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The Mechanism of Action of Hydrocortisone on Mitochondrial Metabolism

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Many studies on the metabolic effects of steroids have been reported. The literature has been adequately reviewed by Gordan, Bentinck & Eisenberg (1951), Hayano & Dorfman (1951) and Lieberman & Teich (1953). Steroids have been shown to inhibit the metabolism of hexoses, phosphorylated glycolytic intermediates, tricarboxylic acid substrates and amino acids and the activity of hexokinase, tyrosinase, urease, ascorbic acid oxidase, D-amino acid oxidase, hyaluronidase, lipase and transaminase.

This study was concerned particularly with the influence of steroids on oxidative metabolism. In general, steroids inhibit the activity of enzymes requiring pyridine nucleotides for electron transfer (Guidry, Segaloff & Altschul, 1952; Sourkes & Heneage, 1952; Cochran & Du Bois, 1954). The succinoxidase system, which does not include a pyridine nucleotide, is unaffected by most steroids.

The aims of this study were to investigate the effect of 17-hydroxycorticosterone (hydrocortisone) on mitochondrial oxidation systems and to determine the mechanism of inhibitions observed. A preliminary account has been published (Gallagher, 1958).

METHODS

Enzyme preparations. Rats were killed by a blow on the neck and sheep by severing the cervical vessels. Tissues were removed rapidly and immersed in ice-cold 0.25 M-sucrose. Homogenates were prepared in 0.25 M-sucrose with glass homogenizers and fractionated by differential centrifuging at 0° (Schneider, 1948). Mitochondria were resuspended in 0.25 M-sucrose unless otherwise indicated.

Hexokinase was prepared from yeast by the Cross, Taggart, Covo & Green (1949) modification of the method of Berger, Slein, Colowick & Cori (1946). The fraction precipitated between 25 and 40% (by vol.) of ethanol was dissolved in aq. 1% glucose and stored in a frozen condition. The optimum addition of this solution for oxidative phosphorylation as determined by assay was 0.01 ml./1.5 ml. final volume.

Reagents. Adenosine triphosphate, adenosine 5-monophosphate, cytochrome c, diphosphopyridine nucleotide (DPN), nicotinamide, reduced glutathione (GSH), citrate, L-glutamate, succinate, L-malate, octanoate, ethylenediaminetetra-acetic acid, semicarbazide hydrochloride and inorganic reagents were obtained commercially. Succinic acid was recrystallized three times from hot water, then dissolved, neutralized and precipitated as the sodium salt with ethanol. The sodium succinate was then redissolved and crystallized from 80% ethanol. Octanoic acid was purified by distillation *in vacuo*. Cytochrome c was

Table 1. *Effect of hydrocortisone on oxidative metabolism by rat-liver mitochondria*

The following system was used: Adenosine monophosphate (mm); MgSO_4 (6.7 mm); KCl (25 mm); sodium potassium phosphate buffer, pH 7.4 (13.3 mm); cytochrome *c* (10 μM), ethylenediaminetetra-acetic acid (mm), mitochondria equivalent to 50 mg. of fresh liver, except for succinate oxidation (25 mg.) added in 0.25 ml. of 0.25 M-sucrose; water to 1.5 ml.; 0.1 ml. of 20% KOH in the centre well to absorb CO_2 ; gas phase, air; temperature, 38°; equilibration period, 10 min. The results given are the means of all experiments.

Additions	No. of experiments	Control	Oxygen uptake ($\mu\text{l./20 min.}$)			
			Ethanol	Hydrocortisone		
				1.0 mm	0.5 mm	0.1 mm
Citrate (10 mm)	6	37	39	19	21	32
Succinate (33 mm)	5	51	48	53	—	—
L-Malate (10 mm)	5	29	28	12	13	19
L-Glutamate (10 mm)	5	40	40	24	25	34
Octanoate (1.67 mm) + L-malate (0.67 mm)	8	49	49	28	32	36

standardized by the method of Potter (1957). Substrates were used as sodium salts. Sodium potassium phosphate buffer, pH 7.4, was prepared by the method of Umbreit (1957). Hydrocortisone, a commercial product free from contamination with heavy metals or calcium, was dissolved freshly before use in a minimal volume of re-distilled ethanol. The ethanolic solution could then be diluted with water to the required concentration. Control reactions with the same addition of ethanol only were carried out with each experiment.

Estimations. Oxygen uptake was measured with Warburg manometers. Inorganic phosphorus was determined by the method of Fiske & Subbarow (1925).

Spectrophotometric measurements. These were made with a Beckman model DU instrument.

RESULTS

Mitochondrial oxidations. Hydrocortisone selectively inhibits oxidative metabolism *in vitro* by liver mitochondria from normal rats (Table 1). Concentrations of the steroid above 0.1 mm inhibit the oxidation of citrate, L-malate, L-glutamate and octanoate. However, the succinoxidase system is not inhibited at mm-hydrocortisone. Ethanol in the concentration used to dissolve the steroid is not inhibitory.

Oxidative phosphorylation. Oxidative phosphorylation by liver mitochondria with L-glutamate as substrate is not affected significantly by 1.0 mm-hydrocortisone, although the rate of oxidation is reduced to nearly one-half the control rate (Table 2).

Effect of respiratory cofactors. Selective inhibition of the pyridine nucleotide-dependent enzymes, as opposed to succinoxidase, which does not require a pyridine nucleotide, suggests that the inhibitory mechanism might involve inactivation or loss of coenzymes. Alternatively, physical disruption of the mitochondria would give the same pattern of inhibition with loss of activity of enzymes other than succinoxidase and cytochrome oxidase. The two latter enzyme systems are associated with

Table 2. *Effect of hydrocortisone on oxidative phosphorylation*

The system was as in Table 1 + L-glutamate (10 mm); glucose (33 mm); hexokinase in 1% glucose (0.01 ml.); NaF (10 mm); mitochondria equivalent to 100 mg. of fresh rat liver in 0.25 ml. of 0.25 M-sucrose; equilibration period, 10 min.; duration of experiment, 30 min. The results given are the means of two experiments.

Additions	O_2 used ($\mu\text{g.atoms}$)	P esterified ($\mu\text{g.atoms}$)	B/A
	A	B	
None	12.2	22.5	1.8
Hydrocortisone (mm)	6.4	9.5	1.5

mitochondrial membranes, the insoluble portion of mitochondria, and are resistant to physical treatments which inactivate other enzymes (Cleland & Slater, 1953; Watson & Siekevitz, 1956).

If the inhibition were secondary to removal of pyridine nucleotides it should be possible to prevent or reverse the effect by the addition of the appropriate nucleotide in excess. Partial reversal of hydrocortisone inhibition of L-malate oxidation was achieved by adding DPN to a concentration of mm in 0.04 M-nicotinamide.

However, satisfactory prevention of hydrocortisone inhibition could not be attained unless GSH was added with the required coenzyme. Significant reversal of L-malate inhibition was provided by the addition of 0.5 mm-DPN, 0.04 M-nicotinamide and 0.67 mm-GSH, but the degree of protection varied greatly from experiment to experiment. Better and less variable reversal at this level of cofactor supplementation was obtained with sheep- than with rat-liver enzymes. More reliable and complete prevention of inhibition of rat-liver enzymes was obtained by supplementing the reaction mixture with mm-DPN (in 0.04 M-nicotinamide) and 1.3 mm-GSH (Table 3). Inhibition of octanoate oxidation was reversed more completely when 0.22 mm-coenzyme A was also added.

Pre-incubation of enzyme and substrate for 30 min. before the addition of hydrocortisone did not prevent the inhibition, which developed very rapidly and was almost completely established in the first 10 min. period (Table 4). A period of 5 min. equilibration was allowed after adding the steroid. Pre-incubation of substrate, enzyme and a supplement of the appropriate respiratory cofactors almost completely prevented the inhibitory effect of hydrocortisone (Table 4). The protection afforded by cofactors in this system was less variable and more complete from experiment to experiment than when the steroid was added to the reaction mixture without pre-incubation of the enzyme and substrate.

Mechanism of inhibition. It was thus established that hydrocortisone inhibits oxidations by dehydrogenases dependent on pyridine nucleotides for electron transfer and that such inhibition can be prevented by the addition of an appropriate supplement of respiratory cofactors. The problem now resolved itself to determining whether (a) the steroid acted directly on pyridine nucleotides and inactivated them by destruction or by the formation of complexes, or (b) the soluble cofactors were lost from the enzyme systems secondary to alteration of mitochondrial structure.

Loss of respiratory cofactors from the mitochondrial enzyme systems would occur readily if hydrocortisone increased the permeability of

Table 3. *Effect of respiratory cofactors on hydrocortisone inhibition*

The system was as in Table 1; mitochondria equivalent to 50 mg. of fresh rat liver were added in 0.25 ml. of 0.25 M-sucrose. The cofactor supplement for citrate and L-malate oxidations was: DPN (mm); nicotinamide (0.04 M); GSH (1.3 mm). The cofactor supplement for octanoate oxidation was: DPN (mm); nicotinamide (0.04 M); CoA (0.22 mm); GSH (1.3 mm). The results given are the means of all experiments.

Additions	No. of experiments	Oxygen uptake (μ l./20 min.)		
		Control	Hydrocortisone	
			1.0 mm	0.5 mm
Citrate (10 mm)	5	29	13	18
Citrate (10 mm) + cofactors	5	32	25	30
L-Malate (10 mm)	3	22	—	12
L-Malate (10 mm) + cofactors	3	27	—	21
Octanoate (1.67 mm) + L-malate (0.67 mm)	5	49	—	28
Octanoate (1.67 mm) + L-malate (0.67 mm) + cofactors	5	54	—	42

Table 4. *Effect of pre-incubation of substrate, enzyme and cofactors on hydrocortisone inhibition of oxidations by rat-liver mitochondria*

The system was as in Table 3. Hydrocortisone was added from the side-arm compartments after 30 min. incubation: equilibration for 5 min. was allowed after mixing. The results given are the means of all experiments. — indicates 'not measured'.

Additions	No. of experiments	Time (min. after mixing-equilibration)	Oxygen uptake (μ l./10 min. interval)		
			Control	Hydrocortisone	
				1.0 mm	0.5 mm
Citrate (10 mm)	4	10	18	5	10
		20	17	3	9
		30	17	2	7
Citrate (10 mm) + cofactors	4	10	23	16	21
		20	22	15	21
		30	23	16	21
L-Malate (10 mm)	2	10	12	—	8
		20	10	—	6
		30	11	—	6
L-Malate (10 mm) + cofactors	2	10	17	—	14
		20	16	—	14
		30	16	—	15
Octanoate (1.67 mm) + L-malate (0.67 mm)	4	10	18	—	9
		20	20	—	11
		30	18	—	11
Octanoate (1.67 mm) + L-malate (0.67 mm) + cofactors	4	10	20	—	18
		20	21	—	17
		30	19	—	16

mitochondrial membranes. An active measure of mitochondrial-membrane permeability may be obtained by following spectrophotometrically the rate of reduction of DPN added externally to a system in which intact mitochondria are oxidizing an exogenous substrate. The rate of reduction of DPN may be measured readily at $340\text{ m}\mu$, the absorption peak of reduced DPN, and is directly proportional in intact mitochondria to the rate of entry of DPN and substrate into the particles. The passage of DPN and substrate through the mitochondrial membranes is in turn proportional to the permeability of the membranes. Thus the rate of reduction of DPN gives a direct measure of membrane permeability (cf. Christie & Judah, 1954; Gallagher, Judah & Rees, 1956).

The permeability of mitochondria in the presence of hydrocortisone was assayed by the spectrophotometric method of Christie & Judah (1953), with

L-malate as substrate and mitochondria in 0.25 M -sucrose as enzyme. Fig. 1 shows clearly that hydrocortisone greatly accelerates the rate of increase of extinction at $340\text{ m}\mu$ ($E_{340\text{ m}\mu}$). The effect is immediate with 2 mM -hydrocortisone and becomes apparent after a short time lag with 1 mM - and 0.5 mM -hydrocortisone. Ethanol in the concentration added with hydrocortisone does not influence the rate. The $E_{340\text{ m}\mu}$ due to hydrocortisone in the complete system (except for L-malate) remains constant and is equal to the initial differences in $E_{340\text{ m}\mu}$.

Suspension of mitochondria in water eliminates the barriers to the passage of DPN and L-malate through mitochondrial membranes and allows full expression of dehydrogenase activity in the presence of excess of substrate and electron acceptor (Christie & Judah, 1953). This provided a simple method with which to assess the importance of the various mechanisms that might accelerate the increase in $E_{340\text{ m}\mu}$. Fig. 2 shows that, when the semi-permeability barriers of mitochondrial membranes have been abolished by

Fig. 1. Reduction of DPN by rat-liver mitochondria in 0.25 M -sucrose. The system was: sodium potassium phosphate buffer, pH 7.8 (33 mM); KCl (25 mM); MgSO_4 (6.7 mM); semicarbazide hydrochloride (neutralized) (0.17 M); DPN (1.5 mM); KCN (0.2 mM); $50\text{ }\mu\text{l}$. of 1% mitochondria in 0.25 M -sucrose; final volume, 3 ml .; gas phase, air; temperature, 22° . ●, 10 mM -L-Malate; □, 10 mM -L-malate + ethanol; ■, 10 mM -L-malate + 2 mM -hydrocortisone; ○, 10 mM -L-malate + 1 mM -hydrocortisone; △, 10 mM -L-malate + 0.5 mM -hydrocortisone; ▲, 2 mM -hydrocortisone.

Fig. 2. Reduction of diphosphopyridine nucleotide by rat-liver mitochondria in water. The system was as in Fig. 1 except that $50\text{ }\mu\text{l}$. of 0.5% mitochondria in water was used. ○, 10 mM -L-Malate; △, 10 mM -L-malate + ethanol; ●, 10 mM -L-malate + 1 mM -hydrocortisone; ■, 1 mM -hydrocortisone.

suspension of the particles in water, the addition of hydrocortisone has no effect on the rate of increase of $E_{340\text{ m}\mu}$. Thus the rapid increase of $E_{340\text{ m}\mu}$ exhibited by mitochondria suspended in 0.25M-sucrose in the presence of hydrocortisone is due to an increased rate of reduction of DPN secondary to greater ease of access of either or both the nucleotide and substrate across mitochondrial membranes. The permeability of mitochondrial membranes is increased greatly by hydrocortisone. A comparison of the two figures will show that, at the highest concentration (2 mM) of hydrocortisone used, membrane permeability is increased as much as by disrupting the particles with water.

Hydrocortisone, therefore, inhibits oxidative metabolism by mitochondria *in vitro* by destroying the selective semi-permeability of mitochondrial membranes. Soluble cofactors of respiration, especially pyridine nucleotides and, to a less extent, glutathione, are leached rapidly from the particles and proportionally inactivate the nucleotide-dependent dehydrogenases. The steroid has no direct effect on dehydrogenases.

DISCUSSION

In common with steroids generally, hydrocortisone has been shown to inhibit oxidative metabolism *in vitro*. The inhibitory effect is specific to those dehydrogenases which require pyridine nucleotides for electron transfer. Similar observations for other steroids were made by Guidry *et al.* (1952), Sourkes & Heneage (1952) and Cochran & Du Bois (1954).

The efficiency of oxidative phosphorylation is not lowered, even though the rate of oxidation of the substrate used is greatly inhibited. This agrees with the finding of Clark & Pesch (1956) that, although cortisone inhibited the oxidation of α -oxoglutarate, P/O ratios were normal. Similarly, Wade & Jones (1956) found that of five steroids tested, only progesterone uncoupled oxidative phosphorylation and activated adenosine triphosphatase.

The studies of Eik-Nes, Schellman, Lumry & Samuels (1954), Westphal (1955, 1957) and others have shown that steroids can combine with protein; this gives rise to the possibility that adsorption on enzyme protein *in vitro* might lead to inhibition. However, the present study suggests that hydrocortisone does not affect the dehydrogenases directly but inactivates the enzyme systems by depriving them of the soluble, respiratory cofactors, in particular the pyridine nucleotides.

Evidence that steroids can form complexes with nucleotides has been presented by Munck, Scott & Engel (1957). Complex formation seemed possibly the mechanism of inhibition. On the other hand,

better protection against hydrocortisone inhibition was achieved when GSH as well as DPN was added to the oxidizing system, and this favoured the loss of soluble cofactors from mitochondria as a likely inhibitory mechanism. It was of course possible that hydrocortisone also formed complexes with or inactivated SH groups. Anderson, Wiesel, Hillman & Stumpe (1951) suggested that deprivation of SH is the process by which cortisone inhibits the hyaluronidase system. Similarly Bacila & Guzman-Barron (1954) suggested that steroids inhibit hexokinase by combining with the SH groups of the enzyme.

The inhibitory effect of hydrocortisone may be prevented by the addition of the appropriate cofactors in excess to the reaction mixture. Such a cofactor supplement is most effective in countering the steroid inhibition if incubated with enzyme preparation and substrate before hydrocortisone is added.

Loss of respiratory cofactors is probably secondary to the effect of hydrocortisone on permeability of mitochondrial membranes. Selective semi-permeability is lost rapidly and extensively on the addition of the steroid to intact mitochondria. When permeability barriers are removed by suspension of mitochondria in water, hydrocortisone has no effect on the activity of L-malic dehydrogenase.

The mechanism of inhibition is thus probably a direct effect of hydrocortisone on the mitochondrial membrane. Permeability is increased rapidly and greatly, leading to the loss of soluble respiratory cofactors, particularly the pyridine nucleotides, from mitochondrial enzyme systems. The activities of pyridine nucleotide-dependent dehydrogenases are secondarily inhibited by lack of their electron acceptor. It seems possible that the same mechanism may be operative for other steroids.

Evidence has been published which favours a mechanism *in vivo* similar to the mode of action defined in this study. Eisenberg, Gordan & Elliott (1949*a, b*) have shown that castration of rats leads to a significant increase in brain respiration, which is reduced to almost normal rates by the administration of testosterone. On the other hand, liver and muscle respiration is not affected by castration, indicating a tissue specificity which may reflect higher sensitivity of brain or increased local concentrations of the steroid in this tissue. Clark & Pesch (1956) found that the administration of cortisone to rats led, among other things, to inhibition of α -oxoglutarate oxidation by mitochondria without lowering the efficiency of oxidative phosphorylation with this substrate. Similarly Hennes, Wajchenberg, Fajans & Conn (1957) found evidence for a block in pyruvate utilization after the administration of adrenal steroids to

normal humans, and Lacroix & Leusen (1958) found that the daily administration of cortisone to rats for 12–17 days considerably depressed the tissue-slice respiration of myocardium.

Such evidence confirms that steroids can inhibit oxidative metabolism *in vivo*. It remains to be shown that the same mechanism is operative *in vivo* and *in vitro*. Hartman (1956) found that mitochondria of the central nervous system became vacuolated after administration of cortisone to rats. This may be explained by an effect of the steroid on the mitochondrial membranes, leading to the loss of selective permeability and rendering them freely permeable. It is possible that, at least in the central nervous system, cortisone influences oxidative metabolism in the same way *in vivo* as hydrocortisone exerts its effect *in vitro*, by increasing the permeability of mitochondrial membranes. Such a mechanism indeed forms an attractive hypothesis for the manner in which adrenal steroids, and probably steroids generally, regulate metabolism.

SUMMARY

1. Hydrocortisone has been shown to inhibit oxidative metabolism *in vitro* by liver mitochondria. Oxidative phosphorylation is not affected.

2. The inhibitory effect is specific to dehydrogenases requiring pyridine nucleotides for electron transfer.

3. The steroid does not inhibit L-malic dehydrogenase directly.

4. Inhibition may be prevented by supplementing the reaction mixture with excess of pyridine nucleotide, nicotinamide, glutathione and, for octanoate oxidation, coenzyme A. The protective effect is greatest if the cofactor supplement is incubated with enzyme and substrate before hydrocortisone is added.

5. Hydrocortisone, in concentrations which produce oxidative inhibition, has been shown to increase the permeability of mitochondrial membranes. Elimination of permeability barriers by suspension of mitochondria in water, instead of in 0.25 M-sucrose, removes completely the effect of hydrocortisone on L-malic dehydrogenase activity.

6. Hydrocortisone is considered to inhibit oxidative metabolism by the following mechanism. The primary effect is to increase the permeability of mitochondrial membranes, allowing loss of soluble respiratory cofactors from mitochondrial-enzyme systems. Dehydrogenases dependent on pyridine nucleotides for electron transfer are thus proportionally inactivated.

7. The applicability of findings *in vitro* to the reported effects *in vivo* of steroids is discussed.

8. Hydrocortisone is postulated as one of the

regulatory factors governing selective semi-permeability of mitochondrial membranes *in vivo* and thereby of tissue metabolism.

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