EXPERIMENTAL STUDY ON THE TOXIC EFFECT OF OVERDOSED VITAMIN A ON THE BONES *

by

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INTRODUCTION

Vitamin A, given in overdoses, has a toxic action in animals and human beings, because vitamin A is not excreted in urine for its fat-solubility and accumulates too excess in body when taken in a large quantity.

Vitamin A intoxication has been clinically described by many investigators[2),9),10),15),26)] since it was noted for the first time in a child by JOSEPH[12)] (1944). KNUDSON and ROTHMANN[15)] have ascertained that vitamin A intoxication presents two different clinical pictures, acute and chronic. The acute type, developing after an excessive intake of vitamin A in a single dose, is manifested as transitory headache, nausea, vomiting, drowsiness, and localized or generalized desquamation of the skin; the chronic type, developed when vitamin A is continually taken in large doses over a prolonged period, is marked first of all with loss of appetite, weight loss and pruritus, and later with painful swelling of one or many bones, dyskinesia, alopecia and bleeding from mucous membrane. Before these clinical observations, the effect of excessive administration of vitamin A had been investigated experimentally by DRIGALSKI[7)] (1933) and others.[3),4),25),33),36)] The conspicuous symptoms noted in rats overdosed with vitamin A were loss of weight, alopecia, diarrhea, bleeding from the conjunctiva and mucous membrane of the nose, and spontaneous fracture of long bones. Among the organic changes noted clinically and experimentally in this type of hypervitaminosis, the bone lesion is required the more accurate examination.

Vitamin A appears to be closly related to the metabolism of minerals, calcium in particular, and inorganic phosphorus. According to BOMSKOVE and SEEMANN,[3)] the bones tend to lose their contents of calcium and inorganic phosphorus in vitamin-A-intoxicated animals, indicating that bone falls fragile in the presence of too much vitamin A as it is in vitamin D deficiency. THOENES[35)] found that the disorders caused by vitamin-D-intoxication could be prevented by an addition of vitamin A to the diet. From these findings vitamin A and D may be antagonistic in their action of controlling the mineral metabolism.

BIFULCO[2)] and GERBER et al[9)] noticed the development of hydrocephalus in the clinical observations of vitamin A intoxication. The mechanism of a development

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of hydrocephalus in vitamin A intoxication has not yet been thoroughly elucidated, while this lesion may be caused by the abnormal acceleration in the production of cerebrospinal fluid[9] or by the failure in a growing balance of skull and brain.[17] A cerebral lesion of this kind has been also encountered in vitamin-A-deficient children[5] and animals by many investigators.[16),18),19),20),21),37]

The principal purpose of our study was to investigat the effect of vitamin A on the central nervous system, but the organic changes in vitamin A intoxication are manifested more markedly in long bones than in the brain, so the effect on the long bone alone will be described in this paper under three headings: (1) The general condition following an excessive administration of vitamin A——The first step towards the elucidation of the toxic action of the hypervitaminosis; (2) The mineral metabolism in hypervitaminotic condition; (3) The histological investigation of the bone in vitamin A intoxication.

EXPERIMENTAL METHODS

The animals used were young rats, male and female, weighing from 50 to 70 grams and in the 4th week of postnatal life, each fed with N. M. C. diet*-1 having the composition as shown in Table. 1. As the source of vitamin, synthetic vitamin

Table 1 Analysis of N.M.C. Diet.[23]

General Constituent (%)		Analysis of Inorganic Composition		Contents of Various Vitamin (in 100 g of Diet)	
Protein	25.81	Ca	1.355	Vitamin A	2,000 I.U.
		P	0.766	Thiamine	700 γ
Fat	5.17	Mg	0.300	Riboflavin	600 γ
		Na	0.276	Pyridoxin	400 γ
Carbohydrate	50.05				
		K	0.474	Pantothenic Acid	2,500 γ
Ash	6.19	S	0.071	Vitamin D	400 I.U.
		Fe	0.064	Vitamin E	3 mg
Miscellaneous	5.78	SiO_2	0.326		

A palmitate*2 dissolved in 20% solution of Tween 80 (polyoxyethylen sorbitan monooleate) was used in almost all of the experiments, while cod-liver oil*3 (30,000 I. U. of vitamin A in 1 g) and vitamin D*4 were also used in a few.

I) For Observation of General Conditions in Vitamin A Intoxication.

A) Toxic dosage of vitamin A and general condition in hypervitaminosis A. Fifty-two rats were divided into following five groups and given various doses of vitamin by oral administration or subcutaneous injection.

*1. N. M. C. diet is manufactured by Oriental Yeast Co Ltd., Tokyo.
*2. Synthetic vitamin A palmitate is manufactured by Sansho Seiyaku Co Ltd., Tokyo.
*3. Cod-liver oil are manufactured by Hayashikane Marine Co Ltd..
*4 Vitamin D is manufactured in Eizai Co Ltd., Tokyo.

Group	Substance given	Daily doses and route of administration
I	Vitamin A	20,000 I.U., 10,000 I.U. or 5,000 I.U. ……subcutaneously
II	Vitamin A	30,000 I.U. or 10,000 I.U. ……orally
III	Cod-liver oil	1 g or 0.3 g……orally
IV	Vitamin A and Vitamin D	15,000 I.U. of vitamin A and 1,500 I.U. of vitamin D……subcutaneously
V (control)	20% Tween 80 solution	0.3 cc ……subcutaneously 0.5 cc ……orally

These experimental animals were weighed every week, and the quantity of food-intake was measured at intervals of 2 days. Radiograms of bones were taken every week.

B) Healing process of artificial fracture in vitamin-A-intoxicated animals.

In 36 rats receiving a daily dose of 10,000 I. U. of vitamin A by subcutaneous injection, an artificial fracture was performed at proximal region of diaphysis of tibia in one side on the 5th day of the administration of vitamin A, and the healing process of these fractured bones were compared in the vitamin-A-intoxicated animals and control animals without the administration of vitamin A.

II) For Estimations of Mineral Metabolism in Vitamin A Intoxication.

i) Vitamin A and Mineral Contents in Various Organs.

Vitamin A was given subcutaneously in a daily dose of 10,000 I. U. for 5 days in one group of rats, for 10 days in a second, for 15 days in a third and for 18 days in a fourth, the animals used totalling 24. Fourteen other rats were used as controls. These animals were sacrificed by ether inhalation 24 hours after final administration of vitamin A; under opening of the chest blood was withdrawn by heart puncture into a syringe for the examination of its different contents.

A) Vitamin A in blood and liver. Serum was saponified by the original method of YUDKIN,[39] and its content of vitamin A was estimated by the FUJITA-AOYAMA's G. D. H. method[8]; the level of vitamin A in the liver was also determined by the same method after saponification and extraction by SOBEL's method.[32]

B) Alkaline phosphatase, calcium and inorganic phosphorus in serum. Alkaline phosphatase in serum was quantitatively determined by KIND-KING's method[13] and the value obtained by their method was converted to be denoted in BODANSKY's unit. Calcium in serum was estimated by phosphate method,[29] and inorganic phosphorus by FISK-SUBBAROW's aminonaphthol sulfonic acid method.[30]

C) Ashes and mineral contents in bones. The femurs were removed immediately after the death of animals, and dried at 95°C for about 2 days and thereafter weighed. The bones were ashed at 750°C for 24 hours, and ashes were weighed and dissolved in 1 N HCl; contents of calcium and inorganic phosphorus in this solution were also determined in the same way as those of serum.

ii) Metabolic Balances of Calcium and Phosphorus.

Eleven experimental animals were individually kept in metabolism-cages provided with a device for separate collection of urine and faeces, as shown by KOYAMA.[14] These animals were divided into three groups. In the first group

using as the control, the animals were allowed to take freely as much food as they required, while in the second group served as control the animals were fed with a daily decreasing amount of food-intake. Those in the third group were given vitamin A subcutaneously in a daily dose of 15,000 I. U. for 12 days and allowed to take food in a sufficient amount, whereas the amount actually taken tended to decrease with the progress of the vitamin A administration. In all groups, the ingested food was weighed out every two days, and the contents of calcium and phosphorus in this food were calculated from Table 1. Faeces were collected every two days and ashed at 700°C for 12 hours. After ashes were dissolved by 1 N HCl, the contents of calcium and phosphorus in this solution were determined by the method using in the determination of those in the bones. Urine were collected every two days, and level of calcium was measured by McCRUDDEN's method[11] and phosphorus was estimated by FISK-SUBBAROW's method.[30]

III) For Histological Examination of Bones in Vitamin A Intoxication.

Vitamin A was given subcutaneously in a daily dose of 10,000 I. U. in totalling 42 growing rats for 5 days in one group, for 10 days in another, for 15 days in a third and for 20 days in a fourth. The rats thus treated were sacrificed 24 hours after the final administration, to be compared with 20 control rats. The tibiae, removed directly after the death, were fixed in neutral 15% formalin solution, electrolyzed for decalcification, embedded in celloidin, prepared into longitudinal section and stained with hematoxylin-eosin to be examined histologically for the injurious effect of excess vitamin A on the bone.

Animals in which spontaneous fractures of tibiae were revealed by the radiological examination were sacrificed in the 5th and 10th day after the first appearances of fractures; the calluses formed in the fractured regions were prepared into the stained section, and submitted to the histological examination. Furthermore, the calluses formed after the artificial fracture in hypervitaminotic rats were also examined histologically in the 4th, 8th and 12th day after the fracture, in comparison with those in control animals.

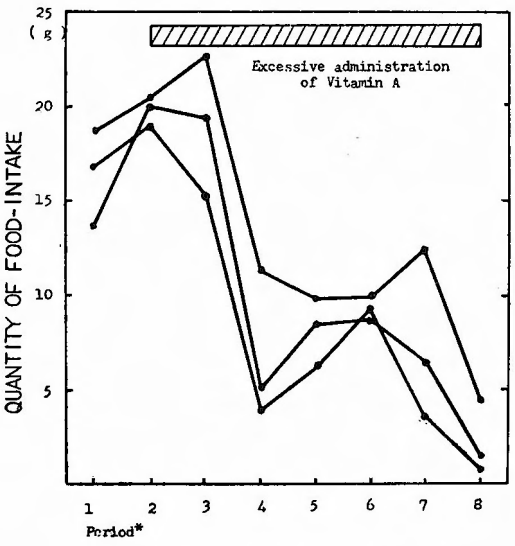
EXPERIMENTAL RESULTS

I) Observations of General Condition in Vitamin A Intoxication.

A) Toxic dosage for vitamin A and general condition in hypervitaminosis A.

The degree in which excessive administration of vitamin A affected the general condition was found to depend on the administered amount of vitamin A. The initial syndroms of hypervitaminosis A were general weakness, anorexia and a failure in growth; the course of reduction of food-intake is shown in Fig. 1, and those of the loss of body weight are as Fig. 2. Subsequently, there appeared various degrees of alopecia, bleeding in conjunctiva or nose and spontaneous fracture of the long bones. Frequencies of the appearance of spontaneous fracture in various experimental groups and the minimal administration-period required for the occurrence of the fracture are summarized in Table 2. Frequencies of the bleeding and alopecia are shown in Table 3.

Among these various groups, the accurate observation were performed in the



* One period corresponds with two days.

Fig. 1 Decreases in the amount of ingested food in three rats given subcutaneously vitamin A in a daily dose of 20,000 I. U.

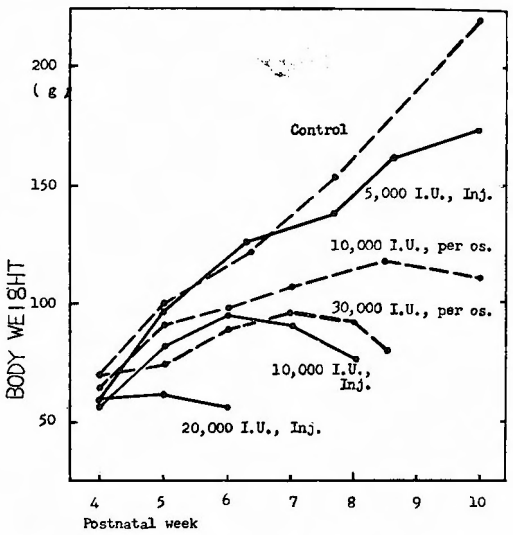


Fig. 2 Failure to gain in body weight in the growing rats given vitamin A in a various doses, orally or subcutaneously.

Table 2. Frequencies of Developments of Spontaneous Fractures in Various Experimental Groups.

Method & Dose of Administration	Number of Animals	Number of Animals developing Spont. Fract. (%)	Minimal Period of Administration (Average Days)	Details of Appearance of Spon. Fract.			
				in all Limbs	in three Limbs	in two Limbs	in one Limb
I: Subcutaneously							
20,000 I.U. of V-A	3	3(100)	7.7	2	1	0	0
10,000 I.U. of V-A	15	12(80)	15.2	5	1	3	3
5,000 I.U. of V-A	4	1(25)	22.0	0	0	1	3
II: Orally							
30,000 I.U. of V-A	9	7(78)	15.7	3	4	0	0
10,000 I.U. of V-A	5	0(0)	—	0	0	0	0
III. Oral Admin. of Cod-liver Oil							
1.0 g	4	0(0)	—	0	0	0	0
0.3 g	4	0(0)	—	0	0	0	0
IV: Subcutaneously 15,000 I.U. of V-A & 1,500 I.U. of V-D	5	5(100)	19.4	3	1	0	1
V: (Control) Admin. of Tween 80	8	0(0)	—	0	0	0	0

Table 3. Frequencies of Developments of Conjunctival Bleeding, Nose-bleeding and Loss of Hair in Various Groups.

Method & Dose of Administration	Number of Animals	Conjunctival Bleeding		Nose-bleeding		Loss of Hair	
		Freq. (%)	Min. Admin Period (Days)	Freq. (%)	Min. Admin. Period (Days)	Freq.	Min. Admin. Period (Days)
I : Subcutaneously							
20,000 I.U. of V-A	3	100.0	7.7	66.0	8.0	100.0	8.0
10,000 I.U. of V-A	15	13.3	14.0	0	—	46.7	15.7
5,000 I.U. of V-A	4	25.0	22.0	0	—	0	—
II : Orally							
30,000 I.U. of V-A	9	33.3	14.0	33.0	16.0	22.2	20.0
10,000 I.U. of V-A	5	0	—	0	—	0	—
III : Oral Admin. of Cod-liver Oil							
1.0 g	4	0	—	0	—	0	—
0.3 g	4	0	—	0	—	0	—
IV : Subcutaneously 15,000 I.U. of V-A & 1,500 I.U. of V-D	5	100.0	19.2	20.0	21.0	20.0	20.0
V : (Control) Admin. of Tween 80	8	0	—	0	—	0	—

animals given vitamin A in a daily dose of 10,000 I. U. by subcutaneous injection. In this group, decrease of general activity could be demonstrated within a few days after the starting of administration, and the food-intake tended to decrease gradually one week after, while any changes in radiograms could not be recognized in this period. Ten days after, general malady more seriously appeared and a failure to gain in body weight could be demonstrable. Two weeks later, some of the animals began to limp. The limping seems to be more frequently recognized in hind-limbs than fore-limbs (Fig. 3). In radiograms, the cortical bones of diaphysis were barely shadowed, whereas in some cases those were not shadowed at all in the position where the spontaneous fractures were frequently observed as described later. On the contrary, the cancellous bones were usually in a normal state in radiograms. These spontaneous fractures were commonly recognized at the proximal region of diaphysis in tibia, and at the distal of diaphysis in radius and ulna, and rarely at the proximal of that in humerus (Fig. 5). Fibula was occasionally broken in several points, probably because the tibia was intensively bended by spontaneous fracture. In epiphysis, however, any radiological changes could not be demonstrated. In the end of the 3rd week, spontaneous fracture appeared in 13 out of 15 rats. Furthermore, the fracture limiting in one limb usually developed in another limbs within 2 or 3 days after its first appearance, and in 5 rats those were recognized in all limbs (Fig. 4). In the radiograms taken at this stage, a fractured bone was often re-united at its two broken ends

by callus formed there. The vertebral column also showed the intensive backward or lateral curvature. In the end of 4th week, most of animals sadly emaciated to death from anorexia.

When vitamin A and D were given together, the frequency in the appearance of spontaneous fracture was similar to that in the group given the equivalent dose of vitamin A only, while the minimal period of administration for its appearance seems to be evidently prolonged. The animals receiving cod-liver oil failed to develop any significant changes of bone, while the half of these animals died from anorexia. In the control animals given Tween 80 solution, any changes were not demonstrated.

B) Healing process of artificial fracture in vitamin-A-intoxicated animals.

In the animals overdosed with vitamin A, the lameness resulting from artificial fracture disappeared in 7 or 8 days later as same as in the control animals without the administration of vitamin A. Radiological examination showed at the same time that there was a bridge-shaped callus formed in the fractured region of bone in the overdosed animals and in controls. In 12 days later, however, the same examination showed that the callus was seen as a deeper shadow in the hypervitaminotic rats than in the controls, indicating presumably that the fracture was healing at a high rate in the former than in the latter animals.

II) Mineral Metabolism in Vitamin A Intoxication.

i) Vitamin A and Mineral Contents in Various Organs.

A) Vitamin A contents in blood and liver.

The level of vitamin A in serum sharply rose with the progress of the administration of large amount of vitamin A. In the experimental animals receiving a daily dose of 10,000 I. U. of vitamin A by injection, the fasting level in serum rose to averaged 271 I. U. per ml in 5 days and 538 I. U. per ml in 15 days, while it averaged no more than 38 I. U. per ml in the controls (Table 4 and Fig. 6).

Vitamin A content of the liver in control animals averaged 257 I. U. per gram

Table 4. Vitamin A Levels in Serum and Liver during Subcutaneous Administration of 10,000 I.U. of Vitamin A per Day.

Duration of Admin.	Number of Animals	Total Amount of administered Vitamin A (I.U.)	Vitamin A Level in Serum (I.U./ml)	Vitamin A Level in Liver		Ratio of Storage in Liver (%)
				(I.U./g*)	(I.U. in total)	
Control	14	——	38 ±**4.2	257 ± 73.0	1,325 ± 245.4	——
5 days	6	50,000	271 ± 10.2	3,710 ± 46.6	12,463 ± 809.0	24.9
10 days	6	100,000	372 ± 13.2	5,324 ±274.4	19,469 ±1,730.6	19.5
15 days	6	150,000	538 ± 20.4	6,705 ±477.8	31,863 ±3,050.8	22.1
18 days	6	180,000	——	7,850 ±786.0	35,718 ±3,168.0	19.8

* Wet weight

** Standard deviations are calculated by following formula.

$$S.D. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

and 1,325 I. U. in entire liver; in the animals given vitamin A for 18 days the vitamin A storage in liver reached intensive high level, such as 7,850 I. U. per gram and 35,718 I. U. in total (Table 4 and Fig. 6). Additionally, it is shown in this Table that the vitamin A storage in the liver was constantly some 20 per cent of the total amount of received vitamin A during the respective experimental period.

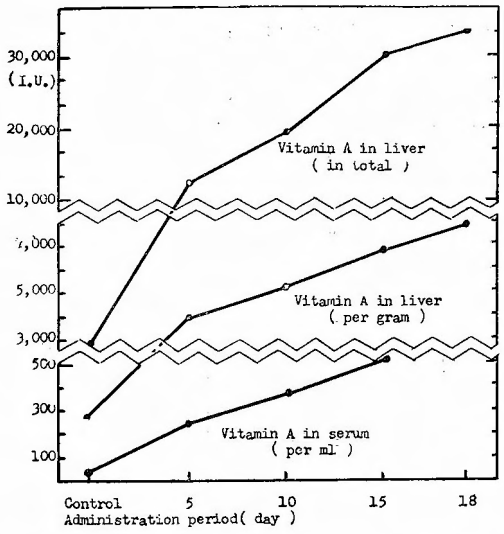


Fig. 6 Levels of vitamin A in serum and liver in rats given subcutaneously vitamin A in a daily dose of 20.000 I. U..

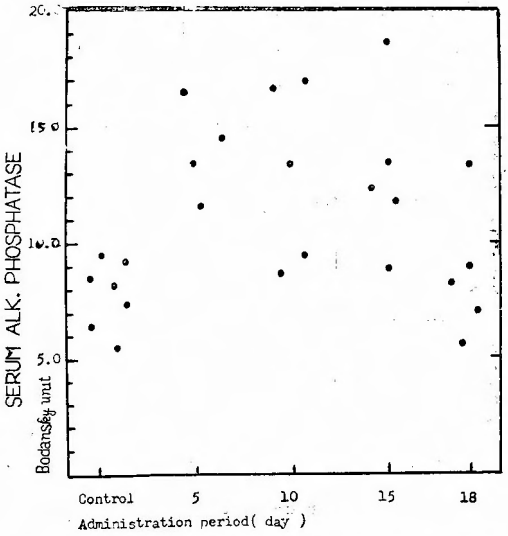


Fig. 7 Levels of alkaline phosphatase in serum in rats given subcutaneously vitamin A in a daily dose of 10,000 I. U.

B) Levels of alkaline phosphatase, calcium and inorganic phosphorus in serum.

In the animals receiving vitamin A the level of serum alkaline phosphatase considerably increased. In the late period of administration, however, this level rapidly began to decrease (Table 5 and Fig. 7).

On the levels of calcium and inorganic phosphorus in serum, no significant differences were demonstrated between the hypervitaminotic animals and controls, while that of inorganic phosphorus seems to be slightly elevated in the late period of administration (Table 5).

C) Ashes, calcium and phosphorus contents in bones.

In the early period of vitamin A administration, no definite reduction in the ashes content of the femurs was demonstrated. After the receiving of vitamin for 18 days, however, the ashes content decreased to 44.8 per cent of bone in weight, while it was 48.8 per cent in controls (Table 6).

The calcium content in bones seems to decrease considerably with the progress of vitamin A administration. In phosphorus content, there was no significant difference between the experimental animals and controls (Table 6).

ii) Metabolic Balances in Calcium and Phosphorus.

In the control animals kept on a non-restricted amount of diet, about 300 mg

Table 5. Levels of Alkaline Phosphatase, Calcium and Inorganic Phosphorus in Serum during Administration of Vitamin A.

Duration of Admin.	Number of Animals	Alkaline Phosphatase (Bodansky unit)	Calcium (mg %)	Inorganic Phosphorus (mg %)
Control	13	8.3 ± 0.92	11.4 ± 0.18	7.7 ± 0.51
5 days	6	14.3 ± 1.08	11.4 ± 0.43	9.7 ± 0.49
10 days	6	12.4 ± 1.29	11.3 ± 0.25	8.8 ± 0.24
15 days	6*	12.8 ± 0.72	11.2 ± 0.36	8.9 ± 0.40
18 days	6**	8.3 ± 1.30	——	——

* Among 6 rats, spontaneous fractures were revealed in 2 rats.

** In all rats, spontaneous fractures were observed.

Table 6. Analysis of Bones in Rats overdosed with Vitamin A.

Duration of Admin.	Number of Animals	Ashes (w %)***	Calcium (w %)	Phosphorus (w %)
Control	13	48.8 ± 0.67	22.3 ± 0.72	10.1 ± 0.24
5 days	6	48.8 ± 1.21	22.6 ± 0.82	10.0 ± 0.29
10 days	6	51.0 ± 0.97	23.8 ± 0.66	9.7 ± 0.28
15 days	6*	48.4 ± 0.62	20.4 ± 1.60	9.1 ± 0.90
18 days	6**	44.0 ± 1.20	19.2 ± 0.30	9.9 ± 0.16

*, **. Same in Table 5.

***. Dry weight.

of calcium was ordinarily ingested in 2 days, and about 200 mg was excreted in faeces and 20 mg in urine. Consequently, 70 to 80 mg of leaving calcium was constantly accumulated in the body for 2 days. Another control animals kept on a restricted amount of diet could also reserve about 50 mg of calcium in 2 days, because the reduction in the amount of ingested calcium was accompanied by a

Table 7. Examination of Metabolic Balances of Calcium and Phosphorus in the Control Rats Kept on a Non-restricted Amount of Diet.

(average level in four rats)

Exp. Period (*)	Dose of Diet (g)	In ingested Diet		In Urine		In Faeces		Storage in Body		Storage Ratio	
		Ca (mg)	P (mg)	Ca (mg)	P (mg)	Ca (mg)	P (mg)	Ca (mg)	P (mg)	Ca (%)	P (%)
1	17.5	236.5	133.8	11.0	12.0	167.0	75.0	59.5	46.8	25.2	35.0
2	20.5	271.0	153.2	20.4	9.4	174.0	75.0	76.6	68.8	28.3	45.0
3	25.3	342.0	193.7	21.2	7.4	258.0	114.0	62.8	71.8	18.0	37.1
4	26.3	355.1	201.2	22.4	6.0	253.5	138.0	79.2	56.6	22.3	28.1
5	28.5	386.2	218.4	11.4	8.9	300.0	114.7	49.8	94.8	13.8	43.3
6	27.4	370.2	209.5	13.4	7.1	288.0	134.3	69.1	68.1	18.7	32.5
7	24.5	332.2	188.3	8.2	7.9	225.0	123.3	99.0	57.1	29.8	30.1
8	21.5	291.4	164.9	15.0	14.7	208.0	114.8	68.4	35.4	23.5	21.5

* One period corresponds with two days.

corresponding decrease in the amont of calcium excreted in faeces, though not in urine. In each animals overdosed with vitamin A, on the contrary, the amount of calcium excreted in faeces was not reduced, while calcium was ingested in a extremely decreased quantity in these cases. After the administration for 8 days in this group, the calcium excreted in faeces and urine was greater in quantity than the calcium contained in the food ingested, and the deficit amounting to 25 mg was shown for 2 days corresponding from 8th to 10th day of administration. These metabolism of calcium in each experimental groups are in details described in Table 7, 8, 9 and Fig. 8, 9, 10.

Table 8. Examination of Metabolic Balances of Calcium and Phosphorus in the Control Rats kept on a Restricted Amount of Diet.

(average level in three rats)

	Exp. Period	Dose of Diet (g)	In ingested Diet		In Urine		In Faeces		Storage in Body		Storage Ratio	
			Ca (mg)	P (mg)	Ca (mg)	P (mg)	Ca (mg	P (mg)	Ca (mg)	P (mg)	Ca (%)	P (%)
	1	17.0	230.3	130.4	9.1	10.6	145.0	68.8	76.2	51.0	33.1	39.2
	2	20.0	271.0	153.2	12.5	9.8	190.0	77.2	68.5	66.2	25.3	43.2
Limitation of Food-intake	3	25.2	347.4	196.6	11.6	7.5	254.5	98.2	51.3	90.8	15.1	46.2
	4	14.0	189.5	107.6	9.7	10.6	148.5	60.3	31.3	36.7	16.5	34.1
	5	8.0	108.4	61.5	6.3	8.6	71.5	40.3	30.6	12.6	28.2	20.5
	6	10.0	135.5	76.6	5.4	5.7	86.0	46.6	44.1	24.3	32.6	31.7
	7	14.0	189.7	107.6	6.2	3.4	87.5	53.9	96.0	50.3	51.1	30.3
	8	5.0	67.5	38.3	7.2	10.1	44.8	18.0	15.5	10.2	23.0	26.6

Table 9. Examination of Metabolic Balances of Calcium and Phosphorus in Rats overdosed with Vitamin A.

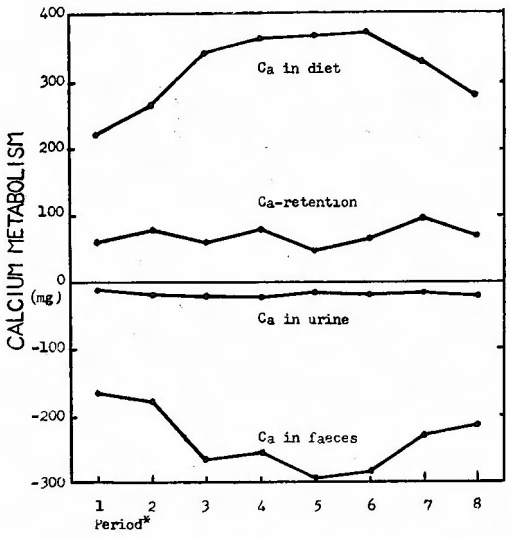
(average level in four rats)

	Exp. Period	Dose of Dies (g)	In ingested Diet		In Urine		In Faeces		Storage in Body		Storage Ratio	
			Ca (mg)	P (mg)	Ca (mg)	P (mg)	Ca (mg)	P (mg)	Ca (mg)	P (mg)	Ca (%)	P (%)
	1	16.2	218.9	124.0	12.8	10.3	149.7	72.3	56.4	41.4	25.8	33.4
	2	19.2	261.0	147.7	11.5	8.3	159.0	67.3	90.5	72.1	34.7	48.8
	3	19.2	258.7	146.8	13.5	5.0	193.3	102.7	41.9	39.1	16.2	26.6
Admin. of Vitamin A	4	6.7	89.8	48.7	9.5	6.2	69.0	31.6	11.3	10.9	12.6	22.4
	5	8.3	112.7	63.9	7.6	8.3	90.7	44.3	14.6	11.3	13.0	17.7
	6*	9.9	133.7	75.5	9.7	8.1	138.0	59.4	−14.0	8.0	−10.5	10.6
	7	7.5	102.4	57.6	7.2	9.5	100.7	52.4	5.0	4.3	5.4	− 7.5
	8**	3.3	44.1	25.1	7.0	12.5	62.3	22.0	−25.2	−14.6	−57.1	−37.5

* In all rats, spontaneous fractures appeared.

** Among four rats, one died from severe malnutrition.

The quantitative ratio of calcium accumulated in the body to that ingested may be clearly exhibited upon the metabolism of calcium in each exprimental



* One period corresponds with two day.

Fig. 8 Calcium metabolism in the control rats kept on a non-restricted amount of diet.

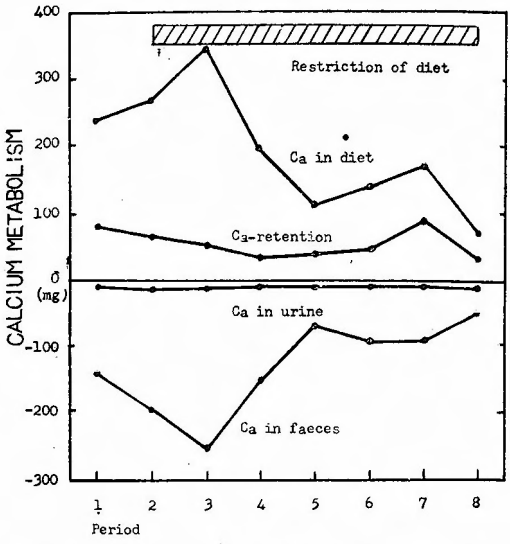


Fig. 9 Calcium metabolism in the control rats kept on a restricted amount of diet.

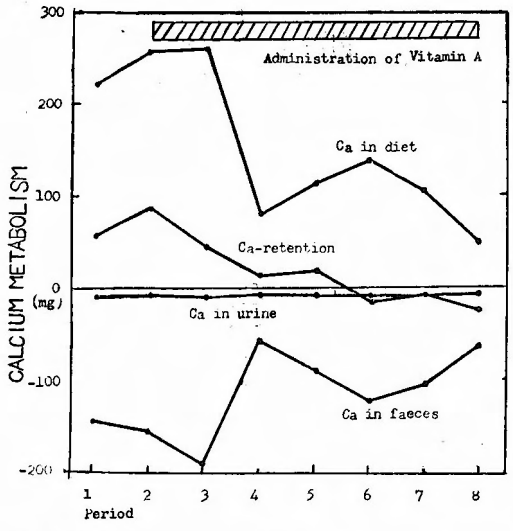


Fig. 10 Calcium metabolism in the rats given vitamin A in a daily dose of 15,000 I. U..

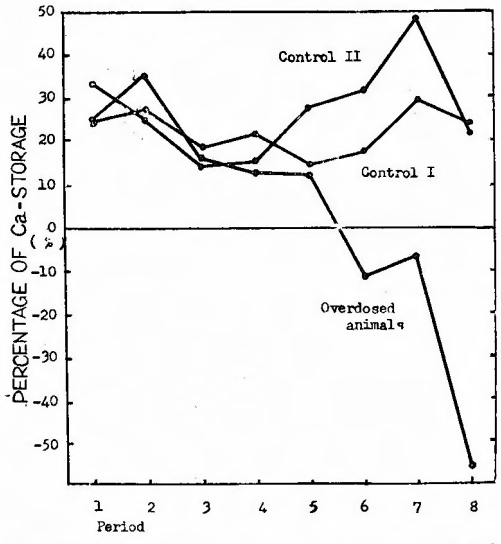
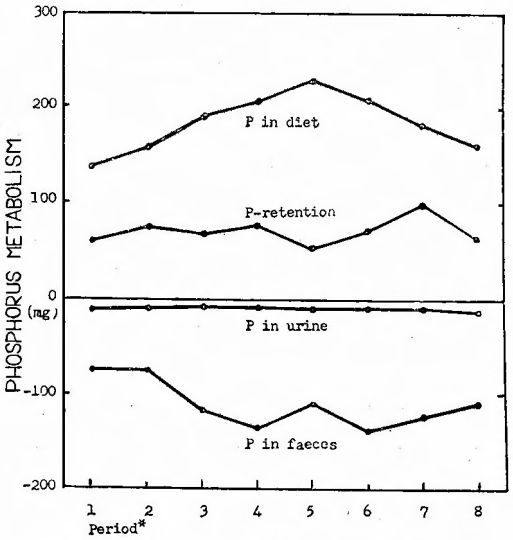


Fig. 11 Quantitative ratio of calcium accumulated in body to that ingested in various experimental groups.

groups. In the control animals, calcium accumulated in the body was 20 to 30 per cent of calcium contained in food ingested, and this percentage tended to rise with a reduction in the amount of ingested food. In the animals overdosed with vitamin A, this ratio did not increase despite of a reduction in the amount of ingested calcium and kept the negative level from the 5th day on (Fig. 11).

On the metabolic balance of phosphorus, the effects of excess vitamin A were

similar to those of calcium, although they were not so serious as the changes in calcium metabolism (Table 7, 8, 9 and Fig. 12, 13, 14, 15).



* One period corresponds with two days.

Fig. 12 Phosphorus metabolism in the control rats kept on a non-restricted amount of diet.

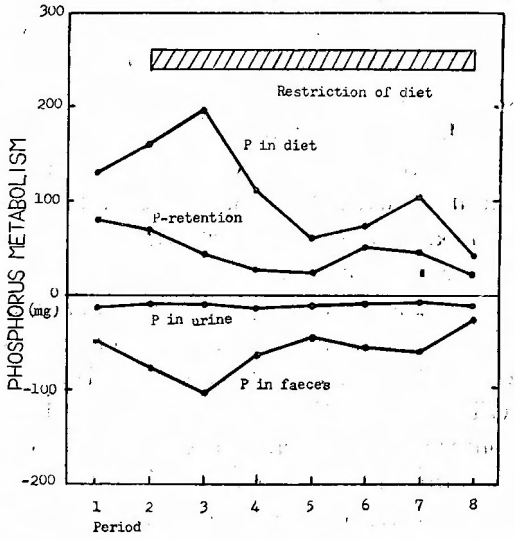


Fig. 13 Phosphorus metabolism in the control rats kept on a restricted amount of diet.

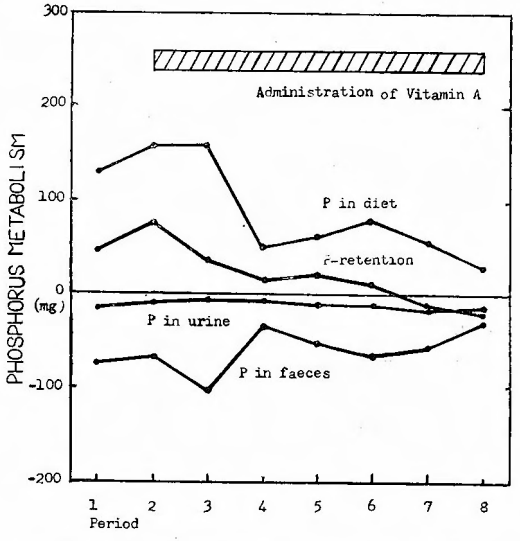


Fig. 14 Phosphorus metabolism in the rats given vitamin A in a daily dose of 15,000 I. U..

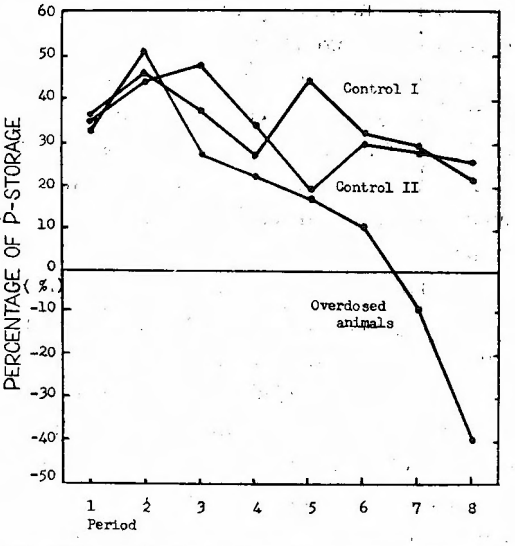


Fig. 15 Quantitative ratio of phosphorus accumulated in body to that ingested in various experimental groups.

III) Histological Changes of Bones in Vitamin A Intoxication.

A) Changes of bones before appearance of spontaneous fracture.

The histological findings of tibiae did't show any significant changes in the

animals given vitamin A of 10,000 I. U. daily for 5 days and for 10 days, while in about half of the animals given in same dose for 15 days some peculiar changes were revealed as will be described later. From the 20th onwards, the bones were remarkably damaged in all animals overdosed with vitamin A.

The changes were revealed mainly in the epiphysis and the cortical bone of diaphysis.

In the proximal epiphysis of tibia, the proliferation and maturation of epiphysial cartilage cells were not revealed; the cartilage cells left there in a smoll number were remarkably shrunken in size, with their nuclei and cytoplasma stained insufficiently. Furthermore, the layer of epiphysial cartilage cells showed a tendency of transformation into the fibrous tissue in its epiphysial side; in the region facing to diaphysis its cartilage matrix stained deeply with hematoxylin from the remarkable calcification. Accordingly, the layer of the epiphysial cartilage cells were extremely reduced in thickness by the degenerating process of these cells (Fig. 19). In the control animals, the layer of the epiphysial cartilage cells were contrarily thick, since the proliferation and maturation of these cells were distinctly maintained (Fig. 18).

The findings of the distal epiphysis of tibia were similar to those of the proximal epiphysis (Fig. 20 and 21).

In the diaphysis, the cortical bone was remarkably decalcified and reduced in thickness, particularly in the region of spontaneous fracture; such decalcified cortical bones tended to be gradually transformed into fibrous tissues as to be barely stained red with hematoxylin-eosin (Fig. 22 and 23). The subperiosteal bleeding was revealed occasionally in the region of the spontaneous fracture which developed apparently just before the examination, and the periost above these bleeding was remarkably thickened with the fibrous tissue proliferated in situ. In the cancellous bone, on the contrary, the deposition of calcium was normal or rather conspicuous than those in controls. Consequently, the cancellous bone was stained deeply violet with hematoxylin-eosin, while the cortical bone was stained only red from decalcification.

B) Observations of healing process after appearance of spontaneous fracture.

Long observation of the healing process for spontaneous fracture was impossible, since the animals affecting the vitamin A intoxication could hardly survive for more than two weeks after the development of fracture. Ten days' observations, however, gave in outline a histological view of the natural process for the healing of a fractured bone.

In the 5th day after the fracture, the two broken ends of the fractured region were completly enveloped by a mass of osteoblasts, producing either from periosteum and endosteum or by the metaplasia of connective tissue cells in surroundings. These spindle-shaped osteoid had in time a large number of capillary vessels and cartilage cells appearing in situ, the cartilage cells appearing more abundantly in the broken end of bone than in any other part, while the bleeding or necrotic mass could be yet found in the fractured regions. Ten days later, the osteoblasts were mostly converted into the cartilage cells, and this cartilage matrix became to be

calcified in the broken end (Fig. 24).

C) Observations of healing process for artificial fracture produced during excessive administration of vitamin A.

In animals overdosed with vitamin A, the fractured region of the tibia became to be surrounded by new osteoblasts in the 4th day after the production of the artificicial fracture. After 8 days the osteoblasts which were found in the broken ends began to be transformed into cartilage cells, accompanied with new capillary vessels appearing simultaneously in the same region. After 12 days the remaining osteoblasts completed their conversion into cartilage cells, and cartilage matrix was calcified in the region where the cartilage cells firstly appeared. These histological findings of bones were the same for the first 4 days in the control animals and in those given excess vitamin A, whereas after 12 days the transformation of osteoblasts into cartilage cells and the process of calcification were less revealed in the former than in the latter animals (Fig. 25 and 26).

DISCUSSION

It has not been ascertained whether the toxic effect of a excessive administration of vitamin A is due to the action of vitamin A itself or the action of the impurities contained in it, because vitamin A used in previous studies were mainly the natural fish-liver oil which simultaneously contained vitamin D or another impurities such as lipid and non-saturated fatty acid. Since the synthetical and water-soluble vitamin A began to be used in current medicine, the possibility of the toxic action of this pure vitamin A has been re-examined. Moore[22] and Rodahl[27] have reported that vitamin A acetate used in excess caused the spontaneous fracture of long bones. Synthesized vitamin A palmitate used in the present experiment also resulted the same effect, proving that vitamin A given in excess, not any of its impurities, has a toxic action on the bone.

The minimal dose in which vitamin A causes the spontaneous fracture has been estimated at 15,000 I. U. per day in rats by several investigators.[22),28] This minimal toxic dose is of course subjected to variation according to the age and sex of animals, the kind of diet, the brand of vitamin A and the method of its administration. It was confirmed in the present experiment that the minimal toxic dose in which vitamin A resulted a toxic effect on a young growing rat was 10,000 I. U. per day in subcutaneous injection and 30,000 I. U. in oral administration. However, this minimal toxic dose in oral administration is not so exact as that in the case of subcutaneous administration, because it was not ascertained whether the diets containing definite amounts of vitamin A were exactly ingested or not. Therefore, it seems that the minimal toxic level of vitamin A is more accurate in the subcutaneous administration than in the oral one.

In the animals receiving the large amount of vitamin A with considerable doses of vitamin D, the spontaneous fractures were demonstrated in all cases, while the minimal administration-period required for the occurence of fractures was evidently prolonged as compared with that in the animals given vitamin A only. Thoenes[35] indicated in his experiment that the development of the vitamin D

intoxication could be prevented by feeding the animal with vitamin-A-added diet. From his experimental data, it is suggested that vitamin A intoxication might be prevented by the administration of vitamin D. But the present experiment failed to show that vitamin D possessed of any property to nullify the fracture-causing action of vitamin A.

The vitamin A level in serum was confirmed to be 15 times higher in the animals overdosed with vitamin A for 15 days than in the control animals not so treated. DAVIS and MOORE[6] had confirmed in their experiments that the severity of vitamin A intoxication developed in animal corresponded with the vitamin A level in the liver of rats. In the present experiment, vitamin A reserved in the livers also showed the remarkable high level, indicating the presence of severe intoxication for vitamin A given in excess.

While the level of inorganic phosphorus in serum was slightly raised in the later half period of vitamin A administration, that of calcium in serum was remained to be constant, as had been observed previously by BOMSKOV and SEEMANN,[3] RODAHL[27] and others.[38] From these results, the mineral contents of blood may be kept constant even when the bone tissue is being absorbed.

The ashes and calcium contents of bones evidently decreased in rats with the spontaneous fracture, while no definite decrease was observed until the development of fracture, and phosphorus content of bones had undergone no significant change.

The metabolic balances of calcium and phosphorus were well maintained in the control animals during the whole course of the experiment. In the controls kept on restricted amount of diet, these minerals excreted in faeces were sufficiently reduced to keep the balance, while in the animals overdosed with vitamin A the excreted minerals were not reduced, but vitamin A given in excess promoted the excretion of calcium and phosphorus. Same results had been shown by NERUKAR and SAHASRABUDHE.[24]

The level of serum alkaline phosphatase was obviously risen in early period of administration, while it was rapidly reduced with the development of spontaneous fractures. It is generally known[32] that the level of alkaline phosphatase in serum is risen either by an increase in the activity of osteoblasts or by an obstructive process in the bile ducts, because the enzyme is formed in the osteoblasts and carried into the blood to be excreted in bile. It may be assumed, therefore, that in the rats overdosed with vitamin A the level of alkaline phosphatase is risen either by an increase in the activity of osteoblasts or as a result of disturbance in liver function. The accumulation of bile in bile ducts and degenerative process of the liver parenchyma have been confiremed histologically in vitamin-A-intoxicated animals, and also a functional disturbance of the liver has been revealed in the excretion of pigments.[1],[25] These physiological changes are considered to occure commonly in the late stadium of intoxication, but in the present experiment the level of alkaline phosphatase in serum was markedly risen in such early stadium, in 5th day after the first administration of vitamin A, as that in which the expected functional disturbance of liver did not yet appear. Therefore, this rise in the level of serum alkaline phosphatase must be ascribed to an increase in

activity of osteoblast. From ROBISON'S studies[28] in which alkaline phosphatase in an osteogenetic region of bone isolates phosphoric acid by separating the phosphoric ester in serum and the liberated phosphoric acid combines with calcium, leaving insoluble calcium as sediments in the region, it should be considered that the mineral contents of bone in vitamin A intoxication are kept at a definite level by an increased activity of alkaline phosphatase even when the absorption of bone exists in the other hand. In the late stadium of vitamin A intoxication, however, the reduction of calcium and phosphorus contents of bone may be appeared by the disappearance or the dysfunction of the osteoblasts resulting from the malnutrition. These opinions on the calcium and phosphorus metabolisms in the vitamin A intoxication may be corresponded with the histological findings as described below.

Chief histological findings on the bones of vitamin-A-intoxicated animals are a disturbance of enchondral ossification and an absorption of the cortical bone in diaphysis.

The normal process through which a bone grows at its epiphysis had been nowadays elucidated sufficiently. According to WOLBACH,[38] the growth in a epiphysis begins with the proliferation and maturation of the cartilage cells arranged in columns on the epiphysial side of bone and with the almost concurrent degeneration of its cartilage cells on the region facing to diaphysis; the next stage in its growth is marked by the penetration of capillary vessels with the osteoblasts into the space produced after the degeneration or disappearance of the cartilage cells on the region facing to diaphysis; at the final stage is seen the deposition of bone matrix on the exposed cartilage matrix. This continuous growth of cartilage cells at the epiphysis results in the formation of thick layer of cartilage cells—a picture invariably presented in any normally growing animal.

However, it was noted in present experiment that the layer of epiphysial cartilage cells in a rat overdosed with vitamin A was remarkably reduced in thickness as a result of the disappearance of the proliferated or matured cartilage cells. STRAUSS[33] has ascertained that there is a decline in the process of enchondral ossification in vitamin A intoxication, while WOLBACH[38] has explained that the growth of bone is promoted abnormally on its course in the animals overdosed with vitamin A and that the consequent rapid consumption of cartilage cells in the epiphysis arrests prematurely the growth of that region and reduces its thickness. Thus, same histological phenomenon is interpreted in two different ways.

Furthermore, cortical bones in the diaphysis were strikingly decalcified in vitamin-A-intoxicated animal, while the cancellous bones were left with their unabsorbed substances. STRAUSS[33] has asserted that these changes are produced by the osteoclasis, while WOLBACH[38] has refuted this view by pointing out the fact that the absorption process of bones in vitamin A intoxication results from an excessive acceleration of the remodelling process, including the absorption of bone substance in the region where the bone needs to be reduced in size and new deposition of bone substance in same region to preserve its continuity of structure. Thus, there are different interpretations to elucidate the changes in diaphysis of vitamin-A-intoxicated animals.

Considering the facts that the calcium and phosphorus contents of bone in hypervitaminosis A were preserved in constant by the increased activity of alkaline phosphatase before the appearance of malnutrition, and that the deposition of calcium in cancellous bones was well maintained in the histological and radiological examinations despite of the development of decalcification in the cortical bone and that the healing process for the artificial fracture was rather accelerated by overdosing with vitamin A, it may be concluded that vitamin A given in excess does not cause the regressive process in the growth of bone and rather abnormally promote the growth of bone. Namely, it seems to be reasonable that the vitamin A given in excess causes the more early-developed closure of the enchondral ossification at epiphysis and the acceleration of the remodelling process at diaphysis, and that in the late period of administration the acceleration of this remodelling process results in the spontaneous fracture of long bone and negative balances of calcium and phosphorus, because the bone substance thus absorbed is insufficiently accompanied by the new deposition of bone substance owing to the progress of malnutrition.

SUMMARY

1) Long excessive administration of pure synthetic vitamin A palmitate resulted in the development of spontaneous fracture in long bones of growing rats. Minimal daily dose in which vitamin A caused the bone lesion was 10,000 I. U. subcutaneously and 30,000 I. U. per os. These disorders in vitamin A intoxication could not be prevented by adding simultaneously with a moderate amount of vitamin D.

2) The levels of vitamin A in serum and liver were remarkably high in animals overdosed with vitamin A. The levels of calcium and inorganic phosphorus in serum were practically the same before and during the excessive administration. The level of alkaline phosphatase in serum was considerably high, but tended to decrease in the late period of the administration. The ashes and calcium contents of each bone in these animals with spontaneous fractures were obviously reduced below the normal, while before the development of the fractures no definite decreases of those were observed. The metabolic balances of calcium and phosphorus were shifted towards the deficit side in the course of excessive administration of vitamin A.

3) Histological examinations of tibia showed that in vitamin A intoxication there were the consumption of the epiphysial cartilage cells and remarkable decalcification of the cortical region of its diaphysis while in its cancellous bone the calcification was well maintained. The formation of callus following the occurrence of artificial fracture was not only normally progressed, but seemed histologically to be more rapidly accomplished in animals overdosed with vitamin A than in normals.

4) Following the discussion in this paper, vitamin A given in excess causes the early-developed closure of the enchondral ossification at epiphysis and the acceleration of the remodelling process at diaphysis. Furthermore, extremely accelerated remodelling process results in the spontaneous fracture of long bone and negative

balances in calcium and phosphorus metabolisms, because the absorbed bone is insufficiently accompanied by the new deposition of bone substance owing to progress of the malnutrition in the late period of vitamin A intoxication.

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Explanation of Photographs and Photomicrographs

Fig. 3 Limping in hind-limb in rat receiving a daily dose of 10,000 I. U. of vitamin A subcutaneously for 13 days.

Fig. 4 Appearance of limping in all limbs and atrophy of hair in rat given with vitamin A in same daily dose for 18 days.

Fig. 5 Radiogram of rat given subcutaneously with vitamin A in same daily dose for 21 days; spontaneous fracture are found in all limbs.

Fig. 16 An spontaneous fracture at proximal region of diaphysis of tibia in vitamin-A-intoxicated rat; immediately after an appearance of fracture.

Fig. 17 Callus formation at the spontaneously fractured region of tibia in rat overdosed with vitamin A; in 8 days after the appearance of fracture.

Fig. 18 A picture of proximal epiphysial cartilage cells of tibia in normal growing rat.

Fig. 19 A picture of proximal epiphysial cartilage layer of tibia in rat receiving a daily dose of 10,000 I. U. of vitamin A subcutaneously for 21 days. The layer of epiphysial cartilage cells is remarkably reduced in thickness as a result of the consumption of cartilage cells.

Fig. 20 Distal epiphysial cartilage layer of tibia in normal growing rat.

Fig. 21 A consumption of the epiphysial cartilage cells in distal epiphysis of tibia in rat given vitamin A in excess.

Fig. 22 & 23 Extreme decalcification in diaphysis of tibia in rats overdosed with vitamin A. M: muscle, C: decalcified cortical bone, E: cancellous bone or endocallus developing in cancellous bone.

Fig. 24 A picture of callus 8 days after a development of spontaneous fracture in rat receiving a daily dose of 10,000 I. U. of vitamin A.

Fig. 25 A picture of callus 12 days after a occurrence of artificial fracture in rat overdosed with vitamin A.

gig. 26 A picture of callus 12 days after the production of artificial fracture in normal rat; its formation is less marked in this rat than in rat overdosed with vitamin A.

Fig. 3

Fig. 4

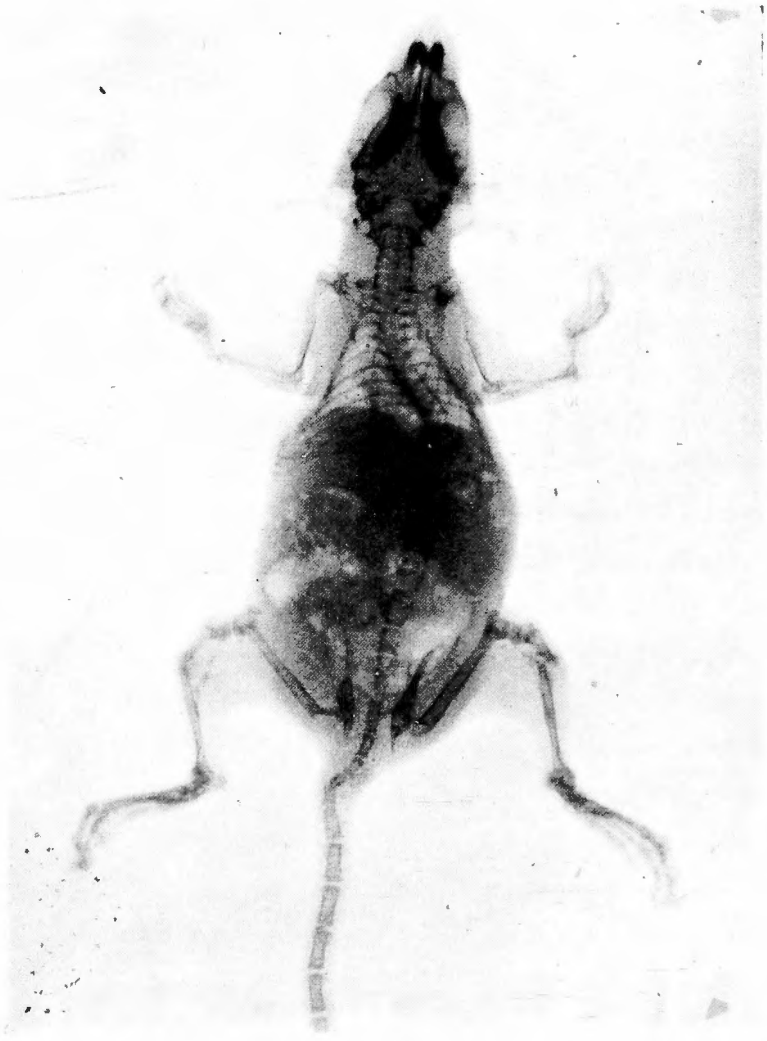

Fig. 5

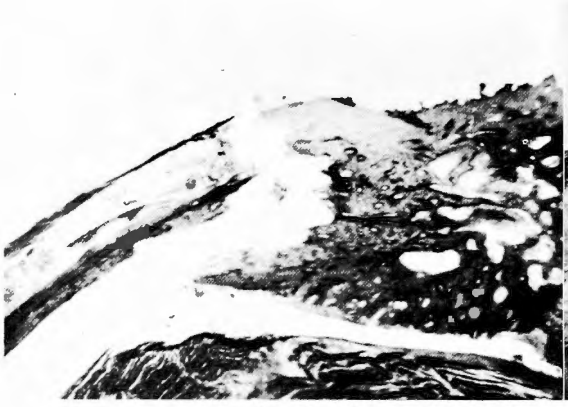

Fig. 16

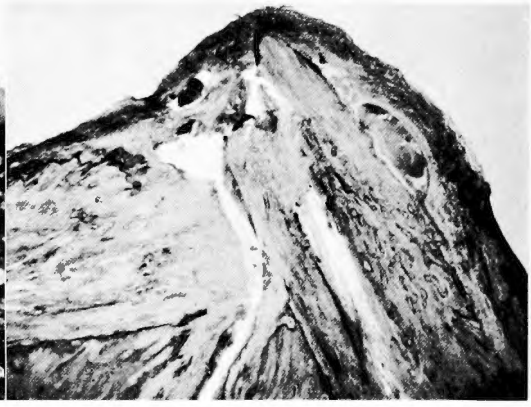

Fig. 17

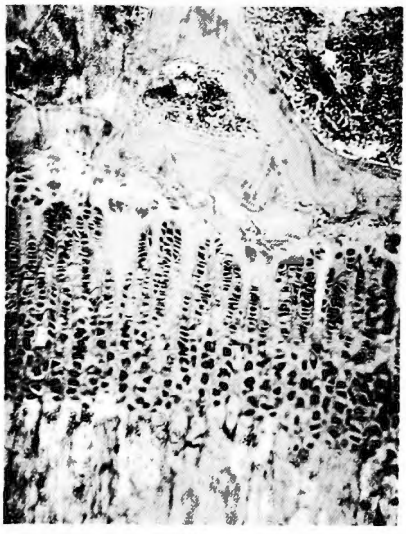

Fig. 18

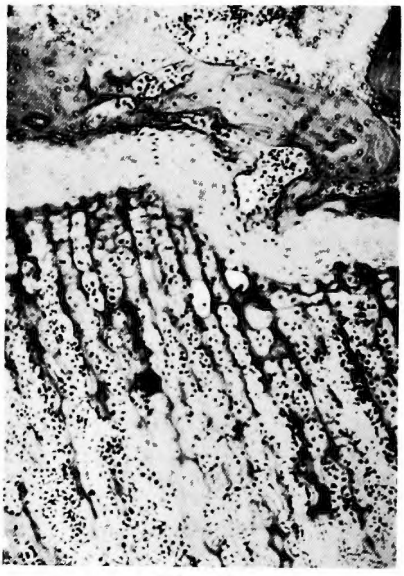

Fig. 19

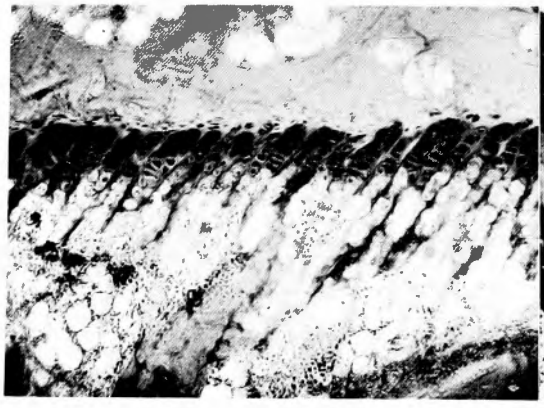

Fig. 20

Fig. 21

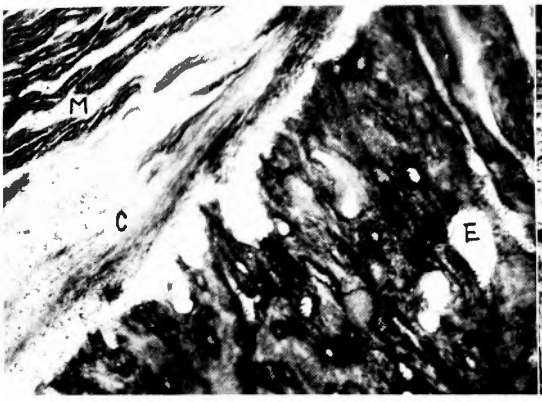

Fig. 22

Fig. 23

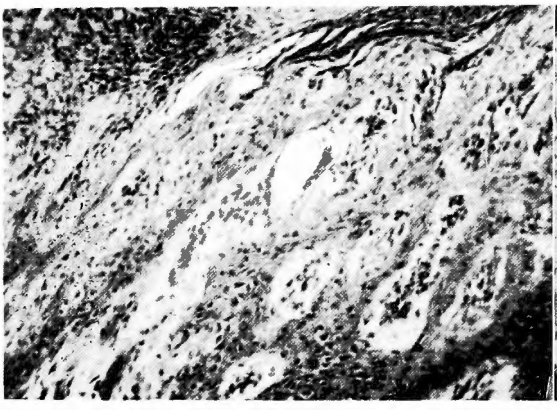

Fig. 24

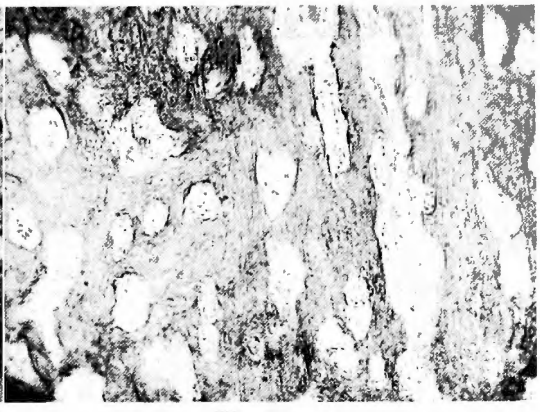

Fig. 25

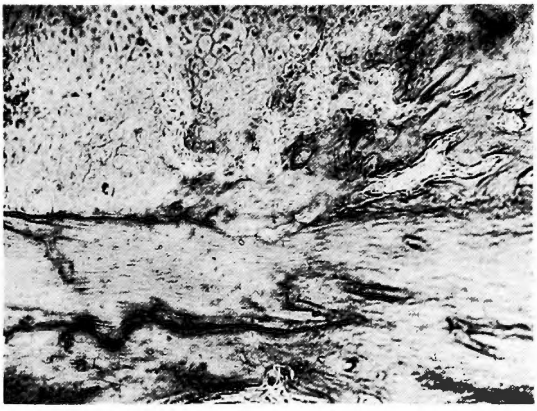

Fig. 26

和文抄録

ビタミンA過剰投与の骨に及ぼす影響（実験的研究）

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1) 合成のビタミンAを生後4週目のラッテに過剰に投与して，長管状骨に特発性骨折の起ることを確めた．骨折を起すに要する最少中毒量は皮下注射では1日1万単位，経口投与では1日3万単位であつて，何れも投与開始後2週前後に特発性骨折が出現した．かゝる骨折の出現は中等量のビタミンDを同時に与えても防ぐことは出来ない．レ線学的に長管状骨の皮質の陰影は菲薄になつているが，骨稜の陰影は正常に保たれている．

2) ビタミンA過剰投与動物の血清並びに肝のビタミンA量は著明に上昇している．血清のCa及び無機燐値には変化を認めない．血清アルカリ・フォスファターゼ値は上昇するが，特発性骨折の出現する頃から急激に減少する．骨の灰分並びにCa量は初期には明らかな変化を示さないが，骨折の発生と共に減少する．Ca及びPの新陳代謝平衡はビタミンA過剰投与の進行と共に負を示して来る．

3) ビタミンA過剰症の骨は組織学的に，骨端軟骨細胞の早期消耗と骨幹部皮質の著明な脱灰を認めるが，海綿状骨の石灰沈着は正常に保たれている．特発性骨折後の仮骨形成は概ね正常に行われている．更に人為的骨折後の仮骨の新生は，ビタミンAの過剰投与によつて促進されることが組織学的に確められた．

4) 以上の所見から，ビタミンAの過剰投与は，Ca及びPの体外への排出を促進し，骨を脆弱にすることが確められた．しかし，骨中のCa及びPの量が中毒症の末期を除いては常に正常値を保つこと，皮質の脱灰はあつても海綿状骨の石灰沈着の良いこと，特発性骨折後にも旺盛な治癒傾向のあること，更に人為的骨折の治癒がビタミンAの過剰投与によつて促進せられること等の事実から，過剰のビタミンAは単に一方的にCa及びPの体外への排出を促しているとは考え難い．従つて大量に投与されたビタミンAは，骨に絶えず繰返されている骨質の吸収並びにその後の再沈着という骨の改変過程を促進するものであつて，大量のビタミンAの長期投与は必然的に食餌摂取量の減少，ひいてはCa及びPの摂取不足を招来し，為に一度吸収された骨への再沈着が伴わなくなり，こゝに特発性骨折を招来するものと解釈される．又，骨端軟骨細胞の早期の消耗も，過剰のビタミンAが軟骨内骨化を異常に促進して，その発育がすでに完了した結果と考えられる．