves vitamin D on one hand and prevents vitamin D toxicity at low levels on the other. The 25-HCC is then "secreted" in a controlled fashion into the blood stream where it appears in the target tissues of its action—namely, bone, intestine, and kidney. In each of these tissues the 25-HCC goes to the nucleus where it induces the formation of enzymes which in each tissue specifically convert the 25-HCC to specific polar metabolites. The polar metabolites in these tissues in some unknown fashion cause the appearance of specific substances which carry out either intestinal calcium transport or bone mineral mobilization or some renal phenomenon. In the intestine the substance finally produced is perhaps the calcium-binding protein which makes its appearance at the brush border surface. Here it combines with a brush border component to bring about an increased rate of calcium transport. This can be measured as an increased calcium-dependent adenosine triphosphatase. This system brings about the increased rate of calcium transfer into the intestinal mucosa. Mitochondria then scavenge the calcium which makes its appearance inside the cell and transports it into granules where it is kept until the mitochondria experience a low calcium environment. This low calcium environment is experienced at the serosal surface where a downhill sodium gradient causes the expulsion of calcium into the serosal fluid with an exchange of sodium ions into the cell. The sodium is then pumped back out of the cell by the sodium-potassium adenosine triphosphatase maintaining this high sodium gradient. The downhill sodium gradient then provides an additional energy push for calcium transport. This mechanism must be taken only as a working hypothesis which can at the present time explain all of the data thus far obtained. Its utility lies only in its remaining pliable for change. Obviously, additional investigations will modify it further and it is in this spirit that it is presented at this time.

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