3. The actomyosin-like protein from rat uterus showed some anomalies, being neither activated nor inhibited by changing the KCl concentration, and being activated by 2:4-dinitrophenol at low as well as high KCl concentrations.

4. In the course of the actomyosin preparation, a cell-particle fraction was separated; its adenosinetriphosphatase activity showed very different characteristics from those of the actomyosin.

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Metabolism of Calcium, Phosphorus and Nitrogen in Hypervitaminosis A in Young Rats

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The production of toxic effects in human beings as well as in experimental animals by the administration of large doses of pure vitamin A is well recognized clinically (Josephs, 1944; Knudson & Rothman, 1953). Among the experimental animals, rats appear to be specially sensitive to these toxic effects. Skeletal fractures and haemorrhages are among the important features in this malady (Herbst, Pevcek & Elvehjem, 1944; Moore & Wang, 1945; Pevcek, Herbst & Elvehjem, 1945). Although the skeletal changes that lead to these fractures have been known for some time, the mechanism remains obscure. Further, since previous workers' preparations of vitamin A contained large amounts of vitamin D, it is not known with certainty whether the recorded effects are due to vitamin A or to vitamin D. Histological studies by Wolbach (1947) suggested that accelerated epiphyseal cartilage sequences and remodelling processes are responsible for the production of fractures, and that these are mediated independently of the pituitary (Wolbach & Maddock, 1952). Fell & Mellanby (1950), in their investigations of tissue cultures of bone, have shown that vitamin A acts directly. In an attempt to elucidate the mechanism of action of pure vitamin A, free from vitamin D, on bones, the effects of hypervitaminosis A on the chemical composition of bones

and on the general metabolism of calcium, phosphorus and nitrogen in rats have been studied. The results are reported in the present paper.

MATERIALS AND METHODS

The experimental animals were male, albino inbred rats, 6–7 weeks' old and fed on a synthetic diet having the following percentage composition: sugar, 34·5; starch, 34·5; casein, 20·0; vegetable fat, 6·7; salt mixture (Hawk & Oser, 1931), 4·0; cystine, 0·2; choline hydrochloride, 0·1. The diet was further supplemented with 10 units of vitamin D, 100 units of vitamin A, 1 mg. of vitamin E, 0·05 mg. of vitamin K and adequate amounts of B complex. Analysis of the diet showed 0·692 % Ca, 0·440 % P and 2·430 % nitrogen.

Synthetic vitamin A palmitate (1 million i.u./g. Hoffmann–La Roche) was used as the source of vitamin A in all these experiments. As this source of vitamin A is free from vitamin D, the possibility of introducing the condition of hypervitaminosis D (Moore & Wang, 1943, 1945) is ruled out in these experiments. Vitamin A (40000 i.u./rat/day) was given to these animals orally.

Metabolic studies were carried out on male rats, kept in pairs in metabolism cages provided with a device for separate collection of urine and faeces. Urine was collected into 10 ml. of a preservative consisting of equal amounts of toluene and conc. HCl. Urine collected during 2 days was pooled for assay purposes. At the end of each collection period, the cages and funnels were washed with hot water acidified with HCl to pH 4·6–4·8 and the washings pooled with the urine. Faeces were collected separately. Samples of urine and faeces were stored at 7–8° until analysed for calcium, total phosphorus and total nitrogen. These experiments were divided into three parts: (1) a preliminary control period of 10 days; (2) a period of administration of excess of vitamin A (10 days); (3) a curative period of 14 days after the administration of excess of vitamin A was stopped. The animals were weighed on alternate days. Food intake was measured daily. An amount somewhat above the daily expected intake was weighed out and put in the cages. At the end of 24 hr., the intake was calculated after weighing the residue. Food waste and its contamination with excreta were reduced by using a special feeding device on the cages. The animals had free access to water.

In another type of experiment, five groups of male albino rats 45–50 days old were used. One group served as the control, whereas the other groups received 40000 i.u. of vitamin A every day, for 3, 6, 9 and 12 days respectively. X-ray photographs of the whole body were taken at intervals. The animals were killed 24 hr. after receiving the last dose, by decapitation, without previous fasting. The chest wall was opened and blood withdrawn from the heart into a heparinized syringe. The levels of calcium, inorganic phosphorus and vitamin A in the serum were investigated. The livers were removed, weighed and placed in 5% ethanolic KOH solution for the estimation of vitamin A content. The femora and tibiae were cut out and cleaned of all the adhering soft tissues. Slits were made longitudinally through the diaphysis and epiphysis to render the extraction more effective. The bones were defatted with two overnight extractions, first with absolute ethanol and then with ether. The dried marrow was removed as completely as possible. The bones, free of fat and marrow, were then dried in an electric oven at 105°, powdered and analysed for Ca, P and N according to the procedure outlined below.

Analytical methods

Nitrogen. N was estimated by the micro-Kjeldahl method as modified by Sobel, Yuska & Cohen (1937).

Calcium. Faeces, food and bones were ashed (A.O.A.C. 1940) before the determinations. The following methods were employed: for faeces and food, the method of Lindner & Kirk (1937): for urine, the same method after a previous treatment with potassium persulphate (Shohl & Pedley. 1922); for bone, titration directly with disodium dihydrogen ethylenediaminetetraacetate with murexide indicator. The procedure was developed by us and its principle is the same as that for the estimation of blood Ca described by Holtz & Seekles (1952). This latter method was used for the estimations in blood.

Phosphorus. Total P in urine, faeces, food or bones was estimated by the method of Fiske & Subbarow (1925), as modified by LePage & Umbreit (1949).

Faeces, food and bones were ashed as for the Ca determinations. Inorganic P in the blood serum was determined by the method of Fiske & Subbarow (1925).

Vitamin A was estimated in liver by the method of Gallup & Hoefer (1946) by saponification and extraction of vitamin A with light petroleum. Vitamin A in blood was estimated according to the technique of Yudkin (1941). Characteristic absorption was measured by means of a Beckman quartz spectrophotometer (Zscheile & Henry, 1942) with Morton & Stubbs' (1948) correction. Vitamin A (U.S.P.) was used as a reference standard (U.S. Pharmacopoeia, 1948).

RESULTS

Administration of single toxic doses of vitamin A resulted in general drowsiness and muscular weakness, accompanied by decreased activity of the animals. If no further excess of vitamin A was given they returned to normal physical condition within 24 hr. Continued administration of vitamin A at toxic dosage for a period of several days caused symptoms such as lack of appetite, loss of weight, muscular weakness, loss of hair, soreness and bleeding of the eyes, stiffness of the limbs, limping, spontaneous fractures, internal haemorrhages and eventually death. Death occurred in a high proportion of animals given more than

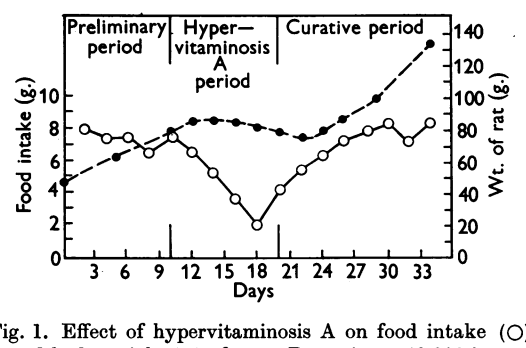

Fig. 1. Effect of hypervitaminosis A on food intake (○) and body weight (●) of rats. Dose given: 40 000 i.u. of vitamin A/day/rat.

Table 1. *Effect of hypervitaminosis A on the excretion of calcium, phosphorus and nitrogen in urine and faeces*

The dose of vitamin A was 40000 i.u./rat/day during the hypervitaminosis A period. The figures are the averages of results from eight rats, Each figure corresponds to a period of 2 days.

	Calcium				Phosphorus				Nitrogen			
	Intake (mg.)	Urinary excretion (mg.)	Faecal excretion (mg.)	Net retention (% intake)	Intake (mg.)	Urinary excretion (mg.)	Faecal excretion (mg.)	Net retention (% intake)	Intake (mg.)	Urinary excretion (mg.)	Faecal excretion (mg.)	Net retention (% intake)
Preliminary period (10 days)*	110·0	7·2	31·3	65·0	70·0	20·6	7·1	60·4	386	61·6	14·5	80·3
	101·0	6·5	27·6	66·4	64·6	20·6	6·3	58·4	357	51·9	17·1	80·7
	102·0	5·5	26·8	68·4	65·1	22·0	5·4	57·7	359	54·8	16·3	80·2
	90·0	5·2	26·9	64·3	57·2	18·7	4·4	59·6	315	53·1	16·8	77·8
	103·0	6·1	28·5	66·4	65·5	21·0	6·4	58·1	362	60·3	20·5	77·6
Period of hypervitaminosis A (10 days)†	90·9	7·1	34·9	53·2	57·1	22·5	6·3	49·5	306	66·8	18·7	72·1
	72·0	8·2	39·3	33·7	45·7	23·0	6·7	35·3	252	89·5	15·8	58·2
	50·5	8·9	39·3	4·37	31·8	22·0	8·0	5·68	177	114·0	14·4	27·7
	25·8	7·2	25·9	−21·0	18·8	22·8	12·5	−82·2	99	118·0	18·0	−51·7
	57·3	15·8	46·0	−7·91	36·5	25·8	12·3	−4·7	201	165·0	20·7	+8·0
Curative period (14 days)‡	75·6	14·5	56·9	5·58	47·8	28·3	10·1	19·6	266	220·0	23·5	8·76
	87·0	14·2	54·7	20·5	57·4	30·5	10·8	27·9	306	167·0	25·2	36·9
	101·0	15·5	65·4	19·8	64·2	27·7	7·6	44·6	355	185·0	21·6	41·7
	110·0	13·7	48·9	43·1	70·6	23·2	9·3	53·9	391	171·0	22·0	50·7
	115·0	11·5	55·3	42·2	73·5	21·7	9·5	58·2	405	147·0	19·8	59·0
	101·0	10·7	41·6	48·2	64·2	18·7	7·6	59·0	354	128·0	18·4	61·4
	118·0	12·7	43·8	52·6	75·3	21·2	11·0	57·2	415	98·0	17·8	72·1

* Preliminary period when animals were maintained on adequate diet.
† Period when the animals were given 40000 i.u. of vitamin A/day in addition to adequate diet.
‡ Period when excess of vitamin A was withdrawn.

twelve doses of vitamin A. In some animals, abnormal posture was observed, with marked bending of the spine, both hind legs appearing paralysed but without any apparent signs of fractures or haemorrhages. The results regarding food intake and growth rate of animals are shown in Fig. 1.

Results of metabolic studies on the urinary and faecal excretions of calcium, phosphorus and nitrogen are given in Table 1. It will be seen that there is a gradual decrease in the percentage retentions of all the components with the continuation of excess of vitamin A administration. There is a negative balance of all the three constituents in the second half of the vitamin A administration period which suggests increased catabolism. The rapidity and the extent of the negative balance closely paralleled the physical condition of the animals. It will be seen from Table 1 that the levels of calcium, phosphorus and nitrogen excretions follow a more or less identical pattern.

Table 2 shows the results of the effect of hypervitaminosis A on the calcium, phosphorus, nitrogen and ash content of the bones. The figures are expressed as percentages of water-free, fat-free and marrow-free bones. The first group of rats, which had received three doses of vitamin A on 3 consecutive days and which were killed on the fourth day were very little different from the control group. The differences become apparent in the second group, which received excess of vitamin A for 6 consecutive days. The maximum effects are seen in the group dosed for 9 days, the group dosed for 12 days showing slightly less difference from the controls than this group. Systematic X-ray examination of the animals at various stages of the experiment showed gradual thinning of the bones. Although bone calcium and phosphorus

Table 2. *Effects of hypervitaminosis A on the composition of bones*

The dose was 40000 i.u. of vitamin A/rat/day. The analyses for calcium, phosphorus and nitrogen are given on dry, fat-free and marrow-free bone.

Treatment of group	No. of animals	Ca (%)	P (%)	N (%)	Ash (%)
Control	12	26·1±0·145	12·4±0·095	4·02±0·027	69·1±0·176
3 doses	6	25·5±0·121	11·8±0·115	4·18±0·045	69·0±0·150
6 doses	6	25·1±0·170	11·5±0·156	4·23±0·030	68·4±0·218
9 doses	6	23·7±0·176	10·3±0·167	5·09±0·051	66·0±0·375
12 doses	6	24·5±0·215	11·0±0·210	4·86±0·042	67·5±0·272

Table 3. *Levels of calcium and inorganic phosphorus in blood serum of normal rats and rats with hypervitaminosis*

The dose was 40000 i.u. of vitamin A/rat/day.

		Serum Ca		Inorganic P	
Treatment of group	No. of animals	Average value (mg./100 ml.)	Range (mg./100 ml.)	Average value (mg./100 ml.)	Range (mg./100 ml.)
Control	12	10·5±0·215	9·21–11·75	4·54±0·093	3·98–5·01
3 doses	6	10·8±0·394	9·65–11·97	4·61±0·197	4·10–5·16
6 doses	6	10·6±0·483	8·81–11·93	4·73±0·169	4·15–5·25
9 doses	6	11·2±0·381	9·92–12·13	4·86±0·206	4·19–5·45
12 doses	6	10·9±0·324	9·85–11·98	5·05±0·178	4·43–5·65

Table 4. *Levels of total vitamin A in the blood and liver of normal rats and rats with hypervitaminosis*

The dose of vitamin A was at the rate of 40000 i.u./day for each animal.

Treatment of group	No. of animals	Total vitamin A received (i.u.)	Blood vitamin A (i.u./ml.)	Liver vitamin A (i.u./g.)	Liver vitamin A (Total i.u.)	Storage in liver (%)*
Control	12	—	66·10±2·84	75±3·02	418±10·8	—
3 doses	6	120 000	184·0±8·36	2750±88·4	13 300±630·0	11·12
6 doses	6	240 000	211·0±7·14	3870±97·0	22 300±930·0	9·30
9 doses	6	360 000	237·0±6·85	5000±120·0	27 300±907·0	7·57
12 doses	6	480 000	262·0±4·92	6000±71·5	31 400±990·0	6·54
15 doses	6	600 000	308·0±5·50	6810±39·5	35 200±445·0	5·86

* Percentage of the administered dose of vitamin A found in liver.

gradually decrease, the levels of calcium and inorganic phosphorus in the serum remain unaltered, as can be seen from Table 3.

Table 4 shows the concentration of total vitamin A (vitamin A alcohol and ester) in the liver and blood after various doses of the vitamin. The purpose of these estimations was to ascertain indirectly whether the absorption of vitamin A is impaired as a result of the massive dosage. Since liver is the chief storage site of vitamin A (Osborne & Mendel, 1918; McCoord & Clausen, 1934) the concentration of vitamin in this organ should be proportional to the intake (Baumann, Riising & Steenbock, 1934) and should serve as a criterion of its absorption (Guggenheim & Koch, 1944; Foy & Morgareidge, 1948). It will be observed from these results that the levels of vitamin A in the blood increased with increasing period of treatment with vitamin A, but no direct correlation could be demonstrated between total amount of vitamin A administered and that present in either the blood or the liver. Although the vitamin A content of the liver in experimental rats increased with the number of doses of vitamin A administered, the absorption, calculated as percentage of the total dose administered, decreased, indicating decreased absorption of vitamin A from the intestine, or increased catabolism of liver vitamin A, or an approach to the saturation limit of the liver. The vitamin A content of the liver in the 15-dose group is greater than that in the previous group (12 doses); thus the liver was not completely saturated.

DISCUSSION

The results described above indicate that pure vitamin A free from vitamin D is toxic when given orally at a dose of 40000 i.u./day. The toxic effects observed were similar to those observed by other workers (Moore & Wang, 1945; Pevcek *et al.*1945). Reduced food intake, loss of weight, skeletal fractures and haemorrhages are in our experience the most characteristic features in genuine hypervitaminosis A. Fractures occur most consistently in young growing rats and were found without exception in rats fed for 12 or more days with 40000 i.u. of vitamin A/day. Vitamin A in the concentrations employed in these investigations appears to interfere in some way with the metabolism of calcium, phosphorus and nitrogen. The retention of these elements in the body is decreased and there is a negative balance. The severity of the interference is more or less parallel with the amount of vitamin A ingested, since the degree of negative balance of calcium, phosphorus and nitrogen becomes more and more pronounced during continued ingestion of the vitamin. This may be due to decreased absorption from the digestive tract or to an increased catabolic excretion from the system or to both; but it is not possible to decide from measurements of the excretion of these elements in urine and in faeces whether absorption from the digestive tract is interfered with or whether there is increased catabolic excretion (Brown & Shohl, 1930). This is particularly true for calcium, because systemic calcium is excreted in urine, by way of bile, etc., and in the faeces. The fraction in the faeces which comes from the catabolism of systemic calcium is known as 'endogenous faecal calcium' to distinguish it from 'exogenous faecal calcium', which is the unabsorbed calcium from the food (Schmidt & Greenberg, 1935). Because of this inherent difficulty in assessing the absorption of calcium from the intestines, we cannot say whether the calcium absorption is impaired or not. But there is no doubt that administration of large doses of vitamin A induces a negative balance in calcium, phosphorus and nitrogen. Recovery from the deleterious effects is evidently slow. The balance has not returned to normal even 10 days after the cessation of administration of excess of vitamin A.

In the early stages of hypervitaminosis A there is a definite rise in the excretion of both urinary and faecal calcium, phosphorus and nitrogen. But in the later stages, although the doses are continued, there appears, contrary to our expectations, to be a fall in the urinary and faecal excretions of all these elements. The reason for this decrease, which coincides with the decrease in the levels of calcium in bones, cannot be ascertained.

The process of bone formation consists of several phases, one of which is the precipitation of calcium phosphate. For this to occur, there must be adequate concentrations of calcium and inorganic phosphorus in the blood stream and alkaline phosphatase in the bones (Howard, 1951). The fact that in our experiments the serum concentrations of calcium and phosphorus are not disturbed and that the bone alkaline-phosphatase activity is increased in hypervitaminosis A (Nerurkar & Sahasrabudhe, unpublished work) suggests that this phase of ossification is not impaired and that the development of fragility and fractures must be ascribed to the disturbances in other phases of bone formation. Our observations that no marked changes in the relative chemical composition of the bones are apparent as a result of hypervitaminosis A are in agreement with those of Moore & Wang (1945) and Rodahl (1950). Gradual thinning of the bones, observed by us in X-ray photographs, has also been reported by Wolbach (1947) and Metre (1947). This gradual thinning of the bones, coupled with the pronounced negative balance, suggests that hypervitaminosis A is draining the inorganic stores of the bones without causing any gross change in their relative composition.

SUMMARY

1. Oral administration of a pure vitamin A preparation at a dose 400 times the normal requirement has been shown to produce effects such as reduced food intake, loss of weight, skeletal fractures and haemorrhages in young albino rats. The degree of toxicity is approximately proportional to the total quantity of vitamin A administered.

2. The effects of administration of large doses of vitamin A on the metabolism of calcium, phosphorus and nitrogen have also been studied. A negative balance for calcium, phosphorus and nitrogen sets in and continues for a considerable period after the administration of large doses of vitamin A has ceased.

3. No changes were detected in the levels of calcium and inorganic phosphorus of the blood.

4. Very little change in the relative mineral composition of the bones was observed. Increased excretion of calcium and phosphorus is therefore probably due to thinning of the bones.

5. Estimation of vitamin A in the liver suggests that administration of amounts of vitamin A, several times greater than the normal requirements, reduces the absorption of vitamin A from the intestine.

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An Improved Method for Measuring Degree of Polarization

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Measurements of the depolarization of fluorescence have recently been used for determining the rotational relaxation times of protein molecules (Weber, 1952*a*, *b*), and they provide a technique of considerable flexibility and usefulness. Such work requires a high degree of accuracy in the measurement of percentage polarization, which is not readily achieved by the methods hitherto described (Perrin, 1929; Lewis, Lipkin & Magel, 1941; Lewis & Bigeleisen, 1943). In connexion with a recent study (Harrington, Johnson & Ottewill, 1956), an improved apparatus has been devised, based upon a method first proposed by Lyot (1929) and later improved by Wright (1934). Its advantages depend upon the principle of matching the intensity and position of a fringe system in two halves of a divided field, rather than judging the

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