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STUDIES OF STEROID MYOPATHY

EXAMINATION OF THE POSSIBLE EFFECT OF TRIAMCINOLONE ON MITOCHONDRIA AND SARCOTUBULAR VESICLES OF RAT SKELETAL MUSCLE*

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Abstract—Weight loss, muscle atrophy and weakness induced by triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnanediene-3,20-dione) could be due to uncoupling of oxidative phosphorylation. To test this possibility, reliable techniques were developed for isolation of skeletal muscle mitochondria. Mitochondria with apparently normal biochemical properties were isolated from skeletal muscle of rats with severe weight loss induced by triamcinolone. Sarcotubular vesicles (fragmented sarcoplasmic reticulum) isolated from the same animals showed no evidence of steroid-induced abnormalities. These data suggest that mitochondria and sarcotubular vesicles are not the site or a steroid-induced defect in muscle biochemistry. The fundamental abnormality of steroid myopathy should be sought in other aspects of muscle biochemistry.

TRIAMCINOLONE (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnandiene-3,20-dione) is reported to uncouple oxidative phosphorylation in rat liver mitochondria *in vitro*,[1, 2] and a recent report[3] indicates that liver mitochondria are uncoupled when isolated from rats treated *in vivo* with cortisone acetate, hydrocortisone acetate and prednisolone. A serious side effect of steroid therapy in humans is muscle weakness and degeneration,[4–6] and it is often suggested that these changes may be a manifestation of steroid-induced changes in skeletal muscle mitochondria. For this reason we have studied the effects both *in vivo* and *in vitro* of triamcinolone on rat skeletal muscle mitochondria.

The sarcoplasmic reticulum regulates the level of ionized calcium in the sarcoplasm and thus is an important component of the system in muscle which governs excitation, contraction and relaxation. The calcium accumulation capacity,[7, 8] calcium-stimulated adenosine triphosphatase (ATPase)[8] and calcium affinity[8] of rat muscle sarcotubular vesicles (fragmented sarcoplasmic reticulum) were studied in steroid-treated rats, because abnormalities of this system might be expected to result in muscle weakness.

METHODS

All chemicals and reagents used were of the highest purity commercially available. The ATP† and sucrose solutions used in the vesicle experiments were treated with

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† Abbreviations used in this paper: ADP (adenosine-5′-diphosphate), ATP (adenosine-5′-triphosphate), EDTA (ethylenediaminetetraacetate), P_i (inorganic orthophosphate), BSA (bovine serum albumin), TES [*N*-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid], TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine), Q_{O_2} (μl oxygen consumed/mg mitochondrial protein/hr), S.E.M. (standard error of mean), AR (acceptor ratio), RCR (respiratory control ratio).

Dowex-50 (H^+) and Dowex-50 (Na^+), respectively, to remove any contaminating calcium or other divalent ions. Triamcinolone was kindly supplied by Dr. I. Ringler of Lederle Pharmaceutical.

Female rats of the Sherman strain weighing from 225 to 280 g were caged in groups of three or four and received Purina Lab Chow and water *ad lib*. The animals were injected on 10 consecutive days with 1·0 cc of triamcinolone (5 mg/ml vehicle) per kg of body weight per day. The vehicle contained 0·5 g carboxymethylcellulose, 0·4 ml Tween 80 and 0·9 g NaCl/100 ml water.[9] Control animals received 1·0 cc of the vehicle per kg of body weight. All injections were subcutaneous in the interscapular region and all animals were killed by a blow to the head 24 hr after the tenth injection.

An intact gastrocnemius muscle from each animal was meticulously dissected, blotted dry and weighed. Hind limb musculature was rapidly removed and placed in an ice-cold medium[10] at pH 7·4 which contained: 50 mM TES, 100 mM KCl, 50 mM P_i, 5 mM $MgSO_4$, 1 mM ATP, 1 mM EDTA and 5 mg BSA (Pentex) per ml. This medium is referred to as "low speed medium". "High speed medium" is identical to it, except that ATP and BSA are omitted. The excised muscle from the hind limbs was carefully trimmed of connective tissue and fat and weighed in a tared beaker containing cold, low speed medium. The muscle was then minced finely with scissors and homogenized with 1·0 mm diameter glass beads in a CO_2-cooled mechanical shaker (Braun, model MSK, Bronwill Scientific, Box 277, Rochester, N.Y., 14601). Mitochondria were obtained by differential centrifugation of the homogenate as previously reported.[10] Mitochondria isolated between 270 and 3500 *g* were washed twice in high speed medium and assayed immediately. Protein was determined by the biuret method[11] on the mitochondrial suspension, which was then adjusted to 10 mg/ml. To ensure complete solubilization of mitochondrial protein, sodium deoxycholate (10 mg/3 ml) was added to the digestion mixture in the biuret method.

Respiration of the mitochondria (Q_{O_2}) was measured with a vibrating platinum electrode polarized at −0·6 V in a GME Oxygraph (Gilson Medical Electronics, Middleton, Wis.) at 26°. The chamber contained 2 ml of a suspension of about 1 mg of mitochondrial protein in 25 mM TES, 30 mM P_i, 8 mM $MgSO_4$, 50 mM KCl, and 0·5 mM EDTA at pH 7·4. The substrates used were as follows: 15 mM pyruvate plus 15 mM malate; 45 mM succinate (+ 5 μM rotenone); and 1·5 mM ascorbate plus 250 μM TMPD. ADP (in 10-μl portions) was added to the chamber to give concentration increments of 150 μM.

Q_{O_2}, respiratory control ratios (RCR) and acceptor ratios (AR) were calculated from the tracings in the conventional manner. The ADP/O ratio was calculated from the oxygen consumed during the phosphorylation of 0·3 μmole ADP. The RCR is the ratio of state 3 respiration rate (ADP present) to the state 4 respiration rate (after the supply of added ADP has been exhausted). The AR refers to the ratio of Q_{O_2} in the presence of added ADP to Q_{O_2} before the first addition of ADP.

Sarcotubular vesicles were sedimented at 40,000 *g* for 90 min from the supernatant remaining after centrifugation of the muscle homogenate at 15,000 *g* for 20 min. The pellet of vesicles was rinsed with sucrose (10 g/100 ml) and suspended in 40 g sucrose/100 ml. Protein was estimated by the method of Lowry *et al.*[12] After storage for about 12–20 hr at 2–4°, the vesicle fraction was adjusted to 0·8 mg protein per ml just prior to use.

The standard calcium accumulation assay was run in a volume of 10 ml with 80 μg

of vesicle protein per ml at 26°, using the method of Martonosi and Feretos.[7] The reaction medium at pH 7·2 contained 5 mM Na oxalate, 5 mM $MgCl_2$, 5 mM ATP, 100 mM NaCl, 5 mM KCl, 40 mM histidine, and 0·1 mM $CaCl_2$ labeled with ^{45}Ca (i.e. 1·25 μmoles Ca^{2+}/mg of protein). The reaction was initiated by the addition of vesicles and aliquots were removed at 1, 2, 5, 10 and 15 min for separation through a Millipore vacuum filter of 0·45 μ pore size. This filtration effectively removed all vesicle protein. An aliquot of the filtrate, which contained no detectable ATPase activity, was then taken for liquid scintillation counting, using 1,4-dioxane containing naphthalene and 2,5-diphenyloxazole.[8] The amount of calcium removed from the medium by the vesicles was obtained by subtraction of the sample counts from the radioactivity contained in a filtered blank to which no vesicles had been added. ATPase activity was estimated by assay of P_i in the filtrate by the method of Rockstein and Herron.[13] Calcium uptake by the vesicles was also measured at 10° with identical medium, but with 40 μg of vesicle protein per ml (i.e. 2·5 μmoles Ca^{2+}/mg of protein).

The ability of the vesicles to lower the calcium concentration of dilute solutions of calcium is referred to as the calcium affinity of the vesicles. It was measured at 26° by incubating vesicles (80 μg protein/ml; 0·25 μmole Ca^{2+}/mg of protein) in the standard medium containing 20 μM calcium labeled with ^{45}Ca.

RESULTS

Body weight, muscle weight and yields of subcellular fractions. A dramatic loss of weight was seen in the triamcinolone-treated animals with an initial weight loss of 10–12 g in the 24 hr after the first injection and a continuous weight loss of about 7–8 g per day over the treatment period of 10 days (Fig. 1). The weight loss of the treated animals ranged from 23 to 31 per cent (mean 28 per cent) of the initial starting weight compared to a mean increase of 1·2 per cent in body weight of the control animals (Table 1).

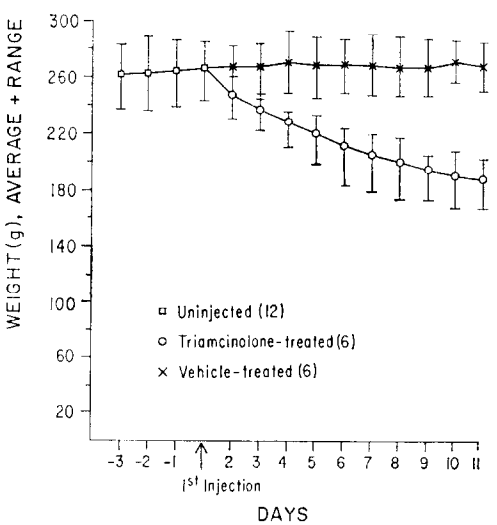

FIG. 1. Weight changes induced by triamcinolone. Six control animals received 1·0 cc vehicle (see Methods) per kg per day subcutaneously for 10 days. Six animals were injected daily with 5·0 mg triamcinolone (in same vehicle) per kg per day. Mean ± range.

TABLE 1. CHANGES INDUCED BY TRIAMCINOLONE TREATMENT*

Rats	Body wt. (g)		
	Initial	After treatment	% Change in body wt.
Treated	264 ± 6	189 ± 5	−28·3 ± 1·3
Control	265 ± 6	268 ± 6	+ 1·2 ± 1·1
	Gastrocnemius muscle		
	Muscle wt. (g)		% Final body wt.
Treated	1·11 ± 0·07		0·58 ± 0·03
Control	1·49 ± 0·02		0·56 ± 0·01
	Yields of subcellular fractions (mg protein/g wet wt. muscle)		
	Mitochondria (270–3500 *g*)		Vesicles (15,000–40,000 *g*)
Treated	1·76 ± 0·26		1·38 ± 0·19
Control	2·01 ± 0·50		1·54 ± 0·31

* Treated animals (six female Sherman rats) were injected subcutaneously with 5 mg triamcinolone (in vehicle described in Methods)/kg of body weight per day for 10 days. Controls (5) were injected subcutaneously with 1·0 ml of vehicle/kg body weight per day for 10 days. Values are means ± S.E.M

TABLE 2. OXIDATIVE PHOSPHORYLATION OF SKELETAL MUSCLE MITOCHONDRIA FROM TRIAMCINOLONE-TREATED RATS*

Group	Substrate(s)	Q_{O_2}	AR	RCR	ADP/O
Control (5)	Pyruvate + malate	128 ± 13	7·2 ± 1·1	3·3 ± 0·2	2·0 ± 0·4
Treated (6)	Pyruvate + malate	109 ± 11	6·5 ± 0·9	4·4 ± 0·5	2·6 ± 0·3
Control (5)	Succinate (+ rotenone)	162 ± 10	3·7 ± 0·3	3·4 ± 0·4	1·5 ± 0·2
Treated (6)	Succinate (+ rotenone)	148 ± 15	3·9 ± 0·3	2·6 ± 0·1	1·5 ± 0·1
Control (5)	Ascorbate + TMPD	126 ± 22	1·5 ± 0·5		
Treated (6)	Ascorbate + TMPD	138 ± 13	1·5 ± 0·1		

* The number of animals in each group is shown in parentheses. Values are given as means ± S.E.M. Q_{O_2} = μl O_2/mg protein/hr. See text for abbreviations.

The weights of the gastrocnemius muscles of the treated animals were always less than those of control animals. The muscle weight loss was proportional to body weight loss, however, so that the ratio of gastrocnemius muscle weight to body weight was unaltered (Table 1).

Table 1 also shows that the yields of mitochondria and vesicles were not significantly different in control and treated animals.

Mitochondria

Representative studies of mitochondrial respiration are summarized in Table 2. There are no significant differences in these parameters between control and treated animals as determined by the Student's *t*-test, using a two-tailed test at the 5 per cent confidence level.[14] Mitochondria isolated between 3500–7000 *g* and 7000–15,000 *g* from the same animals also showed no significant differences in yield, Q_{O_2}, AR, RCR or ADP/O ratios.

The effect of triamcinolone *in vitro* on mitochondria is shown in Fig. 2. This is a

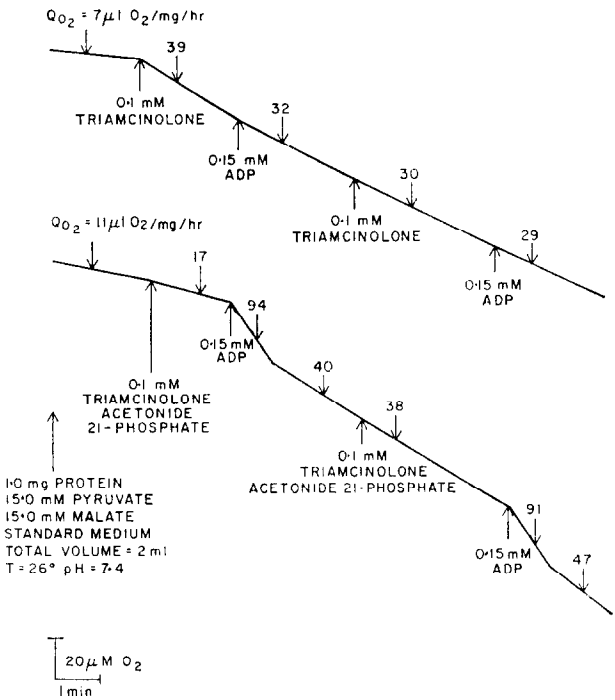

FIG. 2. Uncoupling of oxidative phosphorylation by triamcinolone. Medium is described in Methods. Note failure of the acetonide-21-phosphate derivative of triamcinolone to uncouple. Oligomycin does not inhibit uncoupling by triamcinolone (not shown).

typical oxygraph tracing, illustrating the uncoupling effect of triamcinolone on mitochondrial oxidative phosphorylation. This uncoupling is not prevented by prior incubation with BSA (5 mg/ml) and is not reversed by the addition of BSA subsequent to triamcinolone addition. Triamcinolone (100 μM, but not 10 μM) stimulated oxygen consumption by the mitochondria, and addition of ADP (150 μM) caused only slight further stimulation of respiration. A water-soluble derivative of triamcinolone (triamcinolone acetonide-21-phosphate) had no effect on oxidative phosphorylation of skeletal muscle mitochondria, even in concentrations of 100 μM.

Sarcotubular vesicles

On incubation at 26° with 1·25 μmoles Ca^{2+} per mg of protein, essentially all the calcium was taken up by the vesicles in 2 min. Therefore, in order to measure the total capacity for calcium accumulation and the rate of calcium uptake, we increased the Ca:protein ratio (2·5 μmoles/mg) and lowered the temperature to 10°. Differences in rate of uptake should be more readily detected at lower temperatures because of the greater ease of measurement. In addition, the likelihood of diffusion-limited rates is less at lower temperatures. This is pertinent because calculations[15] show that the rates of Ca^{2+} accumulation attained in this type of assay at 26° cannot account for known rates of muscle relaxation *in vivo*, and it has been suggested that the relatively slow rate of calcium uptake *in vitro* is due to aggregation of the vesicles, leading to hindrance of diffusion to the center of the aggregates. Since the temperature coefficient

of an activated transport mechanism might be expected to be much higher than that of a diffusion process, the rate of uptake at low temperatures might be limited by the active transport step. However, as shown in Fig. 3, these considerations did not influence the conclusions as the assay at 10° with 2·5 μmoles Ca^{2+}/mg shows no significant difference in rate or amount of calcium uptake by vesicles from treated or control animals. Small but not significant variations in ATPase activity are also illustrated.

Since no differences in the calcium uptake by the vesicles were demonstrated with triamcinolone treatment *in vivo*, vesicles from untreated Sherman rats were prepared by the standard procedure and the effect of the triamcinolone on these preparations *in*

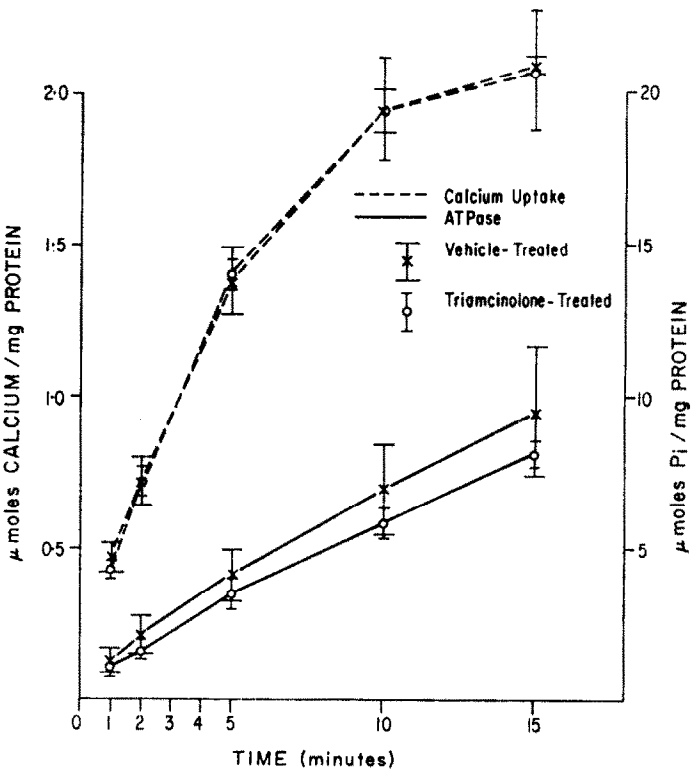

FIG. 3. Calcium accumulation and ATPase activity by sarcotubular vesicles from triamcinolone-treated and vehicle-treated rats. Medium is described in Methods. 100 μM Ca^{2+}, 40 μg vesicle protein/ml, 10°, 2·5 μmoles Ca^{2+}/mg protein. Mean ± S.E.M.

vitro was tested. Calcium uptake and ATPase activity were not significantly changed by the addition of triamcinolone, showing that the steroid has no significant effect *in vitro* on calcium uptake by vesicles or calcium-dependent vesicle ATPase at a concentration of 100 μM (Fig. 4).

Figure 5 shows that vesicles from triamcinolone-treated rats show no defect in their capacity to lower the calcium concentration of dilute solutions (calcium affinity). This property of the vesicles might be expected to be a more sensitive index of adequate functioning of the sarcoplasmic reticulum, as it is the reduction of sarcoplasmic

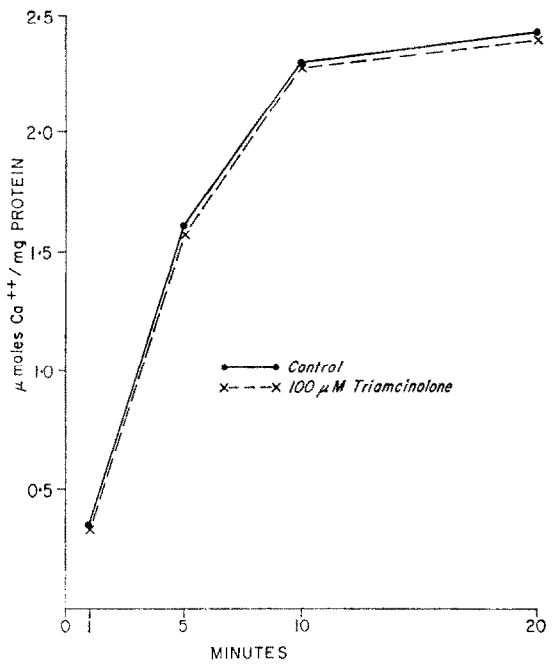

FIG. 4. Failure of triamcinolone to affect calcium accumulation by sarcotubular vesicles. Medium is described in Methods. 100 μM Ca^{2+}, 40 μg vesicle protein/ml, 10°, 2·5 μmoles Ca^{2+}/mg protein. This single experiment is representative of 3 others.

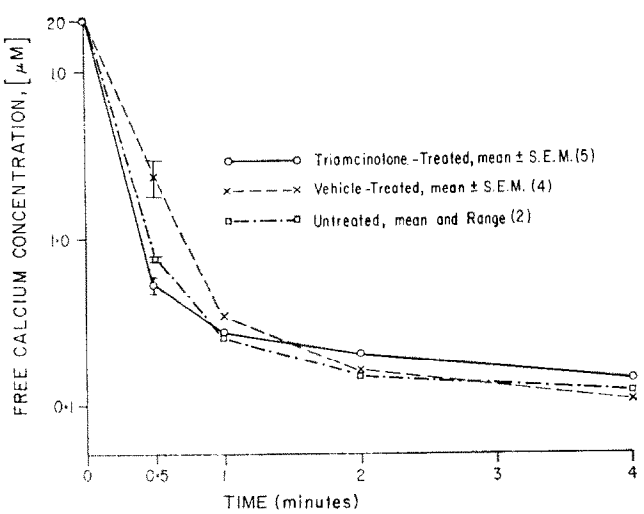

FIG. 5. Calcium affinity of sarcotubular vesicles from triamcinolone-treated and control rats. Medium is described in Methods. 20 μM Ca^{2+}, 80 μg vesicle protein/ml, 26°, 0·25 μmole Ca^{2+}/mg protein.

calcium concentration to less than 1 μM which induces contraction *in vivo*. The significance of the difference at 0·5 min between the vehicle-injected control animals and those injected with triamcinolone in vehicle or with saline alone is not clear, especially since this difference in rate is not apparent in experiments conducted at 10° with 100 μM calcium.

DISCUSSION

The steroid treatment schedule reported herein was patterned after the work of Tonelli *et al.*,[9] who demonstrated striking weight loss in Sherman rats treated with triamcinolone. They reported that 53–65 per cent of such weight loss is due to decreased food intake and the remainder is due to other effects of triamcinolone. In other investigations,[16–22] triamcinolone has been shown to cause severe weight loss, muscle wasting and weakness in man and in various animals.

Induction of weight loss with triamcinolone may be critically dependent on the mode or frequency of its administration. For example, only a relatively small weight loss was found in the same strain of rats when the same dose of triamcinolone dissolved in propylene glycol was administered once daily. The original vehicle employed in this study and developed in the Lederle Laboratories may slow either the absorption or the catabolism of the steroid with consequent induction of weight loss. This observation may be highly pertinent to the clinical use of triamcinolone, if the desired therapeutic effects of this steroid could be as effectively obtained with a single daily dose or by less frequent doses as with divided daily doses, which might accentuate the adverse effects such as weight loss and muscle atrophy.

With reference to triamcinolone and other steroids, it is pertinent to point out that animal experiments show that weight-depressing effects of glucocorticoids parallel thymolytic capacity.[9] There is no substantial evidence to support the commonly accepted notion that the tendency of triamcinolone to cause weight loss or muscle atrophy is in excess of its thymolytic and anti-inflammatory properties as compared with other glucocorticoids.

A possible mechanism for triamcinolone-induced weight loss, muscle wasting and weakness has been suggested by work *in vitro* with rat liver mitochondria where triamcinolone has a dinitrophenol-like uncoupling effect.[1, 2, 23] This same effect has been demonstrated in our work on rat skeletal muscle mitochondria *in vitro*. However, a recent report[23] has shown that optically pure triamcinolone has no effect on rat liver mitochondria, whereas most commercially available triamcinolone (shown to be 95–98% optically pure) exhibits such uncoupling effects as have been reported. These results implicate a potent contaminant as the source of the uncoupling effects *in vitro* on the drug. The absorbance at 239 mμ of our sample of triamcinolone indicated 95 per cent purity, using a molar extinction coefficient of 15,800. On this basis, the effects we found on rat skeletal muscle mitochondria *in vitro* may also be the result of a contaminant rather than of the triamcinolone itself. The failure of triamcinolone acetonide-21-phosphate to affect oxidative phosphorylation *in vitro* is also compatible with the absence of the contaminant presumed to be present in the triamcinolone preparation. However, other differences between triamcinolone acetonide-21-phosphate and triamcinolone itself might also account for the failure of the former to affect oxidative phosphorylation.

Previous studies[24, 25] have shown that addition of albumin to mitochondria exposed

in vivo or *in vitro* to certain uncoupling agents can restore oxidative phosphorylation to normal. It is unlikely, however, that the albumin present in the medium for isolating skeletal muscle mitochondria masked an uncoupling effect *in vivo* of triamcinolone (or an impurity thereof) because albumin does not prevent or reverse the uncoupling effect of triamcinolone *in vitro*. In addition, we have isolated and assayed skeletal muscle mitochondria from triamcinolone-treated rats in the absence of added albumin and found no difference from control animals, although in both groups the ADP/O ratios are sometimes slightly lower than with skeletal muscle mitochondria isolated in the presence of albumin but assayed in its absence.

In preliminary experiments, we have noted decreased Q_{O_2} with both pyruvate–malate and succinate (+ rotenone) in liver mitochondria of triamcinolone-treated rats. Kimberg *et al.*[3] recently reported decreased Q_{O_2} and P/O ratios of liver mitochondria from rats treated with cortisone acetate. Their studies also revealed increased size and decreased number of liver mitochondria. Possibly the liver is more sensitive than muscle to effects of steroids which may be reflected in mitochondrial biochemistry. However, since no changes in mitochondrial metabolism or mitochondrial content were found in our studies of skeletal muscle from treated animals, it is unlikely that the mitochondria are the affected organelles in the triamcinolone-induced muscle wasting.

Previous light and electron microscopic studies gave confusing results as to the type of muscle affected by triamcinolone. Most investigators agree that the morphology of mitochondria is affected by glucocorticoids. Some have found that histologic abnormalities of muscle are most frequent and severe in white, mitochondria-poor fibers[21, 26] but others found severely affected fibers to be the red or mitochondria-rich type.[22] Tice and Engel[17] suggested that triamcinolone has a biphasic effect in which mitochondria first proliferate and then degenerate and decrease in number.

Calcium uptake and ATPase activity of sarcotubular vesicles and the yields of vesicles from triamcinolone-treated rats showed no effect of the drug or any associated contaminant. This together with the lack of effect of triamcinolone added *in vitro* to sarcotubular vesicles strongly suggests that the weakness present in triamcinolone myopathy is not a manifestation of changes in the sarcoplasmic reticulum. This conclusion is considerably strengthened by the apparently normal affinity for calcium of vesicles isolated from triamcinolone-treated animals. Previous studies[8] showed that the calcium affinity assay (which measures the ability of vesicles to lower the calcium concentration of dilute solutions of calcium) is a more sensitive and perhaps more physiologically relevant test of the function of sarcotubular vesicles than is the rate of accumulation or capacity to accumulate large amounts of calcium from concentrated solutions of calcium.

On the basis of experimental results presented here, it is clear that although triamcinolone causes weight loss, muscle wasting and weakness *in vivo*, these effects are not simply manifestations of gross changes in amount or functions of mitochondria and sarcotubular vesicles, as isolated from animals pretreated with large doses of triamcinolone.

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