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Metabolism of Oestriol *in vitro*

COFACTOR REQUIREMENTS FOR THE FORMATION OF 2-HYDROXYOESTRIOL AND 2-METHOXYOESTRIOL

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The conversion of oestriol into 2-hydroxyoestriol and 2-methoxyoestriol by rat- and rabbit-liver slices described in the preceding paper indicated a new metabolic pathway for oestrogens *in vitro*. The results suggested that the oestriol was hydroxylated in the 2 position and this hydroxyl group was then methylated to form 2-methoxyoestriol.

A number of papers (reviewed by Grant, 1956*a*) have described the hydroxylation of neutral steroids *in vitro*, and Mueller & Rumney (1957) have studied the 6' α '-hydroxylation of oestradiol-17 β by rat- and mouse-liver microsomal preparations. These hydroxylations all appear to require molecular oxygen and reduced pyridine nucleotide. The 2-hydroxylation of oestriol represents a major difference from these reactions in that the hydroxyl group is entering a benzenoid ring structure. Brodie *et al.* (1955) have shown that the hydroxylation of aromatic compounds such as acetanilide also requires oxygen and reduced triphosphopyridine nucleotide, so that some similarity exists between the hydroxylation of aromatic and aliphatic compounds. However, Kaufman (1959) has shown that a tetrahydrofolic acid derivative is required for the conversion of phenylalanine into tyrosine,

whereas this vitamin has no effect on the 11 β -hydroxylation of 11-deoxycorticosterone (Tomkins, Curran & Michael, 1958).

The *O*-methylation of catechols is well known (Axelrod & Tomchick, 1958; Pellerin & D'Ionio, 1958), and, like *N*-methylation (Cantoni, 1951; Cantoni & Vignos, 1954), the methyl group can be derived from methionine via *S*-adenosylmethionine.

This paper describes the cofactor requirements of the rat-liver 2-methoxylating system.

EXPERIMENTAL

Enzyme preparations and methods

The rats were killed by a blow on the back of the neck. Liver and kidney homogenates were prepared by mincing the tissue in a Latapie mincer to remove connective tissue and then homogenizing with cold 0.25M-sucrose in a glass homogenizer fitted with a nylon pestle. The ovaries were disrupted in a 0.8 cm. $\times$ 15 cm. glass tube with a hand-operated metal plunger; usually, 20% (w/v) homogenates were used. The uteri were slit longitudinally and the inside surface was blotted dry with filter paper. They were then cut into small pieces and finely minced with a Mickle automatic slicer. This brei was added to the appropriate volume of 0.25M-sucrose to give a 20% (w/v) suspension.

When required, the cell debris and nuclei were removed by centrifuging for 10 min. at 700*g* at 0° in a MSE refrigerated centrifuge and the mitochondria by centrifuging

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for 10 min. at 7000g. The 7000g supernatant fraction was usually obtained by centrifuging the whole homogenate for 10 min. at 7000g. The 105000g supernatant was separated from the microsomes by centrifuging the 7000g fraction for 30 min. at 105000g in a preparative Spinco model L centrifuge. The microsomes were suspended in 0.25M-sucrose by gentle homogenization with a glass ball so that 1 ml. of the suspension was equivalent to 1 ml. of homogenate (equivalent to 200 mg. wet wt. of original tissue).

Duplicate incubations were carried out in glass-stoppered test tubes and each experiment was repeated at least once.

The extraction and estimation of steroids were carried out as described in the preceding paper (King, 1961) except that the alumina-chromatography step was omitted in the experiments in which 2-methoxyoestriol was not estimated. By this modified method 80% recoveries of oestriol could be obtained regularly.

The Fast Black Salt K (diazotized *p*-nitrophenylazodimethoxyaniline) derivatives were prepared by the method of Heftmann (1950). The Girard reaction was carried out as described in the preceding paper. The sodium borohydride reduction was carried out by dissolving the dry material in 1 ml. of methanol and adding 2 mg. of sodium borohydride (British Drug Houses Ltd.). This solution stood at room temperature for 1 hr. and was then diluted with 10 ml. of 2N-HCl plus 39 ml. of water. The resultant solution was extracted three times with portions (50 ml.) of ether and the ether washed twice with 20 ml. of water. The ether extract was evaporated to dryness under vacuum.

Materials

Diphosphopyridine nucleotide (DPN) and barium glucose 6-phosphate were obtained from C. F. Boehringer und Söhne (GmbH., Mannheim, Germany). Sodium glucose 6-phosphate solutions were prepared from the barium salt by precipitating the barium with the calculated amount of sodium sulphate. Calcium lactate was kindly donated by Dr J. K. Grant and the sodium salt prepared from this by addition of the calculated amount of sodium oxalate. Triphosphopyridine nucleotide (TPN), glucose 6-phosphate dehydrogenase and the sodium salt of adenosine triphosphate (ATP) were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). L-Methionine and folic acid were bought from British Drug Houses Ltd. Dihydrofolic acid

was kindly prepared by Dr E. Brode by the method of Futterman (1957). Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid. Adams platinum oxide catalyst (Johnson, Matthey and Co.) (30 mg.) was suspended in 10 ml. of acetic acid and hydrogenated for 3 hr. at room temperature with gentle shaking. Folic acid (100 mg.) was then added and the shaking continued until the yellow suspension changed to a green solution. The catalyst was removed by centrifuging and the tetrahydrofolic acid precipitated with 100 ml. of dry ether. The tetrahydrofolic acid was centrifuged off and rapidly washed twice with portions (50 ml.) of dry ether. The whitish precipitate was dried *in vacuo* and stored under nitrogen in the dark at -17° .

In addition to the steroids mentioned in the preceding paper, the stilboestrol (3:4-di-*p*-hydroxyphenylhex-3-ene) and 17 α -ethynyoestradiol-17 β (17 α -ethynyoestra-1:3:5-triene-3:17 β -diol) were gifts from Dr G. S. Boyd. The oestriol was added to the incubation tubes as a solution in propylene glycol.

Ether (A.R. grade) was distilled once immediately before use. Other solvents and Celite were purified by the methods described by Bauld (1953).

RESULTS

Cofactor requirements for the formation of 2-methoxyoestriol by liver homogenates

Magnesium ions, ATP, L-methionine, DPN and TPN were required for the optimum conversion of oestriol into 2-methoxyoestriol. The pyridine nucleotide requirements of this reaction are shown in Table 1; in the absence of DPN and TPN, the amount of oestriol metabolized is small and very little, if any, 2-hydroxyoestriol is produced. Although the addition of oxidizable substrates (lactate and glucose 6-phosphate) has very little effect, this is probably due to the presence of endogenous substrates which can reduce DPN and TPN, as the experiment described in Table 7 indicates that the reduced form is required for the 2-hydroxylation reaction.

Table 1. *Effect of di- and tri-phosphopyridine nucleotide on metabolism of oestriol by rat-liver homogenates*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 20% (w/v) homogenate in 0.25M-sucrose, 150 μ moles of $MgCl_2$, 5 μ moles of ATP, 60 μ moles of L-methionine, 192 μ moles of potassium phosphate, pH 7.4, 240 μ moles of KCl and 0.52 μ mole (150 μ g.) of oestriol in 0.035 ml. of propylene glycol. Final vol., 3 ml. Incubations were carried out under O_2 for 1 hr. at 37° . G-6-P, Glucose 6-phosphate.

Additions	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
None	63	0	1	64
0.7 μ mole of DPN + 4.5 μ moles of lactate	51	4	1	56
1.4 μ moles of DPN + 9 μ moles of lactate	42	5	1	48
0.7 μ mole of TPN + 4.5 μ moles of G-6-P	37	9	5	51
1.4 μ moles of TPN + 9 μ moles of G-6-P	7	11	6	24
0.7 μ mole of DPN + 0.7 μ mole of TPN + 4.5 μ moles of lactate + 4.5 μ moles of G-6-P	17	18	6	41
0.7 μ mole of DPN + 0.7 μ mole of TPN	19	15	5	39
0.7 μ mole of DPN + 0.7 μ mole of TPN (oestriol added just before extraction)	73	0	0	73

The effect of adding Mg^{2+} ions, ATP and L-methionine is shown in Table 2. No other methyl donors were tested. These three cofactors have a marked effect on the production of 2-methoxy-oestriol without altering the 2-hydroxylase activity. There is an absolute requirement for Mg^{2+} ions and L-methionine, whereas ATP has a pronounced stimulating effect. Dialysis of the homogenate against water at 0° for 18 hr. resulted in the complete loss of 2-hydroxylase activity, whereas pre-incubation of the homogenate for 30 min. at 37° resulted in the complete loss of methylating activity and a partial loss of the hydroxylase system (Table 3).

Conversion of 2-hydroxyoestriol into 2-methoxyoestriol

When 2-hydroxyoestriol was incubated with the optimum methoxylating system described in Table 2 there was a 20–30% conversion into 2-

methoxyoestriol. The omission of DPN and TPN from the incubation medium had no effect, but in the absence of L-methionine no 2-methoxyoestriol was formed.

Substrate specificity of the 2-methoxylating system

Two incubation tubes were set up for each compound to be tested. The complete methoxylating system described in Table 2 was added to tube A of each pair and L-methionine and ATP were omitted from the B series of tubes. The required compound (150 $\mu g.$) was added to the incubation tubes as a methanolic solution (0.025 ml.). Oestrone, oestradiol-17 β , 17 α -ethinyloestradiol-17 β and stilboestrol were used as substrates. After incubation for 1 hr. at 37° an ether extract was prepared as before and divided into three equal parts.

Part 1 was chromatographed on Whatman no. 1 paper in methanol–water–benzene–hexane

Table 2. *Effect of magnesium ions, adenosine triphosphate and L-methionine on metabolism of oestriol by rat-liver homogenates*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 20% (w/v) homogenate in 0.25M-sucrose, 0.7 μ mole of DPN, 4.5 μ moles of sodium lactate, 0.7 μ mole of TPN, 4.5 μ moles of glucose 6-phosphate, 192 μ moles of potassium phosphate, pH 7.4, 240 μ moles of KCl and 0.52 μ mole (150 $\mu g.$) of oestriol in 0.035 ml. of propylene glycol. Where stated, the following additions were made: 5 μ moles of ATP, 150 μ moles of $MgCl_2$, 150 μ moles of $CaCl_2$, 60 μ moles of L-methionine. Final vol., 3 ml. Incubations were carried out under O_2 for 1 hr. at 37°.

Additions	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
150 μ moles of $MgCl_2$	20	0	16	36
150 μ moles of $MgCl_2$ + 60 μ moles of L-methionine	25	6	8	39
150 μ moles of $MgCl_2$ + 5 μ moles of ATP	23	0	16	39
150 μ moles of $CaCl_2$ + 5 μ moles of ATP + 60 μ moles of L-methionine	30	0	15	45
150 μ moles of $MgCl_2$ + 5 μ moles of ATP + 60 μ moles of L-methionine	19	15	5	39
150 μ moles of $MgCl_2$ + 5 μ moles of ATP + 60 μ moles of L-methionine (oestriol added just before extraction)	73	0	0	73

Table 3. *Effect of adenosine triphosphate on the metabolism of oestriol by rat-liver homogenates*

Figures are given as percentages of oestriol added. All incubations contained 0.7 μ mole of DPN, 0.7 μ mole of TPN, 4.5 μ moles of sodium lactate, 4.5 μ moles of glucose 6-phosphate, 60 μ moles of L-methionine, 150 μ moles of $MgCl_2$, 240 μ moles of KCl and 192 μ moles of potassium phosphate, pH 7.4, and 0.52 μ mole (150 $\mu g.$) of oestriol in 0.035 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°. Where stated, the homogenate in sucrose was preincubated for 30 min. at 37° without any additions. 20% (w/v) Homogenates in 0.25M-sucrose were used.

Additions	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
1 ml. of homogenate (oestriol added just before extraction)	72	0	0	72
1 ml. of homogenate	14	3	14	31
1 ml. of homogenate + 5 μ moles of ATP	10	15	8	33
1 ml. of preincubated homogenate	41	0	10	51
1 ml. of preincubated homogenate + 5 μ moles of ATP	19	0	11	30

(70:30:50:50, by vol.) for $3\frac{1}{2}$ hr. With the compounds used in this experiment, this system would separate any *o*-methoxy derivative from the parent phenol. In all of the A series of tubes there was a Folin & Ciocalteu-positive compound which was less polar than the original phenol. With oestrone, this compound had the same mobility as 2-methoxyoestrone. In the A tube of the oestradiol-17 β incubation two compounds were detected which had the same mobility as 2-methoxyoestrone and oestrone respectively. In all of the B series of tubes these less-polar metabolites were absent, apart from the conversion of oestradiol-17 β into oestrone.

Part 2 was chromatographed on Whatman no. 42 paper in the formamide-chloroform system for 10 hr. No compound more polar than oestradiol-17 β was detected in either of the oestrone incubations. In the B but not in the A oestradiol-17 β incubation there was a compound which turned blue with Folin & Ciocalteu reagent in the absence of alkali and which had the same mobility as 2-hydroxyoestradiol-17 β . In the B tube of the 17 α -ethinyloestradiol-17 β incubation, and in both stilboestrol incubations, there were metabolites slightly more polar than 16-epioestriol which, from their chromatographic behaviour and reaction with Folin & Ciocalteu reagent, could have been the *O*-hydroxylated derivatives. With stilboestrol there was about twice as much of this metabolite as in the A tube.

Part 3 was chromatographed on Whatman no. 1 paper for $4\frac{1}{2}$ hr. in acetic acid-water-ethylene dichloride (70:30:100, by vol.) but no phenols more polar than oestriol could be detected.

Metabolism of oestriol by homogenates of different rat tissues

Table 4 shows that the 2-hydroxylase is absent in rat kidney, uterus and ovary and that there is no sex difference in the liver 2-hydroxylase activity. The large amount of oestriol metabolized by kidney homogenates is partly explained by the formation of 16-oxo-oestradiol-17 β and 16-epioestriol (King, 1960).

In this and subsequent experiments a simple potassium phosphate buffer was used instead of the potassium phosphate-magnesium chloride-potassium chloride solution employed in the methoxylation studies. When the phosphate buffer was replaced by an equal volume of 0.35M-2-amino-2-hydroxymethylpropane-1:3-diol(tris)-HCl, pH 7.4, no 2-hydroxyoestriol was formed. This hydroxylase system thus differs from the 11 β -hydroxylase from ox-adrenal mitochondria which is more active in tris than in phosphate (Grant, 1956b).

Intracellular localization of the liver 2-hydroxylase system

The results of experiments with different cell fractions are shown in Table 5. Removal of the

Table 4. *Metabolism of oestriol by rat-tissue homogenates*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 20% (w/v) homogenate in 0.25M-sucrose, 0.7 μ mole of DPN, 0.7 μ mole of TPN, 4.5 μ moles of sodium lactate, 4.5 μ moles of glucose 6-phosphate, 192 μ moles of potassium phosphate, pH 7.4, and 0.35 μ mole (100 μ g.) of oestriol in 0.025 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O₂ for 1 hr. at 37°.

Tissue	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
Female liver (oestriol added just before extraction)	81	0	81
Female liver	16	27	43
Male liver	14	24	38
Female kidney	37	1	38
Uterus	76	1	77
Ovary	70	1	71

Table 5. *Intracellular distribution of rat-liver 2-hydroxylase*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of the cell fraction in 0.25M-sucrose, 0.7 μ mole of DPN, 0.7 μ mole of TPN, 4.5 μ moles of sodium lactate, 4.5 μ moles of glucose 6-phosphate, 192 μ moles of potassium phosphate, pH 7.4, and 0.35 μ mole (100 μ g.) of oestriol in 0.025 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O₂ for 1 hr. at 37°.

Cell fraction	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
Whole homogenate	16	27	43
Minus 700g sediment	9	30	39
Minus 7000g sediment	32	16	48
Minus 7000g sediment + 3 μ moles of ATP	4	29	33

nuclei and cell debris by a short centrifuging at 7000g has no effect on the pattern of oestriol metabolism, but subsequent removal of the mitochondria appreciably lowers the 2-hydroxylase activity. The activity of this 7000g supernatant fraction could be restored to that of the whole homogenate by the addition of ATP. The results shown in Tables 2 and 3 indicate that ATP does not affect the 2-hydroxylase activity of whole homogenates. The presence of ATP or an ATP-synthesizing system in the homogenate is suggested by the small synthesis of 2-methoxyoestriol in the absence of added ATP (Table 2).

The DPN and TPN requirements of the 7000g supernatant fraction (Table 6) differ in some respects from those of the homogenate. Either DPN or TPN can be employed, but the latter is more effective at higher concentrations than the former and under these conditions is as effective as DPN and TPN combined. As rat-liver mitochondria contain a transhydrogenase (Humphrey, 1957), the possibility cannot be excluded that the DPN is in fact only acting indirectly via a transhydrogenation reaction with TPN. Preincubation of the cell fraction for 10 min. at 37° indicated a partial requirement for an oxidizable substrate. The addition of nicotinamide does not influence the hydroxylase but has a pronounced effect on the amount of oestriol metabolized. Fig. 1 shows the time course of this nicotinamide-stimulated oestriol metabolism. This indicates that the lack of effect of nicotinamide on the production of 2-hydroxyoestriol is not due to an increased production of this compound compensated by an elevated destruction. ATP increases both the amount of oestriol metabolized and the formation of 2-

hydroxyoestriol. This stimulation is independent of whether TPN alone or TPN plus DPN is added to the system.

When the 7000g supernatant fraction was separated into microsomal and 105000g supernatant fractions, neither fraction showed any 2-hydroxylase activity in the presence of a reduced TPN-producing system, but the further addition of either tetrahydrofolic acid or ATP to the microsomal

Fig. 1. Effect of varying incubation time on production of 2-hydroxyoestriol by rat-liver 7000g supernatant fraction. All incubations contained 1 ml. of 7000g supernatant fraction, 0.7 μ mole of DPN, 4.5 μ moles of sodium lactate, 0.7 μ mole of TPN, 4.5 μ moles of glucose 6-phosphate, 72 μ moles of nicotinamide, 192 μ moles of potassium phosphate, pH 7.4, and 0.35 μ mole (100 μ g.) of oestriol in 0.025 ml. of propylene glycol. Final vol., 3 ml. Incubations were carried out under O_2 at 37°. ●, 2-Hydroxyoestriol; ○, oestriol.

Table 6. *Effect of di- and tri-phosphopyridine nucleotide and adenosine triphosphate on metabolism of oestriol by rat-liver 7000g supernatant fraction*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 7000g supernatant fraction in 0.25M-sucrose, 192 μ moles of potassium phosphate, pH 7.4, and 0.35 μ mole (100 μ g.) of oestriol in 0.025 ml. of propylene glycol. Final vol., 3 ml. Incubations were carried out under O_2 for 1 hr. at 37°. G-6-P, Glucose 6-phosphate.

Additions	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
0.7 μ mole of DPN + 4.5 μ moles of lactate	56	5	61
1.4 μ moles of DPN + 9 μ moles of lactate	36	8	44
0.7 μ mole of TPN + 4.5 μ moles of G-6-P	56	4	60
1.4 μ moles of TPN + 9 μ moles of G-6-P	32	13	45
1.4 μ moles of TPN + 9 μ moles of G-6-P + 3 μ moles of ATP	3	27	30
0.7 μ mole of DPN + 0.7 μ mole of TPN + 4.5 μ moles of lactate + 4.5 μ moles of G-6-P	32	16	48
0.7 μ mole of DPN + 0.7 μ mole of TPN + 4.5 μ moles of lactate + 4.5 μ moles of G-6-P + 72 μ moles of nicotinamide	7	15	22
0.7 μ mole of DPN + 0.7 μ mole of TPN + 4.5 μ moles of lactate + 4.5 μ moles of G-6-P + 3 μ moles of ATP	4	29	33

Table 7. *Effect of triphosphopyridine nucleotide, adenosine triphosphate and tetrahydrofolic acid on metabolism of oestriol by rat-liver microsomes*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of microsomal preparation in 0.25M-sucrose, 192 μ moles of potassium phosphate, pH 7.4, and 0.35 μ mole (100 μ g.) of oestriol in 0.025 ml. of propylene glycol. Where stated, the following additions were also made: 1.4 μ moles of TPN, 9 μ moles of glucose 6-phosphate (G-6-P), 3 μ moles of ATP, 2.5 μ moles of tetrahydrofolic acid, 0.07 unit of G-6-P dehydrogenase. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°.

Additions	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
TPN + G-6-P + G-6-P dehydrogenase (oestriol added just before extraction)	81	0	81
TPN + G-6-P + G-6-P dehydrogenase	64	1	65
TPN + G-6-P + G-6-P dehydrogenase + ATP	26	4	30
Tetrahydrofolic acid + ATP	74	1	75
Tetrahydrofolic acid + ATP + TPN	—	0	—
Tetrahydrofolic acid + TPN + G-6-P + G-6-P dehydrogenase	47	6	53
Tetrahydrofolic acid + TPN + G-6-P + G-6-P dehydrogenase + ATP	29	11	40

Table 8. *Effect of a number of folic acid derivatives on the 2-hydroxylase activity of rat-liver microsomes*

All incubations contained 1 ml. of microsomal preparation in 0.25M-sucrose, 1.4 μ moles of TPN, 9 μ moles of glucose 6-phosphate, 0.07 unit of glucose 6-phosphate dehydrogenase, 3 μ moles of ATP, 192 μ moles of potassium phosphate, pH 7.4, and 0.35 μ mole (100 μ g.) of oestriol in 0.025 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°.

Additions	2-Hydroxy-oestriol formed (% of oestriol added)
2.5 μ moles of tetrahydrofolic acid	11
2.5 μ moles of dihydrofolic acid	11
2.5 μ moles of folic acid	6
2.5 μ moles of tetrahydrofolic acid (additional 2.5 μ moles added 15 min. after the start of the incubation)	11
None	4

fraction produced small amounts of 2-hydroxy-oestriol (Table 7). The best results were obtained on adding both ATP and tetrahydrofolic acid. The addition of a further 2.5 μ moles of tetrahydrofolic acid 15 min. after the start of the incubation had no effect, which suggests that the folic acid derivative is not rate-limiting. Dihydrofolic acid can replace the tetrahydro compound (Table 8) but folic acid has only a small effect.

When the microsomes were dialysed against ice-cold water for 18 hr. or were washed with 0.25M-sucrose the 2-hydroxylase activity was lost, which suggested that some other cofactor, possibly a metal ion, is also required. This would explain the finding that only about half of the activity present in the 7000g supernatant could be recovered on further fractionation of this cell fraction.

Detection of other phenolic oestriol metabolites

In no case could any 6' α -hydroxyoestriol or 6-oxo-oestriol be detected. The addition of nicotin-

amide to the 7000g supernatant fraction resulted in the detection of two new phenolic metabolites. Both were ketonic, as determined by the Girard reaction. The less-polar compound had the same mobility as 16-oxo-oestradiol-17 β in both formamide-chloroform and acetic acid-water-ethylene dichloride (70:30:100, by vol.) systems. The other metabolite had the same mobility as oestriol in formamide-chloroform but it was slightly more polar than oestriol in acetic acid-water-ethylene dichloride. It was similar to 2-hydroxyoestriol in that it developed the Folin & Ciocalteu blue colour in the absence of alkali.

To characterize these compounds further, 200 μ g. of oestriol was incubated under the conditions shown in Fig. 1 for 1 hr. The oestriol and 2-hydroxy-oestriol were removed by Girard separation and the two ketonic metabolites were separated by paper chromatography with formamide-chloroform for 10 hr. This method of carrying out the Girard reaction does not result in the artifactual formation of 16-oxo-oestradiol-17 β from 16 α -hydroxy- or 16 β -hydroxy-oestrone (Layne & Marrian, 1958).

Half of the less-polar metabolite was converted into the Fast Black Salt K derivative. It had the same mobility as the Fast Black Salt K derivative of authentic 16-oxo-oestradiol-17 β in ethanol-water-toluene-light petroleum (40-60°) (30:70:20:10, by vol.) (Heftmann, 1950). The other half was reduced with sodium borohydride. As judged from the chromatographic behaviour, the main reduction product was 16-epioestriol plus a trace of oestriol. Sodium borohydride reduction of 16-oxo-oestradiol-17 β produces 90% of 16-epioestriol plus 10% of oestriol (Loke, 1958). It thus seems probable that this metabolite is 16-oxo-oestradiol-17 β . Under these incubation conditions there was a 2-3% conversion of oestriol into 16-oxo-oestradiol-17 β . A similar yield was obtained when a 105000g supernatant fraction was used.

The yield of the more-polar metabolite was too small to allow further characterization. It may

be 2-hydroxy-16-oxo-oestradiol-17 β (2:3:17 β -tri-hydroxyoestra-1:3:5-triene-16-one). Another possibility is that it is the *o*-quinone derivative of 2-hydroxyoestriol, but this seems unlikely, as such a compound would strongly absorb u.v. light in the 240 m μ region, whereas this metabolite does not. This would also seem to rule out a *p*-quinol derivative of the type discovered by Hecker & Mueller (1958).

DISCUSSION

This work substantiates the conclusion made in the preceding paper that the conversion of oestriol into 2-methoxyoestriol proceeds via 2-hydroxy-oestriol.

Like other steroid hydroxylases, the 2-hydroxylase requires a reduced pyridine nucleotide. Either DPN or TPN can be utilized, although the latter is more efficient at higher concentrations. The possibility cannot altogether be excluded that DPN is acting only indirectly via a transhydrogenation reaction. It is quite possible that the 7000g supernatant fraction is contaminated with mitochondria, which are known to possess a transhydrogenase (Humphrey, 1957). This explanation would, however, seem unlikely, because, whereas exogenous pyridine nucleotide is required for the formation of 2-hydroxyoestriol, reasonable amounts are formed when DPN is added in the absence of TPN.

The function of the folic acid derivatives in this hydroxylation is not clear from these experiments. There are two possible explanations. The first is that a tetrahydrofolic acid derivative is the direct hydrogen donor and that the reduced pyridine nucleotide is acting only indirectly by reforming the tetrahydro compound. This is similar to the explanation given by Kaufman (1959) to account for his experiments on the conversion of phenylalanine into tyrosine. This would account for the relative effectiveness of folic acid, dihydrofolic acid and tetrahydrofolic acid in the presence of reduced TPN-producing system but does not explain the formation of 2-hydroxyoestriol in the absence of an exogenous folic acid derivative. This may be due to the presence of endogenous material in the microsomes, but as dialysis resulted in complete loss of hydroxylase activity this possibility could not be tested. The ineffectiveness of tetrahydrofolic acid in the absence of reduced TPN-producing system could be explained by the instability of this compound in solution in the presence of oxygen. It would, however, seem to rule out the possibility that the tetrahydrofolic acid and reduced TPN are donating hydrogen to a common hydrogen-accepting cofactor.

The other possibility is that reduced TPN is the direct hydrogen donor and that the added tetrahydrofolic acid is acting by re-forming reduced TPN. This explanation does not account for the

fact that excess of glucose 6-phosphate and glucose 6-phosphate dehydrogenase are present in the incubation medium and that dihydrofolic acid is as effective as tetrahydrofolic acid in stimulating the hydroxylation reaction. It would be difficult to explain the effect of a reduced-DPN-producing system by either of these hypotheses as all of the folic acid reductases so far isolated from normal mammalian tissues are TPN-specific. However, Scrimgeour & Huennekens (1960) claim to have detected a DPN-specific reductase in Ehrlich ascites-tumour cells.

If a folic acid derivative is required for 2-hydroxylation it would provide an interesting link between the observation that folic acid is needed for the oestrogen-stimulated growth of chicken oviduct (Hertz, 1948) and the fact that 2- and 4-hydroxyoestradiol-17 β are the only compounds so far tested which have an effect on uterine protein metabolism *in vitro* (Mueller, 1955).

The exact function of ATP in hydroxylation reactions is not certain. Hayano & Dorfman (1954) found that the DPN and ATP, previously thought to be required for 11 β -hydroxylation, could be replaced by TPN, which indicated that the ATP was converting the DPN into TPN. Similar results have been obtained with the adrenal-microsomal 21-hydroxylase (Ryan & Engel, 1957). ATP stimulates the hydroxylation of acetanilide by liver microsomes in the presence of TPN plus a reduced TPN-producing system but had no effect when reduced TPN alone was added (Mitoma, Posner, Reitz & Udenfriend, 1956). It is difficult to reconcile the results given in the present paper with these explanations. If ATP is acting by phosphorylating DPN it should not stimulate the microsomal 2-hydroxylase in the absence of DPN. Also, in the experiments with the 7000g supernatant fraction, the ATP activation is independent of whether TPN alone or TPN plus DPN is added to the incubation medium. Under the conditions used in these experiments, the reduction of TPN is very rapid in the absence of ATP so it would seem unlikely that it is stimulating the production of reduced TPN. The fact that the 2-hydroxylating system is not affected by nicotinamide rules against pyridine nucleotide breakdown being a rate-limiting reaction, but the possibility cannot be eliminated that the ATP is stabilizing the pyridine nucleotides by some other mechanism. It is noteworthy that Kornberg (1950) has described an enzyme which catalyses the following reaction:

The ATP could also be stimulating the reaction by activating a folic acid compound. Greenberg & Jaenicke (1957) and Whiteley, Osborn & Huennekens (1958) have postulated a phosphorylated

tetrahydrofolic acid compound as an intermediate in the activation of formic acid.

The loss of activity on washing or dialysing the microsome fraction suggests that another cofactor, possibly a metal ion, is required.

As far as can be judged from the experiments with crude homogenates, the 2-hydroxylase does not appear to be very specific.

The small formation of 2-methoxyoestriol in the absence of ATP was rather unexpected. In other cases of *O*-methylation (Axelrod & Tomchick, 1958; Pellerin & D'Ionio, 1958) the methyl group is derived from *S*-adenosylmethionine and it seems likely that the *o*-methyl group of 2-methoxyoestriol has a similar origin. The small amount of 2-methoxyoestriol formed in the absence of ATP is presumably due to the presence of endogenous ATP or an ATP-synthesizing system. The finding that rabbit-liver slices produce less 2-methoxyoestriol but more 2-hydroxyoestriol than do rat-liver slices (King, 1961) suggests that the methylase studied in these experiments is the same as that studied by Axelrod & Tomchick (1958), as they found that rabbit liver had a much lower *O*-methylating activity than rat liver.

The formation of 16-oxo-oestradiol-17 β from oestriol in the liver is very small compared with that in the kidney (King, 1960).

The absence of 6' α '-hydroxyoestriol and 6-oxo-oestriol was rather surprising, especially as the microsomal system used in these experiments is almost identical with that with which Mueller & Rumney (1957) obtained a high yield of 6' α '-hydroxyoestradiol-17 β from oestradiol-17 β . Moreover, Breuer, Knuppen & Schriefers (1960) have recently reported the conversion of oestriol into 6-hydroxyoestriol by rat-liver slices. In view of the large amounts of oestriol which cannot be accounted for, it is possible that small amounts of these compounds are formed but are further metabolized to undetected products. In this respect it is interesting that no 6' α '-hydroxyoestriol or 6-oxo-oestriol could be detected in human pregnancy urine (G. F. Marrian & R. J. B. King, unpublished results).

Graubard & Pincus (1942) showed that oestrogens could be oxidized by a number of plant phenolases and this work has recently been extended by Jellinck (1960). The virtual absence of oestriol metabolism in the absence of DPN and TPN suggests that such reactions do not occur in rat liver.

SUMMARY

1. The quantitative production of 2-hydroxyoestriol and 2-methoxyoestriol has been studied with rat-liver preparations. The 2-hydroxylase requires either a reduced diphosphopyridine nucleo-

tide- or reduced triphosphopyridine nucleotide-producing system and possibly a folic acid derivative. Adenosine triphosphate has a stimulating effect. The 2-hydroxylase is localized in the microsomal fraction.

2. The 2-hydroxylase activity is absent in homogenates of kidney, ovary and uterus. There is no sex difference in the rat-liver activity.

3. The *O*-methylating system requires magnesium ions, adenosine triphosphate and L-methionine. Evidence is presented that the *o*-methylase may be the same enzyme as that studied by Axelrod & Tomchick (1958).

4. Preliminary evidence suggests that oestrone, 17 α -ethynyloestradiol-17 β , stilboestrol and possibly oestradiol-17 β can be methoxylated in the position *ortho* to an existing phenolic group.

5. 16-Oxo-oestradiol-17 β and another unknown compound have been detected as minor metabolites of oestriol. The oestriol-16-hydroxy dehydrogenase is localized in the 105000g supernatant fraction.

6. The relationship of reduced pyridine nucleotide, folic acid and adenosine triphosphate to hydroxylation reactions has been discussed.

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Effects of Metal Ions on the Utilization of Glucose and on the Influence of Insulin on it by the Isolated Rat Diaphragm

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Insulin causes an increased uptake of glucose by the isolated rat diaphragm (Gemmill, 1940), although the mechanism by which insulin exerts its effect has not yet been explained. On the basis of the evidence presented by Levine & Goldstein (1955), Park, Bornstein & Post (1955), Park & Johnson (1955), Park, Johnson, Wright & Batsel (1957) and others, it is, however, now generally agreed that insulin, both *in vivo* and *in vitro*, exerts its effect by increasing the rate of entry of glucose from the extracellular fluid to the interior of the cell, presumably by some effect on the cell membrane. Nothing, however, is so far known regarding the nature of the effect of insulin on the cell membrane.

This paper describes the results of experiments on the effects of physiologically important cations, and of certain other related ions, on the uptake of glucose and on the influence of insulin on it by the rat diaphragm *in vitro*. It is well known that these ions, in addition to their functions of osmotic regulation of tissue fluids and of activation of various enzymes, play essential roles in the preservation of integrity, and the regulation of permeability properties, of cell membranes. Preliminary reports of some of the experiments described in this paper have been published (Bhattacharya, 1959*a, b*).

MATERIALS AND METHODS

Incubation media. The standard incubation medium was the bicarbonate buffer of Gey & Gey (1936). Various other incubation mixtures were used. These latter mixtures

generally consisted of a suitable buffer in iso-osmotic sucrose (0.25M) or in a mixture of iso-osmotic sucrose and iso-osmotic solutions of the chlorides of certain univalent and bivalent metals. 0.154M-Solutions of the chlorides of the univalent metals and 0.11M-solutions of the chlorides of bivalent metals were considered to be iso-osmotic. In most instances the pH of the medium was adjusted to 7.4. The buffers used and their concentrations in the medium were usually as follows: LiHCO_3 , NaHCO_3 and KHCO_3 , 0.02M; Na_2HPO_4 , 0.01M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0.025M. Glucose when present was added to give a final concentration of about 250 mg./100 ml.

Insulin. Crystalline insulin (Wellcome Foundation or Boots Pure Drug Co. Ltd.) was dissolved in 0.033N-HCl to give a concentration of 10 units/ml. This stock insulin was added to the incubation medium to give the required concentration just before each experiment. The final insulin concentration was usually about 0.1 unit/ml.

¹³¹I-marked insulin. This was prepared according to the method of Bournsnel, Coombs & Rizk (1953) as modified by Bournsnel (1958). The final stock preparation was obtained in 0.05M- Na_2HPO_4 buffer, pH 7.4. The insulin concentration was about 0.5 mg./ml. No inactivation of the insulin, as measured by its effect on increased uptake of glucose by the isolated rat diaphragm, could be demonstrated.

Animals. Diaphragm muscle was obtained from female albino-Wistar rats (wt. 100–150 g.) which had been starved 20–24 hr. before use.

Preparation of cut diaphragm. The rat was killed by decapitation and bled. The diaphragm was then removed and put into a beaker containing freshly gassed buffer. After the required number (usually six) of diaphragms had been obtained, each diaphragm was gently blotted and cut into halves, and each half was transferred to a small conical flask containing 1 ml. of the medium. One-half of a diaphragm served as the control and the other was used to determine the effect of insulin or of other treatment. The flasks were then gassed with the required gas mixture (see Table 1), sealed with rubber stoppers and incubated at 38°

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