Studies on an Extract of Rat Testicular Microsomal Fraction that Catalyses the Transformation of Progesterone into 17-Hydroxyprogesterone and Androgens

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The supernatant obtained by centrifugation of Triton N-101-treated freeze-dried rat testicular microsomal fraction at 105000g_{av.} for 2h transformed progesterone into testosterone via 17-hydroxypregn-4-ene-3,20-dione and androst-4-ene-3,17-dione. Hydroxylation at C-17 of 3 β -hydroxypregn-5-en-20-one and deoxycorticosterone was not observed. Non-haem iron protein, cytochrome *P*-450 and material with NADPH dehydrogenase activity were precipitated by 40% saturation of the supernatant with ammonium sulphate; however, it was not possible to establish the participation of these substances in the 17 α -hydroxylase and side-chain-cleavage activities also present in the precipitate. The results of gel-filtration chromatography indicated that the Triton N-101 extract consisted primarily of a suspension of small particles of microsomes and that the progesterone 17-hydroxylase and the 17-hydroxypregn-4-ene-3,20-dione side-chain-cleavage enzyme were not in true solution.

In this paper, the following trivial names are used: androstenedione, androst-4-ene-3,17-dione; cholesterol, cholest-5-en-3 β -ol; corticosterone, 17,21-dihydroxypregn-4-ene-3,20-dione; corticosterone, 11 β ,21-dihydroxypregn-4-ene-3,20-dione; dehydroepiandrosterone, 3 β -hydroxyandrost-5-en-17-one; deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; 17-hydroxypregnenolone, 3 β ,17-dihydroxypregn-5-en-20-one; 16 α -hydroxyprogesterone, 16 α -hydroxypregn-4-ene-3,20-dione; 17-hydroxyprogesterone, 17-hydroxypregn-4-ene-3,20-dione; pregnenolone, 3 β -hydroxypregn-5-en-20-one; progesterone, pregn-4-ene-3,20-dione; testosterone, 17 β -hydroxyandrost-4-en-3-one.

Progesterone 17-hydroxylase [progesterone-NADPH-oxygen oxidoreductase (17 α -hydroxylating), EC 1.14.1.7] and 17-hydroxyprogesterone side-chain-cleavage enzyme are known to reside in the microsomal fraction of the rat testis (Lynn & Brown, 1958; Shikita & Tamaoki, 1965). Young, Bryson & Sweat (1965) observed progesterone 17-hydroxylase and 21-hydroxylase activities and 20 α -hydroxy steroid dehydrogenase activity in supernatant fractions obtained by high-speed centrifugation of suspensions of acetone-dried powders prepared from homogenates of bovine adrenal glands. Little, Shaw & Purdy (1963) reported 1-2% conversion of [¹⁴C]progesterone into androstenedione after incubations of supernatants obtained by

ultracentrifugation of homogenates of human placentae. Attempts to obtain the testicular 17 α -hydroxylase and the 17-hydroxyprogesterone side-chain-cleavage enzyme in the soluble fraction (defined as the supernatant obtained after 2h of centrifugation at 105000g_{av.}) have been hitherto unsuccessful (Lynn & Brown, 1958; Shikita & Tamaoki, 1965; Hagerman, 1967). In this paper we report the properties of these two enzymic activities after their extraction from microsomal preparations into the soluble fraction by the use of the detergent Triton N-101 (the nonylphenyl ether of polyethylene glycol with nine or ten ethylene oxide groups).

MATERIALS AND METHODS

Rats. Long-Evans Hooded rats, weighing approx. 250g and approx. 3 months old, were used for all experiments. Animals obtained from the National Laboratory Animal Breeding Co., Edmonton, Alberta, Canada, and Blue Spruce Farms Inc., Altamont, N.Y., U.S.A., were used for all experiments except the incubation with microsomal fractions. In the latter experiment, the microsomal fractions were obtained from testes of Long-Evans Hooded rats bred at the Cancer Research Centre, University of British Columbia, Vancouver, B.C., Canada.

Tissue preparation. The rats were killed by a blow to the head and the testes were removed and immediately placed in Petri dishes surrounded by crushed ice. Further

procedures were carried out at 4°C. Each testis was quartered and the tissue was homogenized in 0.15M-KCl with a Potter-Elvehjem-type homogenizer (Potter & Elvehjem, 1936). The homogenate was centrifuged at 10500g_{av.} for 25 min and the supernatant was then centrifuged at 105000g_{av.} for 90–120 min to yield the microsomal pellet, which was immediately incubated or freeze-dried. The soluble fraction was obtained by suspending freeze-dried microsomal preparation in a solution (6 ml) of Triton N-101 (Rohm and Haas, Philadelphia, Pa., U.S.A.) in 0.04M-potassium phosphate buffer, pH 7.4. The mixture was swirled several times during the following 15 min and, after centrifugation at 105000g_{av.} for 2 h, the supernatant soluble fraction was collected. Unless otherwise stated, the amount of freeze-dried microsomal preparation used to obtain the soluble fraction was 0.106 g (representing approximately the material derived from two testes) and the buffer solution (6 ml) contained 0.2% (v/v) Triton N-101.

In one experiment, an attempt was made to obtain mitochondrial and microsomal fractions, each relatively free from contamination by other fractions. Twenty rat testes were cut up and homogenized in 0.15M-KCl. The homogenate was centrifuged at 700g for 15 min and the precipitate was discarded. The supernatant was centrifuged at 10000g_{av.} for 45 min; the precipitate constituted the unwashed mitochondrial fraction and the supernatant was centrifuged at 105000g_{av.} for 2 h to obtain the unwashed microsomal pellet. The mitochondrial fraction was suspended in 15 ml of 0.15M-KCl and centrifuged at 700g for 15 min; the precipitate was discarded and the supernatant was centrifuged at 10000g_{av.} for 25 min to obtain the mitochondrial pellet. The mitochondrial pellet was washed twice by suspension in 15 ml of 0.15M-KCl followed by centrifugation at 10000g_{av.} for 15 min. The unwashed microsomal pellet was suspended in 7 ml of 0.15M-KCl and centrifuged at 10000g_{av.} for 45 min and the precipitate discarded. The supernatant was centrifuged at 105000g_{av.} for 90 min; the precipitate constituted the washed microsomal fraction.

Ammonium sulphate fractionation was performed at 4°C. The soluble fraction was adjusted to 40% saturation (di Jeso, 1968) by the addition of solid ammonium sulphate. The material was left for 10 min and was then centrifuged at 10500g_{av.} for 15 min.

Radioactive substrates. [7α - ^3H]Progesterone, [4 - ^{14}C]progesterone, 17-hydroxy[4 - ^{14}C]progesterone and [7α - ^3H]pregnenolone were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [7α - ^3H]Progesterone and 17-hydroxy[4 - ^{14}C]progesterone were purified before use by paper chromatography in the solvent systems light petroleum (b.p. 80–100°C)–propylene glycol (Savard, 1953) and toluene–propylene glycol (Zaffaroni, Burton & Keutmann, 1950) respectively. [4 - ^{14}C]Progesterone was subjected to partition chromatography on a Celite 545 column with a 2,2,4-trimethylpentane–aq. 90% (v/v) methanol solvent system (Ryan & Smith, 1961). [7α - ^3H]Pregnenolone was not chromatographed before use as substrate. [1,2- ^3H]Deoxycorticosterone (a gift from the U.S. Public Health Service) was purified by paper chromatography in the solvent system toluene–propylene glycol before use as substrate. The radioactive compounds used as substrates were transferred to the reaction flasks in methanol solutions, the solvent was evaporated

and the radioactive substrates were redissolved in 0.2 ml of propylene glycol.

Incubation conditions. Incubation mixtures were prepared in Krebs–Ringer phosphate buffer (DeLuca & Cohen, 1964), pH 7.4, in which the Na^+ and K^+ ions were interchanged, or 0.04M-potassium phosphate buffer, pH 7.4. For incubations of the soluble fraction, 5 ml of the soluble fraction and 9 ml of phosphate buffer were added to each reaction flask. In all experiments, NADPH (7.89 μmol) was dissolved in water (1 ml) and added to the incubation mixture immediately before incubation. Unless otherwise stated, the total volume of all incubation mixtures was 15 ml.

Incubations were carried out in a Dubnoff metabolic shaking incubator at 37°C in an atmosphere of air unless otherwise stated.

Extraction, resolution and purification procedures. Incubation reactions were stopped by the addition of ethyl acetate (15 ml) previously chilled at –19°C. Known quantities of purified unlabelled steroids (60–100 μg of each) were added and the contents of each incubation flask were diluted to 40 ml with water and extracted with ethyl acetate (4 $\times$ 40 ml). The organic extracts were evaporated under reduced pressure at 40°C, ethanol was added and the extracts were dried by azeotropic distillation.

Progesterone, 17-hydroxyprogesterone, androstenedione and testosterone were separated and purified by paper chromatography in the solvent systems light petroleum–propylene glycol and toluene–propylene glycol. After initial chromatography, the eluted steroids were treated with pyridine (0.3 ml) and acetic anhydride (0.15 ml) overnight at room temperature and rechromatographed. 17-Hydroxyprogesterone and testosterone do not separate in the chromatographic systems used; hence, the 17-hydroxyprogesterone eluates were treated a second time with pyridine and acetic anhydride to ensure that all the testosterone was acetylated. Pregnenolone, deoxycorticosterone and 20 α -hydroxypregn-4-en-3-one were chromatographed as free compounds and as their acetylated derivatives. Corticosterone, cortisone and deoxycorticosterone were chromatographed on paper in the solvent systems toluene–propylene glycol and toluene–light petroleum (b.p. 80–100°C)–methanol–water (5:5:7:3, by vol.) (Bush, 1952). Other steroids were resolved and purified by paper chromatography in the solvent systems light petroleum–propylene glycol and toluene–propylene glycol. Chromatography of the Δ^4 -3-oxo steroids was repeated until the difference in the specific radioactivities of the compounds for each radioisotope was less than 5% in two successive chromatograms or until less than 1% of the radioactivity of the initial substrate(s) was associated with the steroid. Corrections were made for losses incurred during the isolation procedures. The eluates, after a further addition of the corresponding unlabelled compound, were each submitted to a series of successive crystallizations. The relative specific radioactivities did not vary by more than 8% from those of the eluates from the final chromatograms.

Pregnenolone, 17-hydroxypregnenolone and dehydroepiandrosterone were detected and assayed for radioactivity as follows. Authentic reference compounds were chromatographed simultaneously with the experimental chromatograms and the standards were located by

treatment with ethanolic phosphomolybdic acid (Kritchevsky & Kirk, 1952). The pattern of radioactivity of the experimental chromatograms was determined by scanning (Nuclear-Chicago model 1032 4 π Actigraph II chromatogram scanner) and the results were compared with the locations of the steroids on the standard chromatograms. When it was observed that the zones on the experimental chromatograms corresponding to the locations of standard dehydroepiandrosterone and 17-hydroxypregnenolone contained minimal radioactivity, the zones were excised and bisected longitudinally. One portion was treated with the phosphomolybdic acid reagent to verify the location of the experimental steroid and the other portion was eluted with methanol and the radioactivity of the eluate was measured by liquid-scintillation spectrometry. Before addition as carrier, 20 α -hydroxypregn-4-en-3-one (a gift from Dr R. Neher, Basle, Switzerland) and the 20 β -epimer were purified by paper chromatography in the solvent systems toluene-propylene glycol and light petroleum-propylene glycol respectively.

Column chromatography. Column chromatography was performed on Sephadex G-200, Sephadex G-25 (coarse) and DEAE-Sephadex A-25, obtained from Pharmacia Ltd., Montreal, P.Q., Canada. The Sephadex G-200 was allowed to swell in water for 4 days at 4°C and was then put into a glass tube (2.5 cm $\times$ 45 cm) to a height of 39 cm. The void volume was 65 ml as determined by chromatography of Blue Dextran. The column was run at a pressure of 15 cm of water. The eluent, 0.04 M-potassium phosphate buffer, pH 7.4, was allowed to flow through the column for 6 h before application of the sample. The Sephadex G-25 (coarse) was allowed to swell in 0.04 M-NH₄HCO₃ for 4 h at room temperature before use. The DEAE-Sephadex A-25 was allowed to swell in water and then washed with 0.5 M-NaOH, water, 0.5 M-HCl and finally with water until constant pH of the washings was observed. The DEAE-Sephadex was equilibrated with a solution of 0.04 M-potassium phosphate buffer, pH 7.4, and 0.1 M-NaCl and then packed in a glass tube (1.5 cm $\times$ 30 cm) to a height of 25 cm. All columns were run at 4°C in a cold-room.

Other procedures. Protein was measured by the method

of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955), with crystalline bovine serum albumin as standard. Non-haem iron was determined by the method of Massey (1957) as modified by Simpson & Boyd (1967) but with bathophenanthroline. The molar extinction coefficient for the ferrous iron-bathophenanthroline complex (20100) was determined by measuring the E_{235} of solutions of recrystallized ferrous ammonium sulphate. Haem iron was determined by the method of Diehl & Smith (1952) in the protein that was precipitated during the determination of non-haem iron. NADPH dehydrogenase activity was measured by the method of Omura, Sanders, Estabrook, Cooper & Rosenthal (1966) in which changes in the oxidation-reduction states of dichlorophenol-indophenol are used to measure dehydrogenase activity.

A Sonifier cell disruptor (Heat Systems Co., Melville, N.Y., U.S.A.) was employed for ultrasonic treatment and the specimens were chilled in an ice-methanol bath during the procedure.

17 α -Hydroxylase activity was estimated from the sum of the percentages of substrate [³H]progesterone radioactivity (d.p.m.) recovered as radioactive 17-hydroxyprogesterone, androstenedione and testosterone. 17-Hydroxyprogesterone side-chain-cleavage enzyme activity was estimated from the sum of the percentages of substrate [³H]progesterone radioactivity (d.p.m.) and substrate 17-hydroxy[¹⁴C]progesterone radioactivity (d.p.m.) (in incubations with two radioactive substrates) recovered as radioactive androstenedione and testosterone.

Radioactivity was assayed with a Nuclear-Chicago model 725 liquid-scintillation spectrometer. Steroid samples in methanol solution were pipetted into counting vials and the solvent was evaporated. Each residue was then dissolved in toluene (5 ml) containing 0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis-(5-phenoxyloxazol-2-yl)-benzene and 2% (v/v) ethanol. The counting efficiency was 25% for ³H and 85% for ¹⁴C in samples containing a single radioisotope. In samples containing both labels, the efficiency was 20% for ³H and 40% for ¹⁴C. In samples containing both ³H and ¹⁴C, the d.p.m. for each isotope was

Table 1. *Distribution of radioactivity after incubations of preparations of rat testes*

In the incubations of microsomal fractions (obtained from two testes) and freeze-dried microsomal fractions (0.106 g), [³H]progesterone (4639000 d.p.m., 3.71 μ g) and 17-hydroxy[¹⁴C]progesterone (892200 d.p.m., 3.72 μ g) were used in combination as substrates. The microsomal fractions were transferred to the reaction flask in 5 ml of 0.15 M-KCl, and 5 ml of Krebs-Ringer phosphate buffer, pH 7.4, in which the Na⁺ and K⁺ were interchanged, was added. The freeze-dried microsomal fractions were transferred to reaction flasks and incubated in 10 ml of the Krebs-Ringer buffer.

Steroids isolated	Percentage of substrate radioactivity			
	Microsomal fraction		Freeze-dried microsomal fraction	
	¹⁴ C	³ H	¹⁴ C	³ H
Progesterone	<1	1	<1	2
17-Hydroxyprogesterone	<1	<1	<1	<1
Androstenedione	22	21	24	21
Testosterone	32	31	54	49
Total	54	53	78	72

calculated by the simultaneous-equation method of Okita, Kabara, Richardson & LeRoy (1957).

Extinction was measured with a Beckman DU spectrophotometer with matched quartz cells of 1 cm light-path.

All solvents (A.R. grade) were redistilled before use.

RESULTS

Preparation of a soluble fraction containing 17 α -hydroxylase and side-chain-cleavage enzyme activity. The progesterone 17-hydroxylase and 17-hydroxyprogesterone side-chain-cleavage enzyme activities

Table 2. *Results of incubations of supernatants obtained after high-speed centrifugation of Triton N-101-treated freeze-dried microsomal preparations*

Equal quantities (0.053 g) of freeze-dried microsomal preparations were treated with solutions of Triton N-101 in 0.15 M-KCl. The supernatants (5 ml in each experiment) after centrifugation (105 000 g_{av} , 90 min) were transferred to reaction flasks containing [3H]progesterone (4401 000 d.p.m., 3.64 μ g) and 17-hydroxy[^{14}C]progesterone (723 700 d.p.m., 3.64 μ g) in combination as substrates; 5 ml of Krebs-Ringer phosphate buffer, pH 7.4, in which the Na $^+$ and K $^+$ were interchanged, was added and incubations were performed.

Concn. of Triton N-101 (% v/v)	Percentage of initial radioactivity of substrate [3H]progesterone present as [3H]progesterone after incubation
0.125	56
0.250	45
0.500	71
1.00	86
2.00	93

that were observed in microsomal preparations were only slightly affected by freeze-drying (Table 1). Large percentages of radioactive progesterone and 17-hydroxyprogesterone were converted into C-19 compounds in all experiments. The total values shown in Table 1 are greater than 90% of the initial radioactivity of the substrates when the radioactivity associated with uncharacterized material more polar than 17-hydroxyprogesterone and testosterone is taken into account.

The possibility that progesterone 17-hydroxylase and 17-hydroxyprogesterone side-chain-cleavage enzyme activities could be extracted from freeze-dried microsomal preparations with the detergent Triton N-101 was investigated. The results of preliminary experiments (Table 2) suggested that maximal enzymic activities could be obtained in a soluble fraction prepared by treatment of freeze-dried microsomal preparation with a solution of Triton N-101 of about 0.25% (v/v) concentration. It was later found that a 0.20% solution was as effective as a 0.25% solution. The distribution of radioactivity after incubations of fractions obtained after centrifugation at 105 000 g_{av} for 2 h of freeze-dried microsomal preparations treated with 0.2% Triton N-101 in 0.04 M-potassium phosphate buffer, pH 7.4, is presented in Table 3. Incubation of the precipitate obtained after high-speed centrifugation of detergent-treated freeze-dried microsomal preparations led to very active 17-hydroxylation of [3H]progesterone, a slight decrease in side-chain cleavage of radioactive 17-hydroxyprogesterone and a marked diminution in the reduction of the 17-oxo group of androstenedione by comparison with the results with freeze-dried microsomal

Table 3. *Distribution of radioactivity after incubations of fractions obtained after centrifugation (105 000 g_{av} , 2 h) of freeze-dried microsomal preparations treated with 0.2% Triton N-101*

In all experiments 0.106 g of freeze-dried microsomal fraction was treated with 6 ml of a 0.2% (v/v) solution of Triton N-101 in 0.04 M-potassium phosphate buffer, pH 7.4. [3H]Progesterone (2733 000 d.p.m., 2.35 μ g) and 17-hydroxy[^{14}C]progesterone (372 400 d.p.m., 2.12 μ g) were used in combination as substrates in all experiments. Incubations of the soluble fractions were performed after the addition of 9 ml of Krebs-Ringer phosphate buffer, pH 7.4, in which Na $^+$ and K $^+$ were interchanged. The precipitate was incubated in 14 ml of the same buffer. Sonication (20 kHz, 70 W, 2 min) was performed on the detergent-treated-freeze-dried microsome mixture before centrifugation.

Steroids isolated	Percentage of substrate radioactivity					
	Precipitate		Supernatant (soluble fraction)			
			From microsomal preparation + Triton N-101		From microsomal preparation + Triton N-101 + sonication	
	^{14}C	3H	^{14}C	3H	^{14}C	3H
Progesterone	<1	6	<1	33	<1	47
17-Hydroxyprogesterone	21	19	82	38	89	31
Androstenedione	62	53	10	7	3	4
Testosterone	1	1	3	2	1	1
Total	84	79	95	80	93	83

preparations (Table 1). After incubation of the soluble fraction 47% of the radioactivity of [^3H]-progesterone was present as radioactive 17-hydroxyprogesterone, androstenedione and testosterone; only a small percentage of the original radioactivity of either substrate was associated with androstenedione or testosterone (Table 3).

Attempts were made to increase the enzymic activities present in the soluble fraction by ultrasonic treatment of freeze-dried microsomal preparations suspended in 0.2% Triton N-101 (Table 3). The results suggested that ultrasonic treatment of 30s to 4min duration diminished the progesterone 17-hydroxylase and 17-hydroxyprogesterone side-chain-cleavage enzyme activities of the soluble fraction; ultrasonic treatment for 2min appeared to be least damaging. The enzymic activities were not demonstrable in the soluble fraction obtained after ultrasonic treatment (2min) of freeze-dried microsomal preparations suspended in phosphate buffer without Triton N-101.

The soluble fraction obtained by treatment of freeze-dried microsomal preparations with 0.2% Triton N-101 displayed considerably less side-chain-cleavage activity and androstenedione 17-oxo reduction activity than did freeze-dried microsomal preparations. The possibility that these two enzymic activities in the soluble fraction were inhibited by the presence of Triton N-101 was tested by incubating freeze-dried microsomal preparations in the presence of the detergent. In addition, an experiment was performed to establish that tissue fractions were required for transformations of [^3H]progesterone and 17-hydroxy[^{14}C]progesterone

under the incubation conditions that were employed. No transformation of either radioactive substrate was observed after the incubation of a solution of Triton N-101 and potassium phosphate buffer. The 17 α -hydroxylase and side-chain-cleavage enzyme activities of freeze-dried microsomal preparations were very similar in incubations in the presence or absence of Triton N-101. The results of the two incubations differed in that more radioactive androstenedione and less radioactive testosterone were present after the incubation with Triton N-101.

Other enzymic activities in the soluble fraction. The possible presence in the soluble fraction of NADPH-stimulated enzymic activities other than 17-hydroxylation of progesterone, side-chain cleavage of 17-hydroxyprogesterone and reduction of the 17-oxo group of androstenedione was investigated (Table 4). No 17 α -hydroxylase, 3 β -hydroxy steroid dehydrogenase or steroid Δ -isomerase utilizing pregnenolone as substrate were detected. A 20-hydroxy steroid dehydrogenase was present that reduced progesterone to the 20 α -hydroxy derivative; the corresponding 20 β -epimer was not found. Hydroxylation of progesterone at the C-16 position was not detected, nor was 17-hydroxylation of deoxycorticosterone. In the three experiments in which the conversion of [^3H]progesterone into 20 α -hydroxypregn-4-en-3-one, 20 β -hydroxypregn-4-en-3-one and 16 α -hydroxyprogesterone was investigated, transformation of the radioactive substrates into 17-hydroxyprogesterone, androstenedione and testosterone also occurred to an extent similar to the values given in Table 3 for the supernatant obtained after centrifugation of Triton

Table 4. *Percentage of substrate radioactivity in isolated compounds formed during incubations of the soluble fraction with radioactive steroids*

In all incubations, 0.04M-potassium phosphate buffer, pH7.4, was used. The substrates used singly were [^3H]progesterone (1676000 d.p.m., 1.2 μg) and [1,2- ^3H]deoxycorticosterone (6809000 d.p.m., 3.88 μg), or [4- ^{14}C]progesterone (390000 d.p.m., 4.32 μg) and [7- ^3H]pregnenolone (13080000 d.p.m., 3.99 μg) in combination.

Steroids isolated	Substrate ...	Percentage of substrate radioactivity			
		[^3H]Progesterone	[^3H]Deoxycorticosterone	[^{14}C]Progesterone + [^3H]pregnenolone	
				^{14}C	^3H
Progesterone				64	<1
17-Hydroxyprogesterone				11	<1
17-Hydroxypregnenolone				<1	<1
Dehydroepiandrosterone				<1	<1
Androstenedione				1.8	<1
Testosterone				<1	<1
20 α -Hydroxypregn-4-en-3-one		2.4			
20 β -Hydroxypregn-4-en-3-one		<1			
16 α -Hydroxyprogesterone		<1			
Cortexolone			<1		
Corticosterone			<1		

N-101-treated freeze-dried microsomal preparations. Thus the soluble fraction contains a 17α -hydroxylase for which progesterone is the preferred substrate rather than pregnenolone or deoxycorticosterone, a 17 -hydroxyprogesterone side-chain-cleavage enzyme that does not cleave pregnenolone, a 17 -hydroxy steroid dehydrogenase and a 20α -hydroxy steroid dehydrogenase.

Effects of change of temperature and pH on the 17α -hydroxylase and side-chain-cleavage enzyme activities of the soluble fraction. Both the 17α -hydroxylase and the side-chain-cleavage enzyme displayed maximal activity at 37°C and pH 6.8. At pH 7.4 and at pH 6, one-third to one-quarter of the maximum 17α -hydroxylase activity was observed and the side-chain cleavage activity was negligible. Both enzymic activities were absent at pH 5.5 and pH 8. The activities of the 17α -hydroxylase and the side-chain-cleavage enzyme were only slightly altered in incubations at 23°C , 32°C and 37°C .

Resolution and purification of components of the soluble fraction. The precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate was dissolved in 14 ml of 0.04M-potassium phosphate buffer, pH 7.4, and incubated. Approximately one-quarter of the 17α -hydroxylase activity exhibited by the untreated soluble fraction was observed, i.e. 12–15% of the substrate [^3H]-progesterone was converted into 17 -hydroxyprogesterone (Table 5). At pH 7.4, the activity of the side-chain-cleavage enzyme was negligible in the precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate. However, after incubation at pH 6.7, the conversion of [^3H]-progesterone into androstenedione plus testosterone was 11.4% (Table 5). In the latter experi-

ment, the soluble fraction was prepared from 0.3 g of freeze-dried microsomal preparation rather than the usual 0.106 g. The supernatant after 40% saturation of the soluble fraction with ammonium sulphate was inactive when incubated at pH 7.4 with [^3H]-progesterone and 17 -hydroxy[^{14}C]-progesterone in combination as substrates and was also inactive when incubated after 4 h of dialysis against 0.04M-potassium phosphate buffer, pH 7.4. When the dialysed supernatant was combined with the precipitate the formation of 17 -hydroxyprogesterone was not increased over that occurring when the precipitate was incubated alone.

The specific activity of the 17α -hydroxylase (pmol of progesterone hydroxylated/min per mg of protein) was 10.4 for freeze-dried microsomal preparation and 1.38 for the soluble fraction (Table 6). The specific activity of the 17α -hydroxylase present in the precipitate obtained after 40% saturation of the soluble fraction with ammonium sulphate was determined twice and was 1.73 and 3.89.

Both the soluble fraction and the precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate contained haem iron protein, non-haem iron protein and NADPH dehydrogenase activity. The haemoproteins were shown to include cytochromes *P-450* and *P-420*. A carbon monoxide difference spectrum under reducing conditions exhibited a prominent absorption maximum at 450 nm and a small absorption maximum at 420 nm.

Attempts were made to resolve the non-haem iron protein, the cytochrome *P-450* and the enzyme responsible for the NADPH dehydrogenase activity of the soluble fraction to explore the relationship of

Table 5. *Distribution of radioactivity after incubations of the precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate*

In the incubations at pH 7.4, the soluble fractions were prepared from 0.106 g of freeze-dried microsomal fraction and [^3H]-progesterone (2286 000 d.p.m., 1.96 μg) and 17 -hydroxy[^{14}C]-progesterone (284 660 d.p.m., 1.62 μg) were used as combined substrates in each incubation. In the incubation at pH 6.7, the soluble fraction was prepared from 0.3 g of freeze-dried microsomal fraction and [^3H]-progesterone (1 676 000 d.p.m., 1.45 μg) was used as the sole substrate. All precipitates were dispersed in 14 ml of 0.04M-potassium phosphate buffer, pH 7.4. For the incubation at pH 6.7, the pH of the final incubation mixture was adjusted by the addition of one drop of 2M-HCl.

Steroids identified	Percentage of substrate radioactivity				
	Incubations at pH 7.4				Incubation at pH 6.7 ³ H
	Expt. 1		Expt. 2		
	¹⁴ C	³ H	¹⁴ C	³ H	
Progesterone	<1	80	<1	86	62
17-Hydroxyprogesterone	95	12	100	15	19
Androstenedione	1	<1	<1	<1	5
Testosterone	<1	<1	<1	<1	7
Total	96	92	100	101	93

Table 6. *Specific activity of the progesterone 17-hydroxylase*

Each determination of the specific activity represents a separate experiment in which different weights of freeze-dried microsomal preparation were used as starting material. Specific activity is expressed as pmol of [^3H]progesterone hydroxylated/min per mg of protein in the incubation medium.

Fraction	Treatment	Subfraction	Protein (mg)	% of substrate [^3H]progesterone hydroxylated	Specific activity
Freeze-dried microsomal preparation	Triton N-101		10.5	70	10.4
Freeze-dried microsomal preparation	Triton N-101, centrifugation (105000g, 2h)	Supernatant	9.0	36	1.38
		Precipitate	42.0	57	9.80
Soluble fraction	40% Ammonium sulphate	Precipitate 1*	10.8	12	1.73
		Precipitate 2*	6.0	15	3.89

* The specific activity of the precipitate was determined in two separate experiments.

these compounds to the 17 α -hydroxylase and the side-chain-cleavage enzyme activities that were present. The precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate was subjected to column chromatography on Sephadex G-200 (Fig. 1). One major and one minor E_{280} peak were observed. In separate experiments it was found that a portion of the Triton N-101 present in the soluble fraction was precipitated by 40% saturation with ammonium sulphate. When Triton N-101 was subjected to column chromatography on Sephadex G-200 the detergent was present in the fractions that immediately followed the void volume. The u.v. spectrum of Triton N-101 served as a means of detection of the detergent. The absorption in the region of 280nm interfered with the measurement of protein by measurement of the E_{280} .

Fractions 9–41 from the Sephadex G-200 column (Fig. 1) were pooled and the protein was precipitated by 40% saturation with ammonium sulphate. A portion of the precipitate was dispersed in 15ml of 0.04M-potassium phosphate buffer, pH 7.4, and incubated with [^3H]progesterone and 17-hydroxy[^{14}C]progesterone. No transformations of the radioactive substrates were observed. The remainder of the precipitate was dissolved in the same buffer and rechromatographed on the same column (Fig. 2). Two E_{280} peaks were observed. The position of peak 2 corresponded to the positions of peaks in the profiles of the non-haem iron content and NADPH dehydrogenase activity of the fractions. Haem iron was not demonstrated in the effluent from the column and NADPH dehydrogenase activity was decreased in comparison to the activity present in the precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate.

Non-haem iron protein could be obtained free of NADPH dehydrogenase activity by ion-exchange

Fig. 1. Sephadex G-200 column chromatography of a Triton N-101 extract of rat testicular microsomal preparation. Details are given in the text. The fraction size was 5ml; the void volume was 55ml.

chromatography. The precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate was first subjected to gel-filtration chromatography on Sephadex G-200 as described above. The elution profile in terms of the E_{280} was very similar to that depicted in Fig. 1. The fractions containing peak 2 were pooled and passed through a Sephadex G-25 (coarse) column with 0.04M-ammonium hydrogen carbonate as eluent. The protein-containing fractions were pooled and freeze-dried. The freeze-dried material was dissolved in a minimal volume of 0.04M-potassium phosphate buffer (pH 7.4)–0.1M-sodium chloride and subjected to column chromatography on DEAE-Sephadex A-25 (Fig. 3). The E_{280} peak 4 corresponded in location to the position of a peak in the

Fig. 2. Sephadex G-200 column chromatography of the material present in peak 2 of Fig. 1. NADPH dehydrogenase activity was measured as μg of dichlorophenol-indophenol reduced/min. —, E_{280} ; ○, non-haem iron; ●, NADPH dehydrogenase activity. The fraction size was 5 ml and void volume 55 ml.

Fig. 3. DEAE-Sephadex A-25 column chromatography of the material present in peak 2 of Fig. 1. NADPH dehydrogenase activity was measured as μg of dichlorophenol-indophenol reduced/min. —, E_{280} ; ○, non-haem iron; ●, NADPH dehydrogenase activity. The fraction size was 5 ml and void volume 30 ml.

profile of the non-haem iron content of the fractions. NADPH dehydrogenase activity was low in all fractions in which it was measured and was minimal

in the fraction containing the greatest amount of non-haem iron.

A number of methods for the preparation of

Fig. 4. DEAE-Sephadex A-25 column chromatography of a 105000 g_{av} supernatant obtained after ultrasonic treatment of a suspension of freeze-dried microsomal fraction obtained from rat testes. NADPH dehydrogenase activity is measured as μg of dichlorophenol-indophenol reduced/min. —, E_{280} ; ○, non-haem iron; ●, NADPH dehydrogenase activity. The fraction size was 5 ml and void volume 40 ml.

Fig. 5. Sephadex G-200 column chromatography of the material present in fractions 18–31 of the chromatogram depicted in Fig. 4. —, E_{280} ; ○, non-haem iron. The fraction size was 8 ml and void volume 55 ml.

microsomal non-haem iron protein were evaluated; the following procedure was found to yield the most highly purified product. An acetone-dried powder (Morton, 1955) of freeze-dried microsomal preparation was dispersed in 0.04M-potassium phosphate buffer (pH 7.4)–0.1M-sodium chloride and the suspension was sonicated for 5 min. The supernatant obtained by centrifugation at 105000 g_{av} .

for 2 h was applied to a DEAE-Sephadex A-25 column and elution was performed with a gradient of 0.1–0.7M-sodium chloride in 0.04M-potassium phosphate buffer, pH 7.4 (Fig. 4). A prominent E_{280} peak was observed that coincided in location with the position of a peak in the profile of the non-haem iron content of the fractions. NADPH dehydrogenase activity was minimal in all fractions in

Table 7. *Non-haem iron and protein content of washed and unwashed mitochondria and microsomal fractions*

Experimental details are given in the text.

Tissue preparation	Non-haem iron (μg)	Protein (g)	Non-haem iron ($\mu\text{g/g}$ of protein)
Microsomal fraction			
Unwashed	95.9	8.25	11.6
Washed	2.6	0.05	51.0
Mitochondria			
Unwashed	31.1	1.42	21.9
Washed	1.8	0.05	36.0

which it was measured. Fractions 18–31 from the column were pooled and passed through a Sephadex G-25 (coarse) column, with 0.04M-ammonium hydrogen carbonate as eluent. The protein-containing fractions were pooled and freeze-dried. The freeze-dried material was dissolved in a minimal volume of 0.04M-potassium phosphate buffer, pH 7.4, and subjected to gel-filtration column chromatography on Sephadex G-200 (Fig. 5). Two E_{280} peaks were observed. The column fraction (36) that displayed the greatest E_{280} contained 6.0 μg of non-haem iron.

The procedure used to prepare the microsomal fraction included a minimal number of centrifugation steps and no unusual precautions were taken to exclude contamination of the microsomal fraction by mitochondria. The non-haem iron content of more carefully prepared microsomal and mitochondrial fractions was investigated to establish definitely the subcellular locations of the compound (Table 7). The quantity of non-haem iron per g of protein in washed mitochondria and microsomal fractions was greater than the quantity per g of protein in the corresponding unwashed preparations and the amount of non-haem iron per g of protein in washed microsomal fraction was greater than the amount per g of protein in washed mitochondria.

DISCUSSION

Other workers have attempted to extract the testicular progesterone 17-hydroxylase and 17-hydroxyprogesterone side-chain-cleavage enzyme by treatment of microsomal preparations with detergents (Shikita & Tamaoki, 1965; Hagerman, 1967). The success of the method reported in the present paper may be attributed to the use of freeze-dried microsomal fractions; however, no extractions of microsomes with Triton N-101 were performed. It is possible that the concentrations of the detergents used by other workers precluded the presence or the expression of the enzymic activities in the soluble fraction (Table 2).

The formation of 17-hydroxypregnenolone has been reported in incubations of homogenates of rat

testes with [^3H]pregnenolone (Slaunwhite & Burgett, 1965). Shikita, Kakizaki & Tamaoki (1964), however, were unable to recover radioactive 17-hydroxypregnenolone or dehydroepiandrosterone after incubations of rat testicular microsomal preparations with [^3H]pregnenolone. Shikita and co-workers also performed incubations with [^{14}C]progesterone and [^3H]pregnenolone in combination as substrates and with 17-hydroxy[^{14}C]progesterone and 17-hydroxy[^3H]pregnenolone in combination as substrates. From the results of these experiments they concluded that testosterone is formed from pregnenolone by rat testicular microsomal fraction mainly via progesterone and androstenedione.

The incubation of the soluble fraction with [^{14}C]progesterone and [^3H]pregnenolone in combination as substrates (Table 4) was performed to ascertain the substrate specificity of the 17 α -hydroxylase; [^3H]pregnenolone served as the test substrate and [^{14}C]progesterone served as the control. NAD $^+$, required for the conversion of pregnenolone into progesterone (Koritz, 1964; Kowal, Forchielli & Dorfman, 1964), was omitted from the incubation medium. Negligible conversion of [^3H]pregnenolone into 17-hydroxypregnenolone, dehydroepiandrosterone, progesterone, 17-hydroxyprogesterone, testosterone and androstenedione was observed. The results are in accord with the conclusion of Shikita *et al.* (1964) and also show that progesterone is the preferred substrate for the 17 α -hydroxylase.

A pH optimum of 6.8 was observed for the activities of the 17 α -hydroxylase and the side-chain-cleavage enzyme in the soluble fraction. A pH optimum of 7.0 has been observed for the side-chain-cleavage enzyme activity in incubations of rat (Hagerman, 1967) and guinea pig (Lynn & Brown, 1958) testicular microsomal fraction. Lynn & Brown (1958) reported that the progesterone 17-hydroxylase of guinea-pig microsomal fractions was quite active at pH 8.5. When the activity of the 17 α -hydroxylase is estimated from the sum of the percentages of initial [21- ^{14}C]progesterone present as radioactive acetate plus 17-hydroxyprogesterone, the data of Lynn & Brown (1958) show a pH

optimum of 7.0. Young *et al.* (1965) observed progesterone 17-hydroxylase activity in a supernatant obtained by centrifugation of a suspension of an acetone-dried powder prepared from a homogenate of bovine adrenal glands at 105 000 *g*_{av} for 60 min. The activity of the 17 α -hydroxylase increased as the pH of the incubation media was increased to 9.1.

17 α -Hydroxylase, side-chain-cleavage enzyme and 17-hydroxy steroid dehydrogenase activities were present in the precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate (Table 5). The presence of 17-hydroxy steroid dehydrogenase and side-chain-cleavage enzyme in the precipitate was demonstrated in an incubation performed at pH 6.7 but not in an incubation performed at pH 7.4. The specific activity of the 17 α -hydroxylase was greater in the precipitate obtained by 40% saturation of the soluble fraction than in the untreated soluble fraction; however, considerably less enzymic activity was observed in the precipitate (Table 6). The highest specific activity of the 17 α -hydroxylase was observed in freeze-dried microsomal preparations. The results suggest that the enzyme was partially inactivated during the isolation procedures.

The precipitate obtained by 40% saturation of the soluble fraction with ammonium sulphate contained NADPH dehydrogenase activity, cytochrome *P*-450 and non-haem iron protein. The NADPH dehydrogenase and the cytochrome *P*-450 were unstable and it was not possible to isolate these compounds for further investigations. Kimura & Ohno (1968) extracted non-haem iron protein from an acetone-dried powder of homogenized pig testes. These authors postulated that the non-haem iron protein was present in testicular mitochondria although their method of preparation did not exclude a microsomal source. The results reported in the present paper establish the presence of non-haem iron protein in both microsomal fraction and mitochondria of rat testes. In bovine adrenal mitochondria the 11 β -hydroxylase (Suzuki & Kimura, 1965; Omura, Sato, Cooper, Rosenthal & Estabrook, 1965; Cooper *et al.* 1967; Kimura & Suzuki, 1967) and the cholesterol side-chain-cleavage enzyme (Simpson & Boyd, 1967; Bryson & Sweat, 1968) systems are considered to consist of NADPH dehydrogenase, non-haem iron protein and cytochrome *P*-450. The progesterone 17 α -hydroxylase and the 17-hydroxyprogesterone side-chain-cleavage enzyme of rat testicular microsomal fractions may function by a similar mechanism. This hypothesis cannot be tested conclusively until NADPH dehydrogenase, cytochrome *P*-450 and non-haem iron protein are isolated in active forms.

The results of the column gel-filtration chromatography suggest that the material extracted by treatment of freeze-dried microsomal preparations with Triton N-101 exists largely as a suspension of small particles that remain in the supernatant after high-speed centrifugation. This observation agrees with the results of others who have extracted steroid Δ^5 -isomerase activity from microsomal fractions with deoxycholate (Neville & Engel, 1968) and 21-hydroxylase activity from adrenal microsomal fractions with Triton N-101 (Rosenthal & Narashimhulu, 1969). Nonetheless, an optically clear preparation that catalyses the transformation of progesterone into 17-hydroxyprogesterone and into androgens should provide a useful experimental system for further investigation of the components and properties of the 17 α -hydroxylase and the 17-hydroxyprogesterone side-chain-cleavage enzyme of rat testicular microsomal fractions.

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