The *in vitro* Enzymic Hydroxylation of Steroids

4. THE ROLE OF FUMARATE AND TRIPHOSPHOPYRIDINE NUCLEOTIDE IN THE ENZYMIC 11β-HYDROXYLATION OF 11-DEOXYCORTICOSTERONE*

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Suspensions of ox-adrenocortical mitochondria prepared in sucrose catalyse the 11β-hydroxylation of 11-deoxycorticosterone (DOC), provided that there is concurrent oxidation of a member of the Krebs tricarboxylic-acid cycle (Brownie & Grant, 1954). In contrast with the observations of Hayano & Dorfman (1953) it was found that the mitochondrial preparations show no specific requirement for fumarate. In an investigation of this difference Brownie & Grant (1956) found that the requirement for fumarate and for triphosphopyridine nucleotide (TPN^+) arose when their mitochondrial preparations were treated with hypotonic media or were acetone-dried. It was also concluded from the evidence available that DOC is not dehydrogenated during 11β-hydroxylation, and that fumarate may be required to maintain the concentration of reduced TPN^+ (TPNH). The results of further investigations of the role of fumarate and TPN^+ in the hydroxylation of DOC are now reported. A soluble enzyme preparation, extracted from acetone-dried mitochondria, has been used in these experiments.

A preliminary account of some of the experiments described here has been published (Grant & Brownie, 1955).

EXPERIMENTAL

Enzyme preparations and materials

The following operations were carried out at temperatures near 0°. About 150 g. of ox-adrenals free from adhering fat and connective tissue was ground in a Latapie mincer. The mince was suspended in 350 ml. of 0·25 M sucrose and further disintegrated in a Nelco Blendor (Measuring and Scientific Equipment Ltd., London) run at half maximum speed. Mitochondria were separated as described by Brownie & Grant (1954) from the suspension thus obtained. On this scale it was not possible to exclude other cell components as effectively as in the earlier preparations. Care was taken, however, to wash the mitochondrial fraction thoroughly with sucrose solution in order to remove soluble enzymes known to catalyse 17- and 21-hydroxylation of steroids (Plager & Samuels, 1953). The mitochondria were finally suspended in a small volume of water and dried with acetone and ether as described by Drysdale & Lardy (1953), yielding about 4 g. of a light fawn-coloured powder. This powder could be stored at 0° for several weeks without loss of activity. Enzymes were extracted from the powder by grinding for 1 min. with ten times its weight of 0·154 M-KCl. It was found convenient to use a 50 ml. plastic centrifuge tube and a glass bulb on a rod driven by a stirrer motor for the grinding. The suspension was allowed to stand for 0·5 hr. at 0° and was then centrifuged for 0·5 hr. at $2 \times 10^4 g$. The clear supernatant liquid containing the enzymes to be studied was red–brown in colour. The average N content of a number of preparations was $2{\cdot}5 \pm 0{\cdot}2$ mg./ml. For some experiments the extract was centrifuged for 1 hr. at $1 \times 10^5 g$, giving solutions of slightly lower N content.

The enzyme solution was sometimes prepared from separated cortical tissue.

A suspension of crystalline catalase was prepared according to Sumner & Dounce (1937). Cytochrome oxidase was prepared according to Keilin & Hartree (1938). An acetone-dried preparation of finely ground pig liver was used as a source of uricase (Keilin & Hartree, 1936).

Cytochrome *c* was a lyophilized commercial preparation (L. Light and Co. Ltd., Colnbrook, Bucks.). Assayed by the method of Potter (1949), this was found to be 76% pure. Diphosphopyridine nucleotide (DPN^+) and TPN^+ were prepared and assayed as already described (Brownie & Grant, 1956). For some experiments commercial preparations of DPN^+ and DPNH (C. F. Boehringer and Sons Ltd., Mannheim, Germany) were used. TPNH was prepared by the method of Conn, Kraemer, Pei-Nan & Vennesland (1952).

DOC was prepared from its acetate by the method of Mattox & Kendall (1951). Other steroids were pure crystalline specimens given by those referred to in the acknowledgements.

Incubation conditions and methods of determining steroids

Unless otherwise stated, in all experiments 30 ml. test tubes with ground-glass stoppers were used containing the following reaction mixture: 40 mM potassium phosphate (pH 7·4), 10 mM potassium fumarate, about 0·1 mM TPN^+ and 2 ml. of enzyme solution in a final volume of 3 ml. About 500 μg. of DOC was added in 0·04 ml. of propylene glycol (propane-1:2-diol). Unless otherwise stated, incubations were in air, the mixture being shaken for 1 hr. at 37°.

DOC and corticosterone in reaction mixtures were determined as described by Brownie & Grant (1954), with the exception that the acetone extraction was omitted. After incubation, reaction mixtures were cooled and steroids were extracted directly by vigorous shaking with the benzene–chloroform mixture. Typical results for the

* Part 3: Brownie & Grant (1956).

recovery of DOC and corticosterone added to incubated reaction mixtures and extracted by the procedure described above are shown in Table 1.

Each experiment included 'extraction controls' and 'blank controls' as described by Brownie & Grant (1954).

RESULTS

Selection of enzyme preparation

Typical results obtained with different soluble enzyme preparations are shown in Table 2. Equal portions of the extracted reaction products obtained with each type of enzyme preparation were chromatographed on paper for 5–7 hr. at 18° with the solvent system B_4 of Bush (1952). When the chromatograms were sprayed with blue tetrazolium reagent (Mader & Buck, 1952), four spots which had run to the same relative positions were observed in each case. The spots were of the same relative size. In view of the similarity of these results it was decided as a matter of convenience to use the enzyme preparation from whole adrenals finally centrifuged at 2×10^4 *g* for 0·5 hr. for the experiments described below. This will be referred to as the 'soluble enzyme' preparation.

Investigation of the products of metabolism of DOC by the soluble enzyme

Portions of the benzene–chloroform extracts prepared for DOC analysis were run on paper chromatograms as described above. These chromatograms showed spots which absorbed u.v. light, gave the blue tetrazolium reaction of Mader & Buck (1952) and corresponded in position to DOC and corticosterone run as reference substances on the same paper. Two additional spots were regularly observed by the same means. One, due to a substance referred to below as *X*, was slightly more polar than corticosterone, and gave a smaller spot. This was not referred to in the preliminary report (Grant & Brownie, 1955), since the paper chromatograms were not at first run for sufficient time to resolve corticosterone and substance *X*. A second fainter spot (due to a substance to be referred to as *Y*) appeared about half-way between the corticosterone spot and the origin. Occasionally a very faint spot (substance *Z*) giving the blue tetrazolium reaction was observed very near the origin.

Areas of the paper chromatograms containing metabolic product corresponding in mobility to corticosterone were eluted with chloroform and rechromatographed alone and mixed with authentic corticosterone at 18° in the solvent system B_1 and B_4 of Bush (1952). All chromatograms showed single spots which had run to the same position on the strips. The metabolic product and corticosterone (about 20 μg. of each) were separately treated with 3 ml. of conc. H_2SO_4 for 1 hr. at 37° (Zaffaroni, 1950). Both solutions gave identical absorption spectra with maxima at 285, 326, 372 mμ. and from 455 to 465 mμ. A solution of the metabolic product in ethanol showed an absorption maximum at 240 mμ. Thus although complete identity has not been established it appears likely that the principal product obtained on incubation of DOC with the soluble enzyme preparation is corticosterone.

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

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

DOC			Corticosterone		
No. of expts.	Added (μg.)	Recovery (%)	No. of expts.	Added (μg.)	Recovery (%)
4	528	92·7±3·7	—	—	—
4	296	97·2±1·3	4	245	89·5±2·8
4	162	96·0±1·8	4	132	93·5±4·5

Table 2. *Metabolism of DOC by enzymes extracted from acetone-dried ox-adrenal mitochondria*

The conditions of incubation were as described in the text.

Type of enzyme preparation		DOC metabolized (%)
Origin of mitochondria	Centrifuging of extract from dried mitochondria	
Adrenal cortex	0·5 hr. at 2×10^4 *g* in multispeed attachment of refrigerated centrifuge (Measuring and Scientific Equipment Ltd., London)	90
Whole adrenal	As above	95
Whole adrenal	1 hr. at 10^5 *g* in preparative head of Spinco Model E ultracentrifuge	96

Table 3. *Absorption maxima of substance X and of steroids with similar chromatographic behaviour*

Substance	(*a*) Absorption max. in ethanol (mμ.)	(*b*) Absorption max. in conc. sulphuric acid after 1 hr. at 19° (mμ.)			
X	234	285	340	452	
Corticosterone	240	287	322	465	
17α-Hydroxy-DOC	240	288	338	486	535
6β-Hydroxy-DOC	235	286	343	454	

Table 4. *Recovery of steroids absorbing selectively at 240 mμ. after incubation of DOC with soluble enzyme preparations*

The chromatogram fractions corresponding to DOC and to corticosterone + substance *X* were examined as described in the text.

DOC		Corticosterone + *X* recovered (as μmoles)	Total recovery	
Incubated (μmoles)	Recovered (μmoles)		(μmoles)	(%)
1·63	0·275	1·21	1·49	91

The possibility that substance *X* is a further transformation product of corticosterone formed from DOC was made unlikely by the observation that corticosterone was recovered unchanged after incubation with the usual reaction mixture.

Substance *X* was extracted from the paper chromatograms with chloroform and gave a positive reaction for formaldehyde after periodate oxidation (Daughaday, Jaffe & Williams, 1948). An extract of a similar area of blank paper gave no reaction. The phenylhydrazine reaction (Porter & Silber, 1950) was negative. These results and the positive blue tetrazolium reaction already referred to would be given by a 17-deoxy-21-hydroxy-20-oxosteroid. 6β-Hydroxy-DOC (6β:21-dihydroxypregn-4-ene-3:20-dione) belongs to this class and would be expected to show similar chromatographic behaviour to substance *X*; it has been isolated from incubated adrenal preparations of the hog (Haines, 1952) and ox (Hayano & Dorfman, 1953). A mixed chromatogram of substance *X* and 6β-hydroxy-DOC showed a single spot when run for 5 hr. at 18° with solvent system B_4 (Bush, 1952). Acetylation of substance *X* with acetic anhydride at room temperature for 24 hr. gave a product which showed identical behaviour with 6β:21-diacetoxy-DOC when run on paper chromatograms for 48 hr. at 37° in the hexane:propylene glycol–methanol (50:50, by vol.) system of Burton, Zaffaroni & Keutmann (1951), or for 8 hr. at 18°, in the *iso*octane–*tert.*-butanol (50:22·5, v/v)/methanol–water (22·5:50, v/v) system of Eberlein & Bongiovanni (1955).

It will be seen from Table 3 that solutions of substance *X* and 6β-hydroxy-DOC show the same absorption maxima. Although complete identity of *X* has not been established it appears reasonably likely that this substance is 6β-hydroxy-DOC.

6β-Hydroxy-DOC appears with corticosterone in the eluate fraction from partition-chromatographic columns used for analysis of corticosterone. These two substances together account almost quantitatively for the DOC which is metabolized by the soluble enzyme preparation (Table 4).

Paper chromatograms of metabolic products were run as a routine in all experiments. As judged by the size and colour intensity of spots on the paper, 6β-hydroxy-DOC and corticosterone were formed in the approximately constant proportion of 1:5. The combined quantities of these two substances determined as corticosterone are taken as a measure of DOC hydroxylation in the results now reported.

The identities of the substances *Y* and *Z*, which appeared to be formed in very small amounts, were not extensively investigated. *Y* ran to the same position on paper chromatograms as 19-hydroxy-DOC.

Effect of various substances on hydroxylation of DOC by the soluble enzyme

Added DOC was recovered after incubation with soluble enzyme which had been heated for 5 min. at 55° or after incubation in the absence of air.

It was observed in early experiments that more satisfactory results were obtained when 2-amino-2-hydroxymethylpropane-1:3-diol[aminotrishydroxymethylmethane (tris)] buffer was used in place of phosphate buffer (Fig. 1*a*, *b*). Tris buffer was therefore used in all subsequent experiments.

DOC hydroxylation was completely inhibited by 10 mM Versene (ethylenediaminetetraacetic acid, EDTA) adjusted to pH 7·4 (Fig. 1*c*). This result suggested a requirement for a metal. The soluble enzyme preparation was therefore dialysed for 24 hr. at 0° against 0·154 M-KCl. A part of the same

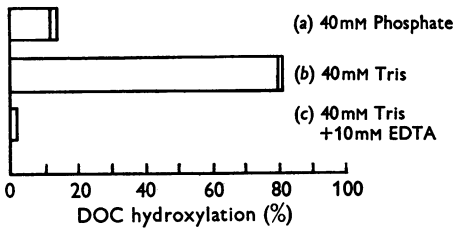


Fig. 1. Inhibition by phosphate and EDTA of hydroxylation of DOC by soluble adrenal enzyme incubated in air for 1 hr. at 37°. Reaction mixture contained 10 mM potassium fumarate, about 0·1 mM TPN^+, 2 ml. of enzyme and the substances shown, in a final volume of 3 ml. Buffers were adjusted to pH 7·4. About 500 μg. of DOC was added in 0·04 ml. of propylene glycol. Double lines at ends of bars indicate duplicate values.

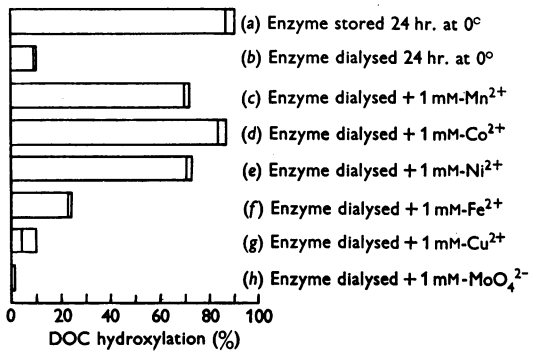


Fig. 2. Influence of metal ions on hydroxylation of DOC by dialysed soluble adrenal enzyme incubated in air for 1 hr. at 37°. Reaction mixture contained 10 mM potassium fumarate, about 0·1 mM TPN^+, 40 mM tris, pH 7·4, 2 ml. of enzyme and the substances shown, in a final volume of 3 ml. About 500 μg. of DOC was added in 0·04 ml. of propylene glycol. Double lines at ends of bars indicate duplicate values.

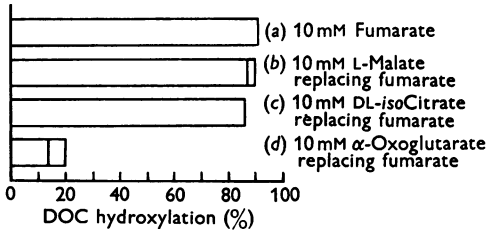


Fig. 3. Effect of replacing fumarate by other members of the tricarboxylic acid cycle on the hydroxylation of DOC by soluble adrenal enzyme incubated in air for 1 hr. at 37°. Reaction mixture contained about 0·1 mM TPN^+, 40 mM tris, pH 7·4, 2 ml. of enzyme and the substances shown, in a final volume of 3 ml. About 500 μg. of DOC was added in 0·04 ml. of propylene glycol. Double lines at ends of bars indicate duplicate values.

preparation was stored for 24 hr. at 0°. The results illustrated in Fig. 2 shows that activity is lost on dialysis and that it is restored by 1 mM-Co^{2+}, -Mn^{2+} or -Ni^{2+} ions. Fe^{2+} ions showed some effect; Cu^{2+} and MoO_4^{2-} ions had no effect.

L-Malate and DL-*iso*citrate could replace fumarate (Fig. 3), but α-oxoglutarate, which was the best 'activator' of DOC 11β-hydroxylation by adrenocortical mitochondria (Brownie & Grant, 1954) was relatively ineffective with the soluble enzyme preparation (Fig. 3*d*). The reduction of TPN^+ is known to be coupled with the conversion of L-malate into pyruvate (Ochoa, Mehler & Kornberg, 1948) and with the oxidation of D-*iso*citrate (Adler, Euler, Gunther & Plass, 1939). The enzymes concerned in both cases are present in mitochondria, require Mn^{2+} ions and are resistant to acetone drying. It appeared probable therefore that in the present experiments L-malate from fumarate and DL-*iso*citrate were concerned with the formation of TPNH. In support of this suggestion it was observed that the addition of fumarate to reaction mixtures containing TPN^+ caused a marked increase in optical density at 340 mμ. (Fig. 4*a*), owing to TPNH formation probably according to the reactions:

$$\text{Fumarate} \rightarrow \text{L-malate}; \quad (1)$$

$$\text{L-Malate} + TPN^+ \rightarrow \text{pyruvate} + CO_2 + TPNH + H^+. \quad (2)$$

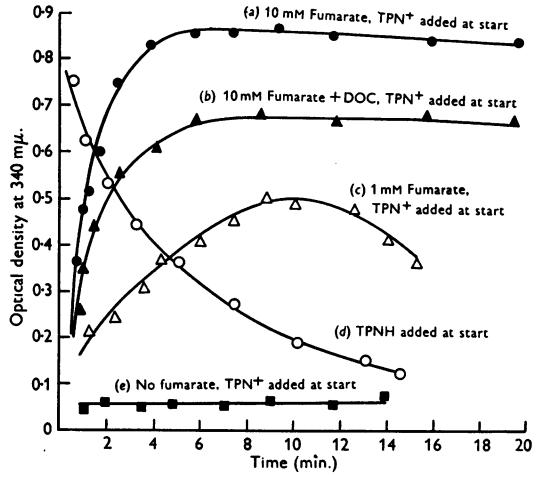


Fig. 4. Influence of fumarate and DOC on the oxidation of TPNH and the reduction of TPN^+ by soluble adrenal enzyme incubated at 37° in cells of Unicam spectrophotometer SP. 500 (Grant, 1955). The reaction mixture contained about 0·1 mM TPN^+, 40 mM tris, pH 7·4, 2 ml. of enzyme and the fumarate shown, in a final volume of 3 ml.; in one case about 500 μg. of DOC was added in 0·04 ml. of propylene glycol.

When DOC was added with the fumarate the increase in optical density was less (Fig. 4*b*). Similar results were obtained with DL-*iso*citrate. In the absence of fumarate or DL-*iso*citrate added TPNH was oxidized (Fig. 4*d*). It would thus appear that the curve obtained at the lower fumarate concentration (Fig. 4*c*) might be explained by the rate of TPN^+ reduction exceeding TPNH oxidation during the first 10 min., after which the fumarate concentration becomes the limiting factor and TPNH oxidation the dominant reaction. It appeared to be significant that the use of 10 mM fumarate, which led to the greater reduction of TPN^+, gave 86% hydroxylation of DOC, whereas mM fumarate gave less TPNH and only 38% hydroxylation.

Need for TPNH in hydroxylation of DOC

The hypothesis that TPNH is required for hydroxylation of DOC was investigated (*a*) by inhibiting TPNH formation, (*b*) by accelerating removal of TPNH from the system used for hydroxylation of DOC and (*c*) by direct measurement of hydroxylation of DOC in the presence of added TPNH.

(*a*) Ochoa (1948) has shown that the following reaction may be reversed by relatively high concentrations of α-oxoglutarate:

$$\text{D-}iso\text{Citrate} + TPN^+ \xrightarrow{Mn^{2+}} \alpha\text{-oxoglutarate} + CO_2 + TPNH + H^+.$$

It has now been observed that the rise in optical density at 340 mμ. due to formation of TPNH in a reaction mixture containing soluble enzyme, *iso*citrate, TPN^+ and tris buffer was inhibited on addition of α-oxoglutarate and bicarbonate. The effect of added α-oxoglutarate and bicarbonate on hydroxylation of DOC is shown in Fig. 5. The poor hydroxylation observed in the controls (Fig. 5*a*) may be attributed to the low concentration of *iso*citrate used to permit demonstration of α-oxoglutarate inhibition and to the displacement of much of the air in the reaction vessel by CO_2 in order to avoid loss of CO_2 from the reaction mixture. Ochoa (1948) drew particular attention to the sensitive dependence of the reaction on the concentration of CO_2. It is evident, however, from Fig. 5*b* that the inhibition of TPN^+ reduction results in failure of hydroxylation of DOC.

(*b*) Acetone drying as used in the preparation of the soluble enzyme is known to destroy cytochrome oxidase (Keilin & Hartree, 1938; cf. Green, Needham & Dewan, 1937). Consequently, the oxidation of TPNH observed with such enzyme preparations (cf. Fig. 4*d*) must proceed slowly via the autoxidation of flavoproteins (cf. Theorell, 1936*a*, *b*). In order to increase the rate of oxidation of TPNH, 0·025 μmole of cytochrome *c* and 0·1 ml. of the cytochrome oxidase preparation were added to 2 ml. of soluble enzyme. On addition of TPNH to give a concentration of 0·1 mM in a final volume of 3 ml. the rate of oxidation of the coenzyme was too rapid to be followed in the spectrophotometer. The inhibiting effect of added cytochrome *c* and cytochrome oxidase on hydroxylation of DOC is shown in Fig. 6*b*. It was also observed (Fig. 6*c*, *d*) that hydroxylation of DOC was inhibited when air was replaced by O_2 as the gas phase in the reaction vessel. This may be explained by the more rapid oxidation of TPNH via flavoproteins at the higher O_2 tension. These results suggest that there is an optimum oxygen tension for hydroxylation of DOC under the conditions of these experiments.

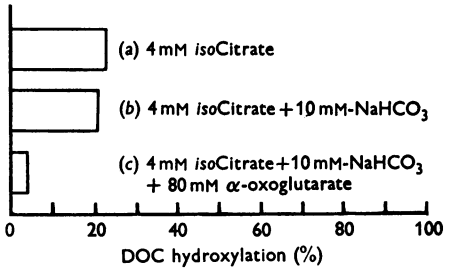


Fig. 5. Effect of inhibiting reduction of TPN^+ by *iso*citrate–*iso*citric dehydrogenase system on the hydroxylation of DOC by soluble adrenal enzyme incubated in air–CO_2 for 1 hr. at 37°. Reaction mixture contained about 0·1 mM TPN^+, 40 mM tris, pH 7·4, 2 ml. of enzyme and the substances shown, in a final volume of 3 ml. Fumarate was omitted. Reaction tubes were briefly flushed with CO_2 and stoppered before incubation. About 500 μg. of DOC was added in 0·04 ml. of propylene glycol. Double lines at ends of bars indicate duplicate values.

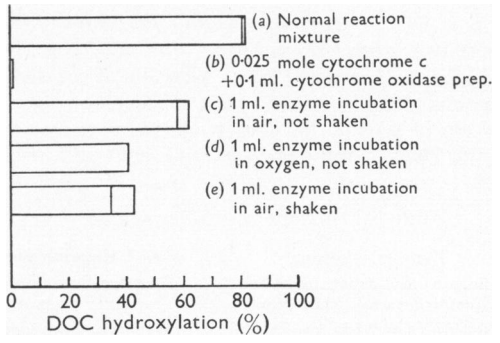


Fig. 6. Effect of stimulating oxidation of TPNH on the hydroxylation of DOC by soluble adrenal enzyme incubated in air or oxygen with and without shaking for 1 hr. at 37°. The reaction mixture contained 10 mM fumarate, 40 mM tris, pH 7·4, about 0·1 mM TPN^+, 2 ml. of enzyme and the substances shown, in a final volume of 3 ml. About 500 μg. of DOC was added in 0·04 ml. of propylene glycol. Double lines at ends of bars indicate duplicate values.

(*c*) The requirement for TPNH for hydroxylation of DOC was confirmed by observations (Fig. 7*c*) that the reaction occurred on the addition of TPNH to reaction mixtures containing no fumarate. The addition of the same concentration of TPN^+ or of DPNH was without effect (Fig. 7*b*, *d*). Hydroxylation of DOC was also achieved on the addition of TPNH to an enzyme preparation which had been dialysed against 0·154 M-KCl for 24 hr. at 0°. In this case, since the 11β-hydroxylation of DOC is no longer dependent on the reaction

$$[\text{malate} + TPN^+ \xrightarrow{Mn^{2+}} CO_2 + \text{pyruvate} + TPNH]$$

the addition of $MnCl_2$ had no effect on the results (Fig. 7*e–g*; cf. Fig. 2).

Investigation of the role of TPNH in hydroxylation of DOC

Brodie *et al.* (1955) have described an enzyme system from rabbit liver which hydroxylates certain aromatic compounds and appears to require TPNH and O_2. Subsequently Brodie (personal communication) showed that H_2O_2 was formed during the oxidation of TPNH in his enzyme preparation, which contained no cytochrome oxidase. He considered that this H_2O_2 might be the hydroxylating agent. It appeared that TPNH might have a similar role in the adrenal enzyme system now under investigation. The inhibition of hydroxylation of DOC with increasing O_2 tension (cf. Fig. 6) is not, however, in accordance with this view. No hydroxylation of DOC occurred on addition of H_2O_2 (0·0001–0·01 M) to the solution of enzyme in the absence of TPN^+. When H_2O_2 was generated slowly in the soluble enzyme preparation by the addition of 0·01 M uric acid and uricase, no hydroxylation of DOC occurred, although the same system effected the coupled oxidation of ethanol described by Keilin & Hartree (1936). Addition of catalase in concentrations up to one part of the suspension of crystals in ten of the final reaction mixtures containing DOC, soluble enzyme, uric acid and uricase was without effect. It would thus appear that under these conditions H_2O_2 is unable to effect hydroxylation of the steroid.

DISCUSSION

In a previous communication (Brownie & Grant, 1956) it was shown that the 'specific' requirement for fumarate (Hayano & Dorfman, 1953) arose when adrenocortical mitochondria which contain the steroid 11β-hydroxylating enzyme were subjected to treatment which disorganized their structure. Experiments with the soluble enzyme described here have indicated that the role of added fumarate is to provide L-malate and that the oxidative decarboxylation of this substance maintains the concentration of TPNH required for steroid 11β- and probably also 6β-hydroxylation. The TPN^+-coupled oxidation of D-*iso*citrate fulfils a similar role. It is probable that fumarate and *iso*citrate have similar functions in other enzyme systems which have been employed to effect steroid transformations (Table 5).

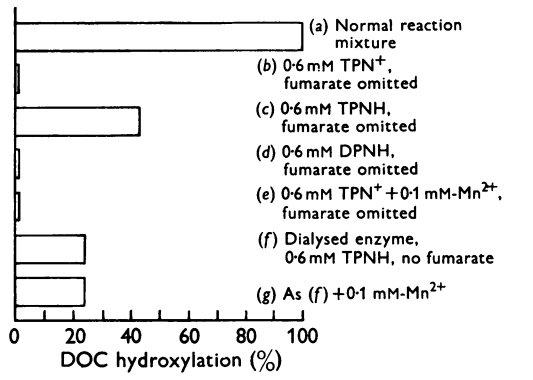


Fig. 7. Influence of DPNH, TPN^+ and TPNH on the hydroxylation of DOC by dialysed and stored soluble adrenal enzyme incubated for 1 hr. at 37°. The reaction mixture contained 40 mM tris, pH 7·4, 2 ml. of enzyme stored or dialysed at 0° for 24 hr. and the substances shown, in a final volume of 3 ml. Fumarate was omitted. About 500 μg. of DOC was added in 0·04 ml. of propylene glycol.

Table 5. *Enzymic transformations of steroids involving fumarate and* iso*citrate as 'activators'*

Type of reaction	Enzyme preparation	'Activator'	Reference
Reduction of C-3 ketone of progesterone and other steroids	Particle-free soluble fraction of rat-liver homogenate	*iso*Citrate, TPN^+	Tomkins & Isselbacher (1954)
Reduction of C-20 ketone of tetrahydrocortisone (3β:17α:21-trihydroxypregnane-3:20-dione)	Rat-liver homogenate	Fumarate, DPN^+	C. de Courcy & J. J. Schneider (unpublished observations)
C-18 and C-19 hydroxylation of DOC	Ox-adrenal homogenate	Fumarate, DPN^+, TPN^+, etc.	Kahnt, Neher & Wettstein (1955)
Conversion of DOC into aldosterone	Ox-adrenal homogenate	Fumarate, etc.	Wettstein, Kahnt & Neher (1955)
C-17 and C-21 hydroxylation of steroids	Particle-free soluble fraction of ox-adrenal homogenate	Fumarate, etc.	Samuels (1953)

Glock & Maclean (1955) have shown that rat adrenals contain high concentrations of TPNH, and Chance & Williams (1955) have demonstrated that in actively respiring rat-liver mitochondria the pyridine nucleotides are very largely in the reduced state. It is therefore probable that ox-adrenal mitochondria, which possess the intact sequence of enzymes concerned in the tricarboxylic acid cycle, would maintain the concentration of TPNH necessary for hydroxylation of DOC in the presence of any member of the cycle without showing a specific requirement for fumarate. This has been demonstrated by Brownie & Grant (1954). In addition, evidence was produced that with the intact mitochondria oxidative phosphorylation was necessary for DOC hydroxylation, and it was suggested that this may be required for 'active transport' of the steroid into the mitochondria. The present investigations have shown that there is no requirement for oxidative phosphorylation with soluble enzyme preparations, which present no permeability barriers.

Sweat & Lipscomb (1955) have also presented evidence that TPNH is a cofactor for 11β-hydroxylation and suggest that it is formed by the following sequence of reactions:

$$\text{Fumarate} \rightarrow \text{L-malate},$$

$$\text{L-Malate} + \text{DPN}^+ \rightarrow \text{oxaloacetate} + \text{DPNH} + \text{H}^+,$$

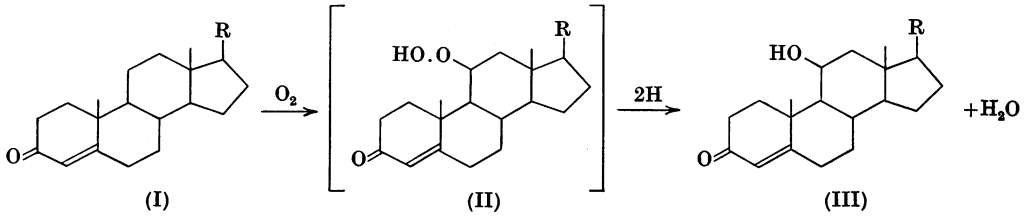
$$\text{DPNH} + \text{TPN}^+ \rightarrow \text{DPN}^+ + \text{TPNH}.$$

The requirement for the coenzyme in the reduced form supports the suggestions of Brownie & Grant (1956) that TPN^+ does not function as a hydrogen acceptor in the 11β-hydroxylation reaction and that the steroid is not dehydrogenated during this reaction.

Brodie *et al.* (1955) reported that TPNH and molecular oxygen are required for the hydroxylation of certain drugs by liver-microsome preparations. Hydrogen peroxide, which was shown to be formed during the oxidation of the TPNH, was thought to be the hydroxylating agent (Brodie, personal communication). The addition of H_2O_2 alone, however, did not effect hydroxylation, an observation which was attributed to the failure of H_2O_2 to enter the microsomes.

Hayano, Lindberg, Dorfman, Hancock & Doering (1955) have recently reported that ^{18}O enters the 11β-hydroxyl group from isotopic molecular oxygen but not from $H_2{}^{18}O$. It would thus appear that the 11β-hydroxylating enzyme is an oxygen-transferase. As such it would be similar to the phenolase complex, whose hydroxylating activity is also coupled with an electron source which may in turn be linked to the usual pathways of metabolism by reduced pyridine nucleotides (Mason, Fowlks & Peterson, 1955). The observations of Hayano *et al.* (1955) have been confirmed by Sweat & Mason (unpublished observation) for the 11β-hydroxylation of 17-hydroxy-DOC (17α:21-dihydroxypregn-4-ene-3:20-dione) and by Bergström (personal communication) for the 7α-hydroxylation of deoxycholic acid (3α:12α-dihydroxycholanic acid).

A possible mechanism for steroid hydroxylation involving direct attack by molecular oxygen is shown in the following scheme.



(I) (II) (III)

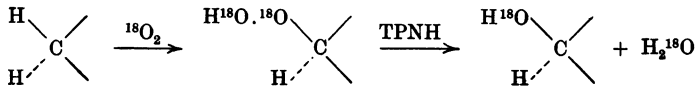
This involves the formation of an unstable hydroperoxide (II) which is reduced to the hydroxysteroid III. Such a scheme may be compared with that proposed for the autoxidation of cholesterol at C-7 (Bergström & Wintersteiner, 1942). The alternative of a steroid trans-annular peroxide involving the 11-position is ruled out, since the subsequent reduction of such a compound would introduce hydrogen into a stable position in the molecule. It is known that no reduction involving the introduction of hydrogen into a stable position occurs during the 11β-hydroxylation of steroids (Hayano & Dorfman, 1954).

The simple autoxidation of DOC in the present experiments is excluded by the observations that hydroxylation does not occur on incubation of the steroid with heat-inactivated enzyme or with active enzyme in absence of TPN^+ or fumarate.

Autoxidation of cholesterol at the 7-position activated by the neighbouring double bond gave rise to both α- and β-hydroxy derivatives (Bergström & Wintersteiner, 1941). With DOC the 6-position is similarly activated by the neighbouring double bond, but the production of the 6β-hydroxy epimer alone must be attributed to the enzymic character of the reaction. Attack at the 11-position in DOC would not be expected in a normal autoxidation. An explanation of the position of attack and its stereospecificity in introducing the hydroxyl group in the 11β-position must again be sought in the enzymic nature of the reaction.

If hydroperoxides are in fact intermediates in steroid 6β- and 11β-hydroxylation it would be reasonable to assume that TPNH is involved in their reduction to hydroxy compounds. (Capp & Hawkins (1953) have found that tertiary amines are generally more effective than secondary or primary amines as reducing agents for hydroperoxides.)

Evidence for the formation of steroid hydroperoxides could be obtained by determination of the isotopic labelling of water resulting from hydroxylation experiments performed in $^{18}O_2$ according to the hypothetical reactions.



The appearance of 6β-hydroxy-DOC among the products of hydroxylation of DOC by the soluble adrenal enzyme is not unexpected, since 6β-hydroxy compounds have been obtained on incubation of steroids with homogenized adrenals of the hog (Haines, 1952), or adrenals (Hayano & Dorfman, 1953), ox corpus luteum (Hayano, Lindberg, Weiner, Rosenkrantz & Dorfman, 1954), and on perfusion of steroids through rat liver (Miller & Axelrod, 1954). Although the physiological significance of 6-hydroxysteroids is obscure, they seem to take some part in the steroid metabolism of intact animals, for they have been isolated from the urine or guinea pigs (Burstein, 1954) and human subjects (Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948).

SUMMARY

1. Incubation of DOC with a soluble enzyme extracted from acetone-dried ox-adrenal mitochondria and supplemented with fumarate and triphosphopyridine nucleotide gives at least four products, two of which are very probably corticosterone and 6β-hydroxy-DOC.

2. The role of fumarate in the 11β-hydroxylation of steroids has been shown to be related to the production of reduced triphosphopyridine nucleotide (TPNH).

3. TPNH and molecular oxygen are the only substances required for the 6β- and 11β-hydroxylation of DOC by a soluble enzyme extracted from acetone-dried ox-adrenol mitochondria.

4. It is suggested that 6β- and 11β-hydroxylation of steroids may proceed via the formation of 6β- and 11β-hydroperoxides, which may then be reduced to the corresponding hydroxy compounds by TPNH.

It is a pleasure to record my appreciation of the interest which Professor G. F. Marrian, F.R.S., has shown in this work and to thank him for his encouragement. I am indebted to Mr D. W. Davidson for skilled technical assistance and to Dr A. C. Brownie for his help with some of the preliminary experiments. Steroids used in this work were generously provided by Dr M. H. Ehrenstein, University of Pennsylvania (11-deoxy-19-hydroxycorticosterone and 11-deoxy-6β:21-diacetoxycorticosterone), Dr W. Klyne, Medical Research Council Steroid Reference Collection (corticosterone), Organon Laboratories Ltd., Newhouse, Lanarkshire (DOCA) and Dr A. Wettstein, Ciba Ltd., Basle (11-deoxy-6β-hydroxycorticosterone). The expenses of the work were defrayed in part by a grant from the Medical Research Council, for which I am most grateful.

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Pentose Phosphate Isomerase and Epimerase from Animal Tissues

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Pentose phosphate isomerase (PPI) was first described by Horecker, Smyrniotis & Seegmiller (1951) as a yeast enzyme which interconverts D-ribose 5-phosphate and D-ribulose 5-phosphate (R 5-P⇌Ru 5-P). The presence of this enzyme in a wide variety of cells explains the formation of R 5-P from the Ru 5-P arising on the hexose monophosphate oxidative pathway (for reviews see Racker, 1954; Gunsalus, Horecker & Wood, 1955; Dickens, 1955). This is one of the key reactions of this cycle, since the R 5-P accepts 'active glycolaldehyde', formed from a pentulose 5-phosphate by transketolase, yielding sedoheptulose 7-phosphate and triose phosphate.

Although the isomerase of spinach leaves has been studied (Axelrod, Bandurski, Greiner & Jang, 1953), and the plant enzyme has been much purified from extracts of alfalfa (lucerne) leaves (Axelrod & Jang, 1954), little work has hitherto been reported on the preparation and properties of PPI of animal tissues. Since Glock & McLean (1954) have shown that R 5-P breakdown occurs rapidly in extracts of many animals cells, the PPI activity in some of these has been studied and is described in the present paper.

Until quite recently it has been generally assumed that Ru 5-P is the only pentulose phosphate concerned in the hexose monophosphate oxidative pathway (cf. Gunsalus *et al.* 1955). Although D-xylulose 1-phosphate (Xu 1-P) has been noted by several workers as a product of aldolase action on dihydroxyacetone phosphate and glycolaldehyde (Hough & Jones, 1952; Glock, 1952; Byrne & Lardy, 1954), this substance has not been clearly shown to be converted into the 5-phosphate, and its metabolism without preliminary re-cleavage by aldolase has not yet been demonstrated (cf. Glock, 1952). The suggestion by McGeown & Malpress (1954) that guinea-pig liver extract can form a ribose phosphate from Xu 1-P needs further supporting evidence, and in attempts to confirm this conclusion we have not yet succeeded in obtaining significant amounts of ribose, such as would demonstrate its formation.

The production of a pentulose phosphate from R 5-P in crude muscle extracts was described by Sable (1952), who believed this to be Ru 5-P. Ashwell & Hickman (1954) found that a D-xylulose phosphate was formed by the action on R 5-P of a fraction from mouse-spleen homogenate; this was the first indication of participation of this ester in animal metabolism. Ashwell & Hickman (1955) also observed the presence of a substance reacting as an *erythro*-3-pentulose among the reaction products: they suggest that the interconversion of the pentulose phosphates may have occurred by way of a 2:3-enediol intermediate compound.