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The *in vitro* Enzymic Hydroxylation of Steroid Hormones

3. AN INVESTIGATION OF THE ROLE OF FUMARATE AND PYRIDINE NUCLEOTIDES IN THE ENZYMIC 11 β -HYDROXYLATION OF 11-DEOXYCORTICOSTERONE*

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Hayano & Dorfman (1953) have claimed 'specificity and absolute necessity for fumarate' for the 11 β -hydroxylation of 11-deoxycorticosterone (DOC) when using 'residue preparations of twice washed ox-adrenal homogenates obtained at 5000 g'. More recently (Hayano & Dorfman, 1954) they have made the same observation with acetone-dried 'homogenate residues' as a source of the hydroxylating enzyme. We have investigated the 11 β -hydroxylation of DOC using ox-adrenocortical mitochondria suspensions prepared in sucrose solution (Brownie & Grant, 1954*a*). With these suspensions 11 β -hydroxylation of DOC occurs readily provided that there is concurrent oxidation of a member of the Krebs tricarboxylic acid cycle. If the simultaneous oxidation of succinate, for instance, is inhibited by malonate, 11 β -hydroxylation does not occur. However, on addition of α -oxoglutarate to the malonate-blocked system, 11 β -hydroxylation of DOC proceeds. From these observations we concluded that there is no absolute and specific necessity for fumarate for 11 β -hydroxylation by intact ox-adrenocortical mitochondria. We also concluded from other evidence presented (Brownie & Grant, 1954*a*) that 11 β -hydroxylation by intact mitochondria requires concurrent oxidative phosphorylation, for which members of the tricarboxylic acid cycle are oxidizable substrates.

In order to investigate the differences between our results and those of Hayano & Dorfman (1953), and in order to obtain further information on the cofactor requirements of the 11 β -hydroxylating enzyme system, we have performed experiments with deliberately damaged mitochondria. The results of these experiments are now reported.

A preliminary account of this work has been communicated to the Biochemical Society (Brownie & Grant, 1954*b*).

EXPERIMENTAL

Enzyme preparations, materials and methods

Suspensions of ox-adrenocortical mitochondria were prepared as already described (Brownie & Grant, 1954*a*; Brownie *et al.* 1954). These will be referred to as 'fresh mitochondria'. Fresh mitochondria were subjected to three types of damaging treatment: (a) They were dispersed in 9 vol. of glass-distilled water and incubated in air with shaking at 37° for 0.5 hr. These preparations will be referred to as 'pre-incubated mitochondria'. (b) Pre-incubated mitochondria were mixed with 1/9 vol. of 1.54 M-KCl to assist subsequent sedimentation, kept for 5 min. at room temperature and centrifuged at 5000 g. The sedimented material was finally dispersed in 9 vol. of 0.154 M-KCl. In this way water-soluble material released on pre-incubation of the mitochondria is removed, and consequently this preparation will be referred to as 'pre-incubated washed mitochondria'. (c) Fresh mitochondria were acetone-dried as described by Drysdale & Lardy (1953).

Diphosphopyridine nucleotide (DPN) was prepared from yeast by the method of LePage (1949*a*) and purified by counter-current distribution (Hogeboom & Barry, 1948). The material obtained was not less than 75% pure as assayed spectrophotometrically following reduction by Na₂S₂O₄ (LePage, 1949*b*). Triphosphopyridine nucleotide (TPN) was prepared from sheep liver by a modification of the method of LePage & Mueller (1949) and purified by gradient elution from an ion-exchange resin column of Dowex-1-formate. The material obtained ranged in purity from 60 to 70% as assayed by the method of Horecker & Kornberg (1948) with isocitric dehydrogenase prepared according to Straub (1942). We are indebted to Professor H. A. Krebs, F.R.S., and to Dr D. D. Davies for providing details of the method of preparation of TPN and for a

* Part 2: Brownie, Grant & Davidson (1954).

sample of dimethyl D-isocitrate lactone from which isocitrate was prepared. Reduced TPN (TPNH) was prepared by heating a solution of 5 mg. of TPN and 5 mg. of $\text{Na}_2\text{S}_2\text{O}_4$ in 1 ml. of 0.45M phosphate buffer, pH 7.4, for 1 min. at 100°, cooling, aerating for 5 min. and diluting to suitable volume. A solution of $\text{Na}_2\text{S}_2\text{O}_4$ without TPN was treated in the same way and will be referred to as the 'reducing solution'.

The methylene blue used was a special zinc-free preparation kindly given by Imperial Chemical Industries Ltd. Dyestuffs Division. Other dyes were ordinary commercial samples. Dicarboxylic acids were used as potassium salts freshly prepared from crystallized samples of the free acids. A commercial preparation of adenosine triphosphoric acid (ATP) (L. Light and Co. Ltd., Slough) was used. 9:11-Anhydrocorticosterone acetate (pregna-4:9(11)-dien-21-ol-3:20-dione acetate), a gift from Professor C. W. Shoppee, was hydrolysed by the method of Mattox & Kendall (1951), to obtain the free alcohol.

The preparation of DOC, incubation conditions and methods of steroid analysis have been described elsewhere (Brownie & Grant, 1954a; Brownie *et al.* 1954).

The reaction mixture employed throughout contained 95 mM-KCl, 4 mM- MgSO_4 , 40 mM potassium phosphate pH 7.4 and other additions described, in a final volume of 3 ml.

The decrease in optical density of solutions at 340 m μ . which accompanies the oxidation of TPNH to TPN was used to measure this reaction. The measurements were made in a Unicam SP. 500 spectrophotometer, using 1 cm. cells and a new design of water-jacketed cell holder (Grant, 1955) maintained at 37°.

RESULTS

11 β -Hydroxylation of DOC by modified mitochondrial preparations

The results illustrated in Fig. 1 show that pre-incubation of ox-adrenocortical mitochondria in hypotonic media diminished their ability to effect 11 β -hydroxylation of DOC (Fig. 1a, b). Further marked loss in activity occurred when the mitochondria were removed from the media in which

Fig. 1. DOC 11 β -hydroxylation by fresh and modified adrenocortical mitochondria incubated in air for 1 hr. at 37°. Reaction mixture was supplemented with 100 mg. wet wt. of mitochondria, 0.04 ml. of propylene glycol (propane-1:2-diol) containing about 500 μ g. of DOC, fumarate and TPN as shown; total vol. 3 ml. Double lines at the ends of bars indicate duplicate values.

they were pre-incubated (Fig. 1c). This activity could, however, be restored if TPN was added with fumarate (Fig. 1d). TPN and fumarate separately were without effect in restoring activity (Fig. 2a, b) and TPN could not be replaced by DPN (Fig. 2c). Malate could replace fumarate but was less effective. Succinate gave very little effect (Fig. 2d, e). α -Oxoglutarate, which was most effective in the stimulation of 11 β -hydroxylation with fresh mitochondria (Brownie & Grant, 1954a), was ineffective with the modified mitochondrial preparations even in the presence of TPN (Fig. 2f).

Fig. 2. Effect of TPN, DPN and various intermediates of the tricarboxylic acid cycle on 11 β -hydroxylation of DOC by pre-incubated washed adrenocortical mitochondria. Final incubations in air for 1 hr. at 37°. Reaction mixture as described in Fig. 1 with additions as shown. Double lines at ends of bars indicate duplicate values.

Fig. 3. DOC 11 β -hydroxylation by acetone-dried adrenocortical mitochondria. Incubations in air for 1 hr. at 37°. Reaction mixture was supplemented with acetone-dried mitochondria equivalent to 300 mg. wet wt. of fresh mitochondria, 0.03 ml. of propylene glycol containing about 375 μ g. of DOC and other substances as shown; total vol. 3 ml. Double lines at the ends of bars indicate duplicate values.

Similar results were obtained with acetone-dried mitochondria (Fig. 3). With this type of preparation DPN supplemented with ATP was almost as effective as TPN in stimulating 11β -hydroxylation in presence of fumarate (Fig. 3c).

Effect of inhibitors on 11β -hydroxylation of DOC by pre-incubated washed mitochondria

2:4-Dinitrophenol (DNP) used in low concentrations which do not inhibit respiration inhibits 11β -hydroxylation of DOC by intact mitochondria (Brownie & Grant, 1954a). The same concentration of DNP was, however, without effect on 11β -hydroxylation by damaged mitochondria in presence of fumarate and TPN (Fig. 4b). The inhibitory effect of malonate previously observed (Brownie & Grant, 1954a) was still obtained with the damaged mitochondrial preparations (Fig. 4c).

Fig. 4. Effect of 2:4-dinitrophenol and of malonate on 11β -hydroxylation of DOC by pre-incubated washed adrenocortical mitochondria. Final incubations in air for 1 hr. at 37° . Reaction mixture as described in Fig. 1 with additions as shown. Double lines at ends of bars indicate duplicate values.

Investigation of products arising from incubation of pre-incubated washed mitochondria with DOC

When fumarate was omitted from the reaction mixture for incubation of pre-incubated washed mitochondria with DOC and TPN no corticosterone was formed and the apparent recovery of DOC accounted for the amounts of this steroid which were added to the reaction mixture. The analytical methods employed in these experiments would not, however, distinguish 9:11- or 11:12-anhydrocorticosterone from DOC. The possibility, that these hypothetical intermediaries of the 11β -hydroxylation of DOC (Hayano, Dorfman & Yamada, 1951) might accumulate on incubation of DOC in absence of fumarate, was therefore investigated. Pre-incubated washed mitochondria (100 mg. wet weight) were incubated for 1 hr. at 37° with 0.1 mM TPN with and without added DOC (500 μ g.). Benzene-chloroform extracts of the incubated mixtures were prepared as described by Brownie & Grant (1954a). DOC and 9:11-anhydrocorticosterone were added individually

and as a mixture to portions of the extract of material incubated without DOC (blank extract). Spots of the DOC incubation extract and of the mixtures of blank extract and of the individual steroids were run on a 40 cm. Whatman no. 42 paper for 5 hr. at 20° using solvent system A of Bush (1952). The bottom of the paper was serrated to facilitate dripping of solvent from the paper. Spots were located on the chromatogram by spraying with blue tetrazolium (Madder & Buck, 1952). DOC and 9:11-anhydrocorticosterone were clearly resolved. The 'DOC incubation extract' showed one spot only, corresponding in position with that of the authentic DOC. 11:12-Anhydrocorticosterone was not used in these chromatograms, but it is probable that it would have been resolved from DOC under the conditions employed. These results suggest that neither 9:11-anhydrocorticosterone nor 11:12-anhydrocorticosterone, the hypothetical unsaturated intermediaries of the 11β -hydroxylation reaction, accumulates on incubation of DOC with modified mitochondria in the presence of TPN and in the absence of fumarate.

Investigation of the role of fumarate in the 11β -hydroxylation reaction with damaged mitochondria

The results described above show that ox-adrenocortical mitochondria, damaged by pre-incubation in hypotonic media or by acetone-drying, develop a specific requirement for fumarate and TPN which is not observed with fresh mitochondria. As a working hypothesis it was assumed that TPN is the H acceptor in the 11β -hydroxylation reaction, and that in the damaged mitochondria with disrupted electron transport mechanisms (see Discussion) fumarate is required to re-oxidize the TPNH formed according to the reaction: $\text{fumarate} + \text{TPNH} + \text{H}^+ \rightarrow \text{succinate} + \text{TPN}^+$ (cf. Slater, 1950). The influence of fumarate on the oxidation of TPNH by pre-incubated washed mitochondria was observed directly by measuring the fall in optical density at 340 m μ . which accompanies the oxidation of the coenzyme. Pre-incubated washed mitochondria (50 mg. wet wt.) were used in these experiments in place of the usual 100 mg., in order to facilitate the setting of the instrument for 100% transmission with the blank cell in the light path. A separate experiment with modified mitochondria (50 mg. wet wt.) incubated for 1 hr. at 37° with 2 mM fumarate and 0.1 mM TPN in the usual reaction mixture gave 60% 11β -hydroxylation of DOC (500 μ g.), compared with 72% obtained with 100 mg. of enzyme under the same conditions. The reaction mixture containing 50 mg. wet wt. of mitochondria was placed in the spectrophotometer cells water-jacketed at 37° . At zero time TPNH solution was

added to the test cell to give a concentration of 0.1 mM in a final vol. of 3 ml., and the same volume of 'reduction solution' was added to the blank cell. The mixtures were stirred and optical densities were read at 1 min. intervals with stirring after alternate readings. The experiment was repeated with 2 mM fumarate in the reaction mixture in each cell, and in the presence of 500 μ g. of DOC added in 0.04 ml. of propylene glycol.

The results shown in Fig. 5 indicate that TPNH was oxidized in the presence of pre-incubated washed mitochondria without the assistance of fumarate. Fumarate appeared to inhibit the oxidation of TPNH. DOC was without effect on these reactions.

Fig. 5. Effect of fumarate and DOC on the re-oxidation of TPNH by pre-incubated washed adrenocortical mitochondria incubated in air at 37°. Reaction mixture supplemented with 50 mg. wet wt. of mitochondria and 0.1 mM TPN/TPNH. I, no additions; II, 500 μ g. DOC in 0.04 ml. propylene glycol; III, 2 mM fumarate; IV, 2 mM fumarate and 500 μ g. DOC in 0.04 ml. propylene glycol.

Attempts were made to replace fumarate in the 11 β -hydroxylation system by a number of different electron acceptors. Ferricyanide (Hunter, 1951) and pyocyanine (Dickens & McIlwain, 1938) are known to react directly with reduced pyridine nucleotides, whereas most other dyes used as hydrogen acceptors require the intervention of flavoproteins (cf. Mahler, Tomisek & Heunneken, 1953). However, neither these substances nor brilliant cresyl blue (Dickens, 1936), methylene blue, or 2:6-dichlorophenol-indophenol added at the start of or slowly during the incubation (Drysdale & Lardy, 1953), were able to replace fumarate in the reaction mixture containing

100 mg. wet wt. of pre-incubated centrifuged mitochondria, 500 μ g. of DOC and 0.1 mM TPN incubated for 1 hr. at 37°.

DISCUSSION

The results reported here show that the specific requirement for fumarate for 11 β -hydroxylation of DOC (Hayano & Dorfman, 1953) arises when ox-adrenocortical mitochondria, which carry the enzymes involved, are subjected to damaging treatment, such as pre-incubation without substrates in hypotonic media, or acetone-drying. More exacting requirements arising when mitochondria are damaged by even milder treatment such as saline washing have already been discussed for the case of octanoate oxidation by liver mitochondria (Drysdale & Lardy, 1953). Green, Loomis & Auerbach (1948) have shown that pre-incubation of kidney mitochondria for 15 min. at 38° permits the 'isolation' of the limited sequence of reactions L-malate $\rightleftharpoons$ fumarate $\rightleftharpoons$ succinate. Other oxidizing enzymes of the tricarboxylic acid cycle are destroyed. This offers an explanation of the observation that malate or succinate but not α -oxoglutarate may replace fumarate in the presence of TPN in the 11 β -hydroxylation reaction. Since it has been shown that 11 β -hydroxylation will occur in mitochondria prepared in sucrose in absence of fumarate and under conditions which prevent the formation of this substance (Brownie & Grant, 1954a) it must be emphasized that the requirement for fumarate by 'damaged' mitochondria may be of somewhat academic interest.

Tsou (1951) suggested that the speed of electron transport may depend on the organization of the enzymes involved. Since organization of the mitochondria is disrupted by pre-incubation or acetone-drying, it appeared that fumarate might be required for the re-oxidation of TPNH, possibly according to the reactions

The apparent inhibition in presence of fumarate of TPNH re-oxidation by pre-incubated centrifuged mitochondria shows that reactions (1) and (2) will not explain the role of fumarate in 11 β -hydroxylation under these conditions. The malonate inhibition of 11 β -hydroxylation by pre-incubated centrifuged mitochondria would, however, suggest that the reduction of fumarate to succinate is involved. The rate of re-oxidation of TPNH in the presence of fumarate and DOC as compared with the rate in absence of DOC gives no evidence that hydrogen is transferred from DOC through the reaction: succinate $\rightleftharpoons$ fumarate to TPN. It is thus not at

present possible to say which is the primary hydrogen acceptor, TPN or fumarate.

The specific requirement for TPN confirms the finding of Hayano, Weiner & Lindberg (1953) and offers an explanation of the requirement for oxidative phosphorylation for 11 β -hydroxylation with intact mitochondria (Brownie & Grant, 1954*a*). Thus ATP arising from such oxidative phosphorylation may be required for the synthesis of TPN according to the reaction $\text{DPN} + \text{ATP} \rightarrow \text{TPN} + \text{ADP}$ (Mehler, Kornberg, Grisolia & Ochoa, 1948) as proposed by Hechter & Pincus (1954). However, the fact that fresh intact ox-adrenocortical mitochondria can oxidize citrate (Brownie & Grant, 1954*a*) and thus contain some TPN suggests that it may be necessary to look for some other role for the oxidative phosphorylation. A possibility is that the entry of DOC into fresh intact mitochondria may be an active energy-requiring process; with the disappearance of permeability barriers on treatment with hypotonic media or acetone-drying, this energy provision would become unnecessary. The 11 β -hydroxylation reaction in such damaged mitochondria would not be inhibited by 2:4-dinitrophenol in accordance with our observations.

The mechanism of 11 β -hydroxylation remains obscure. It has been shown here that the hypothetical intermediate of Hayano *et al.* (1951), 9:11-anhydrocorticosterone, does not accumulate when pre-incubated mitochondria are incubated with DOC and TPN in absence of fumarate. The recent interesting report of Hayano & Dorfman (1954), that no deuterium enters a stable position in the steroid molecule in the course of 11 β -hydroxylation performed in 95% deuterium oxide, precludes the direct entry of water into this reaction. This observation would also indicate that TPN is not involved as an acceptor of hydrogen from the steroid, but is concerned with some side reaction possibly involving fumarate which tends to keep the coenzyme in the reduced state (Fig. 5). The failure of a variety of hydrogen acceptors to influence the 11 β -hydroxylation reaction reported in the present work supports the view that the steroid is not dehydrogenated in the course of 11 β -hydroxylation. In order to obtain further information on these reactions experiments are now proceeding with a soluble 11 β -hydroxylating enzyme extracted from acetone-dried mitochondria.

SUMMARY

1. It has been shown that a specific requirement for fumarate for the 11 β -hydroxylation of steroids arises when ox-adrenocortical mitochondria which carry the enzymes concerned are subjected to damaging treatment such as pre-incubation with-

out substrate in hypotonic media or acetone-drying.

2. Such enzyme preparations also require triphosphopyridine nucleotide.

3. Satisfactory explanations of the role of triphosphopyridine nucleotide and fumarate in the 11 β -hydroxylation reaction cannot be offered at present.

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