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EFFECT OF TRIAMCINOLONE AND OTHER STEROIDS ON THE OXIDATIVE PHOSPHORYLATION REACTION

A. GÓMEZ-PUYOU, A. PEÑA-DÍAS, J. GUZMÁN-GARCÍA and J. LAGUNA

Department of Biochemistry, Facultad de Medicina,
Universidad Nacional, México, D.F., México

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Abstract—The effect of one natural and two synthetic glucocorticoids (hydrocortisone, triamcinolone, and fluoromethyl-prednisolone) on mitochondrial respiration, oxidative phosphorylation, adenosinetriphosphatase activity, and the ^{32}P–adenosine triphosphate exchange reaction was studied.

Fluoromethyl-prednisolone did not affect any of the reactions mentioned above; hydrocortisone inhibited mitochondrial respiration but did not influence the rest of the reactions. Triamcinolone increased respiration, depressed oxidative phosphorylation and the ^{32}P–ATP exchange reaction, and increased the latent adenosinetriphosphatase activity of fresh mitochondria. Triamcinolone also inhibited mitochondrial osmotic swelling.

The effect of triamcinolone on the oxidative phosphorylation reactions studied resembles that of 2,4-dinitrophenol.

DURING recent years, several effects of steroid hormones on mitochondrial respiration or oxidative phosphorylation, or both, have been reported.[1, 2] Kerppola[2] found an inhibition of the coupling reaction in rats treated with cortisone. On the other hand, Gallagher[1] was unable to uncouple oxidative phosphorylation with hydrocortisone *in vitro*. Blecher and White[3] demonstrated an activation of ATPase* under the influence of several steroids.

The present work deals with the effects *in vitro* of three glucocorticoids on a mitocondrial phosphorylating system: hydrocortisone, triamcinolone (9-α-fluoro-11-β,16-α,17-α,21-tetrahydroxy-1,4-pregnandiene-3,20-dione), and a new synthetic fluorinated glucocorticoid, fluoromethyl-prednisolone[4] (21-methyl-9-α-fluoro-11-β,17-α,21-β-trihydroxy-1,4-pregnandiene-3,20-dione,21-acetate). Only triamcinolone was able to affect oxidative phosphorylation, and studies on its action on mitochondrial ATPase and the ^{32}P-ATP exchange reaction were carried out.

The results showed that triamcinolone uncouples oxidative phosphorylation in a manner similar to that of DNP.[5] Hydrocortisone and FMP do not affect this mitochondrial function.

MATERIAL AND METHODS

Animals. Wistar male rats weighing 150 to 200 g were used in all experiments.

Isolation of mitochondria. For the experiments designed to measure oxidative phosphorylation, ATPase activity, and the exchange of ATP with ^{32}P, mitochondria

* The following abbreviations are used: ATPase, adenosinetriphosphatase; DNP, 2,4-dinitrophenol. ATP, ADP, AMP, adenosine tri-, di-, and mono-phosphate respectively; NAD, nicotinamide-adenine dinucleotide. FMP, fluoromethyl-prednisolene.

were prepared according to the method of Schneider and Hogeboom[6] in 0·25 M sucrose; the pellet was washed once after the first high-speed centrifugation. Mitochondria from 200 to 250 mg of liver for oxidative phosphorylation and from 1 g of liver for ^{32}P-ATP exchange reaction were suspended in 1 ml of 0·25 M sucrose. This suspension was kept on ice until the time of the experiment, which in all cases was less than 15 min. For the swelling experiments, mitochondria were prepared in the same way, except that 0·44 M sucrose was employed, and mitochondria from 1 g of liver were suspended in 1 ml of 0·44 M sucrose.

Oxygen uptake. This was determined by the conventional Warburg method at 25° for 30 min after equilibration for 10 min; at the end of this period, hexokinase and glucose were tipped in from the side arm to start the reaction. From the uptake of vessels containing exogenous substrate, the oxygen consumption due to endogenous substrates was subtracted.

Oxidative phosphorylation. For P:O ratios, the oxygen and phosphorus uptakes were corrected by subtracting endogenous consumption in vessels handled in identical conditions.

Adenosinetriphosphatase. The activity of mitochondria from 200 mg of liver suspended in 1 ml of 0·25 M sucrose was assayed at 25° for 20 min in the indicated incubation medium. The reaction was stopped by cold trichloroacetic acid at 7·5%, final concentration. Inorganic phosphorus was determined in the supernatant.

^{32}P–ATP exchange reaction. Mitochondria from 100 mg of liver in 0·1 ml of 0·25 M sucrose were added to 0·9 ml of medium as indicated. After an incubation period of 15 min at 20°, the reaction was stopped by addition of 0·2 ml of 30% trichloroacetic acid for each milliliter of reaction mixture. The tubes were centrifuged, and from the supernatant a known sample was taken for the chromatographic separation of ATP. After the ATP was eluted from the chromatogram, an aliquot was placed on an aluminum plate, dried, and counted with a Tracerlab TGC-14 carbon-counter tube and a Philips PW 4032 scaler.

Chromatography and determination of ATP. Samples were put on no. 1 Whatman paper under a current of air to accelerate drying. The papers were permitted to run for 20 hr at room temperature in the descending technique, the solvent being 96% ethanol and 1 M ammonium acetate in a 75:35 ratio.[7] The chromatograms were dried, and the position of ATP was located and traced by illumination with ultraviolet light;[8] the spot was cut and eluted with 0·01 N HCl. Its absorbancy was read at 260 mμ in a Beckman DU spectrophotometer and the concentration calculated.

Determination of swelling. The optical method employed by many authors was used.[9, 10] Absorbancy changes were followed at 520 mμ in a Beckman DU spectrophotometer for 30 min at room temperature after adding 0·04 ml of mitochondrial suspension to a 1-cm light path cuvet containing 3·0 ml of 0·1 M sucrose buffered with Tris, 0·02 M, pH 7·4. The steroid at the desired concentration was added in 0·1 ml of propylene glycol; the controls were treated with the same amount of the solvent.

Inorganic phosphorus. In the experiments on oxidative phosphorylation and in the measurement of ATPase activity, inorganic phosphorus was determined according to the technique of Lowry and López[11] on the supernatant resulting from precipitation with trichloroacetic acid at a final concentration of 7·5%.

Reagents. All reagents were commercially obtained. Hydrocortisone, triamcinolone, and fluoromethyl-prednisolone were kindly donated by Merck Sharp and Dohme de México, S.A., E. R. Squibb & Sons de México, S.A. de C.V., and Pfizer de México, S.A., respectively.

RESULTS

Varying numbers of experiments, each with a different pool of mitochondria, were done. The number of experiments in each series is indicated in the corresponding table or figure. Since in all cases the same pattern of results was obtained, only typical experiments are presented; it should be mentioned, however, that in the swelling experiments at low steroid concentrations positive results were not always obtained. Determinations in each experiment were made at least in duplicate. Good reproducibility (less than 5 per cent) was obtained in duplicates.

Oxygen uptake

Some of the results reported in the literature were confirmed.[1] Hydrocortisone was found to depress the respiration of mitochondria utilizing substrates which are dependent on NAD. This inhibitory action of hydrocortisone could be confirmed at 6×10^{-5} M, the lowest concentration at which such effect could be obtained. No effect on mitochondrial respiration could be observed with succinate (Table 1).

TABLE 1. EFFECT OF THE THREE STEROIDS ON MITOCHONDRIAL OXYGEN UPTAKE*

Substrate	Control		Triamcinolone	Control		Hydrocortisone	Control		FMP
	(μl of oxygen consumed by mitochondria from 250 mg of rat liver)								
Malate	118	(4)	154	168	(4)	139	168	(4)	155
Glutamate	106	(8)	140	201	(3)	179	91	(2)	82
Succinate	200	(6)	194	179	(2)	191			

* Experimental mixture: 0·01 M potassium phosphate buffer, pH 7·4; $1{\cdot}5 \times 10^{-5}$ M cytochrome C; 0·005 M $MgCl_2$; 0·0015 M ATP; 0·008 M AMP; hexokinase, 0·05 ml of a solution containing 7 mg protein/ml; 0·02 M glucose; 0·02 M KF; 0·0005 M NAD; 0·01 M substrate; 0·1 M sucrose; 4×10^{-4} M triamcinolone, or 6×10^{-4} M hydrocortisone, or 5×10^{-4} M FMP; propylene glycol 0·1 ml; temperature 25°; final volume 3·0 ml; 0·2 ml of 50% KOH in center well; incubation time 30 min. ( ) Number of experiments carried out in triplicate. Figures presented are those of typical experiments. Each set of two values is from a single pool of mitochondria.

Triamcinolone, the synthetic glucocorticoid, was found, on the contrary, to increase the oxygen uptake by mitochondria which had malate or glutamate as substrate. This stimulation was observed at 4×10^{-4} M and 4×10^{-5} M. No effect could be obtained with a lower concentration (4×10^{-6} M). Triamcinolone at a concentration which increased the respiration of mitochondria utilizing glutamate or malate does not stimulate respiration if succinate is the oxidizable substrate. The effect of triamcinolone on oxygen uptake could only be obtained with substrates that in some way are linked to NAD (Table 1).

Fluoromethyl-prednisolone did not affect respiration at similar concentrations.

x

P:O ratio

Another difference between the steroids could be observed when the coupling of oxidation and phosphorylation by mitochondria was studied. Even though hydrocortisone depresses respiration, it had no effect on oxidative phosphorylation; the same negative effect was found with FMP at 5×10^{-4} M. Triamcinolone, however, depressed to a very large extent the phosphorylating activity of mitochondria (Table 2).

TABLE 2. EFFECT OF TRIAMCINOLONE ON P:O RATIO BY RAT LIVER MITOCHONDRIA*

Substrate	Microatoms of oxygen uptake		Micromoles of P uptake		P : O ratio	
	Control	Triam.	Control	Triam.	Control	Triam.
Malate (5)	9·8	13·7	18·4	0	1·8	0
Glutamate (6)	7·4	9·0	22·4	0·2	3·0	0
Succinate (8)	7·3	6·7	12·2	0	1·7	0

* Experimental conditions are the same as for Table 1. Figures presented are those of typical experiments from a single pool of mitochondria studied in triplicate.

TABLE 3. EFFECT OF TRIAMCINOLONE ON ATPASE ACTIVITY*

pH	Control		Triamcinolone	
	Sodium	Potassium	Sodium	Potassium
8·5	2·36	1·31	2·53	5·97
7·4	1·64	0·23	4·35	10·65
6·3	1·56	0·45	2·77	8·62

* Micromoles of P formed in 20 min in a sodium or potassium medium. Experimental mixture: 0·125 M KCl or NaCl; 0·017 M Tris, pH as indicated; ATP, 11 μmole; 0·08 M sucrose; 4×10^{-4} M triamcinolone; propylene glycol 0·1 ml; mitochondria from 200 mg rat liver; final volume, 3·0 ml; temperature 25°. Three experiments, each determination at least in duplicate.

TABLE 4. EFFECT OF THE THREE STEROIDS ON ATPASE ACTIVITY*

	μmole of P formed in 20 min
Control	1·6
Triamcinolone	14·6
Hydrocortisone	2·3
FMP	2·0

* Experimental mixture: 0·05 M KCl; 0·017 M Tris, pH 7·4; ATP, 11 μmole; 0·08 M sucrose; propylene glycol 0·1 ml; 4×10^{-4} M triamcinolone; 6×10^{-4} M hydrocortisone; 5×10^{-4} M FMP; mitochondria from 200 mg rat liver; final volume, 3·0 ml; temperature 25°. Four experiments, each with a different pool of mitochondria; all determinations were made in duplicate.

No incorporation of inorganic phosphate to adequate acceptors occurred. This depressant effect could be observed not only with glutamate or malate, but also with succinate as the oxidizable substrate. The effect of triamcinolone on blocking the incorporation of inorganic phosphate into an appropriate acceptor was observed at a

higher concentration than that required to stimulate respiration. For the uncoupling effect, a 4×10^{-4} M concentration was necessary, whereas the stimulation of respiration could be obtained at 4×10^{-5} M.

Adenosinetriphosphatase

It was decided to study some of the properties of triamcinolone as an uncoupling agent. The results showed that triamcinolone was capable of stimulating the latent ATPase activity of mitochondria at 4×10^{-4} M and at 4×10^{-5} M concentration (Tables 3 and 4). No effect could be obtained at the next lower concentration (4×10^{-6} M). The stimulation of the ATPase activity becomes more important when the incubation medium contains KCl instead of NaCl (both in equimolecular quantities). K^+ in some manner favors the stimulatory effect of triamcinolone. This effect is observed at the three pH's studied under this set of experimental conditions (Table 3).

However, the presence of exogenous K^+ is not necessary for the activation of the ATPase system; as can be seen in Fig. 1, there is a clear-cut stimulation of the activity

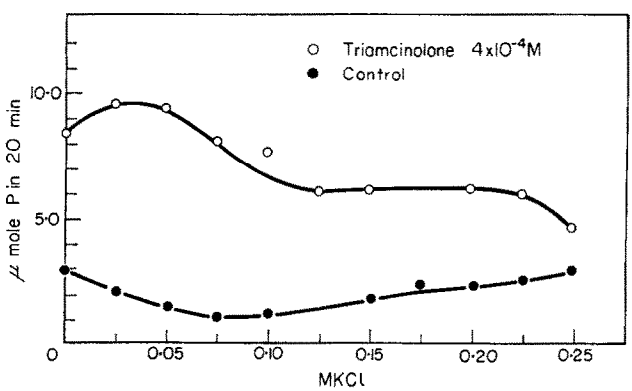

FIG. 1. Effects of different concentrations of KCl on ATPase activity; micromoles of P formed in 20 min. Experimental mixture: 0·017 M Tris, pH 7·4; ATP, 11 μmole; 4×10^{-4} M triamcinolone; 0·1 ml propylene glycol; KCl as indicated; sucrose, 0·08 M; final volume, 3·0 ml; temperature 25°. Three experiments, each determination in duplicate.

when no K^+ is added to the system. Nevertheless, there exists a range of optimum concentrations which lies between 0·05 and 0·075 M KCl. At 0·25 M KCl the stimulatory effect of the steroid tends to vanish.

A study of the action of triamcinolone on ATPase systems of fresh mitochondria at varying pH's showed that the steroid is capable of stimulating the activity over a wide range of (H^+). The optimum pH lies in the region of 7·0 to 7·5; at the highest pH's studied (9·5 to 10·0), there is also a higher activity in the presence of triamcinolone (Fig. 2).

At optimum conditions, hydrocortisone and FMP do not stimulate ATPase activity (Table 4).

In some experiments (Tables 4, 5, 6) the molar amount of inorganic phosphate was larger than the total ATP in the reaction medium when triamcinolone or DNP was present. This apparent anomaly could be explained by the fact that the uncouplers elicited a substantial liberation of inorganic phosphate from ADP (Table 5).

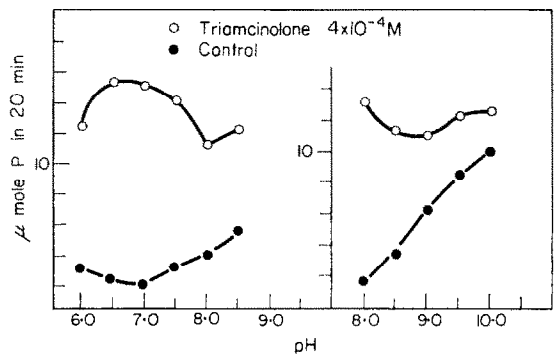

FIG. 2. Effect of pH on ATPase activity; micromoles of P formed in 20 min. Experimental mixture: 0·05 M KCl; ATP, 11 μmole; 4×10^{-4} M triamcinolone; 0·1 ml propylene glycol; 0·017 M Tris, pH as indicated; sucrose, 0·08 M mitochondria from 200 mg rat liver; final volume, 3·0 ml; temperature 25°. Three experiments, each determination in duplicate.

TABLE 5. EFFECT OF TRIAMCINOLONE AND DNP ON THE LIBERATION OF P FROM EITHER ATP OR ADP*

Agent	ATP (μmoles P formed in 20 min)	ADP
None	1·7	0·8
Triamcinolone (4×10^{-4} M)	14·1	5·5
None	1·9	1·9
DNP (10^{-4} M)	15·5	6·4

* Experimental mixture: 0·05 M KCl; 0·017 M Tris, pH 7·4; ATP, 11 μmole; ADP, 11 μmole; 0·08 M sucrose; 4×10^{-4} M triamcinolone dissolved in 0·1 ml propylene glycol; 10^{-4} M DNP dissolved in water; mitochondria from 200 mg rat liver; final volume, 3·0 ml; temperature, 25°. Four experiments, each from a single pool of mitochondria; all determinations in duplicate.

It has been reported that ADP is capable of inhibiting the increase in activity of ATPase;[12] inorganic phosphorus does not possess this property. The effect of ADP on the stimulatory action of triamcinolone was studied; ADP blocked the increase in the activity produced by 4×10^{-4} and 4×10^{-5} M triamcinolone. The same pattern of results was obtained for DNP (Table 6).

^{32}P–ATP exchange reaction

In order to locate the site of the uncoupling action of the steroid on the scheme of oxidative phosphorylation, as proposed by Lehninger *et al.*,[5] the ^{32}P–ATP exchange reaction by fresh mitochondria was studied. Triamcinolone depressed considerably this reaction. The exchange was studied under the influence of triamcinolone at a

TABLE 6. EFFECT OF ADP ON THE STIMULATION OF ATPASE ACTIVITY BY TRIAMCINOLONE OR DNP*

Triamcinolone	ATP	ATP + ADP	DNP	ATP	ATP + ADP
		(μmole P formed in 20 min)			
None	4·1	0·6	None	3·9	0·6
4×10^{-4} M	17·9	2·6	10^{-3} M	16·9	5·0
4×10^{-5} M	7·1	0·8	10^{-4} M	15·1	5·0
4×10^{-6} M	4·7	0·6	10^{-5} M	7·5	2·9

* Experimental mixture: 0·05 M KCl; 0·017 M Tris, pH 7·4; ATP, 11 μmole; ADP, 11 μmole; 0·08 M sucrose; mitochondria from 200 mg rat liver; triamcinolone dissolved in 0·1 ml propylene glycol; DNP dissolved in water; final volume, 3·0 ml; temperature, 25°. Three experiments, each determination in duplicate.

concentration of 4×10^{-4} M. For comparison, DNP at a similar concentration was included in two of a series of experiments. In both cases an inhibition of the exchange reaction was observed; for DNP, this has been reported.[5] To show the specificity of the action of triamcinolone, data with other glucocorticoids are included (Table 7).

TABLE 7. ^{32}P–ATP EXCHANGE REACTION; EFFECT OF TRIAMCINOLONE, DNP, HYDROCORTISONE, AND FMP*

In counts $\times 10^{-3}$ per micromole ATP			
Control†	Triamcinolone	Control‡	DNP
36·2	2·3	30·5	1·3
Control§	Triamcinolone	Hydrocortisone	FMP
45·1	5·7	36·0	41·8

* Experimental mixture: KCl, 11 μmole; $MgCl_2$, 4 μmole; EDTA, 1·5 μmole; Tris, 34 μmole, pH 7·4; phosphate buffer pH 7·4 containing 10^6 counts/min; ATP, 5 μmole; sucrose, 12·5 μmole; mitochondria from 100 mg rat liver; triamcinolone, 4×10^{-4} M; hydrocortisone, 6×10^{-4} M; FMP, 5×10^{-4} M; DNP, 10^{-4} M; final volume, 1·0 ml; temperature, 25°; incubation time, 15 min. The steroids were dissolved in 0·1 ml propylene glycol, DNP in water. All determinations in duplicate.

† Eight experiments.

‡ Three experiments.

§ Two experiments.

It could be expected that the inhibition of the exchange reaction would be the result of a high ATPase activity which would result in a very low level of ATP. However, the experimental fact is that the incubation of ATP in the medium mentioned above does not result in such a marked hydrolysis of ATP. In comparison with its respective control, the ATP level in the presence of the uncouplers falls only to about 50% of

the control values. In comparison with the ATPase experiments the difference is probably due to the different incubating conditions.

During the chromatographic separation of labeled ATP, it was observed that the samples containing either triamcinolone or DNP contained a compound that was absent in the controls; later this compound was shown to be AMP. This is in accordance with the data presented in Table 5. Probably the AMP formed is a result of a myokinase system; that is, the hydrolysis of ATP would result in a high formation of ADP, which in turn would drive the myokinase reaction toward the production of ATP. A high level of ADP plus a low level of ATP would favor this reaction.

Mitochondrial swelling

2,4-Dinitrophenol under certain circumstances prevents osmotic mitochondrial swelling.[9] In order to compare the action of DNP and triamcinolone on mitochondria with a somewhat different approach, the influence of triamcinolone on osmotic swelling was chosen to avoid the complications of respiration and oxidative phosphorylation on this particular property.[13]

The results of typical experiments are presented in Fig. 3. Triamcinolone prevents swelling over a wide range of concentrations; it is to be noted that triamcinolone

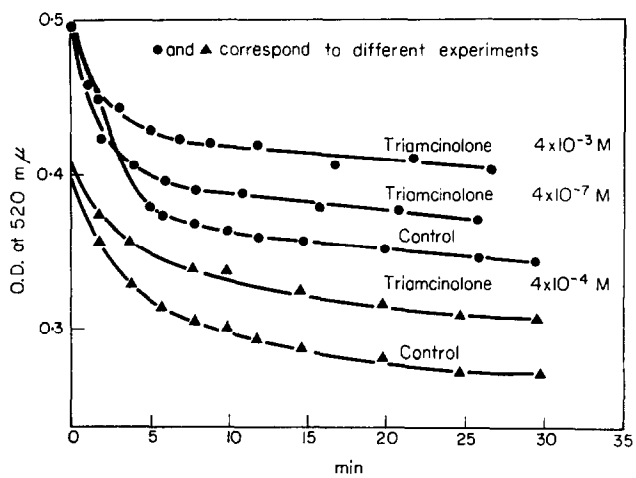

FIG. 3. Effect of triamcinolone on mitochondrial swelling. Experimental mixture: 0·1 M sucrose; 0·02 M Tris, pH 7·4; 0·1 ml propylene glycol; mitochondria in 0·04 ml of 0·44 M sucrose; final volume, 3·0 ml; ● and ▲ are different experiments; 21 experiments were done.

protects against this type of swelling not only at concentrations which uncouple oxidative phosphorylation, but also at molarities having no influence on respiration or oxidative phosphorylation; however, it should be mentioned that the inhibiting effect on swelling at the lowest concentration studied (4×10^{-8} M) appears only in 30% of the experiments. At higher concentrations the effect is consistently observed.

When swelling under the influence of triamcinolone at two concentrations was tested, an apparently paradoxical result was observed, since inhibition was greater with the lower concentration. No explanation at all can be offered for this observation.

DISCUSSION

Steroids of similar physiological action have different effects on certain mitochondrial functions: hydrocortisone depresses respiration, triamcinolone stimulates it, and fluoromethyl-prednisolone does not affect it. Different actions are observed also on the oxidative phosphorylation reaction: triamcinolone inhibits it, and hydrocortisone and FMP do not influence it. These latter steroids do not affect ATPase activity or the ^{32}P–ATP exchange. Since different effects on mitochondria are observed with steroids of similar physiological actions, it is improbable that the primary site of action of glucocorticoids lies in these particular mitochondrial functions.

Racker[14] has defined true uncoupling agents as those that stimulate respiration and at the same time inhibit the incorporation of inorganic phosphate into a suitable acceptor. Triamcinolone falls into this category since it inhibits oxidative phosphorylation and at the same time stimulates respiration.

A very close resemblance exists between the effects of DNP and triamcinolone; DNP is known to stimulate respiration, to depress utilization of inorganic phosphate, to stimulate ATPase activity, and to inhibit the ^{32}P–ATP exchange reaction.[5] Triamcinolone at adequate concentrations shows the same properties.

Nevertheless, on the study of the stimulation of ATPase activity some differences between DNP and triamcinolone were encountered. DNP, as shown by Myers and Slater,[15] has a maximal stimulation of the activity at pH 6·3 with fresh mitochondria; with triamcinolone the most marked effect appears at pH 7·0 to 7·5. Furthermore, triamcinolone stimulates ATPase activity at pH 9·5 and 10·0, whereas DNP does not.

However, the site of action of triamcinolone and DNP, according to the scheme of oxidative phosphorylation proposed by Lehninger *et al.*,[5] would be the same or very close. Since triamcinolone inhibits to a very large extent the ^{32}P–ATP exchange reaction, and because the presence of ADP blocks the stimulation of ATPase activity produced by triamcinolone, it can be assumed that triamcinolone uncouples oxidative phosphorylation by hydrolysis of P ~ C or A ~ C as follows:

$$\begin{aligned}
&AH_2 + C \rightleftharpoons AH_2 - C\\
&AH_2 - C + B \rightleftharpoons A \sim C + BH_2\\
&A \sim C + P \rightleftharpoons P \sim C + A\\
&P \sim C + ADP \rightleftharpoons ATP + C
\end{aligned}$$

DNP presumably acts on this region too.[5]

The action of triamcinolone would be due to the peculiar characteristics of its chemical structure since fluoromethyl-prednisolone, with a similar chemical structure, does not inhibit oxidative phosphorylation or the ^{32}P–ATP exchange reaction, nor does it stimulate ATPase activity.

It can be observed that triamcinolone and DNP have very similar properties. This could be confirmed by a comparison of the effects of the two compounds on experiments carried out under somewhat different conditions. DNP has been reported to protect against mitochondrial osmotic swelling[9] at concentrations which uncouple

oxidative phosphorylation. A similar effect is observed with triamcinolone, which also inhibits osmotic swelling even at concentrations having no effect on respiration or oxidative phosphorylation.

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