METABOLIC ACTIVITIES OF VITAMIN D IN ANIMALS

II. ACTION OF VITAMIN D₃ IN ACTIVE SULFATE METABOLISM OF CHICKS

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In the foregoing paper (1) the present authors studied the relationship between vitamin D potency and sulfate metabolism and higher level of sulfate in left tibia of chicks receiving vitamin D₃ was demonstrated using S³⁵-sulfate.

The researches were further repeated for the action of vitamin D₃ in active sulfate metabolism of chicks. This paper deals with the outline of the results.

In the 1st experiment, the researches were carried out for the action of vitamin D₃ to the metabolic pathway of active sulfate in chicks. One-day-old White Leghorn cockerels, weighing about 30 g, were used in the experiment. The chicks were given 125 μ c of S³⁵-sulfate orally or 25 μ c of it intramuscularly. They were killed and the radioactivities of the left tibia and other tissues were determined by the usual Comar's method (2). The deposition of S³⁵-sulfate in the tissues was found to be greater in the chicks receiving vitamin D₃ than in the group devoid of vitamin D or in the group receiving vitamin D₂.

In the 2nd experiments, the investigations were made for the energy metabolism involving active sulfate by determining the phosphate levels in various tissues of the one-day-old White Leghorn cockerels, weighing about 30 g. After feeding with vitamin D-deficient diet for 5 weeks, 25 μ c of P³²-phosphate was given either orally or intramuscularly, and the chicks were killed. The left tibia and other tissues were collected and the radioactivities were estimated by the usual Comar's method (2). The data showed higher P³²-labelling in the vitamin D₃-treated chicks and lower labelling in the vitamin D-deficient ones.

On the other hand, the activities of liver citrate oxidase and liver pyruvate oxidase were tested using the chicks receiving vitamin D₃, D₂ or D-deficient diet. The increase in citrate-oxidizing ability of liver homogenate was statistically significant, while no influence was found on the oxidative phosphorylating ability of citrate. The conversion of inorganic phosphate to organic phosphorus compound was increased by administration of vitamin D₃. But no influence was observed on the

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pyruvate oxidative phosphorylating ability and pyruvate oxidase activity in liver homogenate by administration of vitamin D₃. With regard to the high level of active sulfate, it was found that the increase of reduced glutathione in blood was statistically significant in the group receiving vitamin D₃, but no influence was recognized on oxidized glutathione in blood and liver.

These findings seem to suggest that there may be some intimate relationship between vitamin D₃ metabolism and energy metabolism relating to active sulfate (Fig. 1).

EXPERIMENTALS AND RESULTS

1. Production of Vitamin D-Deficient Chicks

Day-old-White Leghorn cockerels (about 30 g) were used. The chicks were housed in an electrically heated brooder with a wire floor, and kept away from sunshine and other source of actinic light that might influence calcification.

The chicks were fed on the experimental diet and water *ad libitum*. They were divided at random into three groups, one was fed on a vitamin D-deficient diet, a modification (1) of the Association of Official Agricultural Chemists (AOAC) (3). Other groups receiving orally 500 I.U. of vitamin D₂ or D₃ per kg of the basal ration were served as positive controls.

FIG. 1 Possible Metabolism of Vitamin D₃ in Sulfate Activation of Chicks

2. Tests for Vitamin D-Deficiency

During feeding experiment, the growth curves were recorded and the body weight was estimated at the end of four or five weeks as shown in Table I. (Fig. 2).

After feeding for 4—6 weeks, the chicks were killed, and the left tibia were collected by the ordinary method. Ash was estimated by the AOAC method (3) (Table II).

Pyruvate levels (1) in the total blood was found to be higher in the group receiving vitamin D₃, but scarcely detectable in the kidney and liver. On the other hand, citrate levels (1) in the blood and kidney were tested. The highest level was

TABLE I
Body Weight Gain of Vitamin D-Deficient Chicks

No. of experiment	Date of experiment	Group receiving D ₃	Group receiving D ₂	D-deficient group	Result
1 ^a	Feb. 6, 1961	117.7 ± 31.0 ^c	95.1 ± 23.1	78.0 ± 25.1	A
2 ^a	Oct. 25, 1961	170.4 ± 45.4	147.0 ± 50.1	115.6 ± 34.5	B
3 ^b	Feb. 6, 1962	273.4 ± 97.3	220.7 ± 62.6	188.7 ± 37.6	C
4 ^b	Jun. 1, 1962	151.1 ± 49.7	136.0 ± 36.6	122.6 ± 19.6	D

^a Chicks were 4 weeks old

^b Chicks were 5 weeks old

^c Standard deviation

A, Statistically significant ($p < 0.01$) between D₃ and D₂

" ($p < 0.01$) between D₂ and D-deficient

B, " ($p < 0.05$) between D₃ and D₂

" ($p < 0.01$) between D₃ and D-deficient

C, " ($p < 0.05$) between D₃ and D₂

" ($p < 0.01$) between D₃ and D-deficient

Statistically nonsignificant between D₂ and D-deficient

D, Statistically significant ($p < 0.05$) between D₃ and D₂

" ($p < 0.05$) between D₃ and D-deficient

Statistically nonsignificant between D₂ and D-deficient

FIG. 2 *Vitamin D-Deficient Chicks*

A, D-deficient group; B, group receiving D₂; C, group receiving D₃

seen in the chicks receiving vitamin D₃ and the lowest in the vitamin D-deficient group.

3. Determination of SO_4^{2-} in Tibia and Other Tissues

Chemical analysis of SO_4^{2-} in left tibia were first studied. The highest level was found in the chicks receiving vitamin D₃, while the lowest in the vitamin D-deficient ones (1).

Afterwards, sulfate levels in the left tibia and other tissues using S^{35} -sulfate were tested. Vitamin D-deficient chicks were previously prepared as described above, and after 5-week feeding, the dilute HCl solution of carrier-free S^{35} -sulfate (125 $\mu\text{C}/100$ g of body weight) was introduced into the crop by injection. The chicks were rapidly killed after 3 hours, and the radioactivities of the left tibia, keel and skin were estimated by servometer. (Table III). The radioautograms of keel are presented in Fig. 3 and those of the left tibia were given already in the foregoing paper (1).

In the 2nd experiment, carrier-free S^{35} -sulfate ($25 \mu\text{c}/100 \text{ g}$ of body weight) was injected intramuscularly. The chicks were rapidly killed after 3 hours. The radioactivities of the left tibia, keel and skin were estimated by servemeter (Table IV).

According to the above results, it was proved that the deposition of S^{35} -sulfate in the left tibia, keel and skin was higher in the chicks receiving D_3 than in the

TABLE II
Content of Ash in the Dry Left Tibia

No. of experiment	Group receiving D_3	Group receiving D_2	D-deficient group	Result
	%	%	%	
1	51.6 ± 0.8^a	44.1 ± 6.1	32.9 ± 5.2	A
2	51.8 ± 3.9	43.3 ± 2.7	38.5 ± 3.8	B
3	41.1 ± 3.1	35.8 ± 4.2	32.2 ± 2.2	C
4	44.6 ± 2.2	39.0 ± 2.4	34.5 ± 3.2	D

^a Standard deviation

A, Statistically significant ($p < 0.05$) between D_3 and D_2

" ($p < 0.05$) between D_2 and D-deficient

B, " ($p < 0.01$) between D_3 and D_2

" ($p < 0.05$) between D_2 and D-deficient

C, " ($p < 0.05$) between D_3 and D_2

" ($p < 0.01$) between D_3 and D-deficient

Statistically nonsignificant between D_2 and D-deficient

D, Statistically significant ($p < 0.01$) between D_3 and D_2

" ($p < 0.05$) between D_2 and D-deficient

TABLE III
Radioactivities of Keel, Tibia and Skin in Chicks Receiving Orally S^{35} -Sulfate
 $125 \mu\text{c } S^{35}\text{-H}_2\text{SO}_4$ per 100 g body weight was administered using carrier-free $S^{35}\text{-H}_2\text{SO}_4$ in 0.01 N HCl ($100 \mu\text{c}$ per 0.1 ml). c. p. m. is corrected with background.

	No. of chicks	Body weight at the end of experiment	Volume of S^{35} -sulfate administered	Keel		Left tibia ^a			Skin	
				Weight	Radio-activity	Weight	A	B	C	On ilium On femur
		g	ml	g	c.p.m.	g	c.p.m.			c.p.m.
D-deficient group	1	194	0.242	0.6	300	2.4	437	248	341	169 172
	2	239	0.297	0.9	248	3.0	403	272	286	221 165
	3	248	0.310	0.7	243	2.9	360	266	274	201 178
	Average	240		0.7	264	2.8	400	262	300	197 172
Group receiving D_2	4	200	0.250	0.7	431	2.2	544	386	335	427 354
	5	241	0.301	0.8	376	2.4	512	360	350	443 347
	6	244	0.305	0.5	281	2.4	491	331	320	465 348
	Average	242		0.7	363	2.3	516	362	335	445 350
Group receiving D_3	7	282	0.353	0.7	425	3.2	782	453	497	457 276
	8	264	0.330	—	—	2.8	657	409	693	883 914
	9	326	0.408	0.9	436	3.7	693	404	560	1278 1099
	Average	291		0.8	430	3.5	711	422	583	868 688

^a See the right figure. The same is applicable in the following Tables.

FIG. 3 *Radioautograms of S^{35} -Sulfate in Keel after Oral Administration*

vitamin D-deficient group or in the group receiving vitamin D₂.

4. Studies on Mechanism of Active Sulfate

Phosphate level was studied for the energy metabolism relating to active sulfate formation. At first, phosphate levels in the left tibia and other tissues using P³²-phosphate were orally tested.

TABLE IV

Radioactivities of Keel, Tibia and Skin in Chicks Receiving S³⁵-Sulfate by Injection
25 μ c S³⁵-H₂SO₄ per 100 g body weight was administered using carrier-free S³⁵-H₂SO₄ in 0.01 N HCl solution (100 μ c/0.4 ml).

	No. of chick	Body weight at the end of experiment	Volume of S^{35} -sulfate administered	Keel		Left tibia			Skin		
				Weight	Radio-activity	Weight	A	B	C	On ilium	On femur
D-deficient group		g	ml	g	c.p.m.	g	c.p.m.			c.p.m.	
	1	224	0.22	0.9	23	2.6	31	23	11	18	16
	2	240	0.24	0.8	23	2.9	17	21	9	16	17
	3	210	0.21	0.5	21	2.9	29	23	11	20	21
	Average	231		0.7	22	2.8	26	22	10	18	18
Group receiving D_2	4	286	0.29	0.9	27	4.1	20	32	18	22	25
	5	240	0.24	0.9	23	3.7	26	13	23	22	20
	6	222	0.22	0.9	17	3.6	14	15	13	11	14
	Average	249		0.9	22	3.8	20	20	18	18	20
Group receiving D_3	7	342	0.34	0.7	37	3.4	42	28	23	37	34
	8	300	0.30	0.8	34	3.2	28	26	22	32	29
	9	399	0.40	1.0	55	4.7	35	28	27	28	31
	Average	347		0.8	42	3.8	31	27	24	23	31

TABLE V

Radioactivities of Keel, Tibia and Skin in Chicks Receiving Orally P³²-Phosphate
25 μ c P³²-ortho-phosphate per 100 g body weight using carrier-free P³²-phosphate in dilute HCl solution (5 μ c/0.01 ml).

	No. of chick	Body weight at the end of experiment	Volume of P ₃₂ -phosphate administered	Keel		Left Weight			Skin		Dry residue of liver supernatant	
				Weight	Radio-activity	Weight	A	B	C	On ilium		On femur
D-deficient group		<i>g</i>	<i>ml</i>	<i>g</i>	<i>c.p.m.</i>	<i>g</i>	<i>c.p.m.</i>		<i>c.p.m.</i>		<i>c.p.m./ml</i>	
	1	160	0.08	0.5	914	1.8	20558	17690	8558	1376	1075	
	2	164	0.08	0.4	691	1.5	17913	13034	7528	1384	1326	
	3	220	0.11	0.5	706	2.4	17720	14765	10362	1340	950	
	ave.	181		0.5	770	1.9	18730	15163	8816	1367	1117	3680
Group receiving D ₂	4	130	0.065	0.3	1288	1.2	19832	12111	6802	1977	1739	
	5	176	0.09	0.5	920	1.7	23677	19243	8983	2571	1355	
	ave.	153		0.4	1104	1.5	21755	15677	7893	2274	1547	—
Group receiving D ₃	7	216	0.11	0.5	3281	2.0	14490	12089	10454	3146	1413	
	8	250	0.125	0.6	3261	2.7	20902	18303	13307	3658	2273	
	9	194	0.10	0.5	2934	1.8	23909	15034	12594	1548	1491	
	ave.	220		0.5	3159	2.2	19753	15140	12118	2783	1726	4984

FIG. 4 *Radioautograms of P^{32} -Phosphate in Keel after Oral Administration*

The dilute HCl solution of carrier-free P^{32} -phosphate ($25 \mu\text{c}/100 \text{ g}$ of body weight) was introduced into the crop by injection. After 3 hours, the chicks were rapidly killed, and the radioactivities of the left tibia, keel and skin were estimated by survey meter (Table V). The radioautograms of the keel are presented on Fig. 4.

The livers of three chicks were mixed in the cold. The organ, 3 g, was homogenized with 9 ml of cold 10 % trichloroacetic acid in Waring blender for 3 minutes, followed by centrifugation for 15 minutes at 3000 rpm. 1 ml of the supernatant was dried up on a stainless dish (1 cm^2) by an infra-red lamp, and the radioactivities of the residual preparation were estimated by surveymeter.

In the 2nd experiment, carrier-free P^{32} -phosphate ($25 \mu\text{c}/100 \text{ g}$ body weight) was injected intramuscularly. After 3 hours, the chicks were rapidly killed and the radioactivities of the left tibia, keel, skin and liver dry matter (prepared as mentioned above) were estimated by surveymeter (Table VI). The radioautograms of the keel are presented in Fig. 5. The data showed higher P^{32} -phosphate in the group receiving D_3 and lower in the vitamin D-deficient group.

TABLE VI

Radioactivities of Keel, Tibia and Skin in Chicks Receiving P^{32} -Phosphate by Injection
 $25 \mu\text{c}$ P^{32} -phosphate per 100 g body weight using carrier-free P^{32} -phosphate in dilute HCl solution ($25 \mu\text{c}/0.8 \text{ ml}$).

	No. of chick	Body weight at the end of experiment	Volume of P^{32} -phosphate administered	Keel			Left tibia			Skin		Dry residue of liver supernatant
				Weight	Radio- activity	Weight	A	B	C	On ilium	On femur	
		<i>g</i>	<i>ml</i>	<i>g</i>	<i>c.p.m.</i>	<i>g</i>		<i>c.p.m.</i>		<i>c.p.m.</i>		<i>c.p.m./ml</i>
D-deficient group	1	223	0.178	0.8	749	2.7	15138	13959	11150	3490	2124	
	2	238	0.190	0.8	865	2.9	12736	14537	13463	4783	1432	
	3	197	0.154	0.8	763	2.5	18051	21861	13151	3015	911	
	Ave.	218		0.8	792	2.7	15308	16786	12705	3763	1489	20393
Group receiving D_2	4	250	0.200	0.8	643	3.3	14366	13159	12497	2342	1996	
	6	244	0.195	0.8	699	3.5	10317	10520	9862	3507	2433	
	Ave.	247		0.8	671	3.4	12342	11840	11180	2925	2215	23413
Group receiving D_3	7	214	0.171	0.6	1869	1.8	21635	12975	14982	5217	2808	
	8	352	0.282	0.9	2216	3.5	28258	16109	25507	5953	5370	
	9	214	0.171	0.5	2243	2.2	13071	8479	8439	5396	2433	
	Ave.	260		0.7	2109	2.5	20988	12521	16643	5522	3537	27643

5. Activity Tests for Citrate Oxidase in Liver Homogenate

Warburg's manometric method (4) was used. After feeding experiment, chicks were killed and the liver was collected immediately and perfused with ice-cold isotonic 0.25 M sucrose solution. The tissues were quickly weighed, and homogenized in ice-cold isotonic 0.25 M sucrose solution using the Potter type glass homogenizer. The homogenates were centrifuged at $600 \times g$ for 10 minutes, and the supernatant was used, yielding 10 % of the fresh liver. The sample was added to the ice-cold incubation mixture in a Warburg flask with the reaction system composing of 0.1 ml of 10^{-4} M cytochrome c, 0.1 ml of 0.1 M niacin, 0.1 ml of 0.03 M adenosine triphosphate (ATP), 0.1 ml of 0.02 M nicotinamide-adenine dinucleotide (NAD), 0.1 ml of 0.01 M cocarboxylase, 0.1 ml of 0.04 M MgCl_2 , 0.2 ml of 0.3 M phosphate

FIG. 5 *Radioautograms of P^{32} -Phosphate in Keel after Injection*

buffer (pH 7.3), and 0.1 ml of 0.2 *M* NaF in the main compartment, 0.4 ml of 20 % KOH solution in the center well and 0.2 ml of 0.15 *M* Na-citrate in the side bulb. The final volume was adjusted to 3 ml with distilled water.

Oxygen uptake was measured after 5-minute equilibration period at 37° in air. The data are recorded as microliters O₂ uptake per hour per mg of nitrogen. Nitrogen content was determined by micro-Kjeldahl method (Table VII).

TABLE VII
Activity Tests of Liver Citrate Oxidase

	QO ₂ (N) ^b	P/O ^c
D-deficient group	78.6±30.15 ^a	1.26±0.95
Group receiving D ₂	75.6±22.49	0.97±0.57
Group receiving D ₃	130.8±26.13	1.16±0.44

^a Standard deviation

^b Statistically significant ($p < 0.01$) between D₃ and D₂

” ($p < 0.01$) between D₃ and D-deficient

^c Statistically nonsignificant

Oxidative phosphorylation of citric acid was studied using Extrapolation Method of Cross (5) with the Warburg apparatus. The P:O ratio, micromoles of phosphorus absorbed to the microatom of oxygen consumed, was calculated. Inorganic phosphate of trichloroacetic acid filtrate was determined by Takahashi's method (6). 0.1 ml of 0.3 *M* Na-citrate was used as the substrate, 0.1 ml of 1.0 *M* glucose and 0.1 ml of hexokinase solution (30 mg/ml) were added to the reaction system, 0.2 ml of 80 % trichloroacetic acid solution was given to the side bulb, and 0.4 ml of 20 % KOH solution to the center well. The final volume was adjusted to 3.0 ml with distilled water. Hexokinase was prepared by the method of Maruo *et al.* (7). The reaction was measured after 5-minute equilibration period at 30° in air.

As shown in Table VII, the activities of liver citrate oxidase increased statistically in the chicks receiving vitamin D₃, while no influence was found on the ability of oxidative phosphorylation.

6. Activity Tests for Pyruvate Oxidase in Liver Homogenate

The investigations were performed with the liver homogenate and the procedure of citrate oxidation as mentioned above. 0.2 ml of 0.2 *M* Na-fumarate was added to the reaction system as the sparker and 0.2 ml of 0.15 *M* Na-pyruvate was added to the side bulb (Table VIII).

Oxidative phosphorylation of pyruvate were tested with the liver homogenate. The same procedure as in the case of oxidative phosphorylation of citric acid was

TABLE VIII
Activity Tests of Liver Pyruvate Oxidase

	QO ₂ (N) ^a	P/O ^a
D-deficient group	16.1± 1.67	2.42±0.60
Group receiving D ₂	16.1± 6.62	1.67±0.46
Group receiving D ₃	23.1±10.59	2.06±0.93

^a Statistically nonsignificant

used, and 0.2 ml of 0.2 *M* Na-fumarate and 0.1 ml of 0.3 Na-pyruvate were used as the sparkers and the substrate respectively.

The results given in Table VIII showed no effect of vitamin D₃ both on the activity of liver-pyruvate oxidase and on the ability of oxidative phosphorylation.

7. Tests of Sulfhydryl Group in Blood and Liver

Glutathione was determined by Okuda and Ogawa's method (8) or by Kuroiwa's method (9). The blood was collected with Na-citrate after about 12-hour fasting. To 5 ml of the blood was added 5 ml of 4 % metaphosphoric acid solution, and the volume was made up to 25 ml with 2 % metaphosphoric acid solution. After standing at room temperature for 30 minutes with occasional shaking, the sample was filtered through dry filter paper. The liver, 2.5 g, was placed in a glass mortar and ground with 2.5 ml of 4 % metaphosphoric acid solution. The mixture was filtered and the volume was made up to 50 ml with 2 % metaphosphoric acid solution. It was filtered through dry filter paper. The results are shown in Tables IX and X.

TABLE IX
Content of Glutathione in Blood and Liver

	Blood ^a	Liver ^b
	mg/100 ml	mg/100 g
D-deficient group	52.87 ± 10.36	155.2 ± 28.5
Group receiving D ₂	52.86 ± 11.88	148.0 ± 30.9
Group receiving D ₃	70.74 ± 16.51	133.9 ± 38.0

^a Statistically significant ($p < 0.05$) between D₃ and D₂

" ($p < 0.05$) between D₃ and D-deficient

^b Statistically nonsignificant

TABLE X
Content of Oxidized Glutathione in Blood and Liver

	Blood ^a	Liver ^a
	mg/100 ml	mg/100 g
D-deficient group	17.84 ± 4.84	57.1 ± 23.1
Group receiving D ₂	16.70 ± 6.57	44.5 ± 40.9
Group receiving D ₃	16.10 ± 9.26	50.7 ± 30.8

^a Statistically nonsignificant

The increase of reduced glutathione (GSH) in blood was detected statistically in the group receiving vitamin D₃, while no influence was observed on GSH in the liver.

The contents of oxidized glutathione were statistically nonsignificant in both blood and liver.

SUMMARY

1. The chicks were given S³⁵-sulfate orally and intramuscularly and the radioactivities of the left tibia, keel and skin were tested. The deposition of S³⁵-sulfate in the tissues of the birds receiving vitamin D₃ was found to be higher than those receiving vitamin D₂ or vitamin D-deficient group.

2. Carrier-free P³²-phosphate solution was supplied orally or intramuscularly, and

the radioactivities of the left tibia, keel and skin were estimated. Increased levels of P^{32} -phosphate were always seen in the groups receiving vitamin D_3 .

3. The activity of citrate oxidase increased significantly in the group receiving vitamin D_3 , while no influence was observed on the ability of oxidative phosphorylation of citrate in the liver.

4. No influence was observed on the pyruvate oxidative phosphorylating ability and pyruvate oxidase activity in the liver homogenate by the administration of vitamin D_3 .

5. The glutathione level in the blood was estimated, and the increase was statistically significant in the group receiving vitamin D_3 . But no influence of glutathione in the liver was observed. As for the oxidized glutathione levels, no effects were seen in both blood and liver.

6. From these findings, there seems to be some intimate relationship in chicks between vitamin D_3 metabolism and active sulfate metabolism postulated by Lipmann.

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