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Relative Changes in the Function of Muscle Ribosomes and Mitochondria during the Early Phase of Steroid-Induced Catabolism

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1. Five glucocorticoids, when administered daily to rats for 5–7 days at a dosage of 5mg/kg, were in the following order of effectiveness with respect to their ability to decrease the weight gain of whole animals and the vastus lateralis, vastus medialis and gluteus medius muscles: corticosterone<prednisolone<triamcinolone<betamethasone<dexamethasone<triamcinolone acetonide. 2. The low catabolic potencies of corticosterone and prednisolone were reflected in the high doses (20mg/kg) required to decrease significantly the incorporating activity *in vitro* of ribosomes isolated from the muscles 6h later. However, triamcinolone was as effective as triamcinolone acetonide in causing a significant effect at 0.05mg/kg, although it had a far lower catabolic potency than the acetonide. 3. The relationship between decrease in ribosomal activity *in vitro* and weight loss was better understood by considering the duration of the effect after single doses of steroids. Ribosomes returned to normal function between 6 and 12h after the administration of corticosterone (20mg/kg), but still had decreased activity 12h after a similar dose of prednisolone. Normal ribosomal function was renewed between 12 and 18h after the administration of triamcinolone and betamethasone at 5mg/kg, but decreased activity persisted for more than 48h after similar doses of dexamethasone or triamcinolone acetonide. 4. There was no difference in the rate of incorporation of amino acids into nascent protein on polyribosomes from muscle of control animals and animals treated 6h previously with triamcinolone acetonide or dexamethasone. 5. The rate of weight gain of the heart was not affected by any of the steroids tested and heart ribosomes maintained normal activity although concentrations of steroids in this organ 5min after administration were 2–3 times that in skeletal muscle. 6. Mitochondria, isolated from the muscle of animals that had received triamcinolone acetonide (20mg/kg) 12h previously, were shown, by using a Coulter counter, to be enlarged. Nevertheless oxidation was only slightly uncoupled 12h after drug administration and they had normal function 6h later. Similar results with respect to mitochondrial function were obtained after the administration of dexamethasone (20mg/kg) and betamethasone (20mg/kg). 7. From these results it is concluded that the mitochondria are not functionally involved in the early phase of steroid-induced muscle catabolism. The observed decrease in the incorporating ability of muscle ribosomes *in vitro* appears to be much more closely linked to the initial catabolic event.

Ribosomes isolated from the predominantly white hind-limb muscles of adult male rats have a decreased ability to incorporate amino acids into protein *in vitro* when the animals have previously been treated with the powerful catabolic agent triamcinolone acetonide (9α-fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxypregna - 1,4 - diene - 3,20 - dione) (White, 1967; Bullock *et al.*, 1968; Peters *et al.*, 1970; Bullock *et al.*, 1971). Similar results were obtained when rabbits were given cortisone (Bullock *et al.*, 1968). The ribosomal defect develops comparatively rapidly (4–6h) after the administration of the catabolic agent to rats (Peters *et al.*, 1970) and can be fully or partially reversed by treating the animals with steroids that have an anabolic action (White, 1967; Bullock *et al.*, 1968, 1971). From this it would appear likely that the dysfunctional state of the ribosomes reflects events in the cell that are primary determinants of steroid-induced muscle catabolism. However, there are some omissions that need to be considered before this interpretation of results can be accepted as proven. First, studies so far have only involved the use of two catabolic steroids (cortisone and triamcinolone acetonide) and hence no

attempt has been made to relate the catabolic effectiveness of a range of steroids with their effect on changes in ribosomal activity. Secondly, mitochondrial function as distinct from morphology (Bullock *et al.*, 1971) has not been taken into account soon after steroid administration and therefore the question of lack of energy as a cause of catabolism has not been ruled out. To meet these omissions, the function of isolated skeletal-muscle mitochondria, skeletal-muscle ribosomes and heart ribosomes were measured at various times after different doses of cortisone, prednisolone (11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione), triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16α,21-diacetate), betamethasone (9α-fluoro-11β,17β,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) and triamcinolone acetonide, and related to the different degrees of catabolism engendered by these steroids. The results support the hypothesis that changes in the incorporating ability of isolated ribosomes are nearer to the catabolic event than changes in mitochondrial function. Because of this, further work aimed at understanding the nature of the ribosomal defect is described.

Experimental

Materials

Radioactive compounds. L-[U-^{14}C]Leucine (specific radioactivity 311–344mCi/mmol), a mixture of 16 U-^{14}C-labelled amino acids (specific radioactivity 52mCi/mg-atom of C), [1,2,4-^{3}H]corticosterone (specific radioactivity 31.7Ci/mmol), [G-^{3}H]prednisolone (specific radioactivity 0.5Ci/mmol) and standard $^{3}H_2O$ (specific radioactivity 12.5μCi of ^{3}H/g) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [1,2,4-^{3}H]Triamcinolone acetonide (specific radioactivity 9.5Ci/mmol), [1,2,4-^{3}H]triamcinolone (specific radioactivity 5.0Ci/mmol), [1,2,4-^{3}H]betamethasone (specific radioactivity 5.0Ci/mmol) and [1,2,4-^{3}H]dexamethasone (specific radioactivity 5.6Ci/mmol) were obtained from Centre Etude Nuclear, Department des Radioisotopes, Mol-Donk, Belgium.

Chemicals and enzymes. Potassium phosphoenolpyruvate and pyruvate kinase were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

The dipotassium salt of ATP, the disodium salt of ADP, the sodium salt of GTP, NAD, cytochrome *c*, albumin, and tris were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Sodium deoxycholate was 'Mann Assayed' from Mann Research Laboratories Inc., New York, N.Y., U.S.A., and Lubrol W (cetyl alcohol–polyoxyethylene condensate) was purchased from Imperial Chemical Industries Ltd., Manchester, U.K. α-Amylase (twice crystallized, from hog pancreas), freeze-dried trypsin (200 units/mg) and Azocoll were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. One unit of trypsin hydrolyses 1μmol of *p*-toluenesulphonyl-L-arginine methyl ester/min at 25°C and pH8.1 in the presence of 0.01M-$CaCl_2$.

Prednisolone was obtained from CIBA Laboratories, Horsham, corticosterone and hydrocortisone from Steraloids Ltd., Croydon, Surrey, U.K., betamethasone from Glaxo Laboratories Ltd., Greenford, Middx., U.K., dexamethasone from Merck, Sharp Dohme Ltd., Hoddesdon, Herts., U.K., and triamcinolone and triamcinolone acetonide from E. R. Squibb and Sons Ltd., Speke, Liverpool, U.K.

Animals. Rats were white Wistar (250–300g), CFHB strain from Carworth Europe, Alconbury, Huntingdon, U.K., Bilaterally adrenalectomized male rats were the same weight and from the same source. They were maintained on 1% (w/v) NaCl and were used 1 week after the operation.

Methods

Administration of steroids. These were injected intraperitoneally as a fine suspension in 0.5ml of 0.9% (w/v) NaCl. Control animals received saline only.

Media. Medium A consisted of sucrose (0.15M), tris–HCl buffer (0.1M, pH7.4 at 37°C), KCl (0.185M) and $MgCl_2$ (9mM); medium B, sucrose (0.3M), tris–HCl buffer (0.035M, pH7.4 at 37°C), KCl (0.3M) and $MgCl_2$ (9mM); medium C, tris–HCl buffer (0.035M, pH7.4 at 37°C), KCl (0.1M) and $MgCl_2$ (9mM).

Heart ribosomes, skeletal-muscle ribosomes and glycogen-free skeletal-muscle ribosomes. All preparative procedures, including centrifugation, were done at 4°C. The method used was that described by Bullock *et al.* (1971) except that the medium used as the dense layer during the final centrifugation was medium B in which the KCl concentration was 0.3M. Glycogen-free ribosomes (Peters *et al.*, 1970) were prepared by treating the 2000*g* supernatant with amylase for 20min at 0°C. The amount of of ribosomal RNA obtained by this method was about 10% of the total muscle RNA.

Heavy ribosomes. The muscle was initially processed in the same way as for glycogen-free ribosomes. The 2000*g* supernatant was then layered, after centrifugation at 20000*g* for 15min, in batches of 13ml over medium B (5ml) and was centrifuged for 2h at 105000*g*. Pellets were suspended by very gentle agitation in medium C for density-gradient centrifugation or incubation with energy source and cell sap.

After incubation of glycogen-free ribosomes in large-scale experiments, or with muscle cell sap, heavy ribosomes were prepared in the same way.

Cell saps. Liver (10g) from rats that had been starved overnight was homogenized in medium C (25ml) by using a glass homogenizer with a Teflon pestle. After centrifugation of the homogenate at 14000*g* for 6min the mitochondrial supernatant was centrifuged for 2h at 105000*g*. The liver cell sap was drawn off with a hypodermic syringe.

For the preparation of muscle cell sap, the muscle types used for ribosome preparations were pooled from three animals to give about 15g of tissue. This tissue was homogenized in medium C with an Ultra-Turrax apparatus and then treated in the same way as for the preparation of ribosomes except that the post-20000*g* supernatant was not layered over medium B but was centrifuged at 105000*g* for 2h. Again the supernatant cell sap was removed with a hypodermic syringe.

Incubation of ribosomes. Ribosomes were always incubated at 37°C in duplicate. Each incubation contained, in a total volume of 0.5ml, 0.1ml of ribosome suspension (10–20μg of RNA), $MgCl_2$ (5.5μmol), KCl (60μmol), pyruvate kinase (7.5μg), potassium phosphoenolpyruvate (1μmol), ATP (0.5μmol), GTP (0.1μmol), tris–HCl buffer, pH7.4 at 37°C (23μmol), L-[U-^{14}C]leucine (1.5nmol, 0.5μCi) and liver cell sap (0.3ml) after tenfold dilution with medium C.

In the large-scale incubation mixture the concentration of all ingredients remained the same but the total volume was increased to 50ml.

For stability determinations ribosomes (150–250μg of ribosomal RNA/ml) were incubated with muscle cell saps (3ml) for 10min at 37°C.

Measurement of radioactivity. After the incubation of ribosomes the extent of amino acid incorporation was measured as described by Bullock *et al.* (1968). From the gradient (Fig. 6, Table 4) protein was precipitated by the addition of 10% (w/v) trichloroacetic acid to 2.0ml samples of each fraction. It was washed and counted for radioactivity in the same way as protein precipitated after the normal ribosomal incubation.

Density-gradient sedimentation of ribosomes. Sucrose density gradients (15–47% w/v; 20ml) were prepared from 12.5ml volumes of medium B containing 5% (w/v) and 47% (w/v) sucrose by using the mixing chamber described by Britten & Roberts (1960). A suspension of heavy ribosomes (1.0–1.5ml containing 150–250μg of ribosomal RNA/ml) was layered on the gradient and centrifuged at 103000*g* for 90min in the 3 × 23ml swing-out rotor in an MSE Superspeed 50 centrifuge maintained at 4°C. The rotor was allowed to come to rest at the end of the run without the use of the brake.

Analysis of the gradient was as described by Bullock *et al.* (1968), 60% (w/v) sucrose being used as the pumping medium.

Nitric acid digestion. This was based on the method of Pfeffer *et al.* (1971).

Minced and pooled skeletal muscle (1g) or chopped cardiac muscle (0.5g), after careful removal of traces of blood by washing and blotting, was digested in conc. HNO_3 (1ml; sp.gr. 1.42) by heating in a water bath at 90°C until digestion was complete (20min). After cooling and addition of 0.75M-tris base (10ml), 0.5ml of the mixture was counted for radioactivity in scintillation fluid (Bray, 1960) (10ml) containing Cab-O-Sil (5%).

Isolation of mitochondria. This was done by the enzymic procedure of Bullock *et al.* (1970). An additional precaution, however, was the routine standardization of trypsin through the use of Azocoll. The trypsin at one-tenth of the concentration used for the partial digestion of muscle should allow the release of dye from the protein such that after incubation at 37°C for 15min in 0.1M-potassium phosphate buffer (pH7), the filtered suspension has E^{580}_{1cm} of 0.6.

Measurement of mitochondrial diameter with an electronic particle counter. The Coulter counter (model A) was used with a tube of 50μm aperture. All solutions were passed through Millipore filters (0.3μm pore size) and all glassware was rinsed in filtered solutions before use. The aperture tube was calibrated with puffball spores of 3.28μm diameter and a range of instrument settings was chosen to count material within the diameter range 1.09–6.42μm.

Pellets of mitochondria from 10g of muscle (10–15mg of mitochondrial protein) were combined and suspended in 10ml of 1% (w/v) NaCl and fixed by the addition of glutaraldehyde (1%, w/v). After 30min, Lubrol was added to give a final concentration of 0.01% (w/v) to keep the mitochondria dispersed. A sample (0.6ml) of this suspension was placed on a disc of nylon mesh (pore size 20μm; radius approx. 1cm) stretched over the end of a glass tube. The suspension of fixed mitochondria was then diluted and filtered through the mesh by moving the tube up and down several times in 200ml of 1% (w/v) NaCl. The range of diameters of the mitochondria present were then estimated by using a standard procedure.

Oxidative function of mitochondria. The procedure of Bullock *et al.* (1970) was used.

Results

Relative weight changes brought about by different steroids

The extents to which six different glucocorticoids affected the weight of male rats over a 7-day period are shown in Fig. 1. No decrease in the rate of weight gain resulted from the administration of corticosterone, and the maximum decrease was observed after the administration of triamcinolone acetonide.

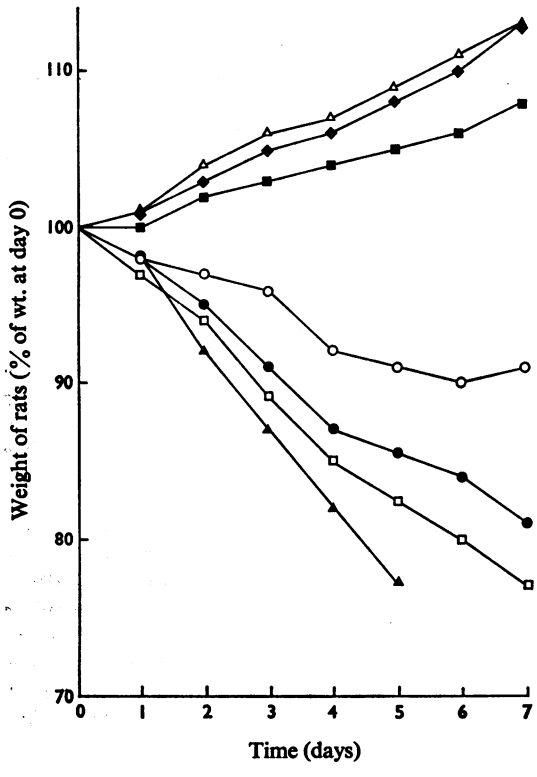

Fig. 1. *Weight changes of rats given steroid (5 mg/kg) daily*

Each group contained five animals and the average weight was recorded daily between 0915 and 1000h. Administration of steroids began on day 0. Triamcinolone (o); triamcinolone acetonide (▲); dexamethasone (□); betamethasone (●); corticosterone (△); prednisolone (■); 0.9% NaCl (♦).

The order of effectiveness of the six compounds with respect to their ability to cause weight loss was therefore triamcinolone acetonide > dexamethasone > betamethasone > triamcinolone > prednisolone > corticosterone. This order was not changed when the animals were pair-fed, nor when weight loss was measured over 6 days after a single dose (5 mg/kg) of each steroid (Fig. 2).

When the relative catabolic effectiveness of the steroids was estimated by measuring the change in weight of the three skeletal muscles from which ribosomes were isolated the order was unchanged (Table 1). In striking contrast, however, the weight of the hearts of the animals did not deviate from control weight at the end of the period of steroid administration (Table 1).

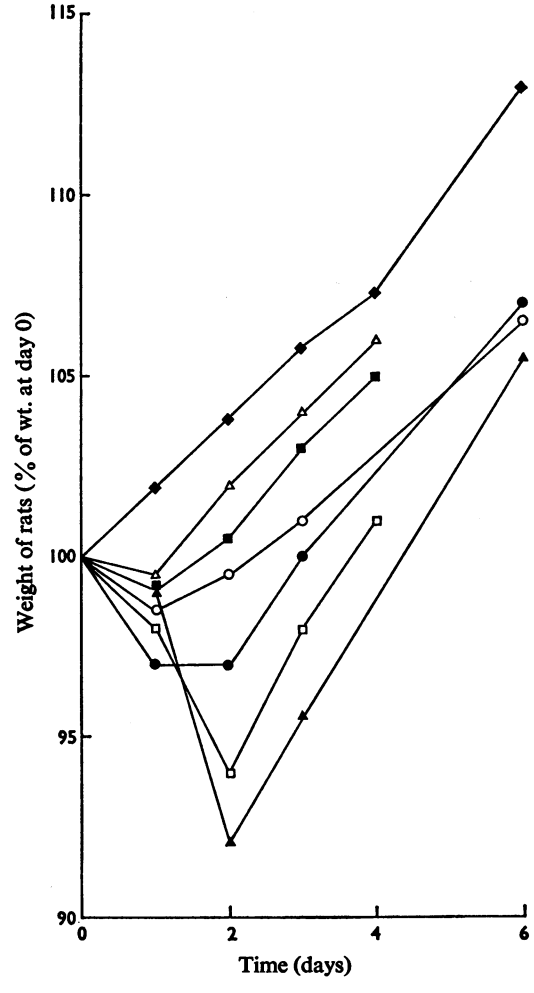

Fig. 2. *Weight changes of rats given steroid (5 mg/kg) on day 0*

Each group contained five animals and the average weight was recorded daily between 0915 and 1000h. Symbols are as in Fig. 1.

Amino acid-incorporating ability of skeletal-muscle and heart ribosomes

The effect of six doses (20–0.01 mg/kg) of the steroid on ribosomal activity was examined 6h after their administration. The minimum effective doses are shown in Table 2, and the full dose–response curves are shown in Fig. 3. These show that the relative potencies of the steroids in decreasing ribosomal activity were not the same as those in causing weight loss since triamcinolone was less catabolic than betamethasone, dexamethasone or triamcinolone acetonide but was as effective as the acetonide

Table 1. *Weight of heart and pooled skeletal muscle from rats that had received daily injections of steroids (5 mg/kg)*

Animals were maintained for 7 days with the exception of those receiving triamcinolone acetonide, which were killed after 5 days. There were five animals in each group and weights were in the range 217–238g on day 0.

	Skeletal muscles		Heart	
Injection	Average wt. (g)	Percentage of average body wt. on day 0	Average wt. (g)	Percentage of average body wt. on day 0
0.9% NaCl	5.04	2.01	0.86	0.34
Corticosterone	5.16	2.09	0.82	0.33
Prednisolone	4.90	2.04	0.81	0.33
Triamcinolone	4.07	1.67	0.78	0.32
Betamethasone	3.63	1.43	0.90	0.35
Dexamethasone	3.05	1.20	0.82	0.32
Triamcinolone acetonide	3.02	1.25	0.85	0.35

in causing a decrease in ribosomal activity at a dose of 0.05mg/kg.

More pronounced differences in relative potencies emerged when duration of action after single doses of steroids was considered (Table 3). There was then a marked difference between the action of triamcinolone and triamcinolone acetonide. Ribosomes returned to their normal function between 12 and 18h after administration of triamcinolone, whereas after triamcinolone acetonide they were still defective after 48h. Similarly corticosterone ceased to be effective at 12h whereas prednisolone still decreased ribosomal function at this time.

Ribosomes from adrenalectomized animals had the same incorporating ability (122±2.75d.p.m./μg of RNA) as those from sham-operated controls (123±3.38d.p.m./μg of RNA). This result emphasized the low activity of corticosterone with respect to the activity of isolated ribosomes. The amino acid incorporation of heart ribosomes *in vitro*, which from control animals was 102.8d.p.m./μg of ribosomal RNA, was not significantly changed 6h after the administration of triamcinolone, betamethasone, dexamethasone or triamcinolone acetonide at a dose of 5mg/kg.

Mechanism of the ribosomal defect

Differences in amino acid-incorporating ability between ribosomes from control and steroid-treated animals might be explained by differences in size distribution. For example, Fig. 4 shows that the sucrose-density-gradient profile of ribosomes obtained from muscle of animals treated with dexamethasone was different from that of control ribosomes. The proportion of polyribosomes was greater when particles were obtained from control rather than from treated animals. The same results were seen after the administration of triamcinolone acetonide.

Such differences in sucrose-density profiles could not be the result of an increase in ribonuclease-like activity within the cytoplasm of cells from treated animals as Fig. 5 shows there was no breakdown of control polyribosomes when they were incubated with muscle cell sap from treated animals.

When the polyribosome fractions (Fig. 6) were examined for the amount of radioactivity incorporated into nascent protein (see Table 4) it was found that this was the same, per unit of ribosomal RNA, irrespective of whether the polyribosomes came from control animals or animals treated with dexamethasone or triamcinolone acetonide. In the monoribosome–diribosome region of the gradient in Fig. 6, there were, however, pronounced differences in incorporating ability, confirming that it is in this region of the gradient that the effects of the drug are most clearly shown.

Size distribution of mitochondria

The mitochondria were prepared by a gentle method, not involving an homogenizer (Bullock *et al.*, 1970), to obtain organelles with a high degree of morphological integrity and consequently a high respiratory-control ratio. Evidence was also found that organelles isolated by this method from animals treated with triamcinolone acetonide had changed with respect to size as expected from electron-microscopic examination of whole tissue after administration of this drug (Bullock *et al.*, 1971). A maximum number of enlarged organelles occurred about 12h after drug administration (Bullock *et al.*, 1971).

Size distribution of isolated mitochondria was

Table 2. *Incorporation in vitro of [U-^{14}C]leucine into protein by skeletal-muscle ribosomes 6h after single injections of different steroids*

Each value represents the mean±S.E.M. of results from five individual animals. Each incubation was done with ribosomes from one control and one treated group prepared simultaneously.

Steroid	Dose (mg/kg)	Radioactivity (d.p.m./μg of ribosomal RNA)	*P*
Corticosterone	None	57.6±1.8	<0.05
	20	50.6±1.9	
	None	98.3±3.1	>0.05
	5	94.1±2.1	
Prednisolone	None	73.4±2.4	<0.001
	20	53.4±2.5	
	None	46.2±1.2	>0.05
	5	41.5±2.4	
Triamcinolone	None	47.2±1.6	<0.01
	0.2	38.1±1.2	
	None	61.9±1.5	<0.02
	0.05	55.0±0.3	
	None	83.0±1.7	>0.05
	0.01	89.2±1.6	
Betamethasone	None	89.1±2.7	<0.02
	5	78.4±2.6	
	None	65.2±3.5	<0.05
	1	54.7±1.6	
	None	83.0±3.2	>0.05
	0.2	74.6±2.8	
Dexamethasone	None	90.1±3.1	<0.001
	0.2	67.9±2.4	
	None	88.8±1.9	<0.05
	0.05	79.6±3.1	
	None	94.7±2.8	>0.05
	0.01	87.5±3.8	
Triamcinolone acetonide	None	84.7±3.2	<0.01
	0.2	70.6±0.9	
	None	65.8±1.6	<0.05
	0.05	59.2±1.7	
	None	100.7±2.3	>0.05
	0.01	91.9±6.4	

measured with an electronic particle counter (Coulter counter). By using a 50μm orifice tube, which had the effect of decreasing the electronic 'noise' it was possible to use 0.9% NaCl after Millipore filtration as a medium in which to suspend the mitochondria for counting. Under these conditions 1.2% of isolated mitochondria were in the range 3.08–6.4μm and 84.6% were in the range 1.09–1.94μm. Examination of the suspension under the electron microscope, by using whole mounts, showed that the mitochondria were mostly broken. However, when the NaCl was made slightly hyperosmotic (1%) the preparation contained whole organelles with virtually no breakage. They were, however, in clumps and therefore unsuitable for counting. To break up the clumps the mitochondria were partially fixed in glutaraldehyde and then different amounts of Lubrol were added to give final concentrations of 0.1, 0.05 and 0.01%. Slight swelling of the mitochondria occurred at 0.05% Lubrol but there was none at 0.01% and no breakage or clumping. With preparations made in this manner control counts became stabilized in the size ranges shown in Table 5. At 12h after the administration of triamcinolone acetonide there was a positive and statistically significant shift in the size distribution of the isolated organelles. These findings showed

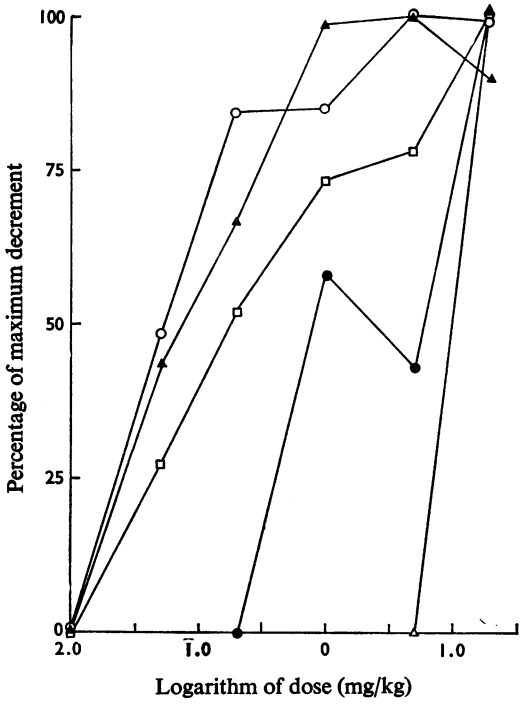

Fig. 3. *Decrement in amino acid-incorporating ability of skeletal muscle ribosomes produced by different doses of steroids*

Results are expressed as a percentage of the maximum decrement achieved in the range 0.01–20mg/kg and were obtained, for each dose, in the same way as those in Table 1. Symbols are as in Fig. 1.

that the mitochondrial populations *in vitro* that were examined for function differed from each other in the same way as *in vivo*. Unfortunately this could only be proved for animals treated with triamcinolone acetonide. With dexamethasone and betamethasone this had to be assumed, because these substances do not cause mitochondrial enlargement over the time-period examined (G. Bullock, D. Bowes & A. M. White, unpublished work).

Mitochondrial function

Results with respect to the functional state of mitochondria isolated 12h after administration of betamethasone, dexamethasone and triamcinolone acetonide are shown in Table 6. At this time, after a large dose, only betamethasone and triamcinolone acetonide caused a significant fall in the respiratory-control ratio of the mitochondria, and then only on glutamate and on glutamate and α-oxoglutarate

Table 3. *Incorporation in vitro of [U-^{14}C]leucine into protein by muscle ribosomes at various times after the injection of a single dose of different steroids*

There were five animals in each group and incubations were done as described in Table 2.

Dose (mg/kg)	Steroid	Group	Time after injection (h)	Radioactivity (d.p.m./μg of RNA)	*P*	Time after injection (h)	Radioactivity (d.p.m./μg of RNA)	*P*
20	Corticosterone	Control	6	57.6±1.8	<0.05	12	46.5±1.8	>0.05
		Test		50.5±1.9			47.1±2.2	
20	Prednisolone	Control	6	73.4±2.4	<0.01	12	26.2±1.6	<0.01
		Test		53.4±2.5			18.6±0.6	
5	Triamcinolone	Control	12	74.3±1.6	<0.01	18	80.1±1.3	>0.05
		Test		64.7±1.1			74.0±4.3	
5	Betamethasone	Control	12	63.3±2.5	<0.05	18	78.2±1.0	>0.05
		Test		53.3±3.4			72.1±3.8	
5	Dexamethasone	Control	24	92.0±1.5	<0.01	48	71.0±3.6	<0.01
		Test		70.2±2.4			55.3±2.0	
5	Triamcinolone acetonide	Control	24	44.8±1.2	<0.01	48	69.5±2.4	<0.01
		Test		35.3±1.9			52.1±2.6	

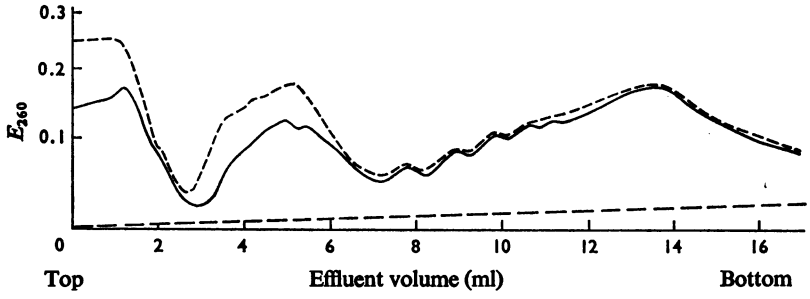

Fig. 4. *Sucrose-density-gradient profiles of heavy ribosomes from skeletal muscle*

Muscle (20g) was pooled from four control animals (——), and from four animals treated 6h previously with dexamethasone (20mg/kg) (----); — — —, sucrose concentration. For experimental details see the text.

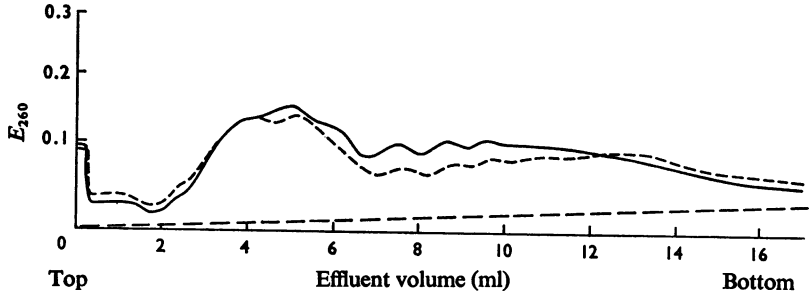

Fig. 5. *Sucrose-density-gradient profiles of heavy ribosomes isolated from skeletal-muscle ribosomes after incubation with muscle cell sap*

Sap was prepared from control animals (——) and from animals treated 6h previously with dexamethasone (20mg/kg) (---); — — —, sucrose concentration. For experimental details see the text.

respectively. Similar experiments done 6h after administration of the same drug showed the mitochondria to be entirely unaffected and measurements of oxygen consumption (Q_{O_2}) and ADP/O ratios at both times and after the three drugs showed no significant deviations from normal values.

Concentration of steroids in tissues

In a separate study by g.l.c. (P. Simpson, unpublished work) it has been shown that radioactivity present in skeletal muscle and heart 5min after injection of labelled steroids represents largely unchanged and non-metabolized material. Absolute amounts of radioactivity were therefore measured to give the concentration of steroids in muscle (Table 7). Presumably the short time between injection and killing the animals leads to poor duplication. Nevertheless the results indicate that all the steroids penetrate the muscles to give high concentrations and that the heart in particular appears to be preferentially well supplied.

Discussion

The order of catabolic effectiveness after daily administration of the steroids (Fig. 1) was similar to that described in dogs (Faludi *et al.*, 1964), mice (Faludi *et al.*, 1966) and rats (Tonelli *et al.*, 1965). The loss in body weight during chronic treatment was paralleled by loss in muscle weight (Table 1), as expected (Tonelli *et al.*, 1965). Neither pair-feeding (P. Elliott, unpublished results) nor measurement of weight changes over a 6-day period after a single injection of each steroid (5mg/kg) altered the order of catabolic potential shown by the steroids. This potency was accurately reflected in the length of time after a single dose *in vivo* over which pre-

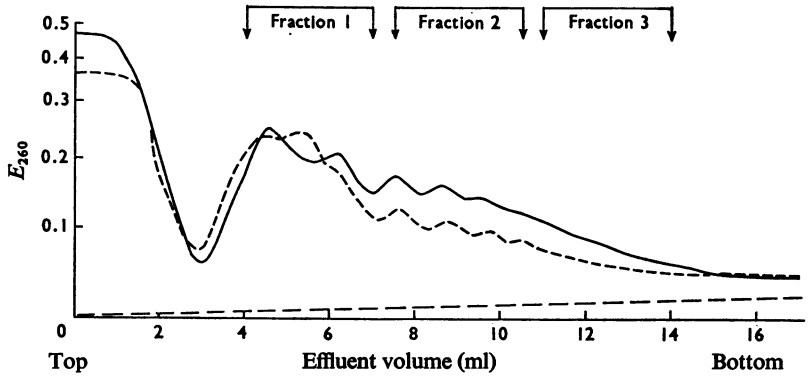

Fig. 6. *Sucrose-density-gradient profiles of heavy ribosomes isolated from skeletal-muscle ribosomes on which the nascent protein had been labelled by large-scale incubation*

Pooled muscle (20g) from four control animals (——) and four animals treated 6h previously with dexamethasone (20mg/kg) (---), — — —, sucrose concentration. Fractions 1, 2 and 3 were counted for radioactivity as described in the Experimental section. Results in Table 4 are taken from this and a similar experiment in which triamcinolone acetonide (20mg/kg) was used.

Table 4. *Amino acid-incorporating ability of muscle ribosomes and polyribosomes 6h after the administration of dexamethasone (20mg/kg) or triamcinolone acetonide (20mg/kg)*

Ribosomes were prepared on the large scale and incubated and fractionated by the method described in the Experimental section. Fractions 1, 2 and 3 are those shown in Fig. 6.

		Incorporation			
		+Dexamethasone		+Triamcinolone acetonide	
Ribosomal population		(d.p.m./μg of RNA)	(% of control)	(d.p.m./μg of RNA)	(% of control)
Fraction 1	Control	50.6	100.0	48.0	100.0
	Treated	21.8	43.1	33.7	70.0
Fraction 2	Control	108.9	100.0	131.6	100.0
	Treated	118.3	108.6	123.0	94.0
Fraction 3	Control	117.2	100.0	127.9	100.0
	Treated	106.9	91.2	121.6	95.2

dominantly white muscles continue to yield ribosomes with decreased ability to incorporate amino acids into protein. The response of the animals to decreasing doses of the steroids at a fixed interval after injection was, in our experimental systems, a poorer indicator of catabolic activity. These points were strikingly illustrated through the comparison between triamcinolone and triamcinolone acetonide. Ribosomes from animals receiving triamcinolone (5mg/kg) returned to normal activity 18h after drug administration, whereas the action of the acetonide persisted for up to 48h. At first sight an explanation for the longevity of action of triamcinolone acetonide with respect to weight loss (Fig. 2) and ribosomal activity (Table 3) might appear to lie entirely in its greater lipophilicity, with consequently increased concentration and retention time in lipid compartments of the cell. However, this factor, although perhaps contributory, does not provide a satisfactory explanation of why the action of corticosterone persists for a shorter time than that of the more polar prednisolone, or why there is a difference in duration of action between betamethasone and dexamethasone, which would be expected to be equally polar. It

Table 5. *Size distribution of mitochondria from animals treated with triamcinolone acetonide (20mg/kg)*

Mitochondrial pellets from the muscles of single animals were prepared and counted as described in the Experimental section. Each value represents the mean±S.E.M. of six counts. Only counts made 12h after administration of triamcinolone acetonide differed significantly from controls ($P<0.05$).

Treatment	Number of particles (% of total) in different size ranges		
	1.09–1.94μm	1.94–3.08μm	3.08–6.4μm
0.9% NaCl	59.4±1.6	25.1±1.2	15.6±0.7
Triamcinolone acetonide (6h)	58.7±3.1	26.4±2.2	15.2±1.1
Triamcinolone acetonide (12h)	44.2±2.6	32.8±2.4	23.1±1.9

seems likely that longevity of action might also reflect the strength of binding to a specific macromolecule. Evidence for the existence of molecules that could fulfil this function in tissues that involute under glucocorticoid action has been derived from work on fibroblasts (Hackney *et al.*, 1970), thymocytes (Schaumburg, 1970) and thymus cells (Wira & Munck, 1970). The lack of such a molecule in the heart, or the presence of one with considerably decreased affinity for glucocorticoids, may explain why this organ did not undergo weight loss [thus confirming other work (Faludi *et al.*, 1964; Krotkiewski *et al.*, 1970)] and why, in agreement with this, heart ribosomes still possessed normal function. However, other less specific explanations may apply, since the heart, as might be expected in the case of a vital organ, is resistant to a number of drugs and is relatively more resistant to starvation (Widdowson *et al.*, 1960) than skeletal muscle. The heart takes up considerably more of the steroids than does skeletal muscle, and therefore a barrier to uptake certainly cannot be invoked as an explanation for its resistance to glucocorticoid-induced involution.

On the basis of previous work (Peters *et al.*, 1970) it had been thought that the ribosomes were possibly being influenced by a cytoplasmic factor, which could even be the steroids themselves, and that this influence was leading possibly to a decreased rate of translation of mRNA. In the light of evidence presented in the present paper, however, this explanation now seems to be untenable. Ribosomes from skeletal muscle, unlike those from liver, contain such a high proportion of monomers and dimers that these are by no means completely removed even after centrifugation through 2M-sucrose (Fig. 3), and therefore in populations of ribosomes from test and control samples of muscle that were centrifuged through sucrose concentrations of 1.5M or less (Peters *et al.*, 1970), those portions were retained that still differed with respect to their incorporating activity per unit of RNA. However, when these were distinguished on a gradient from the polyribosomes in the experiments described here it was seen (Fig. 5, Table 4) that the true incorporation rate into nascent protein was the same, irrespective of whether the polyribosomes were from control or steroid-treated animals. The glucocorticoids do not therefore appear to alter the rate at which mRNA is translated into protein by muscle ribosomes. Nevertheless the sensitivity of the ribosomal system towards glucocorticoids confirms a close relationship between a change in this system and an event *in vivo* that determines muscle weight loss, although it cannot be said with any certainty what this event is. Increased concentrations of ribonuclease-like activity appear not to be involved (Fig. 3). Our results now support those of Young *et al.* (1968), who showed a decrease in the polyribosome content of ribosomes from young rats treated with cortisol.

The mitochondria *in situ* are probably resistant to triamcinolone acetonide, betamethasone and dexamethasone up to 6h after the administration of high doses of these substances, on the usual assumption that the isolated organelles are representative of the state *in vivo*. As our method for isolation of mitochondria is a very gentle one which does not use a homogenizer, this assumption would appear valid although it was only possible to prove it with triamcinolone acetonide and then only at the 12h point. Presumably the method of measuring mitochondrial diameter was not sensitive enough to detect the smaller proportion of enlarged organelles seen under the electron microscope (Bullock *et al.*, 1971) 6h after administration of this drug. However, in view of the very low sensitivity of mitochondrial function compared with that of the ribosomes towards the most catabolic of the steroids tested it can be reasonably assumed that mitochondrial dysfunction is not the primary cause of catabolism in the affected tissues. Nevertheless, in view of the limited uncoupling observed 12h after the administration of betamethasone (20mg/kg) and triamcinolone acetonide

Table 6. *Respiratory-control ratio and oxygen consumption (Q_{O_2}) of skeletal-muscle mitochondria from animals treated with different steroids*

There were six animals in each of the treated groups and twelve in the control group. Steroids (20mg/kg) were administered 12h before killing the animals. Oxygen consumption (Q_{O_2}) is expressed as ng-atom of O/min per mg of mitochondrial protein. *Significantly different from control ($P<0.05$).

Substrate ...	Glutamate (10mM)		α-Oxoglutarate (15mM)		Pyruvate (5mM) + malate (0.5mM)		Succinate (10mM) + amytal (2mM)	
	Respiratory-control ratio	Q_{O_2}	Respiratory-control ratio	Q_{O_2}	Respiratory-control ratio	Q_{O_2}	Respiratory-control ratio	Q_{O_2}
0.9% NaCl	16.4±1	275±12	10.1±1.1	297±15	8.1±0.80	311±43	2.2±0.2	450±20
Betamethasone	12.4±1*	230±31	9.7±1.0	266±25	9.0±1.0	270±15	2.5±0.4	516±25
Dexamethasone	17.5±1.4	237±23	8.5±1.2	255±15	9.5±0.8	277±25	2.1±0.1	504±27
Triamcinolone acetonide	10.6±1.6*	259±17	6.9±1.1*	290±28	6.5±0.3	257±25	2.1±0.2	417±46

Table 7. *Concentration of steroids present in muscle 5min after intraperitoneal injection of labelled substances (5mg/kg)*

The results of two separate animals are given in each case.

Steroid	Concn. (μg/g of tissue)	
	Heart	Skeletal muscle
Corticosterone	5.33	2.91
	1.27	0.81
Prednisolone	1.85	0.60
	1.79	0.51
Triamcinolone	0.76	0.16
	0.81	0.22
Betamethasone	1.47	0.56
	1.20	0.41
Dexamethasone	3.12	0.97
	2.50	0.47
Triamcinolone acetonide	1.99	0.74
	2.12	0.68

(20mg/kg) it would appear that mitochondrial dysfunction plays a part in the chronic myopathic condition. Peter *et al.* (1970) concluded that the muscle mitochondria of rats remained unaffected even after daily treatment with triamcinolone (5mg/kg) for 10 days. However, their technique did not allow the isolation of highly coupled mitochondria, since the controls had a mean respiratory-control ratio of only 3.3 (pyruvate/malate). It could be argued therefore that differences between control populations and populations from steroid-treated animals would no longer be apparent when there were so many damaged organelles present. Nevertheless our findings support their results with triamcinolone *in vivo* and also the results with triamcinolone acetonide 21-phosphate *in vitro* in which it was shown that this derivative has no effect on the coupling of rat muscle mitochondria. Our results also support those of Palmer (1966), who showed that the respiration of muscle slices from young rats was not diminished after cortisol treatment. It is possible that in all experiments involving isolated mitochondria the effects of the steroids were reversed because the mitochondria revert to normal function on the loss of the steroids during isolation or during incubation *in vitro*. Although we have no direct proof that this is not the case the lipophilicity of the substances under investigation would appear to militate against this possibility.

References

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279
Britten, R. J. & Roberts, R. B. (1960) *Science* **131**, 32
Bullock, G., White, A. M. & Worthington, J. (1968) *Biochem. J.* **108**, 417

Bullock, G., Carter, E. E. & White, A. M. (1970) *FEBS Lett.* **8**, 109
Bullock, G. R., Christian, R. A., Peters, R. F. & White, A. M. (1971) *Biochem. Pharmacol.* **20**, 943
Faludi, G., Mills, L. C. & Chayes, Z. W. (1964) *Acta Endocrinol. (Copenhagen)* **45**, 68
Faludi, G., Gottlieb, J. & Meyers, J. (1966) *Ann. N.Y. Acad. Sci.* **138**, 61
Hackney, J. F., Gross, S. R., Aronow, L. & Pratt, W. B. (1970) *Mol. Pharmacol.* **6**, 500
Krotkiewski, M., Krotkiewska, J., & Bjorntorp, P. (1970) *Acta Endocrinol. (Copenhagen)* **63**, 185
Palmer, B. G. (1966) *J. Endocrinol.* **36**, 73
Peter, J. B., Verhaag, D. A. & Worsfold, M. (1970) *Biochem. Pharmacol.* **19**, 1627
Peters, R. F., Richardson, M. C., Small, M. & White, A. M. (1970) *Biochem. J.* **116**, 349
Pfeffer, M., Weinstein, S., Gaylord, J. & Indindoli, L. (1971) *Anal. Biochem.* **39**, 46
Schaumburg, B. P. (1970) *Biochim. Biophys. Acta* **214**, 520
Tonelli, G., Partridge, R. & Ringler, I. (1965) *Proc. Soc. Exp. Biol. Med.* **119**, 136
White, A. M. (1967) *Proc. Int. Congr. Horm. Steroids 2nd. 1966*, 576
Widdowson, E. M., Dickerson, J. W. T. & McCance, R. A. (1960) *Brit. J. Nutr.* **14**, 457
Wira, C. & Munck, A. (1970) *J. Biol. Chem.* **245**, 3436
Young, V. R., Chen, S. C. & Macdonald, J. (1968) *Biochem. J.* **106**, 913