SUMMARY

1. It has been found that in a large proportion of cases the febrile human excretes hydroxykynurenine. This excretion is, in particular, unrelated to tuberculosis or diabetes. It is attributed to a high rate of breakdown of body protein.

2. In states associated with weight loss but not accompanied by fever, hydroxykynurenine excretion could not be detected.

3. The normal human taking an excess of tryptophan by mouth excretes much kynurenine, but no, or negligible, hydroxykynurenine.

4. Possible reasons for this difference in excretory patterns are discussed.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

1. FACTORS INFLUENCING THE METABOLISM OF PROGESTERONE BY RAT LIVER AND THE INVESTIGATION OF THE PRODUCTS OF METABOLISM

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Table 1 lists the steroids closely related to progesterone which have been isolated from human pregnancy urine (see Samuels & West, 1952, for references) and which are therefore assumed to be metabolites of the hormone. By *in vivo* studies, only three of these steroids have been shown conclusively to be derived from progesterone, namely, pregnane-3α:20α-diol (pregnanediol) (Venning & Browne, 1937), pregnan-3α-ol-20-one (pregnanolone) (Dorfman, Ross & Shipley. 1948) and 5α-pregnane-3α:20α-diol (Kyle & Marrian, 1951). In spite of indications from *in vivo* studies that the liver is involved in progesterone metabolism (see Samuels & West, 1952, for references), Zondek (1941) and Engel (1944) were unable to demonstrate inactivation of the hormone by this tissue *in vitro*. However, using more sensitive chemical methods, Samuels (1950) showed that the αβ-unsaturated ketonic group of progesterone underwent some change when incubated with rat-liver mince.

Table 1. *Human urinary steroids related to progesterone*

Pregnane-3:20-dione
5α-Pregnane-3:20-dione
Pregnan-3α-ol-20-one
5α-Pregnan-3α-ol-20-one
5α-Pregnan-3β-ol-20-one
Pregnane-3α:20α-diol
Pregnane-3β:20α-diol
5α-Pregnane-3α:20α-diol
5α-Pregnane-3β:20α-diol
Pregnan-3α-ol

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It was, therefore, decided that further investigation of the *in vitro* metabolism of progesterone should be undertaken. For this purpose a method was developed for the determination of progesterone extracted from incubated tissue preparations. The method, which is based on the partition-chromatographic method of Butt, Morris, Morris & Williams (1951) for the determination of progesterone in blood, is more sensitive and less tedious than those employed by others (cf. Samuels, McCaulay & Sellers, 1947; Hayano & Dorfman, 1953). Contrary to the finding of Wiswell & Samuels (1953) it has been shown that the metabolism of progesterone by rat-liver 'homogenate' is increased by addition of diphosphopyridine nucleotide (DPN), but in confirmation of the finding of these authors, it has been demonstrated that metabolism is increased in the presence of citrate and is not inhibited under anaerobic conditions.

In a preliminary investigation of the products of metabolism, 5α-pregnane-3:20-dione and 5α-pregnan-3α-ol-20-one have been isolated.

EXPERIMENTAL

Materials and methods

Adenosine triphosphoric acid (ATP) and adenylic acid (AMP) obtained from Light and Co. were used without further purification. DPN was prepared in the laboratory by the method of LePage (1949) and its purity as determined spectrophotometrically (LePage, 1947) was 37%. The standard medium used throughout this investigation was the calcium-free phosphate saline (pH 7·4) of Krebs & Eggleston (1940) (subsequently referred to as 'phosphate saline'). Progesterone (Organon Laboratories) was purified by two crystallizations from hexane and one from aqueous ethanol and had m.p. 121–121·5°.

All melting points were determined on an improvised hot-stage apparatus of the Kofler type (Klyne & Rankeillor, 1947) and are corrected for the emergent stem.

Specific rotations were determined for the *D* line using a 0·5 dm. microtube. The errors were calculated as described by Klyne & Paterson (1948).

In partition chromatography, Celite 535 (Johns-Manville Co.) was used as the supporting phase. It was purified before use by heating at 400° for 8 hr., by treatment with 10N-HCl for 12 hr. at room temperature followed by intensive washing with water until free from acid, and finally by air-drying at 130° for at least 12 hr.

Methanol and ethanol were refluxed with solid KOH and twice distilled. Hexane was washed with conc. H_2SO_4, dried over metallic Na and distilled. Benzene (A.R.) and ether (A.R.) were dried over Na and redistilled; $CHCl_3$ was freshly distilled before use. Acid-free acetone was dried by distillation from anhydrous K_2CO_3. In adsorption chromatography, alumina (Peter Spence and Co.) activated by heating at 100° *in vacuo* (activity II, Brockmann & Schodder 1941) was used throughout. The amount of alumina employed was 30 times the weight of material to be chromatographed and columns of length 10 times the diameter were used.

Liver preparations. Experimental animals were female rats of the Wistar strain about 6 months old. They were killed by dislocation of vertebrae in the neck followed by exsanguination. The livers were rapidly removed and placed in chilled 0·15M-KCl. Slices of about 0·3 mm. thickness were prepared as described by Grant & Marrian (1950). Samples were weighed on a torsion balance to the nearest 2 mg.

Suspensions of tissue were prepared in tissue grinders made in the laboratory which had a glass outer tube and a motor-driven, piston-type, nylon pestle about 3·0 cm. long and 1·5 cm. in diameter. The tissue suspensions or 'homogenates', so obtained were found to be uniformly acellular (cf. Brendler, 1951). The tissue (2·5 g.) was ground with 6 ml. ice-cold 0·15M-KCl (unless otherwise stated) for about 1 min. The final suspension or 'homogenate' was diluted to 10 ml. with 0·15M-KCl and was kept at 0° until pipetted into incubation flasks.

Method employed for the determination of progesterone

For the purpose of the present investigation it was necessary to devise a method for the quantitative determination of about 0·1–0·5 mg. progesterone in the presence of 250 mg. tissue as slices or pulp. The extraction procedure was most easily carried out in glass-stoppered test tubes (Quickfit and Quartz, standard B19 ground-glass joint, capacity 30 ml.). Transferences to and from the extraction tubes were made by means of an adaptor constructed from a B19 joint (Fig. 1) into which could be fitted: (*a*) a Hirsch funnel, (*b*) suction tube *A* having a straight tip for transferences to the extraction tubes (see below), and (*c*) suction tube *B* having a U-tip for transferences from the extraction tube (Fig. 1).

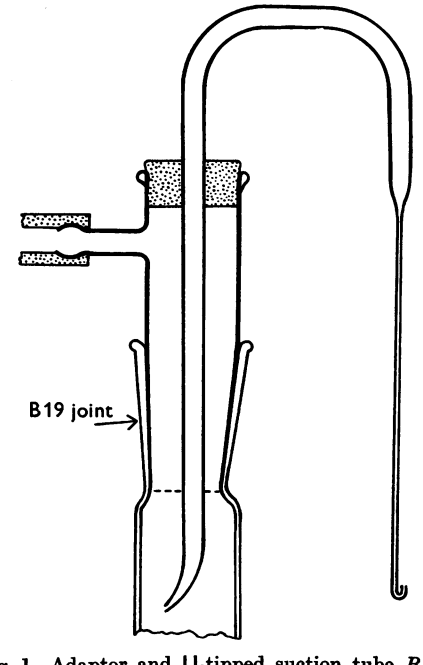

Fig. 1. Adaptor and U-tipped suction tube *B* for transferences from extraction tubes.

Protein precipitation. At the conclusion of the incubation period, 15 ml. acetone were added to each flask and after having been chilled to −20° the contents were filtered with suction through Whatman no. 1 paper into 100 ml. flasks with the aid of the adapter fitted with a Hirsch funnel. The precipitate and paper were transferred to the original flask and washed with 3 × 10 ml. hot acetone; each time before filtration the flasks were chilled to −20°. When slices were used, after two such washings, the slices were ground with 10 ml. acetone and the resultant powder was extracted with 2 × 10 ml. acetone, with chilling to −20° before filtration. The combine filtrates were concentrated to about 3 ml. aqueous residue by removing the acetone in a stream of air at 45°.

Extraction of aqueous residue. To the aqueous residue were added 6 ml. hexane:chloroform (9:1, v/v) and the mixture was refluxed for 5 min. After being cooled, the mixture was transferred, by means of suction tube *A*, to the extraction tube and the flask rinsed with 3 × 5 ml. hexane:chloroform, 2 × 2 ml. water and finally with 2 ml. hexane:chloroform, each rinsing being transferred to the extraction tube. After being centrifuged to separate the emulsions, the upper phase was transferred to the original 100 ml. flask by means of suction tube *B* and the aqueous phase extracted with 2 × 20 ml. hexane:chloroform. The combined extracts were evaporated to dryness in a stream of air at 45°, removal of the last traces of water being facilitated by addition of a little ethanol. The residues were finally dried for at least 1 hr. *in vacuo* over P_2O_5.

Separation of progesterone from lipid extract. Separation of progesterone from the lipid extract was achieved by means of partition chromatography. Equilibration of solvents and preparation and running of columns were carried out in a room thermostatically controlled within the range 16–18° and usually at 17·5±0·5°.

Columns (10 cm. long, 1·0 cm. diameter) were packed in the manner described by Butt *et al.* (1951) in glass tubes about 18 cm. long, one end being fitted with a B 14 standard joint and the other being flat glass with small holes (0·5 mm. diameter) regularly spaced. The first pad, packed effectively, sealed the holes at the base of the tube and no leakage of Celite occurred during the running of the columns. The system methanol:water (7·5:2·5, v/v) (stationary phase) and hexane (mobile phase), having 1·0 ml. stationary phase/g. Celite, was found to be suitable for the separation of progesterone from other material absorbing at 240 mμ. Preliminary experiments showed that progesterone was eluted from columns of these dimensions between 13 and 20 ml.

To each extract obtained as above were added 5·0 ml. mobile phase. Since the incubation and extraction procedures required 1 day to complete, it was convenient to leave the tightly stoppered flasks overnight in order to ensure complete solution of the extract. The columns were packed and 10 ml. mobile phase allowed to run through them. After pouring off the excess of mobile phase from the top of the column, 0·50 ml. extract was added and the walls of the column were rinsed with 3 × 0·5 ml. mobile phase, each washing being allowed to disappear just below the surface of the Celite before the next was applied. During the application of the solution and the washing of the column, the eluate was collected and formed part of the first fraction. About 4·0 ml. mobile phase were then added, care being taken not to disturb the surface of the Celite. A reservoir with a capillary outlet was fitted to the column and the rate of flow checked over a period of 10 min. When the flow rate was outside the limits, 8–12 ml./hr., the column was rejected and a fresh one prepared. The first 10 ml. eluate were rejected and the next 12 ml. (progesterone fraction) collected for analysis. The solvent was removed in a stream of air and 5 ml. absolute ethanol were added to the dry residue. The amount of progesterone present in the solution was determined by measuring the optical density of the solution at 240 mμ. with a Unicam S.P. 500 Spectrophotometer in a 1·0 cm. cell.

As a test of the accuracy and specificity of the method, a series of 'recovery' experiments was carried out in which progesterone was added to incubated liver preparations. These experiments were carried out as follows.

Known amounts of progesterone in propylene glycol solution were added to 250 mg. samples of tissue as slices or homogenate which had been incubated at 37° for 1 hr. Acetone was immediately added and the mixture processed as described above. In 'blank' experiments, progesterone solution was replaced by propylene glycol. The results of these experiments shown in Table 2 indicate that the method is satisfactory for the determination of as little as 0·1 mg. progesterone added to 250 mg. tissue.

The incubation of progesterone with rat-liver preparations

Each experiment was conducted in four parts.

(1) *Incubation experiments.* Duplicate 250 mg. portions of slices were added to 25 ml. conical flasks standing on ice and containing 1·0 ml. 0·15 M-KCl and 2·0 ml. 'phosphate saline'. In the case of 'homogenate', duplicate 1·0 ml. volumes equivalent to 250 mg. tissue were added to 2·0 ml.

Table 2. *Recovery of progesterone added to incubated rat-liver preparations*

(In seven 'blank' experiments, the following quantities of apparent progesterone were recovered: 0·014, 0·016, 0·015, 0·014, 0·015, 0·016 and 0·016 mg. The mean 'blank' value (0·015 mg.) was used to correct the values given below.)

Preparation	Progesterone added (mg.)	No. of results	Progesterone recovered (corrected for 'blank') (%)	Range (%)
Slices (250 mg.)	0·5	8	95±1	92–97
	0·2	4	90±1	89–91
'Homogenate'	0·5	18	97±1	90–100
	0·2	5	95±2	91–98
	0·1	5	89±2	87–95

'phosphate saline'. Unless otherwise stated, additions to the reaction mixture were made with the substance dissolved in 'phosphate saline' and maintaining the volume at 3 ml. by a corresponding reduction in the volume of 'phosphate saline' originally added. A solution of 0·5 mg. progesterone in 0·050 ml. propylene glycol was added to each flask by means of an 'Agla' micrometer syringe (Burroughs, Wellcome and Co.), and when all flasks had been prepared they were shaken for 1 hr. in air (unless otherwise stated) at 37°.

(2) *Steroid recovery controls* (*'controls'*). In these, progesterone was incubated in 'phosphate saline' (with added substances when used) and at the end of the incubation period, incubated tissue was added and the contents of the flask were immediately processed.

(3) *Controls without added steroid* (*'blanks'*). These were introduced in order to ensure that the incubation of liver with propylene glycol alone did not result in production of material absorbing at 240 mμ. and which was not removed by the purification procedure employed. The procedure was as described in (1), except that progesterone solution was replaced by propylene glycol. The values of apparent progesterone obtained from 'blank' experiments were invariably low and consistent. They were therefore not carried out routinely but were checked occasionally.

(4) *Determination of progesterone content of propylene glycol solution.* This determination was carried out on duplicate volumes of the solution added to incubation and control vessels by pipetting 0·010 ml. volumes into tubes containing 3 ml. ethanol and evaporating to dryness. The optical density of the residue was determined at the same time as the residues obtained from the incubation experiment.

It may be seen from the results of these experiments shown in Fig. 2 that progesterone disappears on incubation with tissue slices and cell-free suspensions. The lower activity of cell-free suspensions in metabolizing progesterone is evident from the fact that with these preparations about 80% of the progesterone originally present was recovered, as compared with the recovery of only about 64% progesterone incubated under the same conditions with slices from the same liver. A similar loss of activity on mincing liver has been observed in metabolic studies with oestrogens (Coppedge, Segaloff & Sarrett, 1950), with testosterone (Sweat & Samuels, 1948) and with pregnanediol (Grant, 1952), and has been attributed by these workers to the rapid destruction of DPN in preparations of broken cells from liver and other tissues (Mann & Quastel, 1941). The pH of 7·4 used in the various liver-steroid metabolism experiments reported by others and used in the present investigation is close to the optima of 7·2 (Spaulding & Graham, 1947) and 7·5 (Handler & Klein, 1942) which have been reported for the nucleosidase concerned in the destruction of DPN. This destruction is, however, inhibited by nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942).

Effect of DPN and nicotinamide. In order to determine whether the reduced activity of 'homogenate' could be accounted for by the destruction of endogenous DPN, the experiment illustrated in Fig. 3 was carried out.

It may be observed that there is a decrease in metabolism when 'homogenate' is pre-incubated (bar *C*) compared with 'homogenate' not pre-incubated (bar *B*). This decrease is overcome by adding nicotinamide before (bar *D*) or DPN after (bar *G*), pre-incubation. It is not fully prevented by adding DPN before pre-incubation (bar *F*). The failure of nicotinamide added after pre-incubation to affect markedly the degree of metabolism (bar *E*) shows that

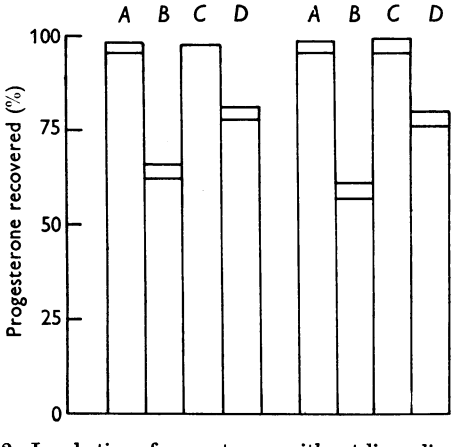

Fig. 2. Incubation of progesterone with rat-liver slices and suspensions of disintegrated tissue ('homogenates'). Reaction mixture: about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline', 1 ml. 0·15M-KCl + 250 mg. liver as slices or 'homogenate'. Progesterone recovered, *A*, from 'controls' (slices); *B*, after incubation with slices; *C*, from 'controls' ('homogenate'); *D*, after incubation with 'homogenate'. In this type of figure, double lines at ends of bars indicate duplicates; single lines coincident duplicates.

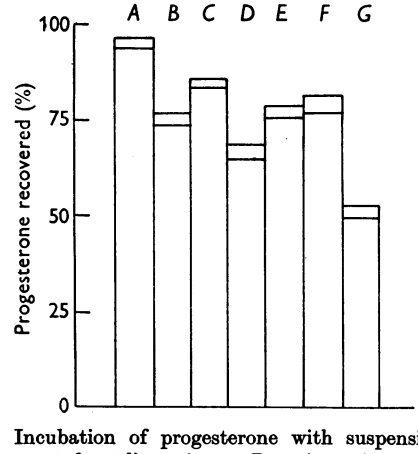

Fig. 3. Incubation of progesterone with suspensions of disintegrated rat-liver tissue. Reaction mixture (RM): about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline' + 1 ml. 'homogenate'. Progesterone recovered, *A*, from 'controls'; after incubation of the following: *B*, RM; *C*, RM using 'homogenate' pre-incubated 0·5 hr.; *D*, RM using 'homogenate' pre-incubated 0·5 hr. with nicotinamide (final concn. 40 mM); *E*, RM using 'homogenate' pre-incubated 0·5 hr. with nicotinamide (final concn. 40 mM) added after pre-incubation; *F*, RM using 'homogenate' pre-incubated 0·5 hr. with DPN (final concn. 3·0 mM) added before pre-incubation; *G*, RM using 'homogenate' pre-incubated 0·5 hr. with DPN (final concn. 3·0 mM) added after pre-incubation.

nicotinamide itself is not responsible for increased progesterone metabolism, but that it exerts its effect by protection of endogenous DPN. The observation that preincubation without added DPN or nicotinamide (bar *C*) did not completely inactivate the 'homogenate' suggests that alternative pathways exist for the metabolism of progesterone and that one or more is not sensitive to DPN.

The effect of various concentrations of DPN and nicotinamide on the metabolism of progesterone by rat-liver 'homogenates' is shown in Fig. 4. It appears that relatively high concentrations of DPN are required to obtain a degree of metabolism comparable with that obtained with the same weight of tissue as slices (Fig. 2). The DPN preparation was only 37% pure, and it is possible that it contained impurity capable of inhibiting the enzymes responsible for progesterone metabolism. Adenylic acid is the chief impurity in the DPN as prepared (LePage, 1949), but when this substance replaced DPN in the reaction mixture in a concentration of 5·0 mM, it had no effect on the activity of the cell-free suspension in metabolizing progesterone. It may be seen from Fig. 4 that an optimum effect was obtained using 0·7 mM DPN and 40 mM nicotinamide.

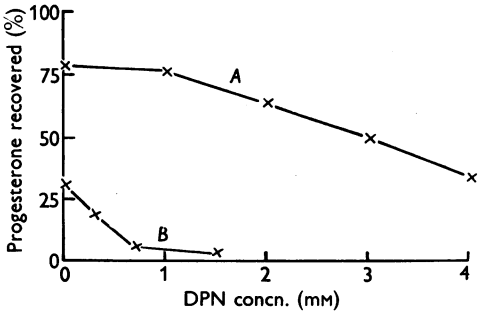

Fig. 4. Incubation of progesterone with suspensions of distintegrated rat-liver tissue. Effect of varying concentrations of DPN. *A*, after incubation with 'homogenate' prepared in 0·15M-KCl; *B*, after incubation with 'homogenate' prepared in 0·15M-KCl – 0·12M nicotinamide.

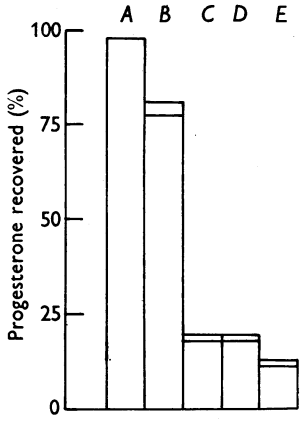

Fig. 5. Incubation of progesterone with suspensions of disintegrated rat-liver tissue; influence of citrate on progesterone metabolism. Reaction mixture (RM): about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline' +1 ml. 'homogenate' prepared in 0·15M-KCl. Progesterone recovered: *A*, from 'controls'; *B*, after incubation of RM; after incubation of RM with citrate; *C*, 1·0 mM citrate, *D*, 2·0 mM citrate and *E*, 3·0 mM citrate.

Effect of other added substances. Fig. 5 illustrates the effect of varying concentrations of citrate on the metabolism of progesterone by rat-liver 'homogenate'. It can be seen that the effect of citrate is almost maximal at a concentration of 1·0 mM. The following substances were tested in the standard reaction mixture and were found to be without effect on the metabolism of progesterone; ATP (0·5 mM), fumarate (1·0 mM), α-oxoglutarate (1·0 mM), malate (1·0 mM), succinate (1·0 mM).

Effect of anaerobic conditions. Replacement of air in the reaction vessels by nitrogen or evacuation of the reaction vessels resulted in no change in the amount of progesterone metabolized (Fig. 6).

Investigation of the products of metabolism

It was considered that incubation of 300 mg. of progesterone with rat liver under optimum conditions would be a convenient scale for the isolation of the major metabolites. At a tissue: steroid ratio of 500:1, this amount of progesterone required 150 g. liver (obtainable from about thirty rats). Since it was intended to include a 'control' (steroid added after incubation) and a 'blank' (no added steroid) the large scale experiment was conducted in two parts. In the first part, 150 mg. progesterone were incubated with tissue suspension and a 'control' treated in identical fashion was run at the same time. The tissue suspension was prepared in small batches as described above. The Waring Blendor, with high-speed rotating blades, was not used for the preparation of the larger quantities of tissue suspension required since its use results in excessive frothing, the temperature is difficult to control, and it has been reported by Stern & Bird (1949) and Lambden (1950) that it is capable of inactivating certain enzymes.

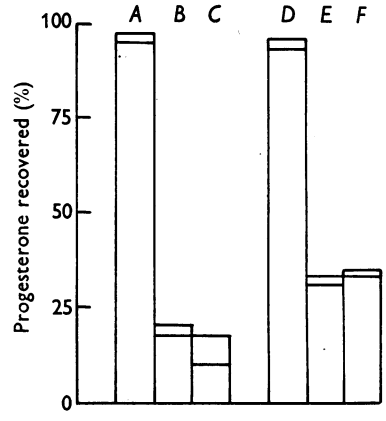

Fig. 6. Incubation of progesterone with suspensions of disintegrated rat-liver tissue. Reaction mixture: about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline' +1 ml. 'homogenate' prepared in 0·15M-KCl containing 0·12M nicotinamide. Progesterone recovered; *A* and *D*, from 'controls': after incubation; *B*, in air; *C*, in nitrogen; *E*, in open Thunberg tube; *F*, in evacuated Thunberg tube.

In the second part, a further 150 mg. progesterone were incubated and a 'blank' was run at the same time.

It was convenient to conduct the incubations in 100 ml. conical flasks; thirty of these were prepared. To each were added 20 ml. chilled 'phosphate saline' and 10 ml. tissue suspension. (The 'homogenizing' was carried out in 0·15 M-KCl containing nicotinamide in sufficient quantity to provide a concentration of 80 mM in the final reaction mixture.) A propylene glycol solution of progesterone (10 mg./ml.) was warmed to 37° to reduce the viscosity of the solvent, and 1·0 ml. volumes were pipetted into each of the 'incubation' flasks. Pure solvent (1·0 ml.) was added to the 'control' and 'blank' flasks. The flasks were not stoppered but were shaken vigorously during the incubation period of 2 hr. After incubation, 'incubation', 'control' and 'blank' experiments were treated in the same manner except that in the case of the 'control', 150 mg. of solid progesterone were added after addition of acetone.

At the conclusion of the incubation period, the contents of the flasks were emptied into a large beaker. Each flask was rinsed with 50 ml. portions of hot acetone until a total of 2 l. acetone had been used, and the washings were added to the beaker. After being vigorously stirred, the mixture was allowed to stand overnight at −20°. The yellow supernatant was filtered and the precipitate returned to the beaker and washed twice with 1 l. volumes of acetone previously chilled to −20°. The bulk of the acetone was removed from the combined filtrates by distillation under reduced pressure (in this and subsequent distillations, the bath temperature was kept below 60°). After dilution with 250 ml. water, the aqueous residue was saturated with NaCl and the yellow oil which separated was extracted with 4 × 250 ml. ethyl acetate. The ester extract was washed successively with 80 ml. volumes water, 0·2 M-$NaHCO_3$, 0·2 N-HCl and finally with water until the washings were neutral. Washing was carried out rapidly so that the ester was in contact with acid or alkali for the shortest possible time. The first water and $NaHCO_3$ washes were combined and back-extracted with 200 ml. ethyl acetate and the same 200 ml. was used to back-extract the HCl and water washes individually. After being dried over anhydrous Na_2SO_4, the combined ester extracts were evaporated to dryness by distillation *in vacuo*.

The resultant brown gum was taken up in 300 ml. methanol and 100 ml. hexane; 100 ml. water were added and two layers separated, the hexane layer being appreciably yellow. The mixture was transferred to a separating funnel and the distillation flask rinsed with 100 ml. 75% (v/v) aqueous methanol and 30 ml. hexane, the rinsings being transferred to the separating funnel. The aqueous methanol was run off and the hexane extracted with 2 × 100 ml. aqueous methanol and the combined extracts washed with 50 ml. hexane. The bulk of the methanol was removed by distillation *in vacuo*, 150 ml. water were added and the aqueous residue was extracted with 2 × 500 ml. $CHCl_3$. The chloroform extract was washed with 100 ml. N-NaOH and then with water until the washings were neutral. After being dried over anhydrous Na_2SO_4, the $CHCl_3$ was removed by distillation *in vacuo*. The weights of the extracts obtained at this stage were: 'incubations', 0·249 g. and 0·223 g.; 'control', 0·30 g. and 'blank', 0·436 g.

The extracts were then subjected to an eight-stage, discrete countercurrent distribution (Craig & Craig, 1950), carried out in separating funnels, using the solvent system methanol:water (80:20, v/v) and benzene:hexane (80:20, v/v) in which the partition coefficient (K) of progesterone was found to be 0·25 $\left(K = \frac{\text{concn. in aqueous methanol}}{\text{concn. in benzene:hexane}}\right)$. A high degree of purification was obtained in this way; the residues from funnels 0–4 of 'incubation' and 'control' extracts were white and semi-crystalline but melting points were not sharp.

The residues from funnels 0–4 were combined and chromatographed on alumina. The material dissolved in a minimum volume of 50:50 (v/v) benzene:hexane was applied to the column, and the following fractions were collected: 5 × 10 ml. benzene:hexane (50:50, v/v); 10 × 10 ml. benzene; 5 × 10 ml. benzene:ether (95:5, v/v); 5 × 10 ml. benzene:ethanol (80:20, v/v) and 25 ml. ethanol.

Isolation of 5α-pregnane-3:20-dione. The benzene:hexane and benzene fractions from the 'control' and 'blank' chromatograms yielded only a small amount of gum, but the benzene fractions from the 'incubations' yielded a total of 40 mg. semi-crystalline material. After two crystallizations from acetone, this material yielded 30 mg. shimmering, white plates, m.p. 199–200° (substance X). A comparison of the properties of this substance with those of a sample of authentic 5α-pregnane-3:20-dione (prepared by reduction of pregn-5-en-3β-ol-20-one with H_2 in presence of Pt followed by oxidation with CrO_3 in acetic acid solution (Marker, Kamm & McGrew, 1937), provided proof that substance X was 5α-pregnane-3:20-dione (Table 3).

Table 3. *Comparison of properties of substance X and of 5α-pregnane-3:20-dione*

		5α-Pregnane-3:20-dione	
	'X'	Found	Reported
M.p.	199–200°	199–200·5°	200–201° (*a*)
Mixed m.p.	198·5–199·5°		
C, H Analysis	Found C: 79·5, 79·5 H: 10·5, 10·4	C: 79·6, 79·5 H: 10·3, 10·3	Calc. for $C_{21}H_{32}O_2$: C, 79·7 H, 10·2
$[\alpha]_D$ in EtOH	+110±1·5° at 19·5° (*c*, 0·537)	+108±2·8° at 19° (*c*, 0·5897)	+117±4° at 24° (*a*) (*c*, 0·255)
Disemicarbazone			
M.p.	>300°	>300°	>300° (*b*)
Mixed m.p.	>300°		

(*a*) Lieberman, Dobriner, Hill, Fieser & Rhoads (1948). (*b*) Kyle & Marrian (1951).

Isolation of progesterone. The ether:benzene fractions from the 'control', after recrystallization from aqueous ethanol yielded 84 mg. progesterone, while from the 'incubation' 12 mg. of this steroid were obtained. Progesterone was identified by m.p., mixed m.p. and u.v. absorption spectrum. No crystalline material was obtained from the 'blank'.

Isolation of 5α-pregnan-3α-ol-20-one. Only the 'incubation' yielded any appreciable material in the ethanol:benzene fractions. The residues were waxy and melted over a wide range; they were therefore chromatographed on alumina a second time. The combined residues were added in benzene and the column eluted with the following series of solvents, 5 ml. fractions being collected: 30 ml. benzene; 35 ml. ether:benzene (1:9, v/v); 15 ml. ether:benzene (5:5, v/v); 20 ml. ether:benzene (9:1, v/v) and finally with 25 ml. ethanol. Two main fractions were obtained; the 1:9 ether:benzene fractions yielded 25 mg. of a semi-crystalline solid, m.p. 165–172° (substance Y) and the 5:5 ether:benzene fractions yielded 22 mg. of a colourless gum which turned white on addition of hexane (substance Z).

Substance Y was recrystallized from aqueous methanol and yielded 20·5 mg. of thin plates, m.p. 173–175°. It gave no precipitate with digitonin and, mixed with 5α-pregnan-3β-ol-20-one (m.p. 193–195°; kindly supplied by Dr J. Y. F. Paterson), the m.p. was 152–167°. A comparison of the properties of this substance with those reported for 5α-pregnan-3α-ol-20-one and the finding that admixture of the acetate with an authentic sample resulted in no depression of the melting point, provided proof that substance Y was 5α-pregnan-3α-ol-20-one (Table 4).

Table 4. *Comparison of properties of substance 'Y' and of 5α-pregnan-3α-ol-20-one*

	'Y'	5α-Pregnanolone*
M.p.	173–175°	174–175°
C, H Analysis	Found C, 79·4, 79·0 H, 10·9, 10·9	Calc. for $C_{21}H_{34}O_2$ C, 79·2 H, 10·8
$[\alpha]_D$ in EtOH	+99·0±1° at 21° (*c*, 0·524)	+96·6±1° at 25° (*c*, 0·755)
Acetate		
M.p.	141–143°	140–142°
Mixed m.p.	140–142°†	
C, H Analysis	Found C, 76·0 H, 9·9	Calc. for $C_{23}H_{36}O_3$ C, 76·6 H, 10·1

* Lieberman *et al.* (1948).
† Sample supplied by Dr H. Hirschmann, Lakeside Hospital, Cleveland.

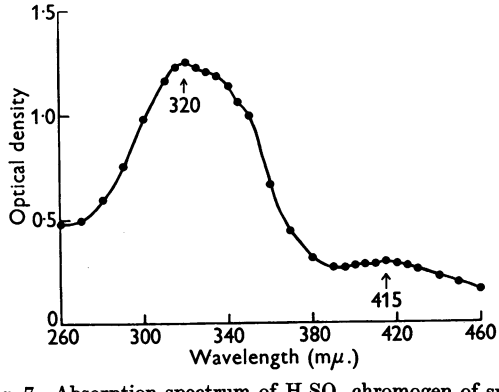

Fig. 7. Absorption spectrum of H_2SO_4 chromogen of substance Z; 320 μg. substance Z + 5·0 ml. conc. H_2SO_4 in 1·0 cm. cell.

Investigation of substance Z. Recrystallization of this substance was attempted from a number of solvents but without success, and so it was rechromatographed; no separation was obtained. The material was eluted from activity II alumina by ethanol:benzene (1:99, v/v) but not by benzene. Fractional crystallization from aqueous acetone yielded three specimens of silky, white needles, m.p.'s 123–130°, 121–130° and 105–124°. Admixture with androstane-3:17-dione (m.p. 132–134°; prepared by oxidation of androsterone with CrO_3) depressed the m.p. in each case. The three specimens were combined and recrystallized from aqueous acetone. The resultant crystals (2·5 mg., m.p. 124–130°) yielded no formaldehyde on oxidation with HIO_4, gave no Zimmermann reaction, had no significant absorption in the ultraviolet region and gave no precipitate with digitonin. With conc. H_2SO_4 a faint yellow colour was obtained at a concn. of 64 μg./ml. conc. H_2SO_4. The absorption spectrum of this solution is shown in Fig. 7.

The mother liquors from the fractional crystallizations were combined and the residue (12 mg.) separated into α and β fractions by means of digitonin. The α fraction (6·7 mg.) could not be crystallized from a variety of solvents, and so was benzoylated and chromatographed on alumina (Brooks, Klyne & Miller, 1953). Recrystallization from $CHCl_3$ gave 1·8 mg. fine white needles which melted at 67°, solidified on further heating, at 110°, and finally melted at 125–126°. Brooks *et al.* (1953) give m.p. 131–133° for 5β-pregnane-3α:20α-diol dibenzoate but do not report a double m.p. Attempts to crystallize the β fraction failed. It is intended to investigate the nature of substance Z more fully by carrying out an isolation experiment on a larger scale.

DISCUSSION

The evidence presented in this paper shows that diphosphopyridine nucleotide and citrate increase the activity of the enzyme system or systems involved in the *in vitro* metabolism of progesterone by rat-liver 'homogenate'. The isolation of two 5α-pregnane derivatives provides evidence that the observed loss of progesterone on incubation of the steroid with rat-liver 'homogenate' is the result, at least in part, of a reductive process. At this stage of the work, however, it would be unwise to attempt to discuss the possible biochemical significance of the enzymic systems involved.

It is of interest that the metabolic reduction of the double bond in ring A was found to be sterically directed to yield only 5α-pregnane derivatives. This finding is in agreement with that of Schneider (1952) who isolated 5α-pregnane derivatives but no pregnane (5β) derivatives after incubation of 11-deoxycorticosterone with rat-liver slices and with that of Caspi, Levy & Hechter (1953) who demonstrated the formation of 5α-pregnane derivatives when cortisone was metabolically reduced by perfusion through rat liver. In the present work,

however, only one of the two possible C-3 epimeric alcohols was obtained, namely, 5α-pregnan-3α-ol-20-one (5α-pregnanolone), whereas Schneider (1952) and Caspi *et al.* (1953) demonstrated that both epimers were formed in their experiments. In the case of deoxycorticosterone (pregn-4-en-21-ol-3:20-dione), the main reduction product was 5α-pregnane-3β:21-diol-20-one and only a small quantity of the 3α epimer was obtained (Schneider, 1952). It would appear that the nature of the C-17 side-chain influences the steric course of the reduction of the C-3 ketonic group of non-benzenoid steroids.

The isolation of 5α-pregnane-3:20-dione as a major metabolite supports the findings of Riegel, Hartop & Kittinger (1950) who demonstrated that after injection of [21-^{14}C]progesterone into rats, most of the radioactivity was found in the faeces in a ketonic, non-alcoholic, neutral steroid which was not progesterone. The failure to isolate any 'pregnanediols' in the present investigation does not necessarily indicate that such substances were not formed. It is not known whether the rat excretes 'pregnanediols' normally or after progesterone administration, but the possibility of detecting such substances is reduced in view of the fact that rat-liver 'homogenate' is able to metabolize pregnane-3α:20α-diol *in vitro* to pregnan-3α-ol-20-one (Grant, 1952).

In the large-scale experiments now reported, only 28% of the progesterone added could be accounted for in terms of the two metabolites and unchanged progesterone isolated. While some of this loss is no doubt manipulative (only 56% of added progesterone was recovered from the 'control'), it is apparent that it reflects enzymic degradation of progesterone to substances not recoverable by the isolation techniques employed. The nature of substance Z requires further investigation.

SUMMARY

1. A method is described for the determination of small quantities of progesterone in incubated tissue preparations.

2. The application of the method to the study of the *in vitro* metabolism of progesterone by rat-liver slices and suspensions of disintegrated tissue ('homogenates') has indicated that this tissue contains an enzyme or enzymes which metabolize this steroid. The metabolism is increased by the addition of either diphosphopyridine nucleotide or nicotinamide or both, and of citrate to the reaction mixture; it is not inhibited under anaerobic conditions.

3. In a large-scale experiment, 28%, of the progesterone incubated with rat-liver 'homogenate' (with added nicotinamide) could be accounted for by the isolation of 5α-pregnane-3:20-dione and 5α-pregnan-3α-ol-20-one and of unchanged progesterone.

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