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Factors Affecting the Activity of Oestradiol Hydroxylase in Rat Liver Microsomal Subfractions

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1. Oestradiol hydroxylase activity, as measured by the formation of water-soluble products, was significantly higher in the smooth endoplasmic reticulum of rat liver than in the rough membrane. 2. The isolated membrane fractions retained their activity for at least 6 months if stored at −30°C and were more stable in tris–HCl than in sodium phosphate buffer. 3. The stability of the oestradiol-hydroxylating system was inversely related to lipid peroxidation and was decreased by phospholipases and deoxycholate, which damage the reticular membranes. Ribonuclease had no effect on this system. 4. Added polyribosomes did not influence the metabolism of oestradiol in the smooth membranes but some inhibition in the yield of water-soluble metabolites was produced by corticosterone. 5. The effect of spermine on microsomal hydroxylation was investigated. It is proposed that this polyamine acts either by direct activation of the enzyme complex or by inhibition of the lipid peroxidase pathway in a linked system rather than by stabilization of the reticular membranes.

The polyamines spermine and spermidine are known to produce a marked increase in the conversion of oestradiol into water-soluble metabolites when added to rat liver microsomal preparations (Jellinck & Perry, 1967). Subsequently, it was proposed (Jellinck & Cox, 1970) that spermine may be stabilizing the membranes of the endoplasmic reticulum which contain the mixed-function oxidases involved in the hydroxylation of steroids and many foreign compounds (Gillette, 1966). This effect might be produced by interaction of the polyamine with RNA, phospholipids or other negatively charged components of the membranes, and the purpose of the studies reported below was to test this hypothesis. However, it is also possible that spermine was affecting directly a component of the oestrogen-hydroxylating system and that ribosomes, to which spermine is known to bind (Raina & Teleranta, 1967), might be involved. It was therefore decided to determine the oestrogen hydroxylase activity of microsomal subfractions and to investigate the effects of spermine and polyribosomes on this system.

EXPERIMENTAL

Materials. ATP, GTP, NADPH, RNA (from yeast), glucose 6-phosphate, GSH, spermine, phospholipases A, C and D, and ribonuclease (type I-A from bovine pancreas), were the highest grade available from Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals used were sucrose (ribonuclease-free) from Mann Research Laboratories, New York, N.Y., U.S.A.; tris, 2-thiobarbituric acid and sodium deoxycholate from Fisher Scientific Co., Toronto, Ont., Canada; and malonaldehyde bis(dimethyl acetal) from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. L-[G-^{14}C]Leucine (316mCi/mmol) and [4-^{14}C]oestradiol (40mCi/mmol) were obtained from Schwarz BioResearch, Orangeburg, N.Y., U.S.A. The [^{14}C]oestradiol was diluted with non-radioactive hormone and stored as a stock solution at 4°C in ethanol. All solvents were redistilled.

Measurement of radioactivity. A Nuclear–Chicago Unilux II scintillation spectrometer was used to determine ^{14}C radioactivity with counting efficiencies of approx. 70% under the conditions employed. The scintillation liquid consisted of 10ml of toluene–ethanol (3:2, v/v) containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene obtained from Amersham–Searle, Toronto, Ont., Canada. Generally the channels ratio of c.p.m. was constant and no quenching correction was necessary.

Preparation of microsomal subfractions. Mature (2–4 months old) male Holtzman rats were used. Starved animals received water only. The rats were anaesthetized by placing them into an atmosphere of CO_2 and killed by cervical dislocation.

The livers were removed rapidly, rinsed and homogenized in ice-cold 0.35M-sucrose in medium B (2.5ml/g wet wt. of tissue) for fractionation by an adaptation of the method of Bloemendal, Bont, de Vries & Benedetti (1967). (Medium B consisted of 50mM-tris–HCl buffer, pH7.6, 25mM-KCl and 10mM-magnesium acetate.) A Teflon–glass homogenizer was used, 10 strokes at 600rev./min being applied.

Nuclei, mitochondria and cell debris were removed by

centrifugation at 15000$g_{av.}$ for 10min in a Spinco model L ultracentrifuge (no. 50 titanium rotor). Portions (6ml) of the supernatant were then layered on top of a discontinuous gradient consisting of 3ml of 2M-sucrose and 3ml of 1.5M-sucrose each in medium B, and centrifuged at 145000$g_{av.}$ for 3.5h.

The 0.35M-sucrose top layer down to about 2cm from the 1.5M-sucrose interface was used as a source of soluble enzyme for the [^{14}C]leucine-incorporation experiments. Surface lipid was discarded. Two bands could be distinguished, one on top and one inside the 1.5M-sucrose layer, and were separated carefully by means of a syringe fitted with a no. 16 gauge needle into a red fraction (smooth membranes) and a yellow fraction (rough membranes). Membrane fractions were isolated as pellets by diluting the sucrose solution to 0.35M with medium B and centrifuging at 145000$g_{av.}$ for 1.5h. The polyribosome pellet was purified by resuspending in medium B and centrifuging at 145000$g_{av.}$ for 1.5h. The resuspended pellets in medium B and sucrose were used immediately. In experiments to test the stability of the system the membrane fractions were stored as a frozen suspension (1g equivalent wet wt. of liver/ml of 0.35M-sucrose in medium B) at −30°C.

Incorporation of [^{14}C]*leucine.* The incubation mixture, adapted from that of Mahler & Brown (1968), consisted of the following: 0.25M-sucrose; 20mM-tris–HCl buffer, pH7.6; 100mM-KCl; 40mM-NaCl; 10mM-magnesium acetate; 5mM-ATP; 0.1mM-GTP; L-[^{14}C]leucine (0.5μCi in 1.58nmol); a portion (0.1ml) of the 145000g-supernatant and 0.05ml of polyribosomes (derived from 0.5g wet wt. of liver) or membrane fractions (derived from 0.25g wet wt. of liver) in a total volume of 1ml.

The mixture was incubated for 30min at 37°C and the reaction stopped by adding 0.1ml of 1M-HCl and placing the tubes in ice. Trichloroacetic acid-precipitable material from 0.4ml samples was prepared for ^{14}C assay by the filter-paper-disc method of Mans & Novelli (1961). A zero-time control was also taken through the complete washing procedure and counted for radioactivity in the usual manner.

The determination of RNA/protein ratios in the microsomal subfractions was carried out as described by Campbell & Sargent (1967) by using the methods of Lowry, Rosebrough, Farr & Randall (1951) and Mejbaum (1939).

Oestradiol hydroxylase assay. Microsomal subfractions derived from 50 or 100mg wet wt. of liver were suspended in 1ml of 0.1M-tris–HCl, pH7.4, and incubated for 1h with constant shaking under O_2 at 37°C with 0.3mM-NADPH, 1mM-GSH and [4-^{14}C]oestradiol (0.125μCi in 37nmol) in a total volume of 4ml.

In experiments to test the stability of oestradiol hydroxylase, the membrane fractions were incubated in the presence of NADPH and GSH for periods up to 1h in tris–HCl or sodium phosphate buffer and also in the presence of hydrolytic enzymes and other compounds (see legends to Figs. 1, 5 and 7 and Table 2) before the addition of [^{14}C]oestradiol and further incubation for 1h.

The reaction was stopped by extracting the incubation mixture three times with equal volumes of peroxide-free ether and the radioactivity in each fraction determined by liquid-scintillation counting as described by Jellinck & Woo (1967). The percentage of added radioactivity remaining in the aqueous medium after extraction with ether has been shown to be proportional to the amount of 2-hydroxyoestradiol formed (Jellinck & Perry, 1967; Jellinck & Garland, 1969) and was therefore used as a measure of oestradiol hydroxylase activity.

A portion of the ether extract was examined by t.l.c. in cyclohexane–ethyl acetate–ethanol (10:9:1, by vol.) (Lisboa & Diczfalusy, 1962) and radioactive material located by radioautography (Lazier & Jellinck, 1965). Oestrogen standards [oestrone, oestradiol, 2-hydroxyoestradiol and oestriol (oestra-1,3,5(10)-triene-3,16α,17β-triol)] were detected by spraying with diluted Folin–Ciocalteu reagent followed by exposure to NH_3 vapour (Mitchell & Davies, 1954).

Lipid peroxide assay. Lipid peroxide was determined by the thiobarbituric acid method of Wilbur, Bernheim & Shapiro (1949) as described by Wills (1966). A portion (1ml) of the mixture used for oestradiol hydroxylase assay was tested at various time-intervals for lipid peroxides. It was mixed with 1ml of 20% (w/v) trichloroacetic acid, 1ml of water and 2ml of 0.67% (w/v) thiobarbituric acid and heated at 100°C for 10min. Suspended material was removed by centrifugation at approx. 1000g for 1min and the E^{1cm}_{535} determined. A standard curve, with malonaldehyde bis(dimethyl acetal) as reference, was also plotted.

Digestion with phospholipase. The smooth-membrane fraction derived from 100mg wet wt. of liver was preincubated for 15min with 0.3mM-NADPH 1mM-GSH, 10mM-$CaCl_2$ and either phospholipase A (0.64 unit), phospholipase C (0.80 unit) or phospholipase D (16.2 units) with or without 0.25mM-spermine in 3ml of 0.1M-tris–HCl buffer, pH7.4. [^{14}C]Oestradiol (37nmol) was then added and the mixture incubated further for 1h.

Digestion with ribonuclease. The mixture used for oestradiol hydroxylase assay was preincubated for various time-intervals with pancreatic ribonuclease (100μg/ml), before the addition of [^{14}C]oestradiol and further incubation for 1h. The activity of the enzyme was checked by measuring the rate of decrease of E^{1cm}_{300} of RNA (Kunitz, 1946), by using a Unicam SP.800 spectrophotometer.

RESULTS

The results (Table 1) show that the ability of the smooth-membrane fraction of the endoplasmic reticulum of rat liver to convert oestradiol into water-soluble metabolites is higher than that of the rough membranes, and that polyribosomes are inactive in this respect. The converse was found for the incorporation of [^{14}C]leucine into protein by these microsomal subfractions.

The hydroxylase activity of the smooth-membrane fraction as measured by the conversion of oestradiol into water-soluble products was greater in tris–HCl than in sodium phosphate buffer (Fig. 1). The system also retained its activity longer after pre-incubation when tris–HCl buffer was used. The rough-membrane fraction showed lower hydroxylase activity and its stability was less influenced by the type of buffer used.

Table 1. *Relation between oestradiol hydroxylase activity and incorporation of* [^{14}C]*leucine into protein in rat liver microsomal subfractions*

The smooth-membrane fraction derived from 100mg of liver was incubated at 37°C for 1h with 0.3mM-NADPH, 1mM-GSH and [^{14}C]oestradiol (0.125μCi in 37nmol) in 4ml of 100mM-tris–HCl buffer, pH7.4, to determine oestradiol hydroxylase activity. [^{14}C]Leucine incorporation was measured by incubating 1ml of a mixture containing 0.05ml of the polyribosomes (from 0.5g of liver) or 0.05ml of the membrane fractions (from 0.25g of liver) with 0.1ml of the 145000$g_{av.}$-supernatant and [^{14}C]leucine (0.5μCi in 1.58nmol) in 0.25M-sucrose, 20mM-tris–HCl buffer, pH7.6, 100mM-KCl, 40mM-NaCl, 10mM-magnesium acetate together with 5mM-ATP and 0.1mM-GTP. After incubation at 37°C for 30min, the reaction was stopped by adding 0.1ml of 1M-HCl and the trichloroacetic acid-precipitable material was prepared for ^{14}C assay by the filter-paper-disc method of Mans & Novelli (1961). The RNA/protein ratios in the microsomal subfractions were carried out as described by Campbell & Sargent (1967) by using the methods of Lowry *et al.* (1951) and Mejbaum (1939). Other conditions are as described in the text.

Microsomal subfraction	RNA/protein ratio	Oestradiol hydroxylase activity/g of liver (% of added ^{14}C remaining in aqueous medium after extraction with ether)	[^{14}C]Leucine incorporated (pmol/mg of protein in microsomal subfraction)
Smooth membranes	0.08	55.2	23
Rough membranes	0.27	10.9	161
Polyribosomes	0.67	3.0	304

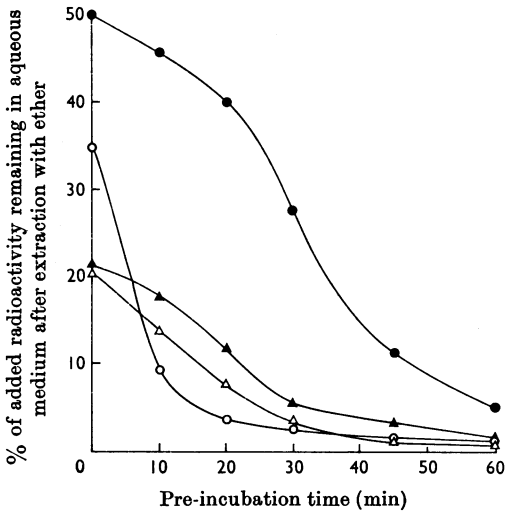

Fig. 1. Stability of oestradiol hydroxylase in microsomal subfractions of rat liver. The smooth (●, ○) and rough (▲, △) membrane fractions (1ml) derived from 50mg of liver were incubated for various time-periods with 0.3mM-NADPH and 1mM-GSH, in tris–HCl (●, ▲) or sodium phosphate (○, △) buffer (0.1M, pH7.4). [^{14}C]Oestradiol (0.125μCi in 37nmol) was then added and the mixture (4ml) was further incubated for 1h before extraction with ether and radioassay.

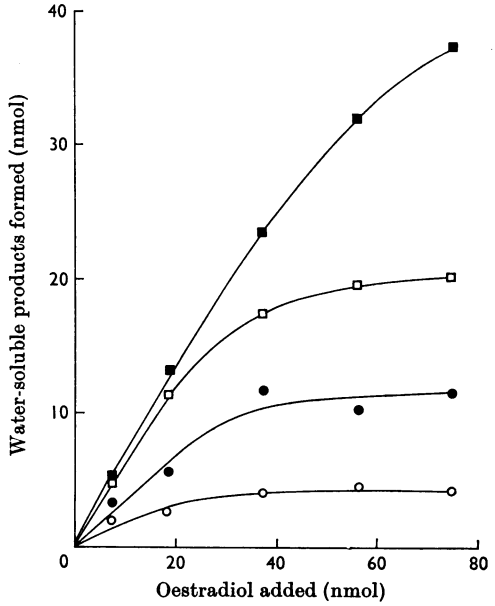

Fig. 2. Effect of enzyme and substrate concentration on the conversion of oestradiol into water-soluble products by smooth membranes of rat liver. The membrane fraction (1ml) derived from different weights of liver (○, 10mg; ●, 25mg; □, 50mg; ■, 100mg) was incubated in 0.1M-tris–HCl buffer, pH7.4, for 1h with 0.3mM-NADPH, 1mM-GSH and various amounts of [^{14}C]oestradiol, before extraction with ether and radioassay.

Although 37nmol of oestradiol did not completely saturate the hydroxylase system in the smooth-membrane fraction derived from 50–100mg wet wt. of liver (Fig. 2), this amount of substrate was chosen for subsequent experiments to provide enough radioactivity for accurate ^{14}C assay and radioautography without exceeding the solubility of the oestrogen.

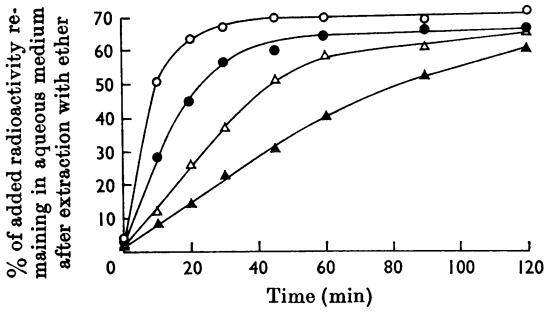

Fig. 3. Effect of spermine on the conversion of oestradiol into water-soluble products by microsomal subfractions of rat liver. Membrane fractions (1 ml) derived from 100 mg of liver were incubated for 1 h with 0.3 mM-NADPH, 1 mM-GSH, [^{14}C]oestradiol (0.125 μCi in 37 nmol) with and without 0.25 mM-spermine, before extraction with ether and radioassay. ●, Smooth-membrane fraction; ○, smooth-membrane fraction + spermine; ▲, rough-membrane fraction; △, rough-membrane fraction + spermine.

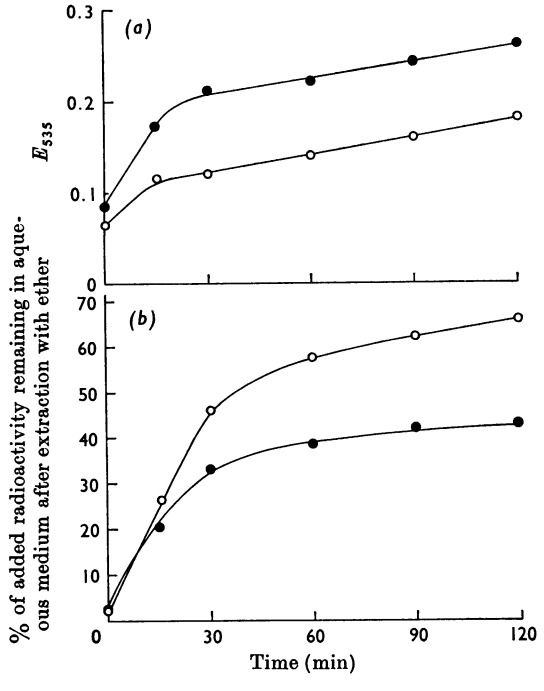

Fig. 4. Relation between lipid peroxidation and oestradiol hydroxylase activity. The smooth-membrane fraction (1 ml) derived from 50 mg of liver was incubated with 0.3 mM-NADPH, 1 mM-GSH, [^{14}C]oestradiol (0.125 μCi in 37 nmol) with or without 0.25 mM-spermine. At various time-intervals samples (1 ml) were tested (*a*) for lipid peroxide formation by measuring E_{535} after heating with thiobarbituric acid (see the Experimental section), and (*b*) the remaining solution was assayed for radioactivity after extraction with ether. ○, With spermine; ●, without spermine.

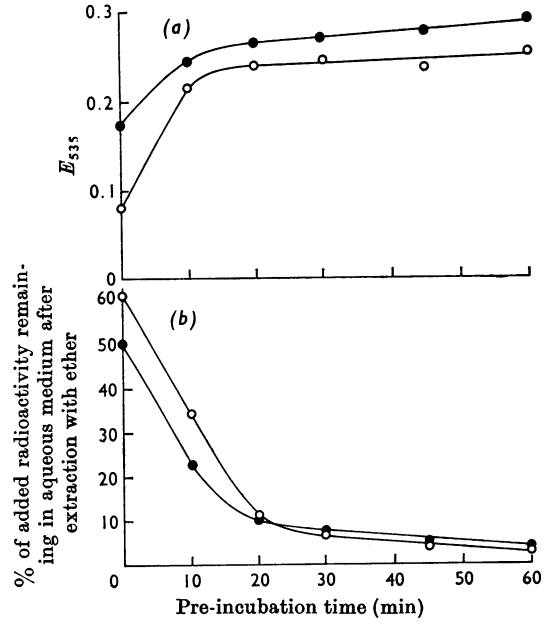

Fig. 5. Relation between lipid peroxidation and stability of the oestradiol-hydroxylating system. The smooth-membrane fraction (1 ml) derived from 50 mg of liver was incubated for various time-periods with 0.3 mM-NADPH, 1 mM-GSH with or without 0.25 mM-spermine. [^{14}C]-Oestradiol (0.125 μCi in 37 nmol) was then added and the mixture (4 ml) was further incubated for 1 h before (*b*) extraction with ether and radioassay or (*a*) lipid peroxide determination. The E_{535} was measured after heating with thiobarbituric acid (see the Experimental section). ○, With spermine; ●, without spermine.

The membrane-bound enzyme system was stable if kept at −30°C and still retained virtually all of its activity after 6 months of storage.

Spermine increased the rate of conversion of oestradiol into water-soluble metabolites in both rough and smooth endoplasmic reticulum (Fig. 3) and affected in a reciprocal manner the hydroxylation and the formation of lipid peroxides (Figs. 4*a* and 4*b*). However, the stability of the membrane-bound system catalysing these reactions was not affected by this polyamine (Figs. 5*a* and 5*b*).

Ribonuclease did not enhance the rate of loss of activity of microsomal oestradiol hydroxylase, but pre-incubation with phospholipases, which damage cellular membranes (Wallach, 1969), resulted in inactivation of oestradiol hydroxylase, which could not be prevented by spermine (Table 2). Similar results were obtained with a detergent such as deoxycholate (Fig. 6).

Polyribosomes did not influence the metabolism of oestradiol in the smooth reticular membrane although some inhibition in the yield of water-

Table 2. *Effect of spermine on the inactivation of oestradiol hydroxylase by phospholipases*

The smooth-membrane fraction derived from 100mg of liver was pre-incubated for 15min with 0.3mM-NADPH, 1mM-GSH, 10mM-$CaCl_2$ and either phospholipase A (0.64 unit), phospholipase C (0.80 unit) or phospholipase D (16.2 units), with or without 0.25mM-spermine, in 4ml of 100mM-tris–HCl buffer, pH7.4. Oestradiol (37nmol) was then added and the mixture further incubated for 1h. Values in parentheses are from experiments without preincubation. Other conditions are as described in the text.

Enzyme added	Oestradiol hydroxylase activity (% of added ^{14}C remaining in aqueous medium after extraction with ether)	
	Without spermine	With spermine
None	53.9 (52.6)	63.4 (69.5)
Phospholipase A	13.2	12.6
Phospholipase C	25.8 (41.6)	26.8 (42.0)
Phospholipase D	24.1	22.2

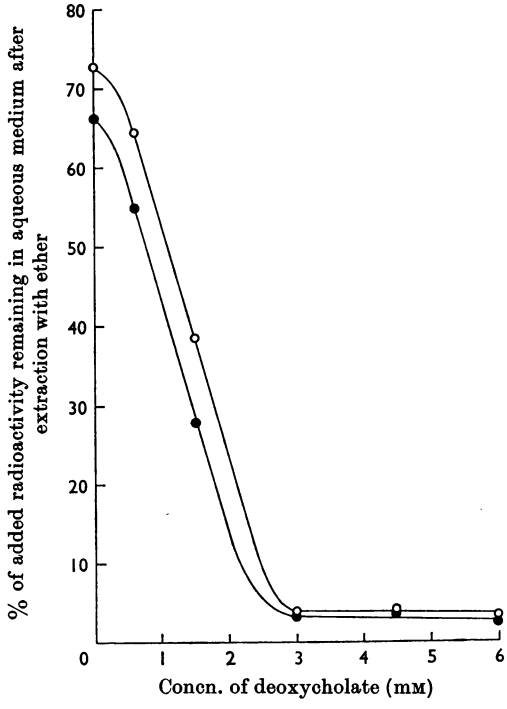

Fig. 6. Effect of deoxycholate on the conversion of oestradiol into water-soluble products by the smooth-membrane fraction of rat liver. The membrane fraction (1ml) derived from 100mg of liver was incubated for 1h with [^{14}C]oestradiol (0.125μCi in 37nmol), 0.3mM-NADPH, 1mM-GSH and various concentrations of deoxycholate in the presence (○) or absence (●) of spermine before extraction of the mixture (4ml) with ether and radioassay.

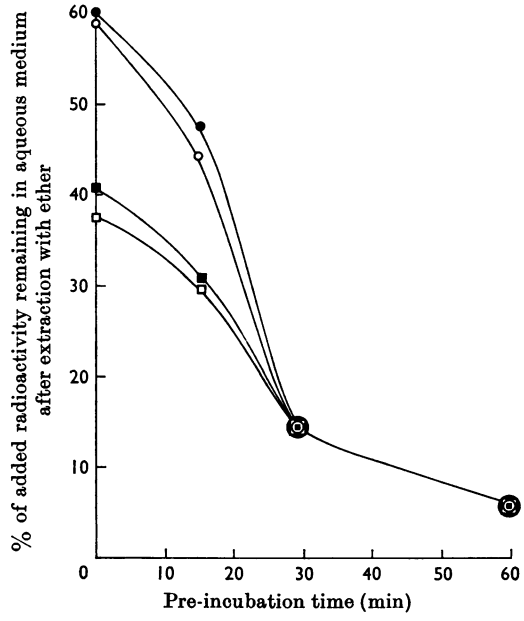

Fig. 7. Effect of corticosterone and polyribosomes on the conversion of oestradiol into water-soluble products by the smooth-membrane fraction of rat liver. The membrane fraction (1ml) and polyribosomes (0.5ml) derived from 100mg of liver were incubated for various time-periods with 0.3mM-NADPH, 1mM-GSH with or without 14.5μM-corticosterone. [^{14}C]Oestradiol (0.125μCi in 37nmol) was then added and the mixture (4ml) was further incubated for 1h before extraction with ether and radioassay. ●, No addition; ○, polyribosomes added; ■, corticosterone added; □, polyribosomes + corticosterone added.

soluble products was observed when corticosterone was present (Fig. 7).

No qualitative difference in the metabolism of oestradiol in the smooth and rough membranous fractions of rat liver was indicated from an examination of the ether-soluble fraction, and as found previously (Jellinck & Perry, 1967; Jellinck & Garland, 1969) the yield of 2-hydroxyoestradiol was proportional to the yield of water-soluble products and was increased by spermine.

DISCUSSION

Previous studies have shown that rat liver microsomal preparations are able to hydroxylate oestrogens and subsequently conjugate the metabolites with GSH or protein to form water-soluble products (Jellinck, Lewis & Boston, 1967; Kuss, 1967). The yield of these conjugates has been shown to depend on a rate-limiting hydroxylation of the aromatic ring, which is under hormonal control (Jellinck & Garland, 1969; Lehmann & Breuer, 1969) and which is believed to be carried out mainly by mixed-function oxidases involving cytochrome *P*-450 (Marks & Hecker, 1968).

Our results support the findings that this hydroxylating system is generally more active in the smooth endoplasmic reticulum than in the rough membrane (Gram, Rogers & Fouts, 1967; Holtzman, Gram, Gigon & Gillette, 1968), which can be due to differences in the concentration or in the rate of reduction of cytochrome *P*-450 in the hepatic microsomal subfractions. However, it is also possible that the ribosomes on the rough membranes may be influencing their rate of hydroxylation by analogy with the findings that disulphide interchange is masked when polyribosomes become attached to the smooth membranes of rat liver microsomal fractions (Williams & Rabin, 1969; James, Rabin & Williams, 1969). These workers showed that the decrease in enzymic activity when smooth membranes are incubated with polyribosomes together with either oestradiol or corticosterone could be correlated with the formation of material having the appearance of rough endoplasmic reticulum. Another factor indicating a possible involvement of ribosomes in controlling hydroxylation was the increase in conversion of oestradiol or 2-hydroxyoestradiol observed with spermine and spermidine (Jellinck & Perry, 1967). There is evidence that these polyamines interact with RNA of ribosomes and of the rough membranous endoplasmic reticulum (Raina & Teleranta, 1967) and also that they inhibit ribonuclease (Feingold & Davis, 1962).

It does not appear that polyribosomes inhibit the membrane-associated hydroxylase, although it was not possible to achieve the concentration of corticosterone used by James *et al.* (1969) because this steroid itself inhibits oestradiol hydroxylation. No effect on membrane-bound enzyme stability was observed with spermine, and the findings that ribonuclease did not affect the activity or lability of the hydroxylase system speaks against a proposal (Jellinck & Cox, 1970) that polyamines enhance oestradiol hydroxylation by removing inhibitor RNA.

Evidence for good morphological separation of membranes with enzymic activity was provided by measuring the RNA/protein ratios and the incorporation of [^{14}C]leucine into protein. The microsomal subfraction containing the smooth membranes with high oestradiol hydroxylase activity was virtually unable to carry out protein synthesis, whereas the isolated polyribosomes were devoid of hydroxylase activity but showed good incorporation of [^{14}C]-leucine. These results also exclude binding of oestrogen to nascent protein unless this reaction occurs in the rough membranous fraction.

Spermine decreased lipid peroxidation and an inverse correlation was found between the stability of the hydroxylating system and the formation of lipid peroxides. This reaction could account for the greater stability of oestradiol hydroxylase in tris–HCl buffer because lipid peroxidation is strongly stimulated by the addition of sodium phosphate (Wills, 1969*a*). The results therefore support the linked-system proposal of Wills (1969*b*) in which the pathways for hydroxylation and lipid peroxidation are similar in part but then diverge at a specific point.

Lipid peroxidation leads to membrane disintegration (Wills, 1969*b*) and the results with phospholipases also point to the possible need for maintaining the integrity of the reticular membrane for oestradiol hydroxylase activity. This is supported by the findings with deoxycholate. However, this detergent also brings about the conversion of cytochrome *P*-450 into inactive cytochrome *P*-420 (Omura & Sato, 1964), whereas the phospholipases would degrade the phosphatidylcholine shown to be required for the enzymic reduction of cytochrome *P*-450 and drug hydroxylation (Strobel, Lu, Heidema & Coon, 1970). The lack of protective effect by spermine therefore militates against its action on the hydroxylase system by preventing changes in cytochrome *P*-450. It is more probable that this polyamine acts by direct activation of microsomal hydroxylation or by inhibition of the lipid peroxidase pathway rather than by stabilization of the reticular membrane. Activation could be achieved by removal of an inhibitor (Jellinck & Cox, 1970) or by physical changes in the hydroxylating system, and it may be relevant that spermine causes the release of secretory enzymes from pancreatic ribosomes (Siekevitz & Palade, 1962).

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