The authors wish to thank Dr A. Kornberg for the DPN preparation and Dr R. G. Bartsch for the preparations of TPN, lithium acetyl phosphate and of sodium and potassium vinylacetates used in these investigations. This work was supported in part by a research grant (RG 607–C4) from the United States Public Health Service, and was carried out during the tenure by one of us (J. L. P.) of a maintenance grant from the U.S. State Department under the Smith–Mundt Act.

REFERENCES

Anfinsen, C. B. & Kielley, W. W. (1954). *Annu. Rev. Biochem.* **23**, 17.
Barker, H. A., Stadtman, E. R. & Kornberg, A. (1955). *Methods in Enzymology*, vol. I, p. 599. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press.
Barker, H. A. & Taha, S. M. (1942). *J. Bact.* **43**, 347.
Bornstein, B. T. & Barker, H. A. (1948). *J. biol. Chem.* **172**, 659.
Chantrenne, H. & Lipmann, F. (1950). *J. biol. Chem.* **187**, 757.
Green, D. E., Mii, S., Mahler, H. R. & Bock, R. M. (1954). *J. biol. Chem.* **206**, 1.
Harrison, K. (1939). *Biochem. J.* **33**, 1465.
Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
Kennedy, E. P. & Barker, H. A. (1951*a*). *J. biol. Chem.* **191**, 419.
Kennedy, E. P. & Barker, H. A. (1951*b*). *Analyt. Chem.* **23**, 1033.
LePage, G. A. & Mueller, G. C. (1949). *J. biol. Chem.* **180**, 775.
Lieberman, I. (1954). *Arch. Biochem. Biophys.* **51**, 350.
Lynen, F. (1953). *Fed. Proc.* **12**, 683.
Lynen, F. & Reichert, E. (1951). *Angew. Chem.* **63**, 47.
Mahler, H. R. (1953). *Fed. Proc.* **12**, 694.
Mahler, H. R. (1954). *J. biol. Chem.* **206**, 13.
Markham, R. (1942). *Biochem. J.* **36**, 390.
Peel, J. L. (1955). *J. gen. Microbiol.* **12**, ii.
Peel, J. L. & Barker, H. A. (1953). *Biochem. J.* **53**, xxix.
Rietz, E. (1944). *Org. Synth.* **24**, 96. Ed. by Drake, N. L. New York: Wiley and Sons.
Seubert, W. & Lynen, F. (1953). *J. Amer. chem. Soc.* **75**, 2787.
Stadtman, E. R. (1952*a*). *J. biol. Chem.* **196**, 535.
Stadtman, E. R. (1952*b*). *J. biol. Chem.* **196**, 527.
Stadtman, E. R. (1953*a*). *Fed. Proc.* **12**, 692.
Stadtman, E. R. (1953*b*). *J. biol. Chem.* **203**, 501.
Stadtman, E. R. & Barker, H. A. (1949*a*). *J. biol. Chem.* **180**, 1085.
Stadtman, E. R. & Barker, H. A. (1949*b*). *J. biol. Chem.* **180**, 1095.
Stadtman, E. R. & Barker, H. A. (1949*c*). *J. biol. Chem.* **180**, 1117.
Stadtman, E. R. & Barker, H. A. (1949*d*). *J. biol. Chem.* **180**, 1169.
Stadtman, E. R. & Barker, H. A. (1949*e*). *J. biol. Chem.* **181**, 221.
Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
Stadtman, E. R. & Lipmann, F. (1950). *J. biol. Chem.* **185**, 549.
Wakil, S. J. & Mahler, H. R. (1954). *J. biol. Chem.* **207**, 125.

The Metabolism of Progesterone by Animal Tissues *in vitro*

3. INVESTIGATION OF THE PRODUCTS OF METABOLISM AFTER INCUBATION OF 5β-PREGNANE-3α:20α-DIOL WITH RABBIT-LIVER HOMOGENATE*

BY W. TAYLOR
Department of Physiology, The Medical School, King's College, University of Durham, Newcastle upon Tyne

(*Received* 26 *September* 1955)

It is well established that when progesterone (pregn-4-ene-3:17-dione) is administered to human subjects a considerable proportion remains unaccounted for by the known progesterone metabolites, mainly 5β-pregnane-3α:20α-diol ('pregnanediol') subsequently recoverable from the urine. Moreover, Sommerville & Marrian (1950) found that when 'pregnanediol' itself was administered orally to human subjects the proportion of administered steroid subsequently excreted in the urine as 'pregnanediol' was no higher than when progesterone was given to the same subjects by the same route. It was suggested by these authors that these low recoveries of urinary 'pregnanediol' might be due to excretion of that steroid by other routes or to conversion into further metabolites as yet unidentified, or to a combination of both processes.

To explore the possibility that further metabolism of 'pregnanediol' occurs in the body, Grant & Marrian (1950) and Grant (1952) investigated the metabolism of 'pregnanediol' dihemisuccinate by rabbit- and rat-liver preparations. Liver slices, acetone powders and homogenates were able to metabolize 'pregnanediol' under aerobic conditions, and addition of diphosphopyridine nucleotide

* Part 2: Taylor (1955).

(DPN) to rat-liver homogenates increased their ability to metabolize 'pregnanediol'. 50% of the 'pregnanediol' incubated with rat-liver homogenate was metabolized and of this amount only 10% was accounted for by the isolation of 3α-hydroxy-5β-pregnan-20-one ('pregnanolone') (Grant, 1952). The fate of the main part of the 'pregnanediol' which disappeared was not known, but it is possible that it can be attributed largely to losses incurred during the extraction and purification processes employed.

It has not been established whether or not 'pregnanediol' is a metabolite of progesterone in the rat; indirect evidence indicates that it is not. Thus Riegel, Hartop & Kittinger (1950) showed that after injection of [21-^{14}C]progesterone into rats, most of the radioactivity was found in the faeces in a ketonic, non-alcoholic steroid which was not progesterone. Furthermore, Taylor (1954) was able to isolate only 5α-pregnane-3:20-dione and 3α-hydroxy-5α-pregnane-20-one after incubation of progesterone with rat liver. It seemed possible, therefore, that Grant (1952) failed to isolate metabolites other than 'pregnanolone' after incubation of 'pregnanediol' with rat liver because in that tissue the enzyme systems responsible for the further metabolism of 'pregnanediol' (except at C-20) are either absent or are specific for steroids possessing the 5α-configuration.

It was therefore decided to investigate the metabolism of 'pregnanediol' by the liver of the rabbit, since in this animal, as in humans, 'pregnanediol' is a major metabolite of progesterone, and when progesterone is incubated with rabbit liver under aerobic conditions 'pregnanediol' is a major metabolite (Taylor, 1955).

'Pregnanediol' dihemisuccinate has been incubated in air with a suspension of disintegrated rabbit liver under conditions similar to those employed for rat liver by Grant (1952). Only two metabolites, 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3:20-dione, have been isolated; these two metabolites along with the unchanged 'pregnanediol' isolated accounted for 54% of the steroid added to the incubation mixture.

EXPERIMENTAL

Materials and methods

Pregnanediol dihemisuccinate (PDHS) (m.p. 147–148·5°) was prepared as described by Grant & Marrian (1950). Authentic 5β-pregnane-3:20-dione (m.p. 119–120·5°) was prepared by oxidation of 'pregnanediol' with CrO_3 in acetic acid solution (Marker, Kamm & McGrew, 1937). Other reference steroids were the specimens previously described (Taylor, 1955).

For adsorption chromatography, alumina (100/150 mesh, Savory and Moore Ltd., London) was inactivated by exposing it to an atmosphere saturated with water vapour at room temperature for 10–14 days. Chromatography was carried out in 2·0 cm. diam. columns containing 30 g. of alumina. The material to be chromatographed was dissolved in the minimum volume of benzene and the column eluted by the gradient-elution technique with ethanol–benzene mixtures ranging from 0 to 2·2% (v/v) of ethanol by a linear gradient over 200 ml., and then finally with 100 ml. of 5% (v/v) ethanol–benzene. Volumes of 10 ml. were collected. Solvents were not dried but were twice distilled in all-glass apparatus shortly before use. Substances isolated were identified by comparison of the following properties with those of authentic steroids: m.p. and mixed m.p., absorption spectra in conc. H_2SO_4 over the range 222–520 mμ. and infrared spectra in CS_2 solution over the 'fingerprint' region of the free steroids or, for hydroxysteroids, their acetates. All melting points are corrected and were determined in a Kofler-type apparatus.

Other materials and methods were as described in previous publications (Taylor, 1954, 1955), unless otherwise stated.

Incubation of PDHS with suspension of disintegrated rabbit liver

The experiment was conducted in four parts, a total of 215 mg. of 'pregnanediol' as PDHS being incubated with 268 g. (wet wt.) of rabbit liver. The following is a description of a typical experiment. PDHS (81·5 mg. containing 50 mg. of 'pregnanediol') was dissolved in 100 ml. of 'phosphate-saline' and 20 ml. of this solution was added to each of five 100 ml. conical flasks. The rabbit liver (61·5 g.) was homogenized for 2 min. at high speed in the Atomix blender (Measuring and Scientific Equipment Ltd., London) with 40 ml. of 0·15 M-KCl containing sufficient nicotinamide to provide a concentration of 0·08 M in the final reaction mixture. The resulting 'suspension' was filtered through two layers of gauze, made up to 100 ml. with 0·15 M-KCl and added in 20 ml. portions to the reaction vessels. The flasks were not stoppered but were shaken vigorously during the incubation for 2 hr. at 37°. A neutral lipid extract was then prepared (Taylor, 1954), and the combined extracts from the four parts were separated into ketonic and non-ketonic, α- and β-fractions by treatment with Girard Reagent T and digitonin as previously described (Taylor, 1955). (Because of the scarcity of 'pregnanediol' it was not possible to carry out a parallel 'control' experiment; the effect of this on the validity of the results is considered in the Discussion section.) The weights of the various fractions thus obtained are given in Table 1.

Table 1. *Weights (mg.) of fractions obtained after treatment of lipid extract with Girard reagent T and digitonin*

Total neutral lipids	426
Ketonic fraction	149
α-Ketonic	116
β-Ketonic	0
Non-ketonic fraction	217
α-Non-ketonic	165
β-Non-ketonic	21

Investigation of fractions

α-Ketonic. This fraction, a dark-brown gum, was separated into three main components by chromatography. Component I (38 mg.) (fractions 1–5) was a dark-brown oil which could not be resolved further either by rechromatography or by acetylation and rechromatography. Component II (7 mg.) (fractions 6–9), after two crystallizations from hexane, yielded 5·3 mg. of thick rods, m.p. 118–120°, identified as 5β-pregnane-3:20-dione. Component III (60·4 mg.) (fractions 10–15), on crystallization from hexane, gave 49 mg. of fine needles, m.p. 146–148°, identified as 3α-hydroxy-5β-pregnan-20-one.

α-Non-ketonic. This fraction was white and semicrystalline. On chromatography only one main fraction (84 mg.) eluted at ethanol concns. from 1·5 to 2·2% (v/v) was obtained. One crystallization from aqueous ethanol and one from benzene yielded 62 mg. of crystals, m.p. 234·5–237°, which were proved to be 'pregnanediol'.

β-Non-ketonic. A quantitative Liebermann–Burchard reaction on this semicrystalline material indicated that it consisted very largely of cholesterol. Direct crystallization from ethanol yielded 17 mg. of fine needles, m.p. 145–148°, which were identified as cholesterol.

Table 2. *Steroids isolated after incubation of* 215 *mg. of 'pregnanediol' with rabbit liver*

Steroid isolated	Wt. (mg.)	As percentage of added 'pregnanediol'
5β-Pregnane-3:20-dione	5·3	2·5
3α-Hydroxy-5β-pregnan-20-one	49	22
'Pregnanediol'	62	29
Totals	116·3	53·5

The results of the experiment are summarized in Table 2. The total recovery of steroids (54%) compares favourably with that obtained in similar experiments in which progesterone was incubated with rat and rabbit liver (Taylor, 1954, 1955) (see Discussion).

DISCUSSION

It has been shown that on incubation with suspensions of disintegrated rabbit liver 'pregnanediol' is metabolized to 5β-pregnane-3:20-dione ('pregnanedione') and 3α-hydroxy-5β-pregnan-20-one ('pregnanolone'). In a similar experiment with rat liver Grant (1952) was able to isolate only 'pregnanolone'. His failure to isolate 'pregnanedione' may have been due to the fact that only a very small amount of this substance was formed. (In the present experiment only 2·5% of the incubated 'pregnanediol' was recovered as the dione.) It is more probable, however, that the oxidation of the 3-hydroxyl group of 5β-pregnane compounds does not occur in rat liver, since in this tissue reduction of progesterone yields only 5α-pregnane derivatives (Taylor, 1954). Rabbit liver, on the other hand, metabolizes progesterone to steroids which are mainly of the 5β-series (Taylor, 1955).

The amount of 'pregnanedione' isolated in the present experiment was small compared with the amount of 'pregnanolone' isolated. This may explain the failure of Taylor (1955) to isolate 'pregnanedione' after incubation of progesterone with rabbit liver.

Since no 20α-hydroxy-5β-pregnan-3-one was isolated in the present investigation, it seems probable that oxidation of the C-20 hydroxyl group must precede oxidation of the C-3 hydroxyl group.

It was not possible to carry out a parallel 'control' experiment, but from the author's previous experience it is considered that the 54% recovery of added steroid precludes the possibility that any other major metabolites were formed. Thus in 'control' experiments in which progesterone was added to incubated rat liver (Taylor, 1954) and rabbit liver (Taylor, 1955) 56 and 52% respectively of the steroid was recovered as crystalline material. It appears, therefore, that under the conditions employed rabbit liver does not convert 'pregnanediol' into substances other than those isolated.

It is recognized that this type of *in vitro* experiment may bear little or no relationship to the metabolism of steroids in the intact animal; nevertheless, it is possible that a large proportion of administered progesterone or 'pregnanediol' unaccounted for as urinary 'pregnanediol' (Sommerville & Marrian, 1950) is excreted in the urine or in the faeces (via the bile) (cf. Marrian, 1954) as 'pregnanolone' and 'pregnanedione'. The determination of these substances in urine and bile or faeces would therefore be of value in attempts to assess disorders of progesterone production and metabolism.

SUMMARY

1. In a large-scale experiment in which 5β-pregnane-3α:20α-diol ('pregnanediol') was incubated with a suspension of disintegrated rabbit liver with added nicotinamide, 54% of the added steroid has been accounted for by the isolation of 5β-pregnane-3:20-dione (2·5%) and 3α-hydroxy-5β-pregnan-20-one (22%) and unchanged 'pregnanediol' (29%).

2. The significance of this finding has been discussed.

The author is indebted to Dr B. Coleby of the Chemistry Department of this College for the determinations of infrared spectra which were carried out at the Courtauld Institute of Biochemistry, Middlesex Hospital, London, by kind permission of Dr A. E. Kellie. This work was carried out during the tenure of a Senior Luccock Research Fellowship.

REFERENCES

Grant, J. K. (1952). *Biochem. J.* **51**, 358.
Grant, J. K. & Marrian, G. F. (1950). *Biochem. J.* **47**, 497.
Marker, R. E., Kamm, O. & McGrew, R. D. (1937). *J. Amer. chem. Soc.* **59**, 616.
Marrian, G. F. (1954). In *La Fonction Lutéale* (Publication des Colloques sur la Fonction Lutéale: April 1954). Paris: Masson et Cie.
Riegel, B., Hartop, W. L. jun. & Kittinger, G. W. (1950). *Endocrinology*, **47**, 311.
Sommerville, I. F. & Marrian, G. F. (1950). *Biochem. J.* **46**, 285.
Taylor, W. (1954). *Biochem. J.* **56**, 463.
Taylor, W. (1955). *Biochem. J.* **60**, 380.

The Oxidation of L-Amino Acids by *Mytilus edulis*

By H. BLASCHKO and D. B. HOPE
Department of Pharmacology, University of Oxford, and Marine Biological Laboratory, Plymouth

(*Received* 5 *August* 1955)

It is now well established that amino acid oxidases are widely distributed in molluscs. The cephalopod liver contains not only a general D-amino acid oxidase (Blaschko & Hawkins, 1951), but probably also a second oxidase that acts upon D-aspartic and D-glutamic acids (Blaschko & Himms, 1955); D-amino acid oxidase also occurs in the digestive gland of *Helix*.

When other molluscan species were examined, it was found that the digestive gland of *Mytilus edulis* also had amino acid-oxidase activity: DL-methionine, DL-α-aminobutyric acid, DL-norvaline and DL-norleucine were found to be oxidized (Blaschko & Hawkins, 1952). In the same year, Roche, Thoai & Glahn (1952) described an enzyme in the digestive gland of *Mytilus* that oxidized L-arginine and a few other amino acids. Since in the earlier experiments with *Mytilus* (Blaschko & Hawkins, 1952) all the compounds tested were racemic mixtures, it seemed desirable to find out whether the *Mytilus* oxidase had acted upon the D- or the L-forms of the racemic amino acids studied.

In the experiments described below, no evidence for the occurrence of a D-amino acid oxidase in *Mytilus* was obtained. On the other hand, many L-amino acids were oxidized. The substrate specificity of the *Mytilus* enzyme shows characteristic differences from other L-amino acid oxidases that are described in the literature.

MATERIAL AND METHODS

Some of the preliminary experiments were carried out at Plymouth, where freshly caught animals were used. Most of the work was done in Oxford, on specimens sent from Plymouth. The animals were dissected soon after arrival; only animals that appeared to be vigorous were used.

In *Mytilus*, the digestive gland contains parts of the intestine which cannot be separated by dissection. The dissected tissue was weighed, frozen in a mortar, cooled to −10° and then thoroughly ground at room temperature. To each gram of fresh tissue 2 ml. of 0·067 M-sodium phosphate buffer at pH 7·15 was added to give a crude suspension.

In the experiments on substrate specificity, the crude suspension was centrifuged at 3000 *g* for 1 hr. at 0°. The sediment was resuspended in the original volume of phosphate buffer and recentrifuged. The sediment was again suspended in the phosphate buffer and dialysed against the same buffer for 24 hr. at 3°. This procedure gave preparations with a satisfactory enzymic activity and a relatively low oxygen consumption in the 'enzyme blank'.

For the manometric determination of enzymic activity 1 ml. of the *Mytilus* preparation was used in the main compartment of a conical flask, together with 0·6 ml. of the phosphate buffer. The inner tube contained a filter paper and 0·3 ml. of N-KOH, the side bulb 0·4 ml. of either water ('enzyme blank') or of a solution containing 10 μmoles of the L-amino acid to be tested, so as to make the initial substrate concentration 0·005 M. Some of the amino acids were not sufficiently soluble; these were prepared as a suspension of the solid material finely ground in a mortar. This was necessary with cystine (L-, *meso*- and DL-cystine), with DL-homocystine, with DL-α-aminocaprylic acid and with DL-tyrosine. When DL-amino acids were used each flask contained 20 μmoles of the acid.

Unless otherwise stated, the material was obtained from commercial sources. We are indebted to Dr Elizabeth Work for the samples of αα′-diaminopimelic acid and to Professor H. A. Krebs for a number of amino acids prepared by Elks, Hems & Ryman (1948); Dr E. P. Abraham gave us a sample of DL-α-aminoadipic acid prepared by Gaudry (1949).

In the preparation of L-canavanine from Jack beans we followed the advice of Dr E. E. Snell and used in the purification procedure a column of Dowex 50 (×8, 200–400 mesh) in its H form. Crystalline L-canavanine acid sulphate was readily obtained upon seeding with a few crystals given by Dr H. Kihara. Another sample of L-canavanine sulphate was kindly given by Dr E. A. Bell and Professor W. R. Fearon. The total yield of analytically pure L-canavanine acid sulphate from 1 kg. of whole Jack beans was 10·3 g.