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Research Article

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Skeletal Muscle Calcium Metabolism and Contractile Force in Vitamin D-deficient Chicks

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ABSTRACT The myopathy associated with vitamin D deficiency has not been well characterized, and it is not known if weakness is a result of a specific effect of vitamin D deficiency on skeletal muscle. Chicks were raised from hatching on a vitamin D-deficient diet, and by 3 wk of age were hypocalcemic and appeared weak. Tension generated by triceps surae during repetitive stimulation of posterior tibial nerve was significantly less than that developed by chicks given vitamin D_3 supplements (309 g tension/g wet weight of triceps surae, SD 60, for vitamin D-deficient chicks; 470, SD 77, for vitamin D_3-treated chicks, $P < 0.01$). Histochemical and electron microscopic examination of skeletal muscles of these chicks showed no abnormalities, and there were no electrophysiologic evidences of motor nerve or neuromuscular junction dysfunction. The concentration of ATP in skeletal muscle of the vitamin D-deficient chicks (5.75 μmol/g wet weight, SD 0.17) was not significantly different from that in vitamin D-treated chicks (5.60, SD 0.50). There was no correlation between strength and serum calcium, serum inorganic phosphate, or skeletal muscle inorganic phosphate. Relaxation of tension after tetanic stimulation was slowed in the vitamin D-deficient chicks (20.6 ms, SD 1.7, vs. 15.4, SD 1.3, in vitamin D-treated chicks and 15.3, SD 1.0, in normal control chicks), and in vitro $^{45}Ca^{++}$ transport by sarcoplasmic reticulum from the vitamin D-deficient chicks was reduced. Calcium content of mitochondria prepared from leg muscles of vitamin D-deficient chicks (24 nmol/mg mitochondrial protein, SD 6) was considerably lower than that of mitochondria from normal control chicks (45, SD 8) or from chicks treated with vitamin D for 2 wk or more (66–100, depending upon level and duration of therapy). Treatment of the vitamin D-deficient chicks from hatching with sufficient dietary calcium to produce hypercalcemia did not significantly raise skeletal muscle mitochondrial calcium content (31 nmol/mg mitochondrial protein, SD 7) and did not prevent weakness. These studies demonstrate objective weakness as a result of myopathy in vitamin D-deficient chicks, and provide evidence that vitamin D deficiency has effects on skeletal muscle calcium metabolism not secondary to altered plasma concentrations of calcium and phosphate.

INTRODUCTION

Vitamin D_3 derived from the diet or synthesized endogenously from 7-dehydrocholesterol (1) is converted by successive hydroxylations in liver and kidney to 1-alpha, 25-dihydroxyvitamin D_3 (1,25-diOHD_3)[1] (2). 1,25DiOHD_3, with parathyroid hormone, regulates plasma calcium and phosphate concentrations through effects on intestine, kidney, and bone (2). Other hydroxylated forms of vitamin D_3 may also be of importance in calcium and phosphate metabolism (3).

Vitamin D deficiency in both humans (4) and experimental animals (5) causes muscle weakness. This presumed myopathy has not been well characterized. Additionally, it is not known whether the weakness is secondary to altered plasma concentrations of calcium and phosphate, or if there is a specific effect of vitamin D deficiency on skeletal muscle.

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[1] *Abbreviations and nomenclature used in this paper*: Ca-Mg-ATPase, Ca^{++}-stimulated, Mg^{++}-dependent ATPase; 1,25diOHD_3, 1-alpha, 25-dihydroxyvitamin D_3; P_i, inorganic phosphate; R_s, Spearman rank coefficient; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; $T_{1/2}$ relaxation time(s), time required for tension to fall from the deflection point at the end of the tetanic plateau to one-half tension value.

We have measured the strength of 3-wk-old chicks, and have found that animals deprived of vitamin D from hatching generated less than one-half the normal muscle tension in response to repetitive electrical stimulation. As previously reported in vitamin D-deficient rats (6), relaxation after muscle contraction was slowed in the vitamin D-deficient chicks. As in vitamin D-deficient rabbits (5), in vitro skeletal muscle sarcoplasmic reticulum calcium uptake of the vitamin D-deficient chicks was reduced. By varying the level and duration of vitamin D therapy, and changing the concentration of calcium in the diet, groups of chicks were obtained with strengths intermediate between the vitamin D-deficient and normal animals. Vitamin D therapy increased skeletal muscle mitochondrial calcium content of vitamin D-deficient chicks; this rise in mitochondrial calcium was correlated with increased force of skeletal muscle contraction. No correlation was observed between increased force of contraction and serum calcium, phosphate, or skeletal muscle inorganic phosphate.

METHODS

White leghorn chicks were raised in a darkened room from hatching. They were handled only with plastic gloves and drank deionized water. The chicks were fed a vitamin D-deficient diet (7) which contained 3.8 g calcium and 3.2 g phosphorus/kg feed. Some chicks received supplementary vitamin D_3 (8,000 IU/kg feed), 7-dehydrocholesterol (provitamin D_3, 2.5 mg/kg feed), sunlight, or calcium chloride (equivalent to 31 g calcium/kg feed) as described in Table I. An additional group of control chicks was fed commercially prepared "starter mash" (Biomedical Service Center, Hatfield, Pa.), which contained 21.5 g calcium, 2.3 g phosphorus, and 8,000 IU of vitamin D_3/kg feed.

To measure the strength of triceps surae in 3-week-old chicks, a leg was restrained by a padded clamp just above the ankle. The ankle was flexed to 90°, and the foot was attached to an adjustable metal band. The band was rigidly connected to a DISA SID 17 force transducer (DISA, Franklin Lakes, N. J.) to measure the tension developed by plantar flexion at the ankle. The force transducer was connected to an oscillator and reactance converter (DISA). Tension was displayed on one beam of a storage oscilloscope (Tektronix Inc., Beaverton, Ore.), and the electromyographic response, recorded with surface electrodes, was displayed on a second beam. Traces were recorded photographically, and measurements were made by use of an enlarger. For nerve stimulation, needle electrodes were inserted percutaneously in the popliteal fossa adjacent to the posterior tibial nerve, which was stimulated with supramaximal square-wave pulses of 0.2-ms duration at frequencies of 100 or 200/s for 200 ms. Stimuli were delivered via a Devices type 2,533 isolated stimulator (Medical Systems Corp., Great Neck, N. Y.) controlled by a Digitimer D4030 gated pulse generator (Digitimer, Welwyn Garden City, Hertfordshire, England). Fig. 1 illustrates representative tracings from chicks in groups 3 (given vitamin D for 1 wk), 4 (given vitamin D for 2 wk) and 5 (given vitamin D for 3 wk). Usually, tensions developed with 100 and 200 stimuli/s were identical. In those few animals where results with the two stimulus rates differed, the greater of the two values was used. Direct muscle stimulation was performed with percutaneous needle electrodes positioned at the triceps surae muscle belly, with square-wave pulses of 1.0-ms duration at 100 and 200/s for 200 ms.

To estimate the rate of relaxation of triceps surae after cessation of electrical stimulation of posterior tibial nerve, the time required for tension to fall from the deflection point at the end of the tetanic plateau to one-half the plateau tension value ($T_{1/2}$ relaxation time) (6) was measured on photographs of the oscilloscope tracings. $T_{1/2}$ relaxation times after stimulation

TABLE I
Chick Treatment Groups

Group	Abbreviation	Supplements given	Interval supplements given
1	noD	none	—
2	noD hiCa	$CaCl_2$	0–3 wk
3	D 1 wk	vitamin D_3	2–3 wk
4	D 2 wk	vitamin D_3	1–3 wk
5	D 3 wk	vitamin D_3	0–3 wk
6	D hiCa 1 wk	vitamin D_3 + $CaCl_2$	2–3 wk
7	proD hiCa 1 wk	sun + 7-dehydrocholesterol + $CaCl_2$	2–3 wk
8	proD hiCa 2 wk	sun + 7-dehydrocholesterol + $CaCl_2$	1–3 wk
9	proD hiCa 3 wk	sun + 7-dehydrocholesterol + $CaCl_2$	0–3 wk
10	starter mash 3 wk	commercial starter mash	0–3 wk

Abbreviations used in this table: D, vitamin D_3; hiCa, high calcium; proD, provitamin D_3.

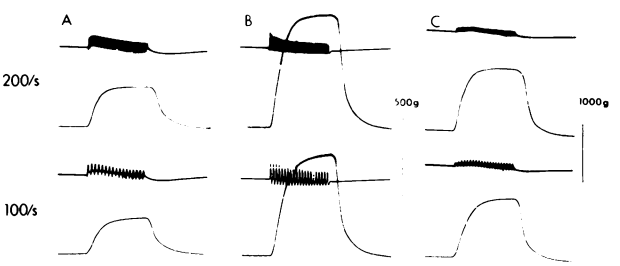

FIGURE 1 Effect of vitamin D_3 therapy on tension generated by triceps surae. Posterior tibial nerve was stimulated by needle electrodes with 200-ms trains of supramaximal 0.2-ms square-wave pulses at frequencies of 100/s (lower traces) or 200/s (upper traces). Electromyograms were recorded with surface electrodes and are displayed above the corresponding tension traces. Vertical bars denote calibrations of the force transducer (note change in calibration in C tracings). Results from a representative chick in each of three groups is shown (pooled data from each group is given in Table III). (A) Group 3 (1 wk of vitamin D_3 therapy); (B) group 4 (2 wk of vitamin D_3 therapy); (C) group 5 (3 wk of vitamin D_3 therapy).

rates of 100 and 200/s were similar, and measurements at each of these frequencies were averaged for each chick.

Statistical analysis of the tension and relaxation data was by one-way analysis of variance (8), and the Studentized range (8, 9) was used to test for the significance of differences between group means. The Studentized-range method minimizes the risk of obtaining falsely significant differences during multiple comparisons.

Other 3-wk-old chicks were killed by decapitation. Samples of triceps surae and pectoralis were obtained for histochemical and electron microscopic examination as previously described (10). Histochemical studies included modified Gomori trichrome, nicotinamide tetrazolium reductase, and myofibrillar ATPase at pH 9.4. Photographs of randomly selected cross-sections of triceps surae from vitamin D-deficient (group 1) and 2-wk vitamin D_3-treated (group 4) chicks, stained for ATPase at pH 9.4, were used to determine average muscle fiber diameters and ratios of lightly:darkly staining fibers. Leg muscles (pooled quadriceps, hamstrings, and triceps surae) were used for ATP and inorganic phosphate (P_i) assays and to prepare sarcoplasmic reticulum (SR) and mitochondria. Serum was collected for calcium and P_i assays.

A freeze-clamp technique (11) was used to obtain muscle specimens for ATP analysis. The frozen muscle was pulverized in 2 vol of frozen 0.9 N perchloric acid with a stainless steel mortar and pestle precooled in dry ice. After thawing and centrifugation (3,000 rpm for 10 min at 5°C in a Beckman J 21 centrifuge with JA 20 rotor [Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.]), ATP in the supernate was assayed using an enzyme-linked spectrophotometric method (12). Muscle and serum P_i were determined colorimetrically (13), and serum calcium was determined by atomic absorption spectroscopy.

For preparation of SR, 2–5 g of leg muscles, pooled from four chicks, was homogenized in 4 vol of 36.2% (wt/vol) sucrose in 0.1 M KCl, 10 mM imidazole, pH 7.3, for 2 min at 5°C in a Waring blender (Waring Products Div., New Hartford, Conn.). The homogenate was centrifuged for 30 min at 10,000 *g* and 5°C, and the supernate was again centrifuged for 30 min at 10,000 *g* and 5°C. The supernate obtained from the second centrifugation was recentrifuged at 48,000 rpm for 1 h in a Sorvall AH-650 swinging bucket rotor (Ivan Sorvall, Inc., Norwalk, Conn.) at 5°C. The resulting pellet was resuspended in 0.3 M sucrose, 1 mM Hepes buffer (pH 7.5). A portion was fixed in glutaraldehyde for electron microscopy (14). Osmium tetroxide was used instead of Dalton's fixative. A portion of the pellet was assayed for protein (15), and the remainder was used for measurements of $^{45}Ca^{++}$ uptake and Ca^{++}-stimulated, Mg^{++}-dependent ATPase (Ca-Mg-ATPase) activity (16). To estimate contamination of the SR fraction by mitochondria, the degree of inhibition of calcium-stimulated ATPase activity by 1 μM oligomycin was determined (17). Also, in some experiments a portion of the preparation was solubilized by boiling in 1% sodium dodecyl sulfate (SDS)/0.1% mercaptoethanol in 10 mM sodium phosphate buffer (pH 7.4) (wt/vol/vol) and examined by SDS polyacrylamide slab gel electrophoresis (18).

Muscle mitochondria were prepared by a modification of the method of Makinen and Lee (19). The leg muscles (2–5 g, pooled from four chicks) were minced in ice-cold 0.15 M KCl and then incubated with stirring for 10 min at 5°C with protease (15 mg/g muscle, type VIII Sigma Chemical Co., St. Louis, Mo.) in Chappell-Perry medium (pH 7.5) that contained 100 U heparin/ml (20) and 1 mM EDTA. The suspension was homogenized for 5 s with a Tekmar hand-held homogenizer (Tekmar Co., Cincinnati, Ohio), and then centrifuged at 600 *g* for 10 min at 5°C. The resulting supernate was then filtered through cheese cloth. The filtrate was next centrifuged at 13,400 rpm in a JA 20 rotor (Beckman J 21 centrifuge) for 10 min at 5°C, the supernate discarded, and fat wiped from the sides of the tube. The pellet was resuspended in 100 mM KCl which contained 0.25 M sucrose, 50 mM Tris (pH 6.9 at 5°C), 1 mM $MgCl_2$, 0.2 mM EDTA, and 100 U heparin/ml. The suspension was centrifuged at 6,600 rpm (JA 20 rotor) for 10 min at 5°C, and the mitochondrial pellet was resuspended in 0.25 M sucrose and 2 mM Hepes-Tris (pH 7.4). Portions of the suspension were taken for electron microscopy as previously described (10), protein assay (15), and for polarographic measurements of oxygen uptake (19). To assay the ATP content of the leg muscle mitochondria, the preparation was carried out as described above, except that all solutions contained 1 μM oligomycin (17) and 5 μM atractyloside (21). The mitochondrial fraction was then homogenized in ice-cold perchloric acid, and ATP was measured by a spectrophotometric method (12).

Mitochondrial calcium content was assayed by atomic absorption spectroscopy after overnight digestion at 25°C in 8 N nitric acid which contained 1.5% (wt/vol) lanthanum chloride. Mitochondrial $^{45}Ca^{++}$ uptake during incubation in 250 mM sucrose, 2 mM Hepes-Tris (pH 7.4) with 2 mM ATP at 30°C was studied by the EGTA quench method of Reed and Bygrave (22). The rate of release of $^{45}Ca^{++}$ from mitochondria from vitamin D-deficient chicks (group 1-noD) and chicks given vitamin D_3 for 2 wk (group 4) was studied by loading mitochondria from the two groups with 50 nmol of $^{45}Ca^{++}$/mg protein during incubation in 250 mM sucrose, 2 mM Hepes-Tris (pH 7.4) with 2 mM ATP at 30°C. Then the mitochondrial suspension was centrifuged at 6,600 rpm (JA 20 rotor) for 10 min at 5°C. The supernate was aspirated, and the pellet was resuspended in calcium-free 250 mM sucrose, 2 mM Hepes-Tris (pH 7.4) which contained 1 mM EGTA, 1 μM oligomycin (17), and 5 μM atractyloside (21). Appearance of $^{45}Ca^{++}$ in the medium during incubation at 30°C was followed by serial sampling of the suspension. Mitochondria were sedimented at 16,000 rpm (JA 20 rotor) for 5 min at 5°C, and $^{45}Ca^{++}$ in the supernate was determined by liquid scintillation spectrometry. The half-time of release of calcium from the mitochondria was estimated by linear-log plots of the data.

RESULTS

As previously reported (23), chicks shielded from sunlight and fed a diet devoid of vitamin D and low in calcium (group 1-noD) gained weight at a slightly slower rate than starter-mash-fed control chicks (group 10). The vitamin D-deficient chicks developed deformities of the sternum and were hypocalcemic, but not hypophosphatemic (Table II). Histochemical and electron microscopic appearance of pectoralis and triceps surae of the group 1-noD chicks were indistinguishable from those of group 4D 2 wk chicks. There was no evidence of myopathy or denervation. Skeletal muscle fiber diameters in triceps surae of the group 1-noD chicks (mean, 13.7 microns; SD 2.0) did not differ significantly from those of chicks in group 4 (mean, 12.5 microns; SD 1.4). In sections stained for myosin ATPase at pH 9.4, the ratio of light:dark fibers in group 1-noD was 0.24; that in group 4-D 2 wk was 0.27.

The vitamin D-deficient chicks appeared weak, they were unable to stand for long periods with their legs

TABLE II
Weight, Serum Calcium and Phosphate, and Muscle Phosphate of 3-wk-old Chicks

Group	Weight	Serum		Muscle phosphate
		Calcium	Phosphate	
	g	*mM*	*mM*	*mM*
1-noD	130 (20)	1.91 (0.84)	2.54 (0.59)	33.4 (8.6)
2-noD hiCa	91 (17)	3.32 (1.15)	2.10 (0.31)	30.1 (3.5)
3-D 1 wk	103 (14)	2.37 (0.15)	1.74 (0.30)	27.3 (3.8)
4-D 2 wk	166 (22)	2.57 (0.82)	2.12 (0.46)	29.6 (5.6)
5-D 3 wk	233 (36)	3.00 (0.56)	2.20 (0.26)	34.9 (3.1)
6-D hiCa 1 wk	96 (16)	4.13 (0.49)	1.75 (0.10)	23.0 (2.1)
7-proD hiCa 1 wk	130 (13)	2.64 (0.34)	1.81 (0.48)	28.9 (8.1)
8-proD hiCa 2 wk	148 (10)	3.22 (1.02)	2.35 (0.42)	27.3 (4.9)
9-proD hiCa 3 wk	145 (30)	2.65 (0.30)	2.54 (0.37)	31.8 (6.1)
10-starter mash 3 wk	149 (31)	2.80 (0.16)	2.11 (0.15)	34.3 (4.9)

Chick treatment group abbreviations are given in Table I. Each value is the mean of results in 10 chicks. Numbers in parentheses are the standard deviations.

fully extended, and they tended to rest their bodies on the floor of the cages. Weakness was confirmed by electrophysiological studies. Force of contraction of triceps surae of the group 1-noD chicks during repetitive stimulation of posterior tibial nerve was less than one-half that of group 10-starter-mash-fed controls (Table III). Tension generated by the muscles of the group 1-noD chicks, like that of vitamin D-treated chicks (Fig. 1) and group 10-starter-mash-fed controls, was well maintained during the stimulus train, without evidence of a decremental response. Also, direct repetitive stimulation of the triceps surae muscle produced no greater tension than repetitive stimulation of posterior tibial nerve (ratio of tension elicited by direct muscle stimulation:tension elicited by stimulation of posterior tibial nerve is 0.98 [SD 0.10, $n = 4$] for group 1-noD and 1.01 [SD 0.07, $n = 4$] for group 4-D 2 wk). Thus, there was no electrophysiological evidence that motor nerve or neuromuscular junction dysfunction was the cause of weakness observed in the vitamin D-deficient chicks.

TABLE III
Tension Generated by Triceps Surae of 3-wk-old Chicks during Repetitive Stimulation

Group	Tension	Difference from 1-noD	Difference from 10-starter mash controls
1-noD	367 (121)	—	$P < 0.01$
2-noD hiCa	544 (162)	$P < 0.01$	$P < 0.01$
3-D 1 wk	322 (83)	NS	$P < 0.01$
4-D 2 wk	774 (83)	$P < 0.01$	NS
5-D 3 wk	906 (173)	$P < 0.01$	NS
6-D hiCa 1 wk	760 (53)	$P < 0.01$	NS
7-proD hiCa 1 wk	415 (77)	NS	$P < 0.01$
8-proD hiCa 2 wk	554 (202)	$P < 0.01$	$P < 0.01$
9-proD hiCa 3 wk	630 (167)	$P < 0.01$	$P < 0.01$
10-starter mash	814 (74)	$P < 0.01$	—

Tension generated by triceps surae was measured in five chicks of each group (see text). Values given are the means, with standard deviations in parentheses. One-way analysis of variance (8) indicated that therapy-altered tension developed during electrical stimulation ($P < 0.005$). Means of the treatment groups were compared with values for Group 1-noD and Group 10-starter mash with the Studentized range (8, 9). Means were different at the 5% level of confidence if >125 g apart, and at the 1% level of confidence if >149 g apart. Abbreviations are given in Table I.

Although the size of individual muscle fibers from triceps surae of group 1-noD and group 4-D 2 wk chicks was similar, it seemed possible that the weakness in the vitamin D-deficient group was a result of reduced muscle mass. To evaluate this possibility, triceps surae were dissected free and weighed after completion of the electrophysiological studies in four animals from group 1-noD and four from group 4-D 2 wk. Chicks from group 1-noD produced 309 (SD 60) g tension/g wet weight triceps surae, whereas chicks from group 4-D 2 wk produced 470 (SD 77) g tension/g wet weight triceps surae. These results were compared by the unpaired Student's two-tailed t test (8); the difference between the means was significant ($P < 0.01$). Thus, when compared on the basis of muscle mass, vitamin D-deficient preparations still exhibited reduced tension development when compared with vitamin D-treated controls.

Chicks fed the vitamin D-deficient diet supplemented with calcium (group 2-noD hiCa) gained weight less rapidly than the vitamin D-deficient group without calcium supplementation. These animals were

hypercalcemic (Table II) and appeared weak. With repetitive stimulation of posterior tibial nerve, group 2-noD hiCa triceps surae tension was greater than that in group 1-noD chicks (Table III; $P < 0.01$) but less than that of group 10-starter-mash controls ($P < 0.01$) or chicks given vitamin D_3 for 2 or 3 wk (groups 4 and 5, $P < 0.01$).

Three vitamin D-treatment regimens (Table I) were instituted at hatching or 1 or 2 wk after hatching. In preliminary experiments, many chicks given both vitamin D_3 and calcium supplements beginning at the time of hatching or a week later died before the age of 3 wk; survivors were hypercalcemic and averaged less than one-half the weight of vitamin D-deficient chicks. Because of this severe toxicity, no further studies were carried out on this group of chicks. Chicks given vitamin D_3 and calcium supplements for only the last week before study (group 6-D hiCa 1 wk) were also hypercalcemic. Although these animals were lighter than group 1-noD, these chicks attained triceps surae contractile forces averaging twice that of group 1-noD (Table III). Chicks exposed to sunlight and given the vitamin D_3 precursor, 7-dehydrocholesterol (1), and supplementary calcium (groups 7–9), or vitamin D_3 without supplementary calcium (groups 3–5) gained weight well and showed increasing triceps surae tensions with increasing duration of therapy (Table III). The best muscle function was attained with vitamin D_3 therapy without supplementary calcium. With 2 or 3 wk of such therapy (groups 4 and 5), tensions were attained that were in the same range as in group 10-starter-mash-fed chicks (Fig. 1).

In contrast to previous studies in vitamin D-deficient rats (24, 25), ATP concentration in leg muscles of vitamin D-deficient chicks (group 1), measured with a freeze-clamp technique (11), was not significantly different from that in 2-wk vitamin D_3-treated chicks (group 1-noD ATP 5.75 μmol/g wet weight of muscle, SD 0.17, $n = 5$; group 4-D 2 wk, 5.60 μmol/g wet weight of muscle, SD 0.50, $n = 5$).

Comparisons of group mean tension data (Table III) with the biochemical data in Table II, using the Spearman rank correlation test (8), showed no significant correlation between triceps surae tension and serum Ca^{++}, serum P_i, or muscle P_i. Although strength tended to be greater in the heavier groups of chicks, this association was not statistically significant (Spearman rank coefficient [8] $[R_s] = 0.545, P > 0.05$).

In a previous study, relaxation of tension after single or repetitive stimulation of skeletal muscle was slowed in vitamin D-deficient rats (6). Though SR calcium metabolism of the rats was not investigated, other investigators found $^{45}Ca^{++}$ uptake by skeletal muscle SR of vitamin D-deficient rabbits to be reduced (5). To determine whether these abnormalities in SR function were also present in the vitamin D-deficient chicks, we measured the $T_{1/2}$ relaxation time (6) of triceps surae after repetitive stimulation, and studied the calcium metabolism of SR isolated from leg muscles. Relaxation after repetitive stimulation was slower in the group 1-

TABLE IV
Triceps Surae Relaxation and SR Calcium Metabolism

Group	$T_{1/2}$	Ca-Mg-ATPase	SR calcium uptake	
			1 min	8 min
	ms	*μmol P_i/mg protein per min*	*μmol calcium/mg protein*	
1-noD	20.6 (1.7)	0.324 (0.065) (0.315 oligomycin)	0.393 (0.101)	1.25 (0.17)
4-D 2 wk	15.4* (1.3)	0.299 (0.029) (0.308 oligomycin)	0.495 (0.080)	1.51* (0.23)
10-starter mash	15.3* (1.0)	0.337 (0.097) (0.322 oligomycin)	0.642* (0.147)	1.93* (0.08)

For the $T_{1/2}$ measurements ([6] see Methods), four chicks in each group were studied; values given are the means, with standard deviations in parentheses. One-way analysis of variance indicated the chicks were from different populations ($P < 0.001$). SR was prepared from groups of four chicks, and Ca-Mg-ATPase and $^{45}Ca^{++}$ uptakes (16) were means of four experiments, with standard deviations in parentheses. Means of two determinations in the presence of 1 μM oligomycin (17) are given below the Ca-Mg-ATPase data. One-way analysis of variance indicated Ca-Mg-ATPase results were drawn from the same population. 1-min $^{45}Ca^{++}$ uptakes were from different populations ($P < 0.05$), as were 8-min $^{45}Ca^{++}$ uptakes ($P < 0.001$). Abbreviations are given in Table I.

* Means were significantly different from those of group 1-noD, using the Studentized range (8, 9), $P < 0.01$.

noD chicks than in group 10-starter-mash controls or in group 4-D 2 wk animals (Table IV). We also prepared SR by the method of Nakamura et al. (26). This procedure gave a fraction of satisfactory purity from the muscles of vitamin D-treated chicks (group 4-D 2 wk) and from starter-mash controls. However, SR protein yield from the vitamin D-deficient chicks (group 1) was considerably higher than from the vitamin D-treated animals (group 4-D 2 wk), and electron microscopy and SDS polyacrylamide slab gel electrophoresis (18) of the fraction from vitamin D-deficient chicks indicated considerable contamination by contractile fibrils. For this reason, the SR isolation method for both the vitamin D-deficient and vitamin D-treated chicks was modified (see Methods) to provide comparable purity. Electron microscopy of fractions obtained by this modified procedure from groups 1-noD, 4-D 2 wk, and 10-starter mash showed SR contaminated by occasional myofibrils and no intact mitochondria. SDS polyacrylamide slab gel electrophoretic patterns (18) of SR proteins from the three groups, stained with Coomassie blue, were indistinguishable. Specific activity of Ca-Mg-ATPase in preparations from group 1-noD chicks was not significantly different from the group 4-D 2 wk and group 10-starter-mash controls, and there was no significant oligomycin-inhibited (mitochondrial) (17) Ca^{++}-stimulated ATPase activity in any of the preparations (Table IV).

Boland et al. (27) reported the specific activity of Ca-Mg-ATPase of SR from the leg muscles of normal 21-d-old chicks to be 0.6 μmol P_i/mg protein per min; this was somewhat higher than the values obtained in our study with SR from group 10-starter-mash controls (Table IV). Ca-Mg-ATPase specific activities of SR from the leg muscles of vitamin D-deficient chicks (group 1) and vitamin D_3-treated chicks (group 4) were not significantly different from the starter-mash controls (group 10). Boland et al. (27) also found that leg muscle SR of normal 21-d-old chicks took up 0.6 μmol, of $^{45}Ca^{++}$/mg SR protein in 1 min, and 2.1 μmol of $^{45}Ca^{++}$/mg SR protein in 10 min. Measuring $^{45}Ca^{++}$ uptakes by a procedure (16) similar to that of Boland et al. (27), $^{45}Ca^{++}$ uptake a 1 min by group 10-starter-mash control leg muscle SR was nearly identical to that previously reported, and 8-min $^{45}Ca^{++}$ uptake was similar to the 10-min uptake. These results suggest that the purity of SR prepared by the modified method used in our study was comparable to that obtained by Boland et al. (27). Both 1- and 8-min $^{45}Ca^{++}$ uptakes by the SR from group 1-noD chicks were below those of the SR from group 10-starter-mash controls. $^{45}Ca^{++}$ uptakes by SR from the group 4-D 2 wk chicks were intermediate between those of the group 1-noD and the group 10-starter-mash control chicks (Table IV).

Mitochondria were prepared from chick leg skeletal muscles. Yield varied between 0.6 and 1.7 mg mitochondrial protein/g wet weight muscle, and no significant differences in protein yields between the treatment groups were noted. Electron microscopy of preparations from group 1-noD, group 4-D 2 wk and group 10-starter mash showed intact mitochondria contaminated by occasional myofibrils and vesicles resembling SR. ADP:O ratio (19) for glutamate: malate was 2.8 (SD 0.2, $n = 4$) for group 1-noD; 2.9 (SD 0.3, $n = 4$) for group 4-D 2 wk; and 2.8 (SD 0.4, $n = 4$) for group 10-starter mash. The respiratory control index for glutamate:malate (19) was 5.1 (SD 0.5, $n = 4$) for group 1-noD; 4.9 (SD 0.4, $n = 4$) for group 4-D 2 wk; and 4.8 (SD 0.6, $n = 4$) for group 10-starter mash. These data indicate that mitochondria prepared from each of these groups were well coupled.

Freshly prepared mitochondria were digested in nitric acid, and calcium content was measured by atomic absorption spectroscopy. Mitochondrial calcium content was greatest in chicks treated for the longest period with vitamin D (Fig. 2). Mitochondrial calcium content did not correlate with serum calcium concentration ($R_s = 0.375$, $P > 0.05$), and was not raised in vitamin D-deficient chicks by calcium supplements sufficient to cause hypercalcemia (group 2-noD hiCa). The mitochondria from group 10-starter-mash control chicks contained 45 nmol calcium/mg mitochondrial protein (SD 8, $n = 6$). This was above the level in group 1-noD and group 2-noD hiCa mitochondria ($P < 0.01$) but below that of chicks given vitamin D for 2 wk or more (groups 4, 5, 8, and 9). There was a significant positive correlation between mitochondrial calcium content and strength ($R_s = 0.746$, $P < 0.01$).

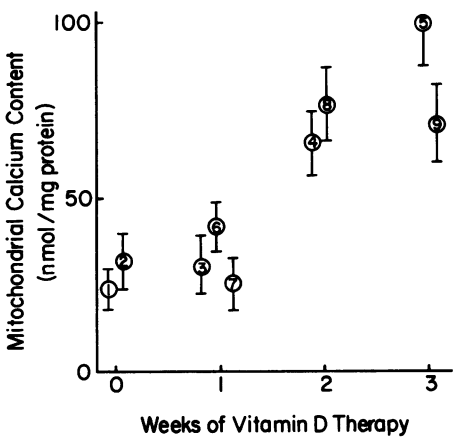

FIGURE 2 Calcium content of chick skeletal muscle mitochondria. Mitochondria were isolated from leg muscles as described in the text, and calcium was assayed by atomic absorption spectroscopy. Values given are the means of four determinations, with vertical bars indicating standard deviations. Numbers within the circles refer to the chick treatment groups listed in Table I.

A number of studies were designed to assess the mechanism by which vitamin D therapy increased the level of calcium in mitochondria isolated from the chick leg muscles. The effect of vitamin D could have been a result of greater uptake of calcium from extracellular fluid by these mitochondria during the isolation procedure. This is unlikely on a number of grounds. First, the mitochondrial calcium content of the hypercalcemic group 2-noD hiCa chicks was below that in vitamin D-treated groups. Second, the solutions used after initial mincing of the muscle specimens during the isolation procedure contained the calcium chelator EDTA. It is conceivable, however, that the mitochondria took up calcium during the muscle mincing in ice-cold 0.15 M KCl. To evaluate the possibility, the isolation procedure was modified by incorporation of 2 μM lanthanum chloride, an inhibitor of mitochondrial calcium transport (22, 28), in the mincing solution and all subsequent solutions used in the isolation procedure. Mitochondria prepared in this way from group 1-noD contained 18 nmol calcium/mg mitochondrial protein (n = 2); those from group 4-D 2 wk, 53 nmol (n = 2); and those from group 10-starter mash, 36 nmol (n = 2). Each of these values was ≅20% lower than that obtained without lanthanum chloride.

Although calcium uptake during the isolation procedure was not responsible for the greater levels of muscle mitochondrial calcium in the vitamin D-treated groups, it is conceivable that the lower calcium content of skeletal muscle mitochondria from the vitamin D-deficient chicks resulted from a greater loss of calcium from these mitochondria during the isolation procedure. We therefore studied the rate of release of $^{45}Ca^{++}$ from mitochondria of vitamin D-deficient chicks (group 1) and chicks given vitamin D_3 for 2 wk (group 4). After isolation, the mitochondria were loaded with 50 nmol of $^{45}Ca^{++}$ in vitro (see Methods). Upon resuspension in a medium that contained EGTA, oligomycin, and atractyloside, the half-time of $^{45}Ca^{++}$ release from the mitochondria of the vitamin D-deficient chicks was almost twice that from the vitamin D-treated animals (Fig. 3). This result suggests that loss of calcium during isolation of skeletal muscle mitochondria is likely to be greater in preparations from vitamin D-treated, not vitamin D-deficient animals.

To determine whether vitamin D therapy increases the rate of active transport of calcium into the mitochondria or the affinity of the transport system for calcium, skeletal muscle mitochondria were isolated from vitamin D-deficient chicks (group 1) and vitamin D-treated chicks (group 4) and then incubated with $^{45}Ca^{++}$ and ATP. No effect of vitamin D therapy on $^{45}Ca^{++}$ uptake was discerned (Fig. 4). Addition of 5 mM P_i to the medium stimulated $^{45}Ca^{++}$ uptake by mitochondria from both vitamin D-deficient and vitamin D-treated chicks by ≅15%. The stimulation of

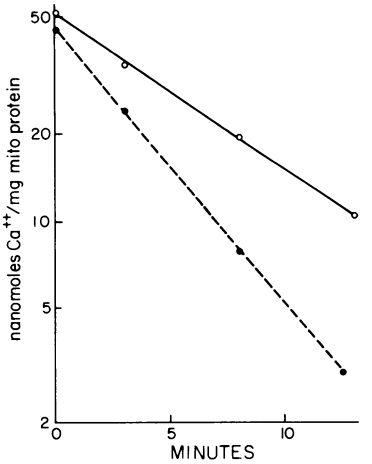

FIGURE 3 Calcium release from skeletal muscle mitochondria (mito). In vitro $^{45}Ca^{++}$ loading was as described in the text. The mitochondria were then suspended in 250 mM sucrose, 2 mM Hepes-Tris (pH 7.4) which contained 1 mM EGTA, 1 μM oligomycin, and 5 μM atractyloside. Portions of the suspension were removed at intervals and cooled, the mitochondria rapidly spun down, and $^{45}Ca^{++}$ in the medium counted in a liquid scintillation spectrometer. The half-time of release of $^{45}Ca^{++}$ from mitochondria of the vitamin D-deficient chicks (group 1) was 5.2 min, whereas that from the vitamin D_3-treated chicks (group 4) was 3.0 min. Values given are the means of triplicate experiments. (○) Vitamin D-deficient chicks, group 1; (●) vitamin D_3-treated chicks, group 4.

mitochondria Ca^{++} uptake by P_i was of a magnitude similar to that reported in rat heart and liver mitochondria (31, 32).

The results shown in Fig. 4 indicate that when in vitro skeletal muscle mitochondrial calcium uptake was driven by ATP, function of the mitochondrial transport system from the vitamin D-deficient chicks was similar to that of mitochondria from vitamin D_3-treated chicks. Further experiments were performed to compare respiratory substrate-driven $^{45}Ca^{++}$ uptakes of mitochondria from group 1-noD and group 4-D 2 wk chicks. Mitochondria were incubated with 5 μM atractyloside (21), 1 μM oligomycin (17), and 2 mM ATP. Sufficient $^{45}Ca^{++}$ was added to bring ionized Ca^{++} to 25 μM. $^{45}Ca^{++}$ uptake at 30 s in the absence of added respiratory substrate was <2 nmol/mg mitochondrial protein for both group 1-noD and group 4-D 2 wk mitochondria; this indicated that the ATP, added to the medium to buffer the ionized calcium concentration, was not available for utilization by the mitochondria. In parallel experiments in which 2 mM succinate was added to the medium, 30-s $^{45}Ca^{++}$ uptakes by group 1-noD mitochondria were 48 and 59 nmol/mg mitochondrial protein on two occasions, whereas uptakes by group 4-D 2 wk mitochondria were 45 and 56 nmol/mg mitochondrial protein. Hence,

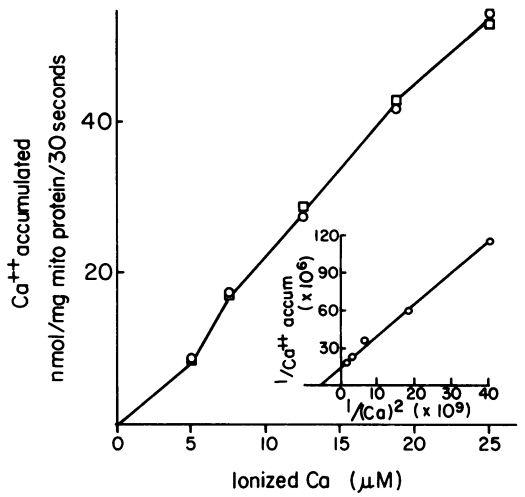

FIGURE 4 $^{45}Ca^{++}$ accumulation by skeletal muscle mitochondria (mito) in vitro. Leg muscle mitochondria from vitamin D-deficient chicks (group 1-noD) and vitamin D_3-treated chicks (group 4-D 2 wk) were incubated in 250 mM sucrose, 2 mM Hepes-Tris (pH 7.4) with 2 mM ATP for 15 min at 30°C. Varying concentrations of $^{45}Ca^{++}$ were then added (29) and incubation was continued for 30 s. The extent of $^{45}Ca^{++}$ accumulation by the mitochondria was determined by the EGTA quench method of Reed and Bygrave (22). Values given are the means of triplicate experiments. (○) Results in vitamin D-deficient chicks (group 1-noD); (□) results in vitamin D_3-treated chicks (group 4-D 2 wk). In the inset, ($^{45}Ca^{++}$ accumulated [accum])$^{-1}$ in (nanomoles $^{45}Ca^{++}$ per milligram mitochondrial protein per 30 s)$^{-1}$ (average of results from groups 1 and 4) is plotted on the y axis, and (ionized Ca^{++} concentration)$^{-2}$ in (moles)$^{-2}$ is plotted on the x axis (30). The x intercept corresponds to an apparent K_m of the skeletal muscle mitochondrial transport system for Ca^{++} of 14 μM. In other experiments (not shown), $^{45}Ca^{++}$ uptake was measured 15, 60, and 120 s after the addition of $^{45}Ca^{++}$ to the medium. Results with skeletal muscle mitochondria from vitamin D-deficient (group 1-noD) and vitamin D_3-treated (group 4-D 2 wk) chicks were not significantly different. Results of experiments in which $^{45}Ca^{++}$ uptake was energized by succinate rather than ATP are described in the text.

$^{45}Ca^{++}$ uptake driven by respiratory substrate closely resembled that energized by ATP, and no differences were noted between the group 1-noD and group 4-D 2 wk mitochondria in respiratory substrate-driven $^{45}Ca^{++}$ uptake.

The ATP content of freshly isolated leg muscle mitochondria from group 1-noD and group 4-D 2 wk chicks was assayed. For this purpose, the mitochondrial isolation procedure was modified by addition of 1 μM oligomycin and 5 μM atractyloside to all solutions. In two experiments, ATP content of group 1-noD mitochondria was 8.9 and 7.1 nmol/mg mitochondrial protein, whereas that in group 4-D 2 wk was 7.3 and 8.3 nmol/mg mitochondrial protein. These values resemble those reported by Kimura and Rasmussen (30) for rat hepatic mitochondria.

DISCUSSION

Vitamin D-deficient chicks (group 1) were weak; tensions generated during tetanic stimulation of a motor nerve were less than one-half that of chicks fed a normal starter-mash diet (group 10). Chicks given vitamin D_3 therapy for 2 or 3 wk (groups 4 and 5) attained strengths equal to the normal starter-mash controls. Therefore, weakness in the chicks maintained on the vitamin D-deficient diet was a result of vitamin D deficiency, not lack of some other essential dietary factor.

The vitamin D-deficient chicks were hypocalcemic, and prevention of the hypocalcemia by addition of supplemental calcium chloride to the diet (group 2-noD hiCa) improved strength. Despite the calcium therapy, however, these chicks remained significantly weaker than starter-mash-fed or vitamin D_3-treated animals. Hence hypocalcemia may have contributed to the weakness of the vitamin D-deficient chicks, but it is clearly not the only factor responsible. Weakness in the vitamin D-deficient chicks was not a consequence of phosphate depletion (33); the chicks were fed a diet containing slightly more phosphorus than starter mash, were not hypophosphatemic, and muscle phosphate (Table II) was not significantly different from that of 3-wk vitamin D_3-treated chicks or starter-mash controls. Although in previous studies vitamin D-deficient rats were found to have lower skeletal muscle ATP concentrations than vitamin D-treated controls (24, 25), comparison of ATP contents of group 1-noD and group 4-D 2 wk chick skeletal muscles by the freeze-clamp technique (11) showed no significant differences in our study. Vitamin D-deficient animals are acidotic (34) and have elevations in plasma parathyroid hormone (35). However, the role of these factors in the pathogenesis of weakness in the chicks is not clear.

Histological studies of the muscles of the vitamin D-deficient chicks indicated that weakness was not a result of denervation or muscle fiber necrosis. In a previous study, the muscle fibers of rats kept vitamin D deficient for 18 wk were smaller than those of normal controls (36). In our study, muscle fibers of group 1-noD and group 4-D 2 wk were similar in diameter. Also, when tensions generated per gram wet weight of muscle of group 1-noD and group 4-D 2 wk chicks were compared, the vitamin D-treated animals were stronger per unit of muscle mass.

Electrophysiological studies of the response of the muscles to repetitive nerve stimulation gave no evidence for an abnormality in neuromuscular junction transmission. Also, tensions produced by direct electrical stimulation of triceps surae were not significantly different from those generated by excitation of posterior tibial nerve. These observations indicate that weakness was not a result of motor nerve or neuro-

muscular junction dysfunction. Because alterations in muscle size and muscle excitation were not the cause of weakness in the vitamin D-deficient chicks, weakness must have been a result of a defect in excitation-contraction coupling.

A number of studies have indicated abnormalities in SR in skeletal muscles of vitamin D-deficient animals. Relaxation of tension after a twitch or tetanus was slowed in vitamin D-deficient rats (6); this also proved to be true of the vitamin D-deficient chicks. Because the rate of relaxation of skeletal muscle is a function of the rate at which SR clears Ca^{++} from muscle fiber cytosol (37), the slowed relaxation in vitamin D-deficient rats and chicks suggests impaired SR Ca^{++} uptake. Uptake of $^{45}Ca^{++}$ by SR prepared from the skeletal muscles of vitamin D-deficient rabbits was below that of vitamin D-treated controls (5); this also proved true of SR from leg muscles of the chicks. Treatment with vitamin D_3 for 2 wk resulted in a rate of skeletal muscle relaxation similar to that in starter-mash controls, and an increase in SR $^{45}Ca^{++}$ accumulation to a level intermediate between vitamin D-deficient chicks and starter-mash controls.

Although we found no abnormalities in electron micrographs of pectoralis and triceps surae of vitamin D-deficient chicks, Sjostrom et al. (38) noted a diminution in the percent area occupied by SR and an increase in the percent area occupied by mitochondria in a quantitative analysis of electron micrographs of skeletal muscle of young vitamin D-deficient rats. Similar alterations were noted in rats fed a calcium-deficient diet that contained vitamin D. The abnormalities in the vitamin D-deficient rats were reversed by vitamin D_3 therapy.

Uremic humans are often weak (39, 40), and, on occasion, weakness is reduced by therapy with 1,2diOHD_3 (39). It is likely that the 1,25diOHD_3-responsive uremics were deficient in this vitamin D metabolite because of a diminution in the capacity of their kidneys to carry out one hydroxylation of 25-hydroxyvitamin D_3 (2). Studies of SR function in such patients have not been reported, but skeletal muscle SR of uremic rabbits has a diminished ability to accumulate $^{45}Ca^{++}$, and this defect is corrected by treatment of the rabbits with 1,25diOHD_3 (41).

Because force of muscle contraction is proportional to the concentration of ionized Ca^{++} in cytosol (42), weakness of the vitamin D-deficient animals could be the result of a diminution in the stores of intravesicular Ca^{++} available for release from SR to cytosol during excitation-contraction coupling (43). This possibility cannot be evaluated by direct analysis of the Ca^{++} content of SR isolated from the skeletal muscles of vitamin D-deficient and vitamin D-treated animals because of the lack of available methods for prevention of calcium flux out of and into SR vesicles during the isolation procedure. Electron probe methods for measurement of calcium content of SR and other subcellular organelles *in situ* are available (44–46), but it remains to be established that such methods are adequate for prevention of Ca^{++} flux into or out of SR during tissue sectioning and processing.

A transitory increase in intestinal mucosal cell mitochondrial calcium content has been noted to occur soon after 1,25diOHD_3 treatment of vitamin D-deficient chicks. This probably is a result of enhanced entry of calcium from intestinal lumen into the intestinal cell, with elevation of intestinal cell cytosolic ionized Ca^{++} concentration (47). If weakness in vitamin D-deficient chicks is a result of reduced cytosolic Ca^{++}, and if a vitamin D metabolite enhances entry of calcium into skeletal muscle fibers as into intestinal cells, then vitamin D therapy might increase skeletal muscle mitochondrial calcium content. Isolation and assay for calcium of skeletal muscle mitochondria might, then, serve as a means for evaluation of the effects of vitamin D deficiency and vitamin D therapy on skeletal muscle intracellular calcium content.

Mitochondrial calcium content of vitamin D-deficient skeletal muscle mitochondria was below that of starter-mash control or vitamin D-treated chicks. Mitochondrial calcium content was not significantly increased in vitamin D-deficient chicks that were given sufficient dietary calcium to become hypercalcemic, but increased progressively with duration of vitamin D_3 therapy. Thus, vitamin D_3 therapy has an effect on chick skeletal muscle calcium metabolism not mediated by changes in plasma calcium concentration.

Early studies suggested an in vitro effect of vitamin D_3 on calcium uptake and release by hepatic mitochondria of vitamin D-deficient animals (48). These studies have not been repeated with physiological concentrations of 1,25diOHD_3 or other vitamin D_3 metabolites, and it is unclear whether these agents have any direct effect on mitochondrial calcium transport or permeability. In our study, 2 wk of prior in vivo vitamin D_3 therapy did not alter the affinity of the mitochondrial transport system for calcium (Fig. 4). As previously reported in hepatic and renal mitochondria (49, 50), prior in vivo vitamin D_3 therapy did cause accelerated in vitro release of $^{45}Ca^{++}$ from mitochondria incubated with inhibitors of ATP utilization and ATP transport (Fig. 3). The faster release of calcium from the mitochondria of the vitamin D_3-treated animals may reflect the greater level of calcium in these mitochondria at the beginning of the in vitro study (Fig. 2), resulting in the loading of $^{45}Ca^{++}$ on lower affinity sites during the in vitro incubation (51), or causing greater calcium-induced changes in cation permeability in the vitamin D_3-treated chicks (52).

Although the more rapid release of $^{45}Ca^{++}$ from the mitochondria from skeletal muscles of vitamin D-treated chicks resembled the acceleration in calcium release from hepatic mitochondria of dexamethasone-treated rats (30), several differences were noted. Whereas the uptake of calcium by mitochondria from vitamin D-treated chicks was virtually identical to that of the vitamin D-deficient ones, both with ATP and with succinate as energy sources, the maximum velocity of succinate-supported calcium accumulation by hepatic mitochondria from dexamethasone-treated rats was considerably reduced. Also, whereas the rapid release of $^{45}Ca^{++}$ from the mitochondria of the dexamethasone-treated rats was correlated with intramitochondrial ATP depletion, mitochondrial ATP contents of skeletal muscle mitochondria from vitamin D-deficient and 2-wk vitamin D_3-treated chicks were nearly identical.

Our study provides objective evidence of weakness resulting from vitamin D deficiency. Abnormalities in muscle relaxation and in SR calcium transport were noted in the vitamin D-deficient chicks that were similar to those previously reported in other species. Of particular interest is the positive correlation between strength and mitochondrial calcium content. This does not necessarily imply a direct role of mitochondria in excitation-contraction coupling. More likely, both the skeletal muscle mitochondrial calcium accumulation and the increased force of contraction of triceps surae are results of greater availability of calcium within the muscle fiber with vitamin D therapy. If so, then vitamin D_3 treatment of vitamin D-deficient chicks causes increased net influx of calcium across sarcolemma, an effect resembling that in intestine (47, 53, 54). Further studies will be needed to determine whether the vitamin D_3 metabolite predominantly affecting skeletal muscle is 25-hydroxyvitamin D_3, as suggested by Birge and Haddad (25), 1,25diOHD_3, or some other hydroxylated form of the vitamin.

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