- Edsall, J. T. (1953). *The Molecular Shapes of certain Proteins and some of their Interactions with other Substances*. A chapter in *Discussions of the Faraday Soc.* no. 13, p. 9. Aberdeen: Faraday Soc.
- Fevold, H. L. (1951). *Advanc. Protein. Chem.* **6**, 187.
- Fevold, H. L. & Lausten, A. (1946). *Arch. Biochem.* **11**, 1.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Francis, G. E. & Wormall, A. (1948). *Biochem. J.* **42**, 469.
- Gettler, A. O. & Bastian, R. (1947). *Amer. J. clin. Path.* **17**, 244.
- Gurd, F. R. N. & Goodman, D. S. (1952). *J. Amer. chem. Soc.* **74**, 670.
- Hanson, H. T. & Smith, E. L. (1949). *J. biol. Chem.* **179**, 789.
- Heath, J. C. (1949). *Nature, Lond.*, **164**, 1055.
- Heath, J. C. & Liquier-Milward, J. (1950). *Biochim. biophys. Acta*, **5**, 404.
- Hoch, F. L. & Vallee, B. L. (1952). *J. biol. Chem.* **195**, 531.
- Johnson, M. J. (1941). *J. biol. Chem.* **137**, 575.
- Jukes, T. H. & Kay, H. D. (1932). *J. exp. Med.* **56**, 469.
- King, E. J. (1932). *Biochem. J.* **26**, 292.
- Markham, R. (1942). *Biochem. J.* **36**, 790.
- Mecham, D. K. & Olcott, H. S. (1949). *J. Amer. chem. Soc.* **71**, 3670.
- Moxon, A. L. & Poley, W. E. (1938). *Poult. Sci.* **17**, 77. Cited from Romanoff & Romanoff (1949).
- Proding, W. (1940). *Organic Reagents used in Quantitative Inorganic Analysis*, p. 130. New York: Elsevier Publishing Company, Inc.
- Romanoff, A. L. & Romanoff, A. J. (1949). *The Avian Egg*. New York: Wiley.
- Rosenfeld, I. & Tobias, C. A. (1951). *J. biol. Chem.* **191**, 339.
- Ruben, S., Frenkel, A. W. & Kamen, M. D. (1942). *J. phys. Chem.* **46**, 710.
- Ruben, S., Kamen, M. D., Allen, M. B. & Nahinsky, P. (1942). *J. Amer. chem. Soc.* **64**, 2297.
- Scott, D. A. (1934). *Biochem. J.* **28**, 1592.
- Scott, D. A. & Fisher, A. M. (1935). *Biochem. J.* **29**, 1048.
- Smith, E. L. (1948). *J. biol. Chem.* **176**, 9.
- Surgenor, D. M., Koechlin, B. A. & Strong, L. E. (1949). *J. clin. Invest.* **28**, 73.
- Tupper, R., Watts, R. W. E. & Wormall, A. (1952a). *Biochem. J.* **50**, 429.
- Tupper, R., Watts, R. W. E. & Wormall, A. (1952b). *Biochem. J.* **51**, ix.
- Tupper, R., Watts, R. W. E. & Wormall, A. (1953). *J. Physiol.* **119**, 13P.
- Vallee, B. L. & Gibson, J. G. (1948a). *J. biol. Chem.* **176**, 435.
- Vallee, B. L. & Gibson, J. G. (1948b). *J. biol. Chem.* **176**, 445.
- Veall, N. (1948). *Brit. J. Radiol., N.S.*, **21**, 347.
- Warburg, O. (1949). *Heavy Metal Prosthetic Groups and Enzyme Action*, p. 191. Transl. by A. Lawson; Oxford: Clarendon Press.
- Warburg, O. & Christian, W. (1941a). *Biochem. Z.* **310**, 384.
- Warburg, O. & Christian, W. (1941b). *Naturwissenschaften*, **29**, 589.
- Yudkin, W. H. & Fruton, J. S. (1947). *J. biol. Chem.* **170**, 421.

The *in vitro* Enzymic Hydroxylation of Steroid Hormones

1. FACTORS INFLUENCING THE ENZYMIC 11β -HYDROXYLATION OF 11 -DEOXYCORTICOSTERONE

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The appreciation of the profound influence of steroid hormones of the adrenal gland on metabolism, and an interest in the biogenesis of adrenocortical hormones stimulated many workers to investigate enzymic hydroxylation of adrenal steroids. Thus Hayano, Dorfman & Prins (1949) and Hayano, Dorfman & Yamada (1951) incubated 11 -deoxycorticosterone (DOC) with adrenal slices, minces and 'homogenates' and observed an increased glycolytic activity in the products which they ascribed to the formation of 11β -hydroxysteroids. Adenosine triphosphate (ATP), fumarate and magnesium ions stimulated the reaction when 'homogenates' were used. Savard, Green & Lewis (1950) obtained evidence for the formation of 11β -hydroxy derivatives from DOC and 17α -hydroxy- 11 -deoxycorticosterone on incubation with adrenal 'homogenates' without added fumarate or other cofactors. McGinty, Smith, Wilson & Worrel (1950) isolated

17α -hydroxycorticosterone as a transformation product of 17α -hydroxy- 11 -deoxycorticosterone incubated with adrenal 'homogenates' supplemented with glucose and fumarate. Kahnt & Wettstein (1951) found that ATP or adenylic acid could replace fumarate as a cofactor in the 11β -hydroxylation reaction. Nicotinamide stimulated the reaction, suggesting a requirement for pyridine nucleotides. Glucose, inorganic phosphate and magnesium ions were without effect. These authors suggested that processes of oxidative phosphorylation may be involved, and that the steroid hydroxylation may be catalysed by enzymes of Green's 'cyclophorase' system (Green, Loomis & Auerbach, 1948). 'Cyclophorase' preparations, however, are not cytoplasmically homogeneous (Harman, 1950). Credit for showing that 11β -hydroxylating enzymes are associated with adrenal-cell particles which by their method of preparation were mainly

mitochondria, is due to Sweat (1951). He isolated crystalline 17α -hydroxycorticosterone after incubation of 17α -hydroxy- 11 -deoxycorticosterone with the adrenal-cell particles in a medium supplemented with glucose, fumarate and magnesium ions. Addition of ATP was not necessary. Recently, Hayano & Dorfman (1953) have extended their studies of steroid hydroxylation using 'residue preparations of twice washed ox-adrenal "homogenates" obtained at 5000 g'. DOC was converted predominantly into corticosterone but other unidentified products were also detected. The authors claim to have demonstrated the 'specificity and absolute necessity for fumarate and magnesium ions and the stimulating capacity of ATP and diphosphopyridine nucleotide (DPN)'. They suggest that fumarate may play some part in an energy-yielding system for the regeneration of ATP, or that it may function as a hydrogen acceptor in the steroid-hydroxylation reaction.

Much of the previous work on enzymic hydroxylation of steroids is open to the criticisms that enzyme preparations and methods of steroid analysis have not been satisfactory. These criticisms will be dealt with fully in the Discussion. Probably on account of these features of earlier investigations, the cofactor requirement for the enzymic *in vitro* hydroxylation of steroids is at present unsettled. The role of cofactors thought to be necessary is unknown and the mechanism of the 11β -hydroxylation reaction remains to be elucidated. As regards the mechanism of hydroxylation, Hayano & Dorfman (1953) have suggested the formation of an unsaturated intermediate which adds on the elements of water, whereas Levy *et al.* (1953) propose a simpler mechanism involving formation of a steroid free radical. As the authors admit, these views are at present no more than speculation.

With a view to studying these outstanding problems a preliminary investigation of the *in vitro* enzymic 11β -hydroxylation of DOC has been made and is now reported. For this purpose a purified mitochondrial preparation from ox-adrenal cortical tissue has been used. Advantage has been taken of a specific, sensitive and accurate method for the determination of DOC and related steroids in tissue preparations which has been developed in this Department (Taylor, 1954).

A preliminary report of this work has been communicated to the Biochemical Society (Brownie, Grant & Taylor, 1953).

EXPERIMENTAL

Preparation of mitochondria

Adrenal glands were obtained from the slaughterhouse from oxen killed by stunning and exsanguination. The glands were removed from the carcasses within 20 min. of death,

stripped of fat and connective tissue and placed in a deep-freeze cabinet at -20° until collected for transport to the laboratory packed in crushed ice. Subsequent operations were performed at $0-4^\circ$ and completed within 3 hr. of the death of the animal. Cortical tissues (15 g.) were separated and finely ground with 45 ml. isotonic (0.25M) sucrose using a loosely fitting pestle of an all-glass 'homogenizer' (Potter & Elvehjem, 1936). Grinding of the tissue was continued in an apparatus of similar design having a glass tube of accurately uniform internal diameter and a well-fitting Nylon pestle grooved longitudinally at three points. This apparatus has the advantage of producing 'homogenates' with few intact cells. It shows no signs of wear in use over long periods (cf. Brendler, 1951). The two-stage preparation of the 'homogenate' was adopted since it was found that adrenocortical cells were not readily broken by procedures required for the preliminary disruption of the tissue. The Waring Blendor type of apparatus was not used since it was found to give a product of low enzymic activity when run at high speed, and unacceptably low yields of mitochondria when run at low speeds. The former observation is in agreement with the findings of Stern & Bird (1949) and of Lambden (1950).

The final 'homogenate' was diluted to 90 ml. with 0.25M sucrose. Nuclei and intact cells were removed by centrifuging for 10 min. at 700 g. The supernatant was carefully removed and centrifuged twice at 5000 g for 10 min. to sediment mitochondria. Care was taken to obtain as pure a mitochondrial preparation as possible rather than a quantitative yield of these particles. Thus poorly packed mitochondria which might contain some microsomes were discarded during removal of the supernatant (cf. Schneider, 1949). The mitochondrial pellets were combined, washed with 0.25M sucrose and resedimented at 21 000 g for 10 min. In later preparations it has been found convenient to obtain firmly packed mitochondria by final sedimentation from isotonic (0.154M) KCl. The wet weight of the mitochondrial pellet (about 2 g.) was determined after decanting the supernatant fluid and carefully drying the walls of the centrifuge tube with filter paper. The mitochondria were finally thoroughly dispersed in 9 vol. 0.154M-KCl. Preparations from the last sucrose wash were occasionally examined under a phase-contrast microscope. Whole cells were never observed, single nuclei very occasionally. Purity was also occasionally checked by a final centrifugation of part of the preparation from a 0.01% solution of janus green B prepared in 0.25M sucrose. On incubation for 5 min. at 37° the blue-stained pellet in the centrifuge tube turned red. This reaction is claimed to be specific for mitochondria (Potter, Rechnagel & Hurlbert, 1951). The pellet is not disturbed in this test. If nuclei and microsomes are present as contaminants they may be distinguished at the lower and upper surfaces of the pellet by their staining characteristics which differ from those of the mitochondria.

Preparation of 11-deoxycorticosterone

11 -Deoxycorticosterone acetate was hydrolysed at 37° by the method of Mattox & Kendall (1951). DOC was recovered from the acid hydrolysate by extraction with CHCl_3 . The residue obtained after distillation of the washed CHCl_3 extract was crystallized from ether and ether:acetone (Reichstein & Euw, 1938) to give a product melting at $138-142^\circ$, unchanged by admixture with an authentic sample of DOC. $[\alpha]_D^{25} + 178.8 \pm 1.2^\circ$ in ethanol (c, 0.493).

Table 1. *Recovery of DOC and corticosterone added to reaction mixtures which had been incubated with adrenocortical mitochondria*

Values have been corrected by subtraction of 'apparent' steroid recovered in blank control experiments in which no steroids were added—20 μ g. for either steroid.

DOC			Corticosterone		
Added (μ g.)	Recovered (μ g.)	Recovery (%)	Added (μ g.)	Recovered (μ g.)	Recovery (%)
524	500	96	125	122	97
470	465	99	99	97	98
36	34	94	36	33	92

Steiger & Reichstein (1937) reported $[\alpha]_D^{22} + 178 \pm 3^\circ$ for DOC in ethanol (*c*, 1.5). (Found: C, 76.3; H, 9.2. Calc. for $C_{21}H_{30}O_3$: C, 76.2; H, 9.1%.)

Incubation conditions

Unless otherwise stated, in all experiments 25 ml. conical flasks were charged with the following reaction mixture: 0.095 M-KCl, 0.004 M-MgSO₄, 0.04 M potassium phosphate, pH 7.4, and a mitochondrial suspension in KCl solution containing about 1.4 mg. total N. The total vol. was 3 ml. Members of the citric acid cycle, cofactors or inhibitors were added as potassium salts wherever possible in solution at pH 7.4. When additions were made to the reaction mixture the amount of KCl present was adjusted to maintain a total cationic concentration of 0.141–0.151 M. A propylene glycol (propane-1:2-diol) solution of DOC, containing about 500 μ g. steroid in 0.04 ml., was added from a Burroughs Wellcome 'Agla' microburette immediately after addition of the mitochondrial preparation. The best results were obtained in this way and it seems likely that the steroid is precipitated out of solution on to the mitochondrial particles and is thus more readily assimilated. Glass-distilled water was used for all solutions and pH values were checked by glass electrode. Unless otherwise stated, incubations were in air, shaking for 1 hr. at 37°.

Determination of DOC and corticosterone

DOC and corticosterone in reaction mixtures were determined by an adaptation of the method described by Taylor (1954) for progesterone. Acetone extracts were not chilled. The aqueous-acetone filtrate obtained after removal of protein was distilled to an aqueous residue from which steroids were extracted by benzene:chloroform (6:1, v/v). DOC in a portion of this extract was separated by partition chromatography on a Celite column using a benzene:hexane (3:7, v/v) mobile phase and methanol:water (7:3, v/v) stationary phase. The eluate fraction from 15 to 25 ml. contained DOC. Corticosterone was separated similarly from another portion of the benzene:chloroform extract using the solvent system benzene:hexane (8:2, v/v), methanol:water (7:3, v/v), and was eluted in the fraction from 18 to 26 ml. Fractions containing DOC or corticosterone were evaporated to dryness under an air stream. The residues were taken up in ethanol and the absorption of solutions measured at 240 m μ . in a 1 cm. cell of a Unicam SP 500 Spectrophotometer. The amounts of steroid present were found by reference to calibration curves.

Typical results for the recovery of DOC and corticosterone added to incubated reaction mixtures are shown in Table 1.

Table 2. *Recovery of steroids absorbing selectively at 240 m μ . in DOC and corticosterone chromatogram fractions after incubation of DOC with adrenocortical mitochondria*

DOC incubated (μ moles)	DOC recovered (μ moles)	Cortico- sterone recovered (μ moles)	Total recovery	
			(μ moles)	(%)
1.72	0.26	1.32	1.58	92
1.42	0.27	1.07	1.34	94.5

In a series of 20 experiments DOC recoveries at the 500 μ g. level were $96 \pm 1\%$.

Every experiment included duplicate flasks to which DOC was added after incubation to permit a check to be made of the extraction procedure ('extraction controls'). 'Apparent' DOC or corticosterone was determined in experiments without added steroid ('blank controls'); the values so found were consistently very low and subsequently were only determined occasionally. A mean blank value was subtracted in all DOC and corticosterone determinations. The concentration of DOC in the propylene glycol solutions used was determined accurately in every experiment by measuring the optical density at 240 m μ . after dilution with ethanol, and by reference to a calibration curve. The values found were used in calculations of percentage DOC recovered in 'extraction controls' and hydroxylation experiments.

RESULTS

Identification of corticosterone as the product obtained on incubation of DOC with adrenocortical mitochondria

For the experiments to be described in this section DOC was incubated with adrenocortical mitochondria in reaction mixtures containing 0.002 M fumarate. The metabolic product eluted from Celite columns in the 'corticosterone fraction' described in the Experimental section absorbed selectively at 240 m μ . and accounted quantitatively for the amount of DOC which had disappeared. Typical results are shown in Table 2.

In support of this suggestive evidence that the single metabolic product is corticosterone, the material eluted in the 'corticosterone fraction' was run on paper-strip chromatograms using the solvent systems *B*₁, *B*₄ and *B*₅ of Bush (1952). Mixed chromatograms of authentic corticosterone and

metabolic product, metabolic product alone and authentic corticosterone alone were run and developed with the 15% phosphoric acid reagent of Neher & Wettstein (1951). All chromatograms showed spots with a green fluorescence in the ultra-violet, which had run to the same position on the strips. Approximately 50 μ g. quantities of metabolic product, authentic DOC and corticosterone were treated with 4 ml. conc. sulphuric acid for 1 hr. at 37° (Zaffaroni, 1950), and absorption curves of the chromogens were determined in a Unicam SP 500 Spectrophotometer. Curves with metabolic product and corticosterone showed maxima at 285, 326, 372 m μ . and between 455 and 465 m μ .

Thus although complete proof has not been obtained, it appears very probable that the predominant or single metabolic product obtained on incubation of adrenocortical mitochondria with DOC is corticosterone.

Citric acid cycle intermediates as activators of DOC 11 β -hydroxylation

The results of typical experiments with and without intermediates of the citric acid cycle (called for brevity 'cycle intermediates') (0.002M) added to the reaction mixture are shown in Fig. 1. It is evident that the presence of certain 'cycle intermediates' will permit the 11 β -hydroxylation of DOC. Pyruvate has a negligible effect, whereas citrate, oxaloacetate, L-malate, fumarate, succinate and α -ketoglutarate (α -oxoglutarate) are effective. 0.0005M-ATP or adenosine diphosphate (ADP) alone are without effect but these substances enhance the hydroxylation obtained with added 'cycle intermediates'. A typical result with succinate supplemented with ATP is included in Fig. 1.

The observed requirements for 'cycle intermediates' in the DOC hydroxylation reaction suggested the possibility of a relationship between steroid hydroxylation and oxidation of the added 'cycle intermediate'. This was investigated by oxygen-consumption measurements made by the usual Warburg technique (Umbreit, 1949). From the results shown in Fig. 1, it may be seen that the 'cycle intermediates' which are oxidized more rapidly are the more effective activators of DOC hydroxylation. ATP which increased the rate of oxidation of 'cycle intermediates' has a corresponding stimulating effect on their ability to activate DOC hydroxylation. Similar results were obtained with ADP. These respiration studies are complicated by the observation that the oxidation of the 'cycle intermediate' is inhibited by the DOC present. In circumstances, under which the inhibition by DOC is sufficiently marked, steroid hydroxylation may be completely suppressed. Such a case is illustrated in Table 3. The use of oxaloacetate as

Fig. 1. DOC 11 β -hydroxylation and oxygen consumption by adrenocortical mitochondria incubated in air for 1 hr. at 37°. Reaction mixture contained 0.095M-KCl, 0.004M-MgSO₄, 0.04M potassium phosphate (pH 7.4); 'activators' added as 0.002M potassium salts (pH 7.4), ATP as 0.0005M potassium salt (pH 7.4), mitochondria (about 1.4 mg. total N) and 0.04 ml. propylene glycol containing about 500 μ g. DOC; total volume, 3 ml.

Table 3. Influence of DOC on oxidation of tricarboxylic acid cycle intermediates in relation to DOC hydroxylation

DOC was added as a solution in 0.04 ml. propylene glycol. Propylene glycol alone was added to vessels without DOC.

DOC added (μ g.)	DOC recovered (μ g.)	DOC recovered (%)	'Cycle intermediate' (0.002M)	-Q _{O₂}
0	—	—	Oxaloacetate	5.6
490	460	94	Oxaloacetate	0.6
0	—	—	L-Malate	10.6
500	5	1	L-Malate	7.8

'activator' normally permits about 40% hydroxylation of DOC (see Fig. 1).

The circumstances giving rise to the differing results reported in Table 3 and Fig. 1 are at present not clear. It is possible that the difference may be attributed to variations in the enzyme preparation or to the age of the adrenal glands used.

In the present case suppression of oxidation has resulted in failure of DOC hydroxylation. When malate was used as 'activator', oxidation was not as markedly suppressed by the added steroid, and hydroxylation of DOC was almost complete.

The effect of magnesium ions on 11 β -hydroxylation of DOC

Cohen (1953) observed an increased requirement for magnesium ion when citrate oxidation is used as an energy source for certain synthetic reactions in liver 'homogenates'. As this offered an explanation for the poor activation of DOC hydroxylation by citrate, the effect of varying the magnesium ion concentration was studied. The results shown in

Fig. 2 indicate no increase in DOC hydroxylation in presence of citrate with increased concentration of magnesium ion. In view of the claims of Hayano & Dorfman (1953) it was surprising to observe (Fig. 2) that DOC hydroxylation was diminished but not abolished when added magnesium ion was absent from the reaction mixture using succinate as activator. A 2 g. mitochondrial pellet containing 30 mg. total N was ashed and found to contain less than $10\text{ }\mu\text{g.}$ magnesium oxide by spectrographic analysis. Thus the usual reaction mixtures containing 1.4 mg. mitochondrial total N might be expected to contain less than $3.8 \times 10^{-6}\text{ M}$ magnesium ion.

Effect of different concentrations of fumarate and succinate on 11 β -hydroxylation of DOC

If fumarate functions as a hydrogen acceptor for DOC oxidation, according to the reaction $\text{fumarate} + 2\text{H} \rightarrow \text{succinate}$, an adequate concentration of fumarate might be expected to permit 11 β -hydroxylation to proceed in the absence of oxygen. Similarly, increasing amounts of succinate might be expected to have an inhibitory effect. To test the first possibility, anaerobic experiments with varying concentrations of fumarate were performed in evacuated Thunberg tubes. Results shown in Fig. 3 (a) indicate that no hydroxylation occurs in absence of oxygen with concentrations of fumarate up to 0.01 M. The extensive metabolism of DOC in the control tubes opened to the air after evacuation demonstrates that the mitochondrial enzymes involved are undamaged by subjection to low pressures.

Fig. 2. Effect of Mg^{2+} on 11 β -hydroxylation of DOC by adrenocortical mitochondria incubated in air for 1 hr. at 37°. Reaction mixture contained 0.095 M-KCl, 0.04 M potassium phosphate (pH 7.4); 'activators' added as 0.002 M potassium salts (pH 7.4), ATP as 0.0005 M potassium salt (pH 7.4), mitochondria (about 1.4 mg. total N), MgSO_4 in concentration shown and 0.04 ml. propylene glycol containing about 500 $\mu\text{g.}$ DOC; total volume, 3 ml. Double lines at the ends of bars indicate duplicate values.

The effect of increasing the concentration of succinate (Fig. 3 (b)) was to increase DOC metabolism.

The effect of malonate and the activation of DOC hydroxylation by the oxidation of α -oxoglutarate to succinate

The results of experiments described in the previous sections indicate that the concurrent oxidation of certain 'cycle intermediates' will enable 11 β -hydroxylation of DOC to proceed. These experiments, however, have not eliminated the possibility of some special requirement for fumarate since this substance will be formed from other intermediates by operation of the citric acid cycle. The formation of fumarate may be prevented by the use of malonate.

Further, it appeared to be of interest to determine whether the 'one step' reaction α -oxoglutarate $\rightarrow$ succinate could activate 11 β -hydroxylation of DOC. The results shown in Fig. 4 indicate that the

Fig. 3 (a). Effect of varying fumarate concentrations on 11 β -hydroxylation of DOC.

Fig. 3 (b). Effect of varying succinate concentration on 11 β -hydroxylation of DOC in adrenocortical mitochondria. Reaction mixtures contained 0.095 M-KCl, 0.004 M-MgSO₄, 0.04 M potassium phosphate (pH 7.4); 'activators' added as potassium salts (pH 7.4), mitochondria (about 1.4 mg. total N) and 0.04 ml. propylene glycol containing about 500 $\mu\text{g.}$ DOC; total volume, 3 ml. Incubation for 1 hr. at 37°. Double lines at the ends of bars indicate duplicate values.

activation of hydroxylation by 0.002M succinate is almost completely abolished by 0.01M malonate. With succinate oxidation blocked in this way it was observed (Fig. 4) that the 'one step' oxidation α -oxoglutarate $\rightarrow$ succinate enabled DOC hydroxylation to proceed. It thus appears that any specific role for fumarate is excluded.

Effect of 2:4-dinitrophenol on 11 β -hydroxylation of DOC

Since the 'cycle intermediates' might be involved in processes of oxidative phosphorylation resulting in the activation of DOC for hydroxylation, the effect of 2:4-dinitrophenol on the reaction was investigated. This substance is well known to 'uncouple' oxidative phosphorylation (Loomis & Lipmann, 1948). The addition of 2×10^{-4} M dinitro-

Fig. 4. Effect of malonate on 11 β -hydroxylation of DOC by adrenocortical mitochondria incubated in air for 1 hr. at 37°. Reaction mixtures contained 0.095M-KCl, 0.004M-MgSO₄, 0.04M potassium phosphate (pH 7.4), ATP as 0.0005M potassium salt (pH 7.4), malonate as potassium salt (pH 7.4), at concentration shown, mitochondria (about 1.4 mg. total N) and 0.04 ml. propylene glycol containing about 500 μ g. DOC; total volume, 3 ml. 'Activators' added as 0.002M potassium salts (pH 7.4). Double lines at the ends of bars indicate duplicate values.

Fig. 5. Effect of 2:4-dinitrophenol on 11 β -hydroxylation of DOC and oxygen consumption. Reaction mixture contained 0.095M-KCl, 0.004M-MgSO₄, 0.04M potassium phosphate (pH 7.4), fumarate added as 0.002M potassium salt (pH 7.4), ATP as 0.0005M potassium salt (pH 7.4), 2:4-dinitrophenol as 0.0002M potassium salt (pH 7.4), mitochondria (about 1.4 mg. total N) and 0.04 ml. propylene glycol containing about 500 μ g. DOC; total volume, 3 ml. Incubation in air for 1 hr. at 37°.

phenol caused marked inhibition of DOC hydroxylation without having any significant effect on the observed oxygen uptake (Fig. 5).

DISCUSSION

In the preliminary investigation of the *in vitro* enzymic hydroxylation of steroids now reported, a method for the determination of DOC has been used which permits the recovery of $96 \pm 1\%$ of this steroid added to preparations of mitochondria. After incubation of DOC with the mitochondria, recoveries of unchanged DOC plus metabolic product as measured by specific absorption at 240 m μ . were of the same order. The percentage recoveries are based upon the actual amount of DOC added to the reaction mixture. These results compare favourably with those obtained by analytical procedures previously reported. For example, Hayano & Dorfman (1953) have made use of a method which permits the recovery of 86% DOC in unincubated control experiments. After incubation the recovery of unchanged DOC plus metabolic products was $80 \pm 5\%$. These authors state that 'these figures were taken as indices of 100 in the calculation of the percentage of products formed'. Percentage recovery of metabolic products would thus appear unduly high.

In the present investigation the identity of the metabolic product with corticosterone and the complete absence of other products have not been proved with certainty. Nevertheless, the evidence obtained supports the view that DOC hydroxylation in adrenocortical mitochondria may be largely restricted to the 11 β position. This concept may be of more general application, since Plager & Samuels (1953) have shown that hydroxylation of progesterone at the 17 and 21 positions is catalysed by enzymes in the 'supernatant' fraction of adrenal 'homogenates', and that further hydroxylation at the 11 β position is brought about by the mitochondrial fraction.

The enzyme system responsible for steroid 11 β -hydroxylation appears to be associated with the mitochondria of adrenocortical cells (Sweet, 1951). It is not yet known which cells are concerned nor have the enzymes been obtained in soluble form. Although the best methods available at present for the preparation of morphologically and biochemically intact mitochondria are far from ideal, they are preferable to the procedures frequently used in preparation of adrenal tissue for steroid-hydroxylation studies. Thus, for example, adrenal cell 'homogenates' have been prepared in water or saline by the Waring Blendor (Hayano & Dorfman, 1952; Hayano *et al.* 1951), which when used without speed reduction is known to break up nuclei and mitochondria as well as whole cells (Hogeboom, 1951; Sarkar, Beinert, Fuld & Green, 1952). The

mitochondria in such preparations exposed to water or hypotonic media sustain damage resulting in the release of water-soluble proteins, nucleotides, enzymes and cofactors (Kennedy & Lehninger, 1949; Huennekens & Green, 1950; Schneider, 1949; Berthet, Berthet, Appelmans & Duve, 1951). Incubation of whole 'homogenates' results in extensive destruction of pyridine nucleotides by enzymes released from damaged cell components (Mann & Quastel, 1941). Saline-washed sediments are relatively free from soluble proteins including released nucleotidases. Nevertheless, they contain a mixture of nuclei and mitochondria, intact and damaged by the Waring Blendor and by exposure to saline, and of microsomes agglutinated by the electrolytes present (Schneider & Hogeboom, 1951). The use of such preparations in the study of steroid hydroxylation (Hayano & Dorfman, 1953) must incur the danger of producing artificial steroid metabolic products by interruption of normal metabolic pathways in damaged-cell component particles or by abnormal interaction of enzymes from different cell components. Experiments with washed sediments can give no indication of the location of steroid-hydroxylating enzyme systems in the cell.

The use of mitochondria isolated in sucrose solutions offers several advantages in the preliminary study of steroid hydroxylation. Whole cells, nuclei and soluble proteins may be eliminated so that their effect need not be considered when interpreting results. Contamination by microsomes is not extensive. Schneider (1953) has estimated this to be about 5% of the volume of the mitochondrial sediment. The reduction in the amount of cellular material employed simplifies steroid analysis. Isolated mitochondria retain many dehydrogenases with intact hydrogen- and electron-transporting mechanisms and can catalyse oxidative phosphorylation (Potter, Lyle & Schneider, 1951). Hayano & Dorfman (1953), using washed sediments, were unable to demonstrate 11 β -hydroxylation of DOC in absence of added magnesium. The 11 β -hydroxylation of DOC observed in experiments now reported in which added magnesium was not required may reflect the intact state of the mitochondria employed. With mitochondria, corticosterone has been obtained as the probable single product of DOC hydroxylation. Hayano & Dorfman (1953) obtained corticosterone and other unidentified products. It remains to be proved that these other products are not artifacts produced by the washed sediment enzyme preparation used by these workers.

Although the use of isolated mitochondria has advantages for the preliminary study of steroid hydroxylation it must not be forgotten that other cell components may have an influence on steroid hydroxylation as it occurs in the intact adrenal cell. 11 β -Hydroxylating enzymes may occur in other

parts of the cell but may be dependent on mitochondria for energy production or for the provision of terminal electron-transporting mechanisms. These possibilities might be investigated by determining the 11 β -hydroxylating capacity of the whole cell as compared with that of mitochondria isolated in maximum yield. Schneider & Hogeboom (1951) and Schneider (1953) have drawn attention to the importance and to the difficulties of obtaining evidence of this nature.

Any explanation of steroid 11 β -hydroxylation must account for the role of members of the citric acid cycle in the reaction. The results of the present investigation have confirmed the observations of other workers that the concurrent oxidation of members of the citric acid cycle permits steroid 11 β -hydroxylation to proceed. It has now been shown that 11 β -hydroxylation is inhibited if succinate oxidation is blocked by malonate. The 'single step' oxidation of α -oxoglutarate to succinate in the malonate-blocked system has been shown to permit 11 β -hydroxylation. This observation eliminates the possibility that fumarate has some specific role in the reaction. The failure of fumarate to stimulate 11 β -hydroxylation under anaerobic conditions, and the failure of high concentrations of succinate to inhibit 11 β -hydroxylation do not support the suggestion that fumarate acts as a hydrogen acceptor. It is very likely that added fumarate enters the citric acid cycle and thereby provides substrates for oxidative reactions coupled with 11 β -hydroxylation.

A number of explanations may be offered for the variation in efficiency of different substances as activators of 11 β -hydroxylation. Oxaloacetate is not an efficient activator. Low concentrations of this substance inhibit oxidation of succinate (Pardee, Potter & Lyle, 1948) and malate (Elliott, 1941). Less efficient functioning of the citric acid cycle and less rapid 11 β -hydroxylation of DOC would thus be expected when oxaloacetate is added as activator as compared with succinate which has no such inhibitory actions. The poor activation obtained with citrate is not explained by the ability of this substance to remove magnesium ions. The observation that citrate does not readily pass through mitochondrial membranes (Schneider, 1953) offers a probable explanation of the poor effect of added citrate. In addition, it is known that the isocitric dehydrogenase activity of isolated mitochondria is low (Hogeboom & Schneider, 1950). These observations indicate a limitation of the *in vitro* technique since mitochondria in the intact cell are not normally confronted with the necessity to utilize high concentrations of individual members of the citric acid cycle.

Kahnt & Wettstein (1951) suggested that oxidative phosphorylation might be involved in

steroid 11β -hydroxylation, but hitherto no evidence has been produced to support this suggestion. It has now been shown that 2:4-dinitrophenol inhibits 11β -hydroxylation of DOC without decreasing the oxidation of the activator. 2:4-Dinitrophenol used in low concentrations, which do not inhibit respiration, is well known to 'uncouple' phosphorylation from oxidation (Loomis & Lipmann, 1948). The role of members of the citric acid cycle in the 11β -hydroxylation of DOC would thus appear to be that of oxidizable substrates for processes of oxidative phosphorylation possibly involved in activation of the DOC before hydroxylation.

In view of the successful linking of 11β -hydroxylation of DOC with the 'single step' oxidation of α -oxoglutarate to succinate, it is interesting to note that Copenhaver & Lardy (1952) observed the highest 'phosphorus esterified/oxygen consumed' ratio for this particular step of the citric acid cycle.

Only those oxidations involving members of the citric acid cycle as substrates have been considered in this report. It is possible, however, that other oxidations coupled with phosphorylation occurring in the adrenal cortex cells are equally capable of 'activating' 11β -hydroxylation of DOC. Since it is probable that in many cases oxidative phosphorylation is associated with hydrogen- and electron-transporting steps of biological oxidation rather than with the primary dehydrogenation (Hunter, 1951), oxidation of reduced pyridine nucleotide coenzymes would be expected to activate 11β -hydroxylation of steroids. Indeed the linking of such hydroxylations with processes of oxidative phosphorylation calls for care when discussing cofactor requirements. It will be necessary to distinguish between cofactors involved in energy-producing reactions and those concerned more directly with steroid hydroxylations. In this connexion the observation of Hayano, Wiener & Lindberg (1953) is of interest. They found that triphosphopyridine nucleotide alone was able to replace the three cofactors—magnesium ions, adenosine triphosphate and diphosphopyridine nucleotide—which were required to regenerate the steroid-hydroxylating activity of enzyme preparations from stored adrenal tissue. A probable interpretation of this result is that triphosphopyridine nucleotide, required for some step of the hydroxylation reaction, is washed out of or destroyed by damaged particles prepared from the stored tissue cells. The enzyme preparation may still retain the ability to synthesize triphosphopyridine nucleotide from diphosphopyridine nucleotide and adenosine triphosphate with magnesium ion as cofactor by reactions described by Kornberg (1951). It remains to be proved however that triphosphopyridine nucleotide acts as a hydrogen acceptor for a reaction in which DOC is directly involved.

It has usually been found necessary to add magnesium ions to reaction mixtures when studying oxidative phosphorylations (Lehninger, 1951). If such reactions are required for activation of 11β -hydroxylation of DOC, the hydroxylation reported above with very low magnesium ion concentrations would be difficult to explain. It is, however, equally difficult to explain the failure to inhibit oxidative phosphorylation by 0.02M ethylenediaminetetraacetic acid (Lehninger, 1951) since this substance is known to form magnesium complexes with extremely low dissociation constants.

The observation that added adenosine triphosphate alone is unable to activate 11β -hydroxylation of DOC may be related to the similar observation of Kennedy & Lehninger (1948, 1951) in the case of the 'priming' of fatty-acid oxidations. This may find an explanation in the fact that added adenosine triphosphate is less readily available for intramitochondrial reactions, than that generated within the mitochondria (Siekevitz & Potter, 1953).

It has frequently been observed that certain adrenocortical steroids inhibit biological respirations *in vitro*. In particular, Sourkes & Heneage (1952) found that DOC inhibited the oxidation of certain citric acid cycle intermediates by rat-adrenal slices. Hayano & Dorfman (1953) noted the inhibitory effect of increasing concentrations of DOC on the 11β -hydroxylation of this steroid by adrenal-tissue preparations. The significance of these observations in relation to 11β -hydroxylation may be more fully appreciated now that it has been shown that the hydroxylation reaction depends upon concurrent oxidation of suitable substrates, as for instance in the operation of the citric acid cycle, coupled with phosphorylation. The rate of steroid hydroxylation may thus be controlled by the amount of steroid present, the control being effected by the influence of the steroid on oxidations of the citric acid cycle. It is not yet possible to decide whether such a control mechanism has any physiological significance. It points, however, to the possibility that hormones influence enzyme reactions while they themselves are undergoing metabolic changes.

In view of the obvious complexity of the enzymic 11β -hydroxylation of steroids it seems premature to adopt the name ' 11β -hydroxylase' proposed by Hayano & Dorfman (1953). The term ' 11β -hydroxylating enzyme system' would be more appropriate at present.

SUMMARY

1. A preliminary investigation of the *in vitro* 11β -hydroxylation of 11-deoxycorticosterone (DOC) by ox-adrenocortical mitochondria has been made.
2. The reaction has been shown to require concurrent oxidative phosphorylation, for which added

citric acid cycle intermediates are oxidizable substrates. The relative efficiency of the intermediates in this role is discussed.

3. Fumarate is not specifically required and the reaction will proceed in the absence of added magnesium ions.

4. The preparation of cell fractions containing steroid 11 β -hydroxylating enzymes is discussed. Cofactor requirements are discussed in relation to energy-producing and steroid-hydroxylating reactions.

5. Attention has been drawn to the manner in which certain steroids may influence their own hydroxylation through their effect on oxidative reactions of the citric acid cycle.

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REFERENCES

- Berthet, J., Berthet, L., Appelmans, F. & Duve, C. de, (1951). *Biochem. J.* **50**, 182.
- Brendler, H. (1951). *Science*, **114**, 61.
- Brownie, A. C., Grant, J. K. & Taylor, W. (1953). *Biochem. J.* **55**, xxii.
- Bush, I. E. (1952). *Biochem. J.* **50**, 370.
- Cohen, S. (1953). *J. biol. Chem.* **201**, 93.
- Copenhaver, J. H. & Lardy, H. A. (1952). *J. biol. Chem.* **195**, 225.
- Elliott, K. A. C. (1941). *Physiol. Rev.* **21**, 267.
- Green, D. E., Loomis, W. F. & Auerbach, V. H. (1948). *J. biol. Chem.* **172**, 389.
- Harman, J. W. (1950). *Exp. Cell Res.* **1**, 382.
- Hayano, M. & Dorfman, R. I. (1952). *Arch. Biochem. Biophys.* **36**, 237.
- Hayano, M. & Dorfman, R. I. (1953). *J. biol. Chem.* **201**, 175.
- Hayano, M., Dorfman, R. I. & Prins, D. A. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 700.
- Hayano, M., Dorfman, R. I. & Yamada, E. Y. (1951). *J. biol. Chem.* **193**, 175.
- Hayano, M., Wiener, M. & Lindberg, M. C. (1953). *Fed. Proc.* **12**, 216.
- Hogeboom, G. H. (1951). *Fed. Proc.* **10**, 640.
- Hogeboom, G. H. & Schneider, W. C. (1950). *J. biol. Chem.* **186**, 417.
- Huennekens, F. M. & Green, D. E. (1950). *Arch. Biochem.* **27**, 418.
- Hunter, F. E. (1951). In *Phosphorus Metabolism*, **1**, 297, eds. W. D. McElroy and B. Glass. Baltimore: The Johns Hopkins Press.
- Kahnt, F. W. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 1790.
- Kennedy, E. P. & Lehninger, A. L. (1948). *J. biol. Chem.* **173**, 753.
- Kennedy, E. P. & Lehninger, A. L. (1949). *J. biol. Chem.* **179**, 957.
- Kennedy, E. P. & Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 361.
- Kornberg, A. (1951). In *Phosphorus Metabolism*, **1**, 392, eds. W. D. McElroy and B. Glass. Baltimore: The Johns Hopkins Press.
- Lambden, M. P. (1950). *Fed. Proc.* **9**, 193.
- Lehninger, A. L. (1951). In *Phosphorus Metabolism*, **1**, 344, eds. W. D. McElroy and B. Glass. Baltimore: The Johns Hopkins Press.
- Levy, H., Jeanloz, R. W., Marshall, C. W., Jacobsen, R. P., Hechter, O., Schenker, V. & Pincus, G. (1953). *J. biol. Chem.* **203**, 433.
- Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
- McGinty, D. A., Smith, G. N., Wilson, M. L. & Worrel, C. S. (1950). *Science*, **112**, 506.
- Mann, J. P. C. & Quastel, J. H. (1941). *Biochem. J.* **35**, 502.
- Mattox, V. R. & Kendall, E. C. (1951). *J. biol. Chem.* **188**, 287.
- Neher, R. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 2278.
- Pardee, A. B., Potter, V. R. & Lyle, G. G. (1948). *J. biol. Chem.* **176**, 1075.
- Plager, J. E. & Samuels, L. T. (1953). *Arch. Biochem. Biophys.* **43**, 476.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Potter, V. R., Lyle, G. G. & Schneider, W. C. (1951). *J. biol. Chem.* **190**, 293.
- Potter, V. R., Recknagel, R. O. & Hurlbert, B. (1951). *Fed. Proc.* **10**, 646.
- Reichstein, T. & Euw, J. von (1938). *Helv. chim. acta*, **21**, 1197.
- Sarkar, N. K., Beinert, H., Fuld, M. & Green, D. E. (1952). *Arch. Biochem. Biophys.* **37**, 140.
- Savard, K., Green, A. & Lewis, L. A. (1950). *Endocrinology*, **47**, 418.
- Schneider, W. C. (1949). In *Manometric Techniques and Tissue Metabolism*, p. 148, by W. W. Umbreit, R. H. Burris and J. F. Stauffer. Minneapolis: Burgess Publishing Co.
- Schneider, W. C. (1953). *J. Histochem. Cytochem.* **1**, 212.
- Schneider, W. C. & Hogeboom, G. H. (1951). *Cancer Res.* **11**, 1.
- Siekevitz, P. & Potter, V. R. (1953). *J. biol. Chem.* **201**, 1.
- Sourkes, T. L. & Heneage, P. (1952). *Endocrinology*, **50**, 73.
- Steiger, M. & Reichstein, T. (1937). *Helv. chim. acta*, **20**, 1164.
- Stern, R. & Bird, L. H. (1949). *Biochem. J.* **44**, 635.
- Sweat, M. L. (1951). *J. Amer. chem. Soc.* **73**, 4056.
- Taylor, W. (1954). *Biochem. J.* **56**, 463.
- Umbreit, W. W. (1949). In *Manometric Techniques and Tissue Metabolism*, p. 1, by W. W. Umbreit, R. H. Burris and J. F. Stauffer. Minneapolis: Burgess Publishing Co.
- Zaffaroni, A. (1950). *J. Amer. chem. Soc.* **73**, 3828.