STUDIES IN

PROGESTERONE METABOLISM

by

Lewis Malcolm Atherden, B.Sc.

Thesis presented for the Degree of Doctor of Philosophy, University of Edinburgh.

Sept. 1956.

ACKNOWLEDGMENTS

The author wishes to record his appreciation of the constant advice and guidance of Professor G.F. Marrian, F.R.S., and Dr J.K. Grant, under whose supervision these investigations were carried out.

For gifts of reference materials, he is indebted to Professor F.S. Spring, F.R.S., Dr A. Klopper, Dr W. Taylor, the Medical Research Council and Dr W. Mitchell of Stafford Allen & Sons, Ltd.; thanks are due also to Mr C. Bell for making special glassware and to Mr R. Bayne for purification of Celite and solvents.

CONTENTS

FIGURES

TABLES

GENERAL INTRODUCTION

The biochemistry of progesterone (pregn-4-ene-3:20-dione) has been reviewed by Lieberman and Teich (1953) and by Fukushima and Rosenfeld (1954); more recent reviews are those by Roberts and Szego (1955) and Dorfman (1955a). For older reviews, Pearlman (1948), Marrian (1949), Bradbury, Brown and Gray (1950) and Samuels and West (1952) may be consulted. Certain aspects of some recent papers will be found here and in the general discussion.

Of the 5β-pregnane (pregnane) and 5α-pregnane (allopregnane) derivatives which have been isolated from human pregnancy urines, only 5β-pregnane-3α:20α-diol (pregnanediol), 3α-hydroxy-5β-pregnan-20-one (pregnanolone) (Dorfman, Ross and Shipley, 1948) and 5α-pregnane-3α:20α-diol (allopregnanediol) (Kyle and Marrian, 1951) have been shown conclusively to be progesterone metabolites.

Dobriner, Lieberman, Rhoads and Taylor (1948) observed that 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) was excreted in the urine of a normal woman only during pregnancy; before and after pregnancy, none was excreted. Strong evidence that allopregnanolone is also a metabolite of progesterone was presented by Dobriner and Lieberman (1950) who injected progesterone into a normal man and examined the urine. The former

steroid was found only as a result of the administration of progesterone; before and after this administration period, none was present. These results have been confirmed by Davis and Plotz (1956) who observed excretion of allopregnanolone in the urine of pregnant women; none was found in the urine of the same women when not pregnant. Furthermore, Plotz and Davis (1956) found increased urinary excretion of this steroid by pregnant women when they were treated with progesterone. This leaves little doubt that allopregnanolone is a metabolite of exogenous progesterone; of course this is not complete proof that it is produced normally from progesterone, in vivo.

Pregnanediol only accounts for about 20% of the administered progesterone, while pregnanolone accounts for about 3% and allopregnanediol and allopregnanolone for even less.

In vivo metabolism of progesterone

Considerable evidence is available that the liver is the major site of inactivation of progesterone; Selye (1941) found that the anaesthetic and toxic effects were very much greater in partially hepatectomized rats than in normal rats. Kochakian, Haskins and Bruce (1944) implanted progesterone pellets in the muscle, subcutaneously, in a fold of the mesentery (progesterone absorbed here would be

transported to the liver) and in the kidney of female rabbits. Three mg. of progesterone were absorbed from the pellet implanted in the muscle and this led to a strongly positive endometrial response; twenty mg. of progesterone were absorbed from the pellet implanted in the mesentery, but no endometrial response was observed. In support of this, Masson and Hoffman (1945) found that the dose of progesterone required to induce endometrial proliferation in rabbits was eight times as high in normal animals as that required in partially hepatectomized animals.

The importance of the liver has been confirmed by studies with progesterone-21-C^{14}. Riegel, Hartop and Kittinger (1950) found that after intraperitoneal injection into rodents, the greatest amounts of radioactivity were in the liver; the concentration per gram of tissue was highest in the pituitary and adrenals, but these are small organs and the values may not be very significant. Shen and Doisy (1953) found that after intravenous injection into rats, most of the radioactivity was found in the liver and intestinal tract. In experiments with rats with bile fistulas, Grady, Elliott, Doisy, Bocklage and Doisy (1952) found, after intramuscular injection, 56-84% of the activity in the bile; in similar experiments but using intragastric administration, Shen, Elliott, Doisy and Doisy (1954) reported

57-65% of the activity in the bile; Doisy, Bocklage, Shen and Valcourt (1952) reported 56-96% activity in the bile of rats with bile fistulas.

However, other organs have been shown to be involved, both in vivo and in vitro; Wiest, Berliner and Woods (1954) injected progesterone-4-C^{14} intravenously into totally eviscerated male rats and found that after one hour no evidence of progesterone could be found in any of the tissues, including blood.

Marker, Wittle and Lawson (1938) found that the bull excretes pregnanediol in the urine but Marker (1939) found that the steer does not. Thus the testes must enter into the metabolism of progesterone in some way.

In vitro metabolism of progesterone

Some of these in vivo observations have been confirmed by in vitro studies, but as is generally found in attempting to correlate in vivo and in vitro work, some differences have arisen also. These correlations and differences will be dealt with in the general discussion.

That the placenta plays some part was shown by Hagopian, Pincus, Carlo and Romanoff (1956) who perfused progesterone-4-C^{14}, in bovine blood, through a near-term human placenta and obtained 6-ketoprogesterone; Berliner and Salhanick (1956) incubated the

same substrate with near-term human placenta and obtained 6β-hydroxyprogesterone.

Hayano, Lindberg, Wiener, Rosenkrantz and Dorfman (1954) found that the 20-ketone group of progesterone was reduced to a 20β-hydroxy group by incubation with a beef corpus luteum homogenate.

The adrenal gland has a complicated role in the metabolism (and biosynthesis) of progesterone and other steroids; Levy, Jeanloz, Jacobsen, Hechter, Schenker and Pincus (1954) showed that isolated bovine adrenal glands will hydroxylate perfused progesterone in the 11β, 17α and 21 positions; Levy and Kushinsky (1955) using the same technique achieved 19-hydroxylation of progesterone. Heard, Jacobs, O'Donnell, Peron, Saffran, Solomon, Thompson, Willoughby and Yates(1954) incubated progesterone with rat adrenal glands and identified cortisone(17α,21-dihydroxy-pregn-4-ene-3:11:20-trione) as one of the products. Thus the adrenal gland effects hydroxylation and oxidation, leaving untouched the double bond and ketonic groups.

Lynn (1956) obtained an enzyme from rat and bovine testicular extracts which split off the side-chain of progesterone; 17α-hydroxyprogesterone was isolated as a probable intermediate. This work has been substantiated by Slaunwhite and Samuels (1956) who incubated rat testis tissue with progesterone

and obtained 17α-hydroxyprogesterone, androst-4-ene-3:17-dione and testosterone(17β-hydroxy-androst-4-en-3-one).

Rongone, Bocklage, Strength and Doisy (1956) found that blood itself metabolizes progesterone, since bovine blood albumin yielded 5β-pregnane-3:20-dione (pregnanedione) after incubation with progesterone. This appears to be confirmation of the work by Hooker and Forbes (1949) who observed that plasma stored at body temperature rapidly lost its initial progestational activity; it is possible, though, that some of this activity is not due to progesterone since chemical studies (Haskins, 1950; Hinsberg, Pelzer and Seuken, 1956) have shown lower progesterone levels in blood than those indicated by bio-assay (Hooker et al., 1949).

Many studies have been made of the effect on progesterone of liver and liver cell fractions of various species; these will be dealt with in the general discussion.

Thus it can be seen that progesterone is subjected to attack by many tissues, both in the male and the female, and these tissues, either singly or together, perform reduction, oxidation, hydroxylation and degradation.

Taylor (1953) investigated the in vitro metabolism of progesterone with rat liver homogenate and isolated metabolic products. The problem set before

the present author was to separate the homogenate into nuclear, mitochondrial, microsomal and soluble fractions and to find the fraction or fractions responsible for the reduction of Ring A. While this work was proceeding, Tomkins and Isselbacher (1954) published their results and so further investigations in this field were, for the time being, abandoned.

At this point, as a result of publications in the clinical field, Dr Grant became interested in the role of glycyrrhetinic acid (GA) in steroid metabolism, believing it to be an inhibitor of steroid inactivation. The author investigated this in vitro and found GA to be a powerful inhibitor of Ring A reduction.

Subsequently, Professor Marrian desired an investigation into the metabolic products of progesterone in the rat in vivo and so rat excreta were examined for these after injection of progesterone.

GENERAL METHODS AND MATERIALS

A) Experimental Animals

To prepare homogenates, Taylor (1953) used 6-12 months old female rats of the Wistar strain. Since the present *in vitro* work was an extension of Taylor's investigations, it was decided to continue using rats of the same age, sex and strain. The animals were killed by dislocation of cervical vertebrae, the livers removed rapidly and placed in an ice-cold medium, the same as that in which the homogenization was to be carried out, in general, 0.25M-sucrose containing 0.12M-nicotinamide.

B) Liver Preparations

1) Homogenates

The liver tissue was removed from the medium, placed on filter paper and weighed into ice-cold medium in the glass mortar of the homogenizer which had been inserted previously into an outer vessel packed with crushed ice and water. A nylon pestle was used; this had the advantage over a glass one in that wear of the pestle and of the mortar was very greatly reduced and thus the clearance between the shearing surfaces remained uniform for many months. In addition, Brendler (1951) showed that the resulting homogenates were more uniform and contained fewer intact cells than those prepared in all-glass homogenizers. The tissue was ground for one minute, with ten passages of the pestle. The homogenate was diluted to the

appropriate volume with more ice-cold medium and pipetted immediately into the incubation flasks, unless it was to be used for fractionation studies. In general, a 10% homogenate was prepared, that is, x g. of moist liver were diluted, after homogenization to 10x ml. For certain experiments, some of this was used as homogenate and the remainder separated into cell fractions.

2) Cell fractions

Fractionation procedures were based on the method of Schneider and Hogeboom (1950) for homogenates prepared in 0.25M-sucrose. Centrifugation was carried out using the superspeed unit with four place angle head for the M.S.E. Major refrigerator centrifuge, with a bowl temperature of 0°.

Nuclei and unbroken cells were sedimented at 700g for ten minutes. The supernatant fraction (supernatant) was removed carefully and the mitochondrial fraction obtained by centrifuging twice at 5,000g for ten minutes. On removing the supernatant fluids (supernatants) from these sediments, as far as possible the loosely packed 'fluffy layer' was allowed to remain behind since Laird, Nijgaard, Ris and Barton (1953) have shown that this layer is mitochondrial rather than microsomal in nature.

The combined supernatants were centrifuged at 25,000g for one hour in order to obtain a microsomal

pellet. Schneider et al. (1950) used 57,000g for one hour to sediment microsomes, and the former procedure only yields part of the microsomal fraction. This was, however, the maximum centrifugal force which could be obtained with the centrifuge available. On a few occasions it was possible to obtain the use of a Spinco Model E ultracentrifuge. With the aid of the preparative rotor A, a complete yield of microsomes was obtained by centrifuging at 150,000g for one hour and the resulting supernatant was microsome-free.

When a nuclear, mitochondrial or microsomal fraction was to be used in an investigation, it was washed by re-suspending in the ice-cold homogenizing medium and again centrifuging. The fraction was then suspended in ice-cold medium by means of a glass ball pestle, which fitted the centrifuge tube closely, and the suspension was diluted with more medium to the same volume as the original homogenate; in this way the activity of various cell fractions could be compared with each other and with the original homogenate. Similarly, the final supernatant was also diluted to the original homogenate volume.

C) Dialysis

When supernatants from rat liver homogenates required to be dialysed, they were placed in a sac of Visking tubing and dialysed in the cold room at

0-1°, usually against one litre of solution, the solute varying with the experiment. A magnetic stirrer was used and fresh solution was provided after a few hours.

D) Incubation Media

All solutions used in enzymic experiments were prepared in distilled water from an all-glass still and all reagents were of Analytical Reagent (A.R.) grade, where available.

In most experiments, 25 ml. conical flasks were used as incubation vessels; in some of the later experiments, Quickfit & Quartz test tubes, 150 mm. long with B19 sockets were used. The reaction mixture varied but unless otherwise stated was as follows:- 0.05M-KCl; 0.005M-$MgSO_4$; 0.01M-potassium phosphate, pH 7.4; 0.0005M-adenosine triphosphate (ATP) sodium salt, pH 7.4, and 0.002M-sodium citrate, pH 7.4. One ml. of enzyme preparation was used and the final volume was normally 3 ml., but for some experiments was 2 ml. As far as possible other substances added were adjusted to pH 7.4 and potassium salts were used when applicable.

E) Measurement of pH

Measurements were at first made on a battery-operated pH meter (Type D-417, Muirhead & Co., Ltd.) but in later experiments a Pye universal pH meter and millivoltmeter (Cat. No. 11066) was used.

F) Addition of Steroids

Propylene glycol (propane-1:2-diol) has a high solvent power for steroids and has come to be used increasingly as a vehicle, since steroids can be added in very small volumes (Taylor, 1954; Brownie and Grant, 1954). Progesterone and 11-deoxycorticosterone (21-hydroxy-pregn-4-ene-3:20-dione) were added to incubation mixtures as solutions in propylene glycol containing about 0.5 mg. in, respectively, 0.050 ml. and 0.040 ml.; these were measured from an Agla micrometer syringe (Burroughs, Wellcome & Co.).

Duplicate 0.010 ml. volumes of these solutions were measured into test tubes, dissolved in 10 ml. ethanol and the solutions analysed for steroid content in the spectrophotometer. In this way, the amount of steroid added to incubation flasks could be calculated from a calibration curve. Propylene glycol from an Agla micrometer syringe was added to all vessels, whether containing steroids or not, in such amounts that the final volume of propylene glycol was the same in all vessels.

G) The General Arrangement of Incubation Experiments

Most experiments included incubation, 'control' and blank vessels, in duplicate, and except where otherwise stated, the following procedures were adopted:

1) Incubations

Into these vessels was placed the particular reaction mixture desired. It was found by experiment that homogenate, stored at 0°, lost appreciable activity in one hour, whereas cell fractions were relatively stable. Thus for incubations in which homogenate was to be used, either alone or in conjunction with cell fractions, the homogenate was added to the vessels as soon as prepared, steroid added and incubations begun at once. In some experiments, comparison was made more complete by including incubations both with fresh homogenate and with homogenate stored in the cold room during the fractionation.

In experiments involving cell fractions these, as prepared, were stored in the cold room until the final fraction was ready when all enzyme preparations and steroids were added to the incubation vessels. Incubations were carried out on a Warburg bath at 37° with shaking at a frequency of about 100/minute, in general for one hour, in air.

2) Blanks

These were carried out in the same way as incubations except that, instead of steroid solution, solvent only was added to vessels. The resulting apparent steroid content was consistently low.

3) Steroid recovery controls ('Controls')

These were carried out in the same way as incubations, except that the steroid solutions were added after incubation; extraction procedures were begun immediately and in this way a check was made on the steroid recovery levels.

H) Steroids

1) Progesterone (Organon) was recrystallized twice from n-hexane and once from aqueous ethanol to give prisms (α-progesterone) melting at 127.5-128°.

2) 11-deoxycorticosterone (DOC) was prepared from 11-deoxycorticosterone acetate (Organon); the product melted at 138-142°.

3) 5β-pregnane-3α:20α-diol was a pure substance melting at 235.5-236°.

4) Sodium 5β-pregnane-3α:20α-diol 3-glucuronidate (NaPG), from human pregnancy urine, melted with decomposition at 269-270°.

5) 5β-pregnane-3α:20α-diol diacetate, kindly provided by Dr A. Klopper, Clinical Endocrinology Research Unit, Edinburgh, had melting points of 165-166° and 180-181°.

6) 5α-pregnane-3α:20α-diol diacetate, was gifted by Dr A. Klopper and had a melting point of 141-142°

(Hartmann and Locher, 1935, reported 141.5-142.5°). Fifty mg. of this were refluxed for 30 minutes with 5 ml. of a 5% solution of KOH in methanol, and the mixture poured into 50 ml. of cold water. This was extracted with ether, the ether extract washed with water, dried with Na_2SO_4, filtered and evaporated to give 33 mg. of crude allopregnanediol. By recrystallization from the minimum amount of boiling methanol, 16 mg. of crystals, which melted sharply at 247-247.5°, were obtained (Hartmann et al.,1935, reported 248-248.5°) and this material was used for recovery experiments from rat urine and from rat liver homogenates.

7) 5α-pregnane-3:20-dione melted at 196-200° and was kindly supplied by Dr W. Taylor, University of Durham.

8) 5β-pregnane-3:20-dione, 3α-hydroxy-5α-pregnan-20-one and coprostan-3β-ol were supplied by the Medical Research Council Steroid Reference Collection and had melting points of 120-121°, 168-172° and 101-103°, respectively.

I) Adenosine Triphosphate (ATP)

This was a commercial preparation of the sodium salt (Light & Co., Ltd.), containing at least 40% adenosine diphosphate .

J) Diphosphopyridine Nucleotide (DPN)

This was prepared from yeast by the method of Le Page (1949a) and purified by counter-current distribution (Hogeboom and Barry, 1948). It was obtained also as a by-product of the preparation of triphosphopyridine nucleotide (TPN) from sheep liver, the DPN being eluted from the ion-exchange resin column before the TPN. The material obtained was not less than 45% pure as assayed spectrophotometrically following reduction by fresh sodium dithionite (Le Page, 1949b). Occasionally, DPN bought from C.F. Boehringer & Soehne GMBH, Mannheim, and reputed to be 100% pure was used. DPN was stored in a stoppered tube at -20°.

Reduced diphosphopyridine nucleotide (DPNH) was either bought from the same company as the yellow sodium salt, 64% pure, or prepared as a solution only, by reducing DPN with sodium dithionite (Hogeboom and Schneider, 1950).

K) Triphosphopyridine Nucleotide (TPN)

This was prepared from fresh sheep liver by a modification of the method of Le Page and Mueller (1949) and purified by gradient elution from an ion-exchange resin column of Dowex-1-formate. The material obtained was not less than 43% pure as assayed by the method of Horecker and Kornberg (1948) using isocitric dehydrogenase prepared according to Straub (1942). Isocitrate for this assay was prepared from dimethyl-D-isocitric

lactone. TPN was stored in an evacuated tube at -20°.

Reduced triphosphopyridine nucleotide (TPNH) was prepared as a solution only, by reducing TPN with sodium dithionite by the same method as that used for the preparation of DPNH (Hogeboom et al., 1950).

L) Melting Points

Melting points were determined on a modified hot-stage apparatus of the Kofler type (Klyne and Rankeillor, 1947), and are uncorrected.

M) Determination of Progesterone and DOC in Liver Preparations

1) Introduction

Progesterone present in liver preparations was determined by the method of Taylor (1954) and where applicable, modifications of the extraction procedure introduced by Brownie (1955) were used; DOC was determined by the method of Brownie (1955).

2) Protein precipitation

Fifteen ml. cold acetone were added to each 25 ml. incubation flask to terminate the reaction and precipitate protein. In experiments involving homogenate, it was necessary to chill the flasks to -20° before filtration and to use hot acetone for further extraction. When cell fractions were used, the reduced phospholipid content made it possible to omit the chilling of the vessels, and hot extraction was found to be unnecessary;

three extractions with 10 ml. volumes of acetone at room temperature were sufficient.

Latterly, with cell fractions, it was found that steroid recoveries were not lowered if the addition of acetone was omitted and the extracting solvent mixture (see 3) below) was added directly to the incubation mixture. For this purpose, incubations were carried out in Quickfit & Quartz test tubes; at the end of the incubation the extracting solvents were added, the tubes stoppered and shaken vigorously.

3) Extraction of aqueous phase

For progesterone, n-hexane:chloroform (9:1, v/v) was used; for DOC, benzene:chloroform (6:1, v/v). The test tubes in which these extractions were carried out were always centrifuged routinely irrespective of the degree of emulsification, before the organic phase was drawn off.

4) Purification of steroid extract

Celite was purified, partition columns prepared, chromatography carried out and steroids determined as described by Taylor (1954) and Brownie (1955).

5) Accuracy of the method

Recovery of progesterone from rat liver homogenates was of the same order as that found by Taylor (1954); recovery from rat liver fractions, in experiments

in which the use of acetone was omitted, gave recoveries ranging from 94-99%. Recovery of DOC from rat liver homogenates was of the same order.

Results

a) Recovery of Progesterone from rat liver homogenates

Progesterone added (µg.)	Progesterone recovered (corrected for blank) (µg.)	(%)
515	488	95
	488	95
	494	96
	509	99

b) Recovery of progesterone from rat liver fractions (use of acetone omitted)

Progesterone added (µg.)	Progesterone recovered (corrected for blank) (µg.)	(%)
525	504	96
	504	96
520	505	97
	515	99
505	475	94
	475	94
	495	98
	500	99
495	475	96
	490	99
485	456	94
	470	97
	470	97
	470	97
460	450	98
	455	99
445	431	97
	436	98

c) Recovery of DOC from rat liver homogenates

DOC added (µg.)	DOC recovered (corrected for blank) (µg.)	(%)
610	573	94
	592	97

These results show that as far as cell fractions are concerned, the precipitation of proteins with acetone may be omitted without deleterious effect on the recovery of progesterone.

6) Specificity of the method

As far as progesterone is concerned, this has been discussed by Taylor (1953); however, since the submission of Taylor's thesis, Hayano et al.(1954), as mentioned in the introduction to this thesis, have isolated 20β-hydroxy-pregn-4-en-3-one after incubating progesterone with beef corpus luteum homogenate. Furthermore, Caspi, Lindberg, Hayano, Cohen, Matsuba, Rosenkrantz and Dorfman (1956) showed that liver tissue may reduce the 20-keto group of a pregnene derivative to a 20-hydroxyl without affecting Ring A when they incubated DOC with hog liver homogenate and isolated 20α:21-dihydroxy-pregn-4-en-3-one. Thus it is possible that rat liver may yield a 20-hydroxy-pregn-4-en-3-one as a metabolite of progesterone; even if this is so, the former steroid does not appear to contaminate the progesterone fraction since the author

has obtained occasionally complete destruction of progesterone as measured by absorption at 240 mμ.; if a 20-hydroxy-pregn-4-en-3-one had been a metabolic product in these experiments, and had been eluted with the progesterone fraction, it would have yielded significant absorption at 240 mμ. That this contamination is unlikely is confirmed by the results of Hayano et al. (1954) who found that 20β-hydroxy-pregn-4-en-3-one was eluted from a silica gel column after the complete elution of progesterone.

With regard to DOC, Schneider (1952) isolated four metabolites after the incubation of DOC with rat liver slices, but none of these, even if possessing similar partition coefficients to DOC, retain the 3-keto-4-ene group and therefore would not absorb significantly at 240 mμ.

Possible contaminants of the DOC fraction are the 20:21-dihydroxy-pregn-4-en-3-ones and as noted above when discussing the specificity of the method for progesterone, 20α:21-dihydroxy-pregn-4-en-3-one was isolated by Caspi et al. (1956) after incubation of DOC with hog liver homogenate. These authors found that its running rate on paper in a toluene-propylene glycol system was only about one sixth that of DOC. Thus it is probable that the retention volume of the former on a Celite column using the DOC solvent system (Brownie et al., 1954) would be sufficiently greater than

that of DOC to prevent contamination of the latter. It is therefore concluded that the method is probably specific for the determination of DOC after incubation with rat liver preparations.

7) Ultraviolet spectroscopy

For this purpose, a Unicam Photoelectric Quartz Spectrophotometer, S.P. 500 (Unicam Instruments, Cambridge, Ltd.) was used. A slit width of 0.90 mm. was employed as this gave optimum sensitivity; otherwise, the technique described by Taylor (1953) was complied with.

SECTION I

THE DESTRUCTION OF PROGESTERONE BY RAT LIVER PREPARATIONS

A) Introduction

In this introduction an attempt will be made to present a concise summary of some salient features concerning the role of the main cell fractions and their interdependence in cell metabolism. It is realized that this account will suffer from the defects common to all summaries but, for a fuller account, which cannot be given here, the reader is referred to Runnström (1952). For a discussion of the technique of differential centrifugation and the significance of the localization of enzymes, De Duve and Berthet (1954) may be consulted.

The living cell is a highly complex structure, the nucleus and cytoplasm of which have complicated inter-relationships. Experiments with enucleated Amoeba (Lorch and Danielli, 1950) have shown that, for a period, the cell appears to carry on all its activities as before. Sooner or later, however, metabolism dwindles and the cell dies. Comandon and de Fonbrunne (1939) found that A. sphaeronucleus which had been enucleated for three days, completely recovered when another nucleus was transplanted into it. This was not the case, however, if the new nucleus was exposed to any medium other than cytoplasm; that is, the transfer had to be made through apposed cell surfaces. This implies strongly that isolated nuclei are never normal. If a medium can be found which will preserve nuclei so that

they can restore enucleated cells, then it will be the medium of choice for isolating nuclei.

Comandon et al.(1939) found that if a nucleus was restored to an Amoeba which had been enucleated for six days, then only partial recovery took place. Seemingly some alteration had taken place in the cytoplasm which the nucleus could not then restore, showing that the nucleus is not independent of the cytoplasm.

Confirmation of this is shown by the fact that energy yielding processes, for example, the glycolytic system and the Krebs cycle, are absent in nuclei; they depend on the cytoplasm for their energy supply. Conversely, the nucleus provides the cytoplasm with DPN for various enzymic reactions which occur there (Hogeboom and Schneider, 1952a). This DPN-synthesizing enzyme is one of the few which has been shown definitely to reside in the nucleus.

The nucleus has also been shown to be active in synthesizing ribose nucleic acid and it is known that this passes into the cytoplasm (Goldstein and Plaut, 1955) where it associates with the mitochondria and microsomes (Jeener and Szafarz, 1950); as a possible confirmation of this Duryee (1950) observed that nucleoli, migrating from the chromosomes, apparently may fuse with the nuclear membrane and evert their contents into the cytoplasm. The main role of the nucleus may be said to be a long-term dependence of

mechanism?

cell function on nuclear contributions. For a review of nuclear physiology, the reader is referred to Mazia (1952) and for a recent discussion of some nuclear functions see Mazia (1956).

Many particles are found in the cytoplasm. The mitochondria are the largest and contain many enzymes. Not only is the Krebs cycle carried on here but the energy thus obtained is stored up in the form of phosphate bonds as in ATP. The enzymes which produce ATP are found to be in close liaison with those requiring ATP for synthetic purposes (Green, 1954). Thus, operation of the Krebs cycle leads to ATP production which enables fatty acids, awaiting oxidation, to form an active fatty acyl coenzyme A, which undergoes β-oxidation and then enters the Krebs cycle. Thus, once initiated by ATP, the process of fatty acid oxidation is self-perpetuating, since the final stages of the cycle result in the production of more ATP. This led Green (Green, Loomis and Auerbach, 1948) to regard the enzymes in mitochondria not as being a random conglomeration but as an organized complex, which he called the cyclophorase system. This concept is supported by the fact that all enzymes required for the Krebs cycle are found in the mitochondria and in such proportions that any member of the cycle can be oxidized completely without any excess accumulation of intermediate products. Further, the coenzymes required are found to be bound to their apoenzymes and some of the cyclophorase enzymes are

found not to function if they are isolated from the complex (Green, 1952). A useful discussion on the biochemistry of mitochondria is that by Schneider (1953).

The other main particle fraction consists of the microsomes. DPN-cytochrome c reductase is one of the few enzymes which has been demonstrated to be present (Hogeboom et al., 1950). Liver microsomes, with the aid of the soluble fraction, detoxify, by various methods, drugs and other substances foreign to the cell (Brodie, Axelrod, Cooper, Gaudette, La Du, Mitoma and Udenfriend, 1955). They are also at least partly responsible for the reduction of steroids (Glenn, Siu and Recknagel, 1956) and the synthesis of cholesterol (Bucher and McGarrahan, 1955) and proteins (Littlefield, Keller, Gross and Zamecnik, 1955). Adrenal microsomes, with TPNH and oxygen, can hydroxylate steroids, including progesterone, in the 21 position (Ryan and Engel, 1956). Microsomes are thought by some to be disintegration products of mitochondria but there is evidence that microsomes are formed in the Golgi region (Zollinger, 1950) and that they may be converted gradually into mitochondria.

In centrifugation studies, intermediate particles are found, forming the 'fluffy layer'. Tracer techniques have shown that these are not microsomes; they appear to be small mitochondria (Laird et al.,1953). No clear cut classification of

cytoplasmic particles can be made at present; thus mitochondria have been shown to consist of at least four types of particles (Paigen, 1954).

The supernatant remaining after removal of particles from the cytoplasm contains many proteins and a correspondingly large number of enzymes. One of the main groups is that responsible for anaerobic glycolysis; most of the activity of liver homogenates as measured by lactic acid production has been recovered in the supernatant, but its activity was increased by addition of particulate fractions (Le Page and Schneider, 1948). This implies that the supernatant contains most of the enzymes involved and the enhancement by other fractions is a good example of the integrated activity of cell structures. Oxidizing enzymes and enzymes capable of reducing steroids are also found in the supernatant, though their coenzymes must either be added as such, or provided from other cell fractions.

To summarize, no cell fraction can be regarded as autonomous; all are dependent on one another for survival and for effecting cell metabolism.

Taylor (1953) showed that progesterone is metabolized both by rat liver slices and homogenate and isolated metabolic products after incubation of progesterone with homogenate.

Dr Grant, of this Department, thought it would be of great interest to fractionate rat liver homogenates

and to find the cell fraction responsible for the metabolism of progesterone; this was, therefore, made the prime aim; a complete fractionation study was not attempted.

Homogenization was carried out in 0.25M-sucrose containing 0.12M-nicotinamide, thus giving a final concentration of 0.04M-nicotinamide; in certain experiments, as indicated, 0.25M-sucrose was used, and nicotinamide was added to the incubation medium to provide a final concentration of 0.04M-nicotinamide.

Further work in this section was stopped, for the time being, when Tomkins et al. (1954) published their findings with regard to the reduction of steroids by a purified supernatant enzyme prepared from rat liver homogenate.

B) Nuclear Fraction

A 25% homogenate was prepared and part of it was separated into a nuclear fraction and a 700g supernatant.

Results

After incubation	Progesterone recovered * (µg.)	(%)
Homogenate	150	29
	159	31
'Control'	467	91
Nuclear fraction	454	88
	472	92
'Control'	472	92
700g supernatant	237	46
	258	50
'Control'	463	90
Progesterone added	515	

Conclusions

(i) Metabolism of progesterone does not occur with the nuclear fraction to any significant extent.

(ii) Metabolism takes place with the supernatant but to a lesser degree than with the homogenate.

* All recoveries are corrected for the blank

A 25% homogenate was prepared and 2 ml. were used immediately for incubations; a few ml. were stored in the cold room until all other enzyme preparations were ready, and the remainder was divided into two equal parts. These were both separated into a nuclear fraction and a 700g supernatant; the nuclei and supernatant from one tube were used as such, and the nuclear fraction in the other tube was suspended back into its own supernatant.

Results

After incubation	Progesterone recovered (µg.)	(%)
Fresh homogenate	122	25
	134	27
Homogenate stored at 0°	146	30
	152	31
700g supernatant	196	40
	206	42
Nuclear fraction	451	92
	456	93
700g supernatant + nuclear fraction	172	35
	177	36
'Controls'	456	93
	461	94
Progesterone added	490	

Single Expt?

Conclusions

(i) Metabolism of progesterone does not occur with the nuclear fraction.

(ii) The lower activity of 700g supernatant as compared with homogenate is not simply an ageing effect; this loss in activity is only partially restored by the addition of the nuclear fraction.

C) Mitochondrial Fraction

1) Intact mitochondria

Results

After incubation	Progesterone recovered (µg.)	(%)
Homogenate	300	54
	311	56
Mitochondrial fraction	538	97
	544	98
5,000g supernatant	495	89
	495	89
'Controls'	527	95
	532	96
Progesterone added	555	

After incubation		
Homogenate	94	18
	101	22
Mitochondrial fraction	488	94
	488	94
5,000g supernatant	322	62
	337	65
'Controls'	488	94
	494	95
Progesterone added	520	

After incubation	Progesterone recovered (µg.)	(%)
Homogenate	56	12
	60	13
Mitochondrial fraction	438	94
	446	96
5,000g supernatant	321	69
	325	70
'Controls'	432	93
	442	95
Progesterone added	465	

Conclusions

(i) The mitochondrial fraction does not metabolize progesterone

(ii) Metabolism occurs with 5,000g supernatant, but to a lesser extent than with homogenate.

In the following experiment, after the homogenate had been prepared, 2 ml. were used immediately for incubations, a small portion was stored in the cold room until all other enzyme preparations were ready, and the remainder was separated into a nuclear fraction, which was discarded, and a 700g supernatant, a small portion of which was stored in the cold room. The remainder was divided into two halves and both were centrifuged to yield a mitochondrial fraction and a 5,000g supernatant. The mitochondrial fraction and 5,000g supernatant in one centrifuge tube were used as

such; the mitochondrial fraction in the other tube was suspended back into its own supernatant with the aid of a ball pestle.

Results

After incubation	Progesterone recovered (µg.)	(%)
Fresh homogenate	56	12
	61	13
Homogenate stored at 0°	195	42
	205	44
700g supernatant	233	50
	247	53
Mitochondrial fraction	436	94
	445	96
5,000g supernatant	321	69
	325	70
5,000g supernatant + mitochondrial fraction	288	62
	293	63
'Controls'	432	93
	441	95
Progesterone added	465	

Conclusions

(i) The lower activity of 700g supernatant as compared with homogenate is not simply an ageing effect.

(ii) 5000g supernatant is less active than 700g supernatant; this loss of activity is only partially restored by the addition of the mitochondrial fraction which, by itself, is inactive.

Nuclear and Mitochondrial fractions combined

The nuclear and mitochondrial fractions have been found to be inactive with regard to progesterone metabolism. The following experiment was carried out to determine if these two fractions had any activity when combined. An homogenate was prepared, 2 ml. were used immediately for incubations and a small portion was stored in the cold room until all other enzyme preparations were ready. The remainder was divided into three equal amounts, these being centrifuged to yield nuclear fractions and 700g supernatants. The nuclear fraction and 700g supernatant in one tube were used as such. The 700g supernatant from a second tube was centrifuged to yield a mitochondrial fraction and a 5,000g supernatant; the undisturbed third tube was recentrifuged at the same to give a 5,000g residue (nuclear + mitochondrial fractions) together with a 5,000g supernatant (direct).

Results

After incubation	Progesterone recovered (µg.)	(%)	
Fresh homogenate	193	39	
	198	40	
Homogenate stored at 0°	223	45	
	228	46	
Nuclear fraction	461	93	
	465	94	
700g supernatant	272	55	
	277	56	
Mitochondrial fraction	475	96	
	480	97	
5,000g supernatant	332	67	1 X
	337	68	
5,000g residue (nuclear + mitochondrial fractions)	366	74	
	372	75	
5,000g supernatant (direct)	303	61	1 Y
	312	63	
'Controls'	475	96	
	490	99	
Progesterone added	495		

Conclusions

(i) The 5,000g residue (nuclear + mitochondrial fractions) shows definite activity, though the nuclear and mitochondrial fractions show little or none.

(ii) The 5,000g supernatant prepared direct (thus combining the nuclear and mitochondrial fractions) exhibits higher activity than the 5,000g supernatant prepared in the usual manner. c/ X and Y. Is the diff significant?

2) Damaged mitochondria

A 25% homogenate was prepared and a portion of it was separated into a mitochondrial fraction and a 5,000g supernatant. The mitochondrial pellet was suspended in 0.25M-sucrose and divided into two equal portions. Both portions were recentrifuged and the resulting pellets were made up to the appropriate volume with, respectively, 0.25M-sucrose and distilled water. The former suspension was divided into three equal volumes; one portion was used as such, a second portion was placed in a deep freeze cabinet at -20° for eight days, after which it was thawed out by immersion in water at 37°, and the third portion was frozen and thawed three times by placing alternately in a solid CO_2-acetone mixture and water at 37°.

From the same liver, another 25% homogenate was prepared, but 0.12M-nicotinamide was used as the homogenizing and diluting medium instead of the usual 0.25M-sucrose containing 0.12M-nicotinamide.

Results

After incubation	Progesterone recovered (µg)	(%)
Homogenate prepared in 0.25M-sucrose, 0.12M-nicotinamide	0	0
	0	0
Homogenate prepared in 0.12M-nicotinamide	0	0
	5	1
'Controls'	495	98
	500	99
Mitochondrial fraction	474	94
	474	94
Mitochondrial fraction aged 8 days at -20°	458	91
	464	92
Mitochondrial fraction frozed and thawed 3 times	454	90
	464	92
Mitochondrial fraction lysed in water	454	90
	454	90
'Controls'	486	96
	495	98
Progesterone added	505	

Conclusions

(i) An homogenate prepared in the absence of sucrose still retains high activity.

(ii) The damaged mitochondrial fractions appear to show a little activity.

D) Microsomal Fraction

A 25% homogenate was prepared and a microsomal fraction obtained from it by centrifugation at 25,000g for 1 hr.

Results

After incubation	Progesterone recovered (µg.)	(%)
Microsomal fraction	463	85
	463	85
Microsomal fraction with 0.001M-DPN	426	78
	437	80
Microsomal fraction with 0.001M-DPNH	366	67
	382	70
'Controls'	517	95
	528	97
Progesterone added	545	

Conclusions

(i) The microsomal fraction metabolizes progesterone.

(ii) The metabolism is increased by added DPN.

(iii) The metabolism is still further increased by DPNH.

A 25% homogenate was prepared and from it a 5,000g supernatant was obtained; the major portion of this was separated into a microsomal fraction and a 150,000g supernatant. DPNH (0.001M) was added to the incubation flasks.

Results

After incubation	Progesterone recovered (µg.)	(%)
5,000g supernatant	0	0
	5	1
Microsomal fraction	127	26
	152	31
150,000g supernatant	471	97
	476	98
'Controls'	476	98
	485	100
Progesterone added	485	
After incubation		
5,000g supernatant	11	2
	16	3
Microsomal fraction	312	58
	318	59
150,000g supernatant	491	91
	491	91
'Controls'	496	92
	496	92
Progesterone added	540	

Conclusions

In the presence of DPNH:

(i) The microsomal fraction metabolizes progesterone;

(ii) 150,000g supernatant does not metabolize progesterone;

(iii) 5,000g supernatant, that is, one containing microsomes, metabolizes progesterone to a greater extent than the microsomal fraction alone.

Inhibition of the microsomal fraction

A 25% homogenate was prepared and a microsomal fraction obtained from it; 0.001M-DPNH was added to the incubation flasks.

Results

After incubation	Progesterone recovered (µg.)	(%)
Microsomal fraction	132 145	27 30
Microsomal fraction with 0.001M-p-chloromercuri-benzoate	446 456	92 94
Microsomal fraction with 0.001M-p-chloromercuri-benzoate and 0.001M-reduced glutathione	223 243	46 50
'Controls'	446 456	92 94
Progesterone added	485	

Conclusions

In the presence of DPNH:

(i) The microsomal fraction metabolizes progesterone;

(ii) this metabolism is inhibited by 0.001M-p-chloro-mercuribenzoate; the inhibition is partially reversed by 0.001M-reduced glutathione.

E) Supernatant Fraction

This will be considered under three headings, namely 5,000g supernatant, 25,000g supernatant and 150,000g supernatant.

1) 5,000g supernatant

As can be seen from sections C and D, supernatant is less active than homogenate but is more active than the microsomal fraction.

a) Effect of storage at 0°

A 25% homogenate was prepared and from it a 5,000g supernatant was obtained; this was divided into three equal portions, one being used immediately, the second portion being used after ½ hr. storage and the third portion after 1 hr. storage.

Results

After incubation	Progesterone recovered (µg.)	(%)
Fresh 5,000g supernatant	41	7
	46	8
5,000g supernatant stored ½ hr.	52	9
	52	9
5,000g supernatant stored 1 hr.	52	9
	52	9
'Controls'	546	95
	563	98
Progesterone added	575	

Conclusion

5,000g supernatant, unlike homogenate, is stable when stored at 0°.

b) Enzyme lability

In the following experiment, a 5,000g supernatant was divided into three equal portions; one was stored at 0° and the other two were centrifuged at 25,000g. The supernatant from one tube was used as such and the microsomal pellet in the other was stirred back into its own supernatant.

Results

After incubation	Progesterone recovered (µg.)	(%)
Homogenate	49	9
	60	11
'Control'	518	94
5,000g supernatant	193	35
	214	39
'Control'	518	94
25,000g supernatant	457	83
	463	84
25,000g supernatant with microsomal fraction	259	47
	270	49
Progesterone added	550	

Conclusions

(i) 5,000g supernatant is less active than homogenate but more active than 25,000g supernatant.

(ii) Addition of the microsomal fraction to 25,000g supernatant only partly restores its activity to that of the 5,000g supernatant.

c) Effect of DPN and DPNH

Results

After incubation	Progesterone recovered (µg.)	(%)
5,000g supernatant	333	62
	333	62
5,000g supernatant with 0.001M-DPN	264	49
	285	53
5,000g supernatant with 0.001M-DPNH	177	33
	177	33
'Controls'	515	96
	515	96
Progesterone added	538	

Conclusions

(i) Metabolism of progesterone by 5,000g supernatant is increased by addition of 0.001M-DPN.

(ii) Activity is increased further by 0.001M-DPNH.

d) Effect of dialysis

A 25% homogenate was prepared and from it a 5,000g supernatant was obtained. A few ml. were taken for immediate incubation and the remainder was divided into four equal portions; one portion was stored in the cold room at 0-1° for 4 hr. A second portion was dialysed for 2 hr. against 1 litre of a 0.12M solution of nicotinamide in 1.15% KCl solution; the third and fourth portions were dialysed against a similar solution for 4 hr. and 20 hr. respectively. The nicotinamide solution was changed after 2 hr. and after 4 hr.

Results

After incubation	Progesterone recovered (µg.)	(%)
5,000g supernatant	10	2
	31	6
After 2 hr. dialysis	189	37
	208	41
After 4 hr. dialysis	396	78
	407	80
After 4 hr. storage	36	7
	62	12
After 4 hr. dialysis, 0.001M-DPN added	46	9
	67	13
After 20 hr. dialysis	474	93
	494	97
'Controls'	480	94
	485	95
Progesterone added	510	

Conclusions

(i) Enzyme activity diminishes as a result of dialysis.

(ii) This diminution in activity is not caused by loss of nicotinamide or by ageing.

(iii) Activity is restored by addition of DPN.

e) Effect of replacing magnesium ions with manganous ions

The incubation mixture as given on p. 11 was used except that 0.04M-2-amino-2-(hydroxy-methyl)-propane-1:3-diol (Tris) buffer, pH 7.4 replaced phosphate buffer in order to avoid precipitation of manganous phosphate; 0.0002M-DPNH was added and, in the second pair of flasks, 0.005M-manganous sulphate replaced 0.005M-magnesium sulphate.

was dialysed enzyme used?

Results

After incubation	Progesterone recovered (µg.)	(%)
5,000g supernatant with Mg^{++}	165	34
	184	38
5,000g supernatant with Mn^{++}	44	9
	63	13
'Controls'	469	97
	474	98
Progesterone added	483	

Conclusion

Manganous ions provide a more effective co-factor than do magnesium ions.

f) Comparison of citrate with Versene (ethylene diamine tetra-acetic acid)

In order to test the chelating role ascribed to citrate by Wiswell and Samuels (1953), the effect of Versene was tried.

Results

After incubation	Progesterone recovered (µg.)	(%)
5,000g supernatant	421	81
	437	84
5,000g supernatant with 0.004M-Versene	442	85
	442	85
5,000g supernatant with 0.002M-citrate	302	58
	307	59
'Controls'	520	100
	520	100
Progesterone added	520	

Conclusions

(i) Versene does not affect metabolism appreciably.

(ii) Citrate increases metabolism.

2) 25,000g Supernatant

As can be seen from p. 42 , 25,000g supernatant is less active than 5,000g supernatant and if the microsomal fraction is incorporated back into it, its activity is only partially restored.

a) Effect of citrate and coenzymes

The incubation medium (p. 11) was present in the following flasks, except for one pair in which water replaced citrate.

Results

After incubation	Progesterone recovered (µg.)	(%)
5,000g supernatant	366	70
	387	74
5,000g supernatant with 0.001M-DPN	273	52
	278	53
5,000g supernatant with 0.001M-DPNH	105	20
	121	23
25,000g supernatant	446	85
	451	86
25,000g supernatant with 0.001M-DPN	435	83
	441	84
25,000g supernatant with 0.001M-DPNH	367	70
	383	73
25,000g supernatant with 0.0003M-TPNH	110	21
	110	21
25,000g supernatant with 0.0003M-TPNH but without added citrate	304	58
	315	60
'Controls'	509	97
	515	98
Progesterone added	525	

Conclusions

(i) 25,000g supernatant metabolizes progesterone to a small extent only.

(ii) This activity is increased greatly by TPNH, especially in the presence of citrate.

(iii) Activity is also increased to a lesser extent by DPN and DPNH.

b) Effect of increasing TPNH concentration

A 25,000g supernatant was prepared and half was stored in the cold room while the remainder was dialysed against 1.15% KCl solution for 22 hr. Incubation medium: 0.05M-KCl; 0.005M-$MgSO_4$; 0.01M-potassium phosphate pH 7.4, 0.04M-nicotinamide.

Results

After incubation	Progesterone recovered (µg.)	(%)
Stored 25,000g supernatant with 0.0003M-TPNH	356 356	62 62
Dialysed 25,000g supernatant with 0.0003M-TPNH	489 489	85 85
Dialysed 25,000g supernatant with 0.0006M-TPNH	425 442	74 77
Dialysed 25,000g supernatant with 0.0012M-TPNH	391 397	68 69
'Controls'	569 575	99 100
Progesterone added	575	

The ability of this dialysed supernatant to reduce TPN was measured in the spectrophotometer. The cells contained the same mixture of substances as in the incubation medium, together with 1 ml. of dialysed supernatant.

Results

	Log I_o/I at 340 mμ after 1 min.
No additions	0.245
0.0012M-TPN added	0.250
0.025M-isocitrate added	0.950

Conclusions

(i) Dialysis reduces the ability of 25,000g supernatant to metabolize progesterone.

(ii) Increasing the concentration of TPNH increases metabolism by dialysed supernatant.

(iii) Dialysed supernatant is unable to reduce TPN; addition of isocitrate enables reduction to proceed.

c) Reduction of TPN and oxidation of TPNH by supernatant

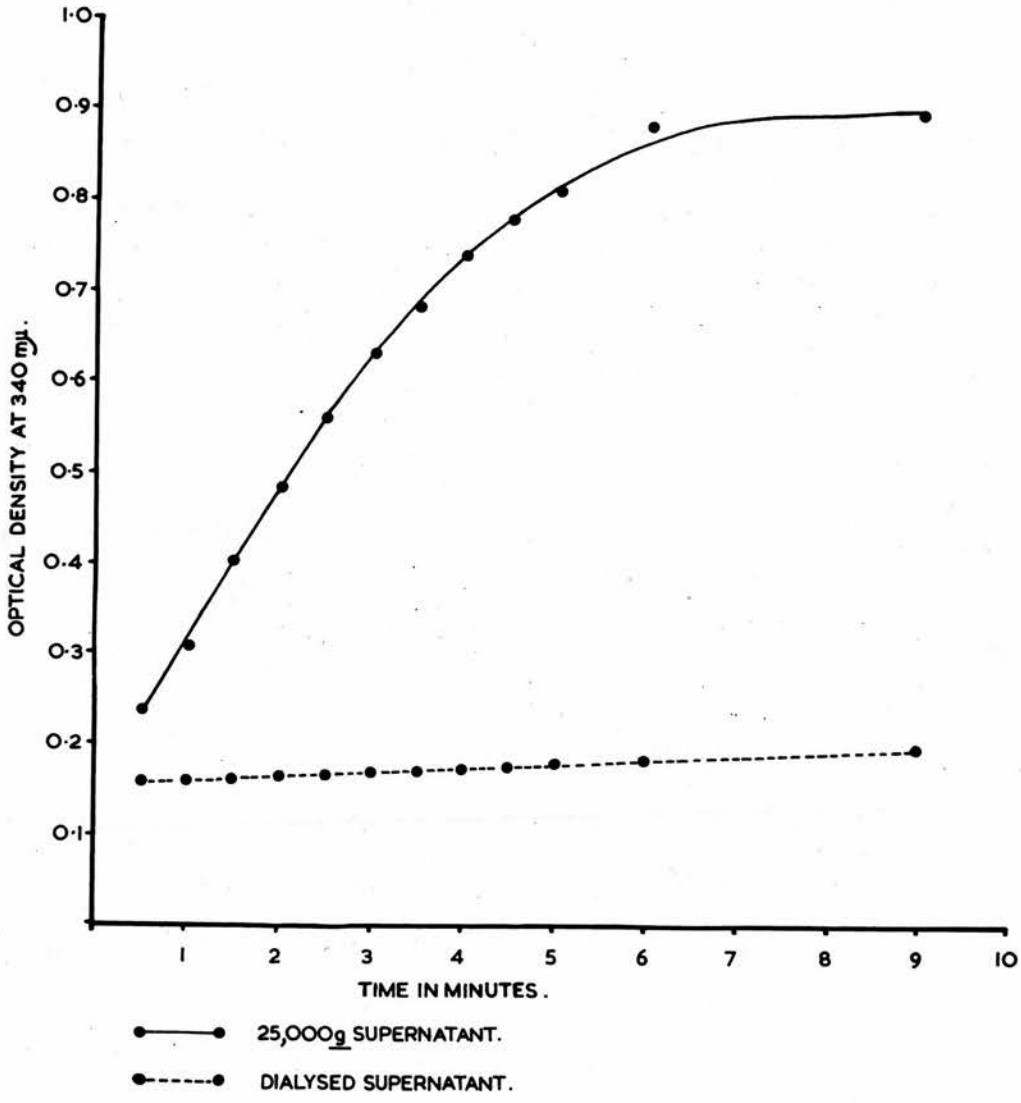
It was shown on p. 49 that dialysed supernatant was unable to reduce TPN unless isocitrate were added. Since Tomkins et al. (1954) had shown that TPNH was required for the reduction of steroids, it seemed possible that undialysed supernatant would be capable of reducing TPN; further, that dialysed supernatant would oxidize TPNH.

(i) Comparison of reduction of TPN by supernatant and by dialysed supernatant

An homogenate was prepared in 0.25M-sucrose and a 25,000g supernatant obtained from it. Half of this was stored in the cold room, while the remainder was dialysed against 1 l. of 1.15% KCl for 18 hr., fresh solution being provided after 4 hr.

The following volumes (in ml.) of fluids were pipetted into 1 cm. cells and these were placed in the cell-holder of the Unicam S.P. 500. The TPN was added last, the cell contents mixed, the time noted and optical densities at 340 mμ determined at intervals.

FIGURE 1 . THE REDUCTION OF TPN BY 25,000g SUPERNATANT AND BY DIALYSED SUPERNATANT.



	Cell 1 (blank)	Cell 2	Cell 3 (blank)	Cell 4
0.06M-$MgSO_4$	0.25	0.25	0.25	0.25
0.06M-KCl	0.25	0.25	0.25	0.25
0.12M-potassium phosphate pH 7.4	0.25	0.25	0.25	0.25
Supernatant	1.00	1.00	-	-
Dialysed supernatant	-	-	1.00	1.00
Water	1.25	0.25	1.25	0.25
0.0002M-TPN	-	1.00	-	1.00
	3.00	3.00	3.00	3.00

The results are shown in Fig. 1.

Conclusion

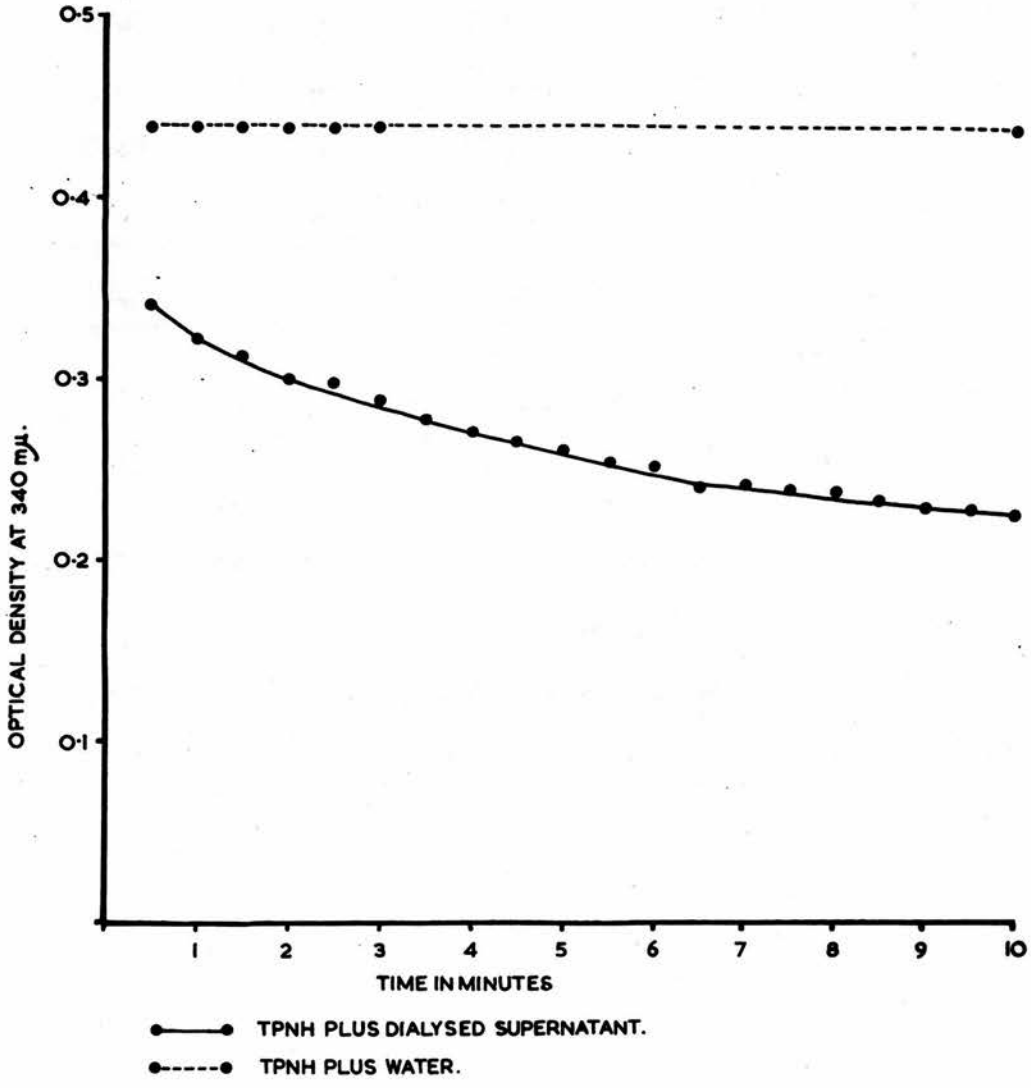
The rate of reduction of TPN is very much lower with dialysed supernatant than with supernatant.

(ii) Oxidation of TPNH by dialysed supernatant

An homogenate was prepared in 0.25M-sucrose and a 25,000g supernatant obtained from it. This was dialysed against 1 l. of 0.04M-Tris buffer for 22 hr., fresh solution being provided after 4 hr. Since it was possible that aerobic oxidation of TPNH might occur, a control was included in which water replaced the supernatant.

The following volumes (in ml.) of fluids were pipetted into 1 cm. cells and these were placed in the thermostat cell-holder (Grant, 1955) equilibrated

FIGURE 2. THE OXIDATION OF TPNH BY DIALYSED 25,000g SUPERNATANT.



at 37°. The TPNH was added last, the cell contents mixed, the time noted and optical densities at 340 mμ determined at intervals. This was carried out in two stages, since the thermostat cell-holder accommodates only two cells.

	Cell 1 (blank)	Cell 2	Cell 3 (blank)	Cell 4
0.48M-Tris buffer, pH 7.4	0.25	0.25	0.25	0.25
Dialysed supernatant	1.00	1.00	-	-
Water	1.75	0.75	2.75	1.75
0.0001M-TPNH	-	1.00	-	1.00
	3.00	3.00	3.00	3.00

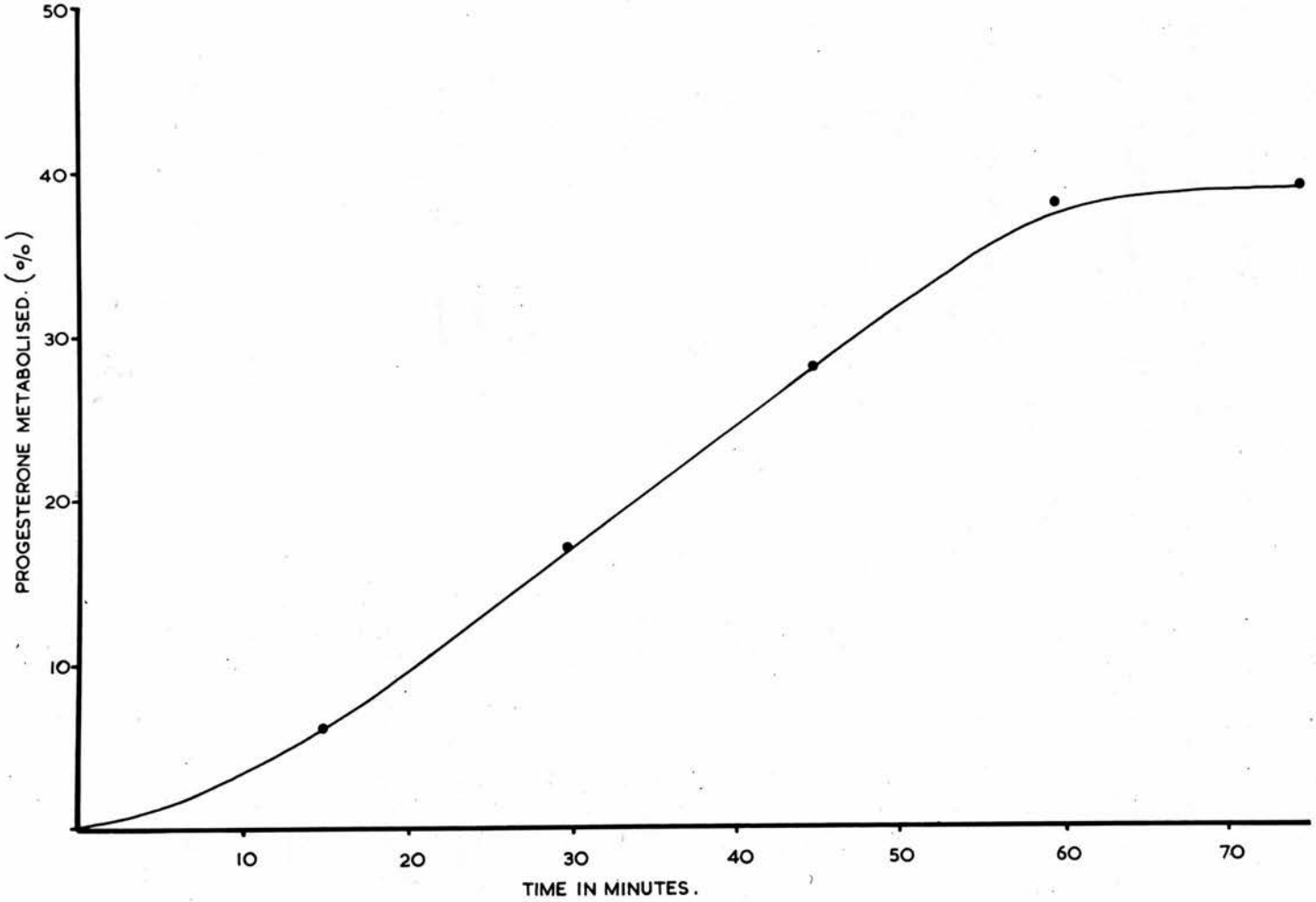
The results are shown in Fig. 2.

Conclusions

(i) TPNH is stable at 37° in aqueous solution at pH 7.4, in the presence of air.

(ii) Dialysed 25,000g supernatant oxidizes ~~oxides~~ TPNH rapidly at first and then more slowly.

FIGURE. 3. METABOLISM OF PROGESTERONE WITH TIME BY DIALYSED 25,000g SUPERNATANT.



d) Effect of different incubation times

An homogenate was prepared in sucrose and from it a 25,000g supernatant was obtained; this was dialysed against 1.15% KCl solution for 22 hr. Citrate was omitted and 0.001M-TPNH was added to all flasks.

Results

After incubation	Progesterone recovered (µg.)	(%)
Incubated 15 min.	419	88
	428	90
Incubated 30 min.	357	77
	366	79
Incubated 45 min.	297	64
	321	69
Incubated 60 min.	256	55
	274	59
Incubated 75 min.	251	54
	265	57
'Controls'	437	94
	446	96
Progesterone added	465	

Conclusion

Metabolism of progesterone is complete in about 60 min.

e) Precipitation of enzyme with ammonium sulphate

A 25,000g supernatant was prepared, and a portion of this stored in the cold room; to the remainder was added sufficient solid ammonium sulphate (Analar) to produce 50% saturation; solution was effected by gentle inversion of the tube and it was allowed to stand for 15 min. in the cold room. The precipitate was centrifuged off and solid ammonium sulphate was added to the supernatant to produce 70% saturation; the above procedure was repeated and the precipitate again centrifuged off. This precipitate was dissolved in water and made up to one tenth the volume of its own supernatant. A portion of this solution was dialysed against 0.025M-potassium phosphate buffer, pH 7.4, for 2 hr. This dialysed enzyme and the stored supernatant were then used for incubations; 0.0003M-TPNH was added to the flasks.

Results

After incubation	Progesterone recovered (µg.)	(%)
25,000g supernatant	257	46
	268	48
Dialysed enzyme	404	72
	409	73
'Controls'	538	96
	544	97
Progesterone added	560	

Conclusions

(i) Some of the enzyme activity in 25,000g supernatant responsible for the metabolism of progesterone is precipitated between 50 and 70% saturation with ammonium sulphate.

(ii) Less than one tenth of the original activity of the supernatant was recovered

f) Paper chromatography of metabolic products

Solvent system A (Bush, 1952) was used, except that n-hexane replaced light petroleum. Thus the mixture used was 200 ml. water, 800 ml. methanol and 1,000 ml. n-hexane. Chromatograms were run in glass tanks, similar to those used by Bush (1952), and chromatography was carried out in a temperature controlled room at about 20°. Sheets of Whatman No. 1 filter paper were used to saturate the atmosphere of the tank and Whatman No. 42 filter paper (46 cm. long, 15 cm. broad) was used for chromatograms; the general technique was that of Bush (1952). The dried sheets were dipped into 2.5N-KOH in freshly distilled ethanol, excess reagent removed by thorough blotting and the sheets then sprayed with 2% m-dinitrobenzene (purified after Callow, Callow and Emmens, 1938) in ethanol; colours were developed by placing in an oven at 65° (cf. Savard, 1953).

Single chromatograms of progesterone (10 µg.) and 5α-pregnane-3:20-dione (allopregnanedione) (20 µg.), a mixed chromatogram of the two and a chromatogram of allopregnanedione together with material from a blank incubation, were run for 5 hr. Progesterone could be detected as a fluorescent spot by means of ultraviolet light and gave the same R_F value in single and mixed chromatograms; allopregnanedione was detected after spraying and drying, as a blue spot, and gave the same

R_F value in all three chromatograms.

The partially purified solutions of metabolites remaining in the flasks after incubations with 5,000g and 25,000g supernatants had been carried out, were evaporated to dryness, dissolved in 0.5 ml. ethanol, and 0.01 ml. used for paper chromatography. A spot was detected with precisely the same R_F value as that given by authentic allopregnanedione, and giving the same colour; this was obtained with both the 5,000g and 25,000g supernatants.

Conclusions

(i) With this solvent system, progesterone has an R_F value of 0.72 and 5α-pregnane-3:20-dione has an R_F value of 0.89.

(ii) 5α-pregnane-3:20-dione has been identified tentatively as a metabolite of progesterone after incubation with 5,000g and 25,000g supernatants.

3) 150,000g supernatant

As can be seen from p. 39 , 150,000g supernatant was inactive in the presence of 0.001M-DPNH. On p. it was shown that Mg^{++} and Mn^{++} effected a marked difference in metabolism; to investigate whether this still held in the absence of citrate, cis-aconitate or isocitrate, the following experiment was carried out. An homogenate was prepared in 0.25M-sucrose and an 150,000g supernatant obtained from it; this was dialysed against 1 l. of 0.04M-Tris buffer, pH 7.4, for 22 hr., fresh solution being provided after 4 hr. The incubation medium consisted of 0.04M-Tris buffer, pH 7.4, and 0.04M-nicotinamide.

Results

After incubation	Progesterone recovered (µg.)	(%)
No additions	465	92
	475	94
With 0.0003M-TPNH	343	68
	368	73
With 0.0003M-TPNH and 0.005M-$MgSO_4$	353	70
	358	71
With 0.0003M-TPNH and 0.005M-$MnSO_4$	364	72
	368	73
'Controls'	475	94
	475	94
Progesterone added	505	

Conclusions

(i) Dialysed 150,000g supernatant is inactive in the absence of added coenzyme.

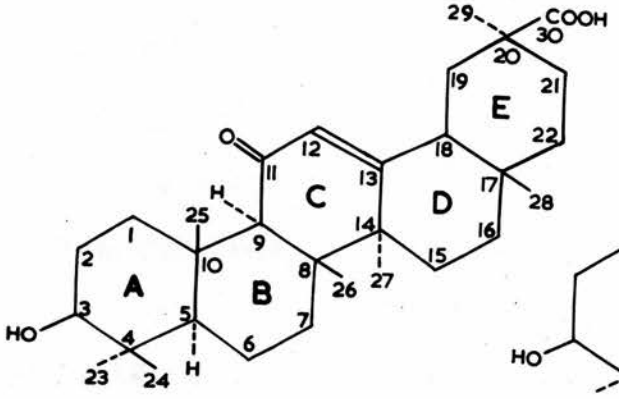
(ii) It is active in the presence of 0.0003M-TPNH.

(iii) The absence or presence of Mg^{++} or Mn^{++} with dialysed 150,000g supernatant in the presence of 0.0003M-TPNH does not affect metabolism.

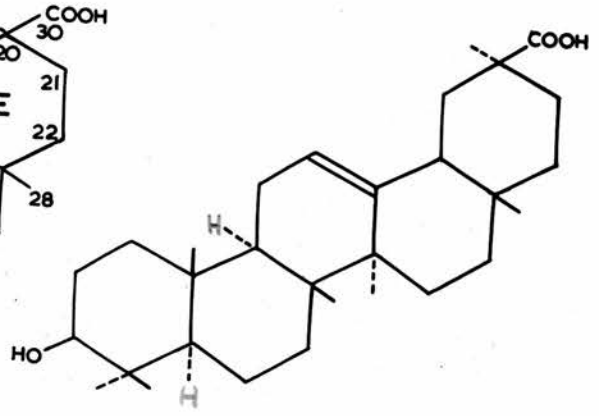
SECTION II

INHIBITION BY GLYCYRRHETINIC ACID AND OTHER SUBSTANCES OF THE DESTRUCTION OF PROGESTERONE AND 11-DEOXYCORTICOSTERONE BY RAT LIVER PREPARATIONS

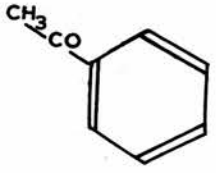
FIGURE .4.



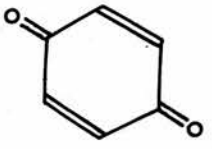
GLYCYRRHETINIC ACID.



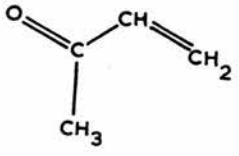
11-DESOXOGLYCYRRHETINIC ACID.



ACETOPHENONE.



p-QUINONE.



METHYL VINYL KETONE.

A) Introduction

Glycyrrhizinic acid, which is responsible for the sweet taste of liquorice, is the diglucopyruronide of glycyrrhetinic acid (GA) (Lythgoe and Trippett, 1950) which has a triterpenoid structure (Ruzicka, Jeger and Ingold, 1943) as in Fig. 4 (Beaton and Spring, 1955). Hydrogen atoms in β positions at ring junctions have not been drawn in; it can be seen that as far as the junctions of Rings A, B, C and D are concerned, the stereochemistry is the same as in allopregnane.

Clinical interest in liquorice was first aroused when Revers (1946, 1948, 1951) reported that it relieved the pain of gastric ulcers, but if given in too great a dose caused oedema; it has been shown since that the former effect is due to the glycyrrhizinic acid-free part of liquorice extract (Revers, 1956). Molhuysen, Gerbrandy, De Vries, De Jong, Lenstra, Turner and Borst (1950) showed that liquorice and its active principle, glycyrrhizinic acid, have a DOC-like action in that they promote sodium and water retention and potassium loss, in normal humans, but when tried on one severe case of Addison's disease, did not have these effects.

Groen, Pelser, Willebrands and Kamminga (1951) described two Addisonian patients who responded to treatment with deoxycorticosterone acetate and who could be maintained in correct electrolyte balance

with liquorice or glycyrrhizinic acid alone, contrary to the findings of Molhuysen *et al.* (1950).

Pelser, Willebrands, Frenkel, Van der Heide and Groen (1953) showed that GA, when administered orally to Addisonians, was more effective than its diglucuronide, glycyrrhizinic acid. Pelser, Willebrands and Groen (1953) found that during prolonged treatment of Addisonians with liquorice or GA, the maintenance dose could be reduced after some time; in fact, if it were not reduced, oedema set in. Groen, Pelser, Willebrands, Veer and Van der Kerk (1953) investigated the effect of derivatives and analogues of GA on the electrolyte balance of Addisonians. They concluded that the keto group and/or the carboxyl group (either as such, or as a ketol-acetate group) were essential for activity.

Dr Grant suggested to the present author that the effect of GA might be due to its inhibiting the metabolism of DOC *in vivo*. It was decided, therefore, to investigate the action of GA on steroid metabolism *in vitro*.

A recent review of the inhibition of enzymes by drugs has been given by Quastel (1956).

B) Materials and Methods

1) Materials

a) GA was a gift from Stafford Allen & Sons, Ltd., London; it had a melting point of 278-282°. As it was found to be insoluble in water but soluble in propylene glycol, it was added to the incubation vessels from an Agla micrometer syringe as a solution in propylene glycol. An equivalent quantity of this solvent was added to blank and 'control' vessels and to vessels containing smaller quantities of GA, in such amounts that the final volume of propylene glycol was the same in all vessels.

b) 11-desoxoglycyrrhetinic acid (DGA) was kindly provided by Professor F.S. Spring, Royal Technical College, Glasgow; it had a melting point of 320-323°. As it was found to be insoluble in water and not sufficiently soluble in propylene glycol, it was added to incubation flasks as a solution in ethanol.

c) Methyl vinyl ketone was the product of Light & Co., Ltd., and was not purified further.

d) p-quinone was purified by sublimation and had a melting point of 113-114°.

e) Commercial acetophenone was purified by distillation, the fraction boiling at 198-199° being collected.

f) Glucose-6-phosphate, barium salt, B.D.H. Laboratory Reagent, was converted to the soluble sodium salt for enzyme experiments.

g) Glucose-6-phosphate dehydrogenase was the commercial product of the Sigma Chemical Co., St. Louis, Missouri. For experimental use, it was dissolved in fresh 0.1% (w/v) $NaHCO_3$ solution.

2) Recovery of Steroids in Presence of GA

It was necessary to ascertain whether or not GA was carried through the analytical procedure for steroids and appeared in the same final fraction as progesterone or DOC, particularly in view of the fact that a solution of GA in ethanol gives a peak at 248 mμ. (van Katwijk and Huis in't Veld, 1955). For this purpose, duplicate blank incubations, duplicate progesterone 'control' incubations and single DOC 'control' incubations were carried out in the absence and in the presence of 0.001M-GA.

Results

Recovery of Steroids in presence of GA

Progesterone added (μg.)	GA concn. (M)	Apparent Progesterone recovered (μg.)
0 (blank)	-	15 23
0 (blank)	0.001	20 23

		Progesterone recovered (μg.)	(%)
395	-	364 368	92 93
395	0.001	368 384	93 97

DOC added (μg.)		DOC recovered (μg.)	(%)
610	-	573	94
610	0.001	591	97

Conclusion

Blank values and recoveries of progesterone and DOC are unaffected by the presence of 0.001M-GA.

C) The Inhibition by GA of the Metabolism of Progesterone by Rat Liver Homogenate

1) In the absence of added DPNH

Results

After Incubation	GA concn. (M)	Progesterone recovered (µg.)	(%)
Homogenate	-	307	64
		322	67
"	0.0002	403	84
		418	87
"	0.0005	467	97
		470	98
"	0.0010	475	99
		475	99
"	0.0020	475	99
		480	100
'Control'		480	100
Progesterone added		480	

Conclusions

(i) GA inhibits the metabolism of progesterone by rat liver homogenate.

(ii) At these enzyme, coenzyme and substrate concentrations, complete inhibition occurs with a GA concentration of 0.0005M.

2) In the presence of added 0.001M-DPNH

Results

After Incubation	GA concn. (M)	Progesterone recovered (µg.)	(%)
Homogenate	-	55	11
		70	14
"	0.0002	290	58
		295	59
"	0.0005	395	79
		420	84
"	0.0010	435	87
		445	89
'Controls'		490	98
		500	100
Progesterone added		500	

Conclusions

(i) Inhibition occurs in the presence of added 0.001M-DPNH.

(ii) Increasing the inhibitor concentration causes increased inhibition.

D) The Inhibition by GA of the Metabolism of DOC by Rat Liver Homogenate

0.001M-DPNH was added to all incubations.

Results

After Incubation	GA concn. (M)	DOC recovered (µg.)	DOC recovered (%)
Homogenate	-	104	17
		116	19
"	0.0002	360	59
		384	63
"	0.0005	458	75
		464	76
'Controls'		573	94
		591	97
DOC added		610	

Conclusions

(i) 11-deoxycorticosterone is destroyed by rat liver homogenate in the presence of 0.001M-DPNH.

(ii) GA inhibits this destruction and higher concentrations cause increased inhibition.

(iii) Inhibition by GA of the reduction of Ring A is possibly a general phenomenon for steroids with an αβ-unsaturated ketonic group.

E) The Inhibition by GA of the Metabolism of Progesterone by Rat Liver Supernatant

A 25,000g supernatant was prepared in 0.25M-sucrose and dialysed against 1.15% KCl solution for 4 hr., the KCl solution being changed after 2 hr.; this procedure removes citrate, cis-aconitate and isocitrate almost completely; 0.04M-nicotinamide and 0.0003M-TPNH were added to all incubation vessels. Citrate was omitted in all cases and 0.0001M-GA was added to alternate incubations; 'controls' were included for each of the four progesterone concentrations.

Results

Progesterone concn. (M)	% recovery of progesterone	
	GA absent	GA present
0.0002	54	93
'Control' "	94	
0.0003	70	98
'Control' "	97	
0.0004	84	96
'Control' "	101	
0.0005	86	101
'Control' "	94	

Conclusions

(i) The metabolism of progesterone by 25,000g supernatant in the presence of TPNH and in the absence of added citrate is inhibited by 0.0001M-GA.

(ii) Under these conditions, complete inhibition occurs at all the substrate concentrations tested.

F) The Inhibition by GA and Other Substances of the Metabolism of Progesterone by Rat Liver Homogenate

The fact that GA contains an αβ-unsaturated ketone group, in common with progesterone and DOC was suggestive that this might be the active moiety of the inhibitor. To investigate this possibility, the effect on progesterone metabolism of various substances was tried.

Ethanolic solutions (0.1 ml.) of DGA, methyl vinyl ketone, p-quinone and acetophenone were used; the acetone was added as an aqueous solution. The final concentrations of these substances and of GA were 0.0005M. The effect of 0.1 ml. of ethanol alone was investigated, and progesterone recovery 'controls' were carried out in the presence of 0.0005M-DGA, methyl vinyl ketone and p-quinone.

Results

After incubation	Progesterone recovered (µg.)	(%)
No additions	321	63
	331	65
With GA	475	93
	486	95
With acetone	347	68
	352	69
With 0.1 ml. ethanol	377	74
	377	74
With acetophenone	383	75
	387	76
With DGA	398	78
	403	79
With methyl vinyl ketone	500	98
	510	100
With p-quinone	505	99
	510	100
'Controls'		
No additions	480	94
With DGA	490	96
With methyl vinyl ketone	495	97
With p-quinone	486	95
Progesterone added	510	

Conclusions

(i) Progesterone recovery is unaffected by the presence of 0.0005M-DGA, methyl vinyl ketone or p-quinone.

(ii) Acetone causes a slight inhibition of metabolism.

(iii) Ethanol inhibits metabolism to some extent.

(iv) Acetophenone causes a further slight inhibition.

(v) DGA produces a small degree of inhibition.

(vi) Methyl vinyl ketone and p-quinone effect complete inhibition.

FIGURE .5. TPN REDUCTION BY CONCURRENT OXIDATION OF GLUCOSE-6-PHOSPHATE IN THE ABSENCE AND IN THE PRESENCE OF GA.

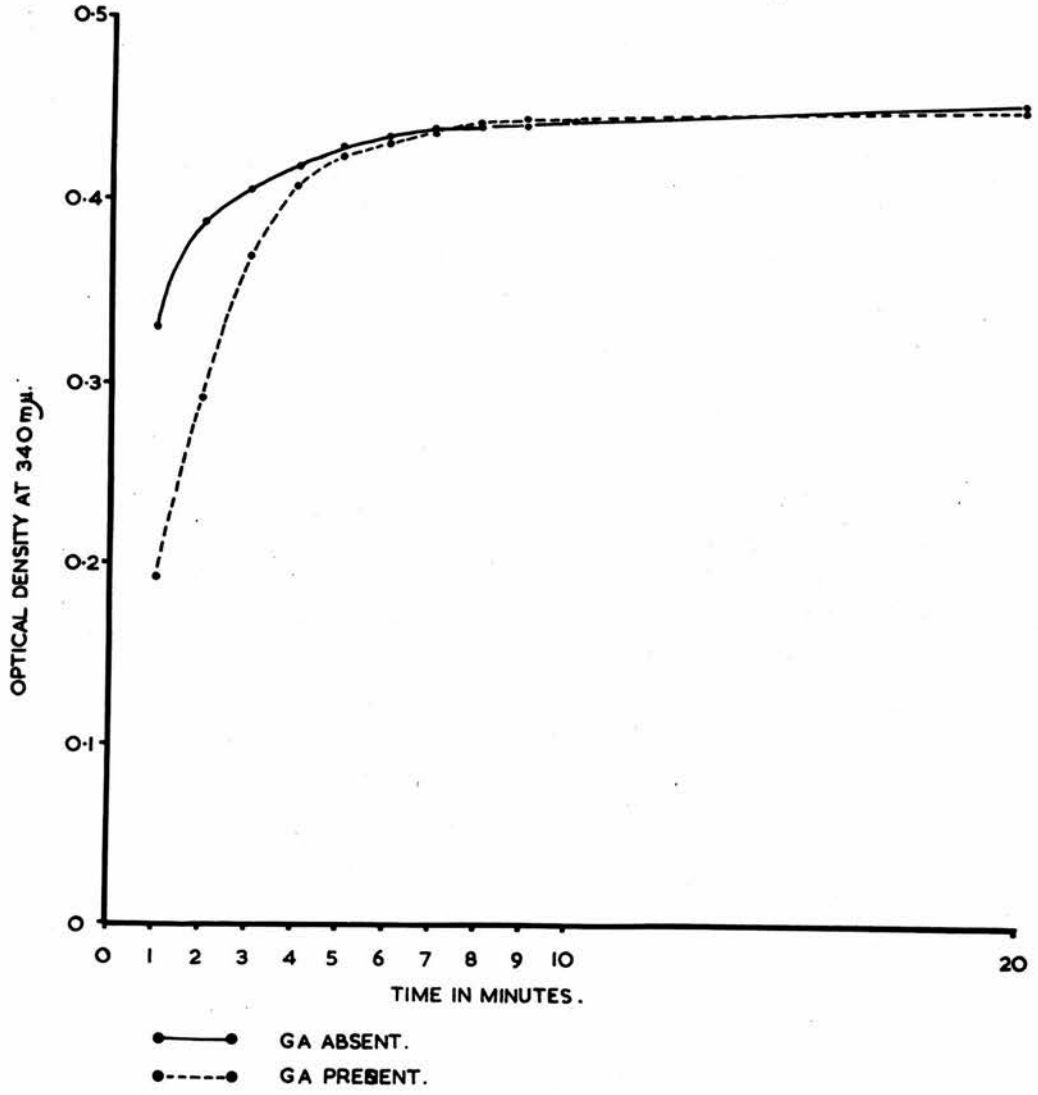
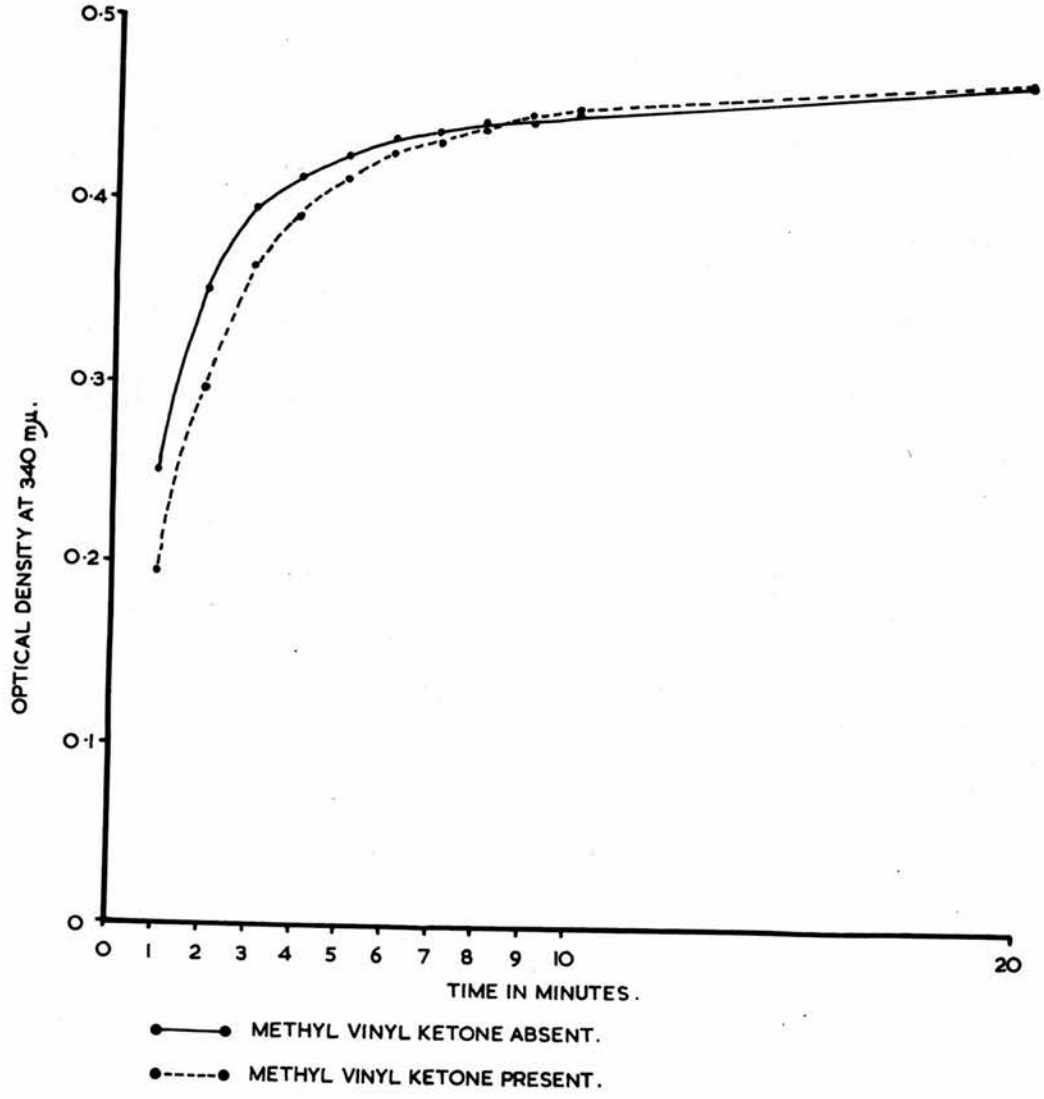


FIGURE. 6. TPN REDUCTION BY CONCURRENT OXIDATION OF GLUCOSE-6-PHOSPHATE IN THE ABSENCE AND IN THE PRESENCE OF METHYL VINYL KETONE.



G) The Effects of GA and Methyl Vinyl Ketone on the Oxidation of Glucose-6-phosphate

It was necessary to determine whether GA and methyl vinyl ketone were general or specific enzyme inhibitors. The concurrent reduction of TPN, as glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase, was chosen as a means of measuring any inhibition of this enzyme. The following volumes (in ml.) of solutions were pipetted into 1 cm. cells and placed in the cell carrier of the Unicam S.P. 500. The glucose-6-phosphate was added last, the cell contents mixed, the time noted, and the optical densities at 340 mμ determined at minute intervals for 10 min. and finally at 20 min.

	Cell 1 (blank)	Cell 2	Cell 3 (blank)	Cell 4
0.0001M-TPN	1.0	1.0	1.0	1.0
Fresh $NaHCO_3$ (1% w/v)	0.5	0.5	0.5	0.5
Glucose-6-phosphate dehydrogenase (Conc? 20 mg. crystals per 3.5 ml.)	0.1	0.1	0.1	0.1
Water	1.4	1.0	1.35	0.95
0.03M-GA	-	-	0.05	0.05
0.045M-glucose-6-phosphate	-	0.4	-	0.4
	3.0	3.0	3.0	3.0

In the case of methyl vinyl ketone, 0.1 ml. of a 0.015M solution of it replaced 0.05 ml. of GA solution and the volume of water was adjusted accordingly. The results are shown in Figures 5 and 6.

Conclusion

GA and methyl vinyl ketone both cause an initial lag in the rate of reduction of TPN, but the final values attained are the same.

H) The Effects of Methyl Vinyl Ketone and p-quinone on the 11β-hydroxylation of DOC

These experiments were carried out as a further test of specificity for these inhibitors. The following incubation medium was used: 0.0002M-TPN; 0.1 ml. glucose-6-phosphate dehydrogenase; 0.006M-glucose-6-phosphate; 0.04M-Tris buffer, pH 7.4. The hydroxylating enzyme solution was prepared as described by Grant and Brownie (1955) and 2 ml. were added to each incubation vessel, the final volume being 5 ml.; 0.1 ml. of ethanolic solutions of methyl vinyl ketone and p-quinone were used, giving molarities of 0.0005.

Results

After incubation	DOC recovered (µg.)	(%)
No additions	13	3
With 0.1 ml. ethanol	36	8
With methyl vinyl ketone	49	11
With p-quinone	107	24
'Controls'	436 445	98 100
DOC added	445	

Conclusions

(i) Ethanol causes some inhibition of 11β-hydroxylation.

(ii) Methyl vinyl ketone increases this inhibition slightly.

(iii) p-quinone causes marked inhibition.

SECTION III

THE METABOLISM OF PROGESTERONE IN THE RAT *IN VIVO*

A) Introduction

Taylor (1954) isolated allopregnanedione and allopregnanolone after incubation of progesterone with female rat liver *in vitro*. In order to attempt to correlate these results with *in vivo* metabolism, it was decided to investigate rat urine and faeces for the possible presence of progesterone metabolites after intraperitoneal injection of progesterone. In particular, use was made of a specific, sensitive and accurate method for the determination of pregnanediol in urine, devised by Klopper, Michie and Brown (1955), and of a modification of this method for the determination of allopregnanediol.

B) Materials and Methods

1) Materials

Materials used for pregnanediol determinations were prepared by the methods given by Klopper *et al.* (1955). Girard's Reagent T and 2:4-dinitrophenylhydrazine (B.D.H. Laboratory Reagents) were used without further purification; glacial acetic acid (Analar) was redistilled before use. Alumina, for chromatographic analysis (Savory & Moore, Ltd., London; 100/150 mesh), was activated by heating at 100° *in vacuo* for 2 hr.; its activity was tested by the method of Brockmann and Schodder (1941). Deactivated alumina was

prepared by the method given by Klopper et al. (1955).

Six months old male rats of the Wistar strain were used for most of the work in order to avoid, as far as possible, endogenous progesterone metabolism.

2) Collection of urine and faeces

Twenty four rats were weighed individually and divided evenly into 4 groups, so that each group weighed 1665 g., the average weight of each rat being 278 g.

The 4 groups of 6 rats were placed in metabolism cages which had wire mesh floors and stood over zinc funnels with perforated zinc tops to collect the faeces. The urine was collected in beakers containing 0.5 ml. toluene to act as preservative. In order to avoid contamination of excreta with rat cake, a diet was prepared from powdered rat cake, gelatin and water, which gave a firm jelly (Wilson, 1954). It was given to the rats ad lib. in screw capped jars, with circular apertures 3.7 cm. diameter in the lids; drinking water was also provided. The urine and faeces were collected daily and the latter stored for 3 days in an evacuated desiccator over P_2O_5 (Riegel et al., 1950) in the cold room. They were powdered finely and kept in screw capped jars in a deep freeze cabinet at -20°. The urine was worked up daily and the residues stored in evacuated desiccators at room temperature.

3) Injection of progesterone

Twelve rats as controls (control rats) were each injected intraperitoneally with 0.1 ml. sterilized olive oil at 9 a.m. each day and the remaining 12 rats ('metabolic' rats) were injected in the same way with a sterilized solution of 2 mg. of progesterone in 0.1 ml. olive oil.

4) Determination of pregnanediol

This was carried out by the method of Klopper et al.(1955) with the following modifications introduced by Dr Grant. In place of sintered glass discs in chromatogram tubes, plugs of solvent-washed cotton wool were used; three extractions with toluene were performed instead of two and emulsions which formed during these extractions were broken routinely by slow filtration on a Buchner funnel.

5) Determination of allopregnanediol

A search for allopregnanediol was carried out separately after the pregnanediol determinations were concluded. The fact that no pregnanediol had been detected greatly simplified the task of adapting the method of Klopper et al.(1955) for the measurement of allopregnanediol. The elution pattern for this steroid given in Table 1 (Klopper et al.,1955) showed that it began to be eluted from the first alumina column after

the passage of about 15 ml. of 0.8% ethanol in benzene. During the determinations of pregnanediol in rat urine, it had been observed routinely that a fairly narrow band of brown pigment began to travel down the column when this solvent was added and it was found that 12 ml. was the minimum volume required to elute this pigment completely. Hence this fraction was discarded and a further 30 ml., as indicated by the same Table, was found to be sufficient to recover the allopregnanediol. When this eluate was evaporated to dryness, white allopregnanediol could be seen together with a little gum when recovery experiments were being carried out at the 50 µg. level. After acetylation and rechromatography, the petroleum ether and benzene fractions were examined for allopregnanediol and this was found to be in the latter fraction.

Five ml. of Analar concentrated sulphuric acid were used for the formation of chromogens which were read at 400, 430 and 460 mµ. on a Unicam S.P. 600 spectrophotometer, using 1 cm. glass cells, as in the pregnanediol method. Allopregnanediol has a similar spectrum in sulphuric acid to that of pregnanediol with a peak at 430 mµ. (Bernstein and Lenhard, 1955).

C) Examination of urine for pregnanediol

1) After injection of olive oil

The 24 rats were allowed to become accustomed to the diet and to the metabolism cages; olive oil

injections were then begun. The urine from 6 rats, collected for 24 hr. was strained through glass wool and made up to 100 ml. with distilled water. Forty ml. of toluene were added and 10 ml. of concentrated hydrochloric acid were used for hydrolysis; 20 ml. of toluene were used for the second and third extractions, and the oxidation was carried out by shaking for 15 min. with 25 ml. of freshly prepared 4% $KMnO_4$ in N-NaOH. For recovery experiments, pregnanediol in ethanolic solution was added to the urine before addition of toluene, prior to hydrolysis.

Klopper et al.(1955) obtained 72-104% recovery after addition of 25 µg. of pregnanediol to 1/20th of a 24 hr. specimen of human male urine. In order to extend the sensitivity of the method, if possible, recovery experiments were carried out after addition of 50 µg., 25 µg. and 12 µg. of pregnanediol to a urine specimen.

On the first day, urine from groups 1 and 2 was used for blank determinations and urine from groups 3 and 4 for recoveries; on the second day, urine from groups 3 and 4 was used for blanks and urine from groups 1 and 2 for recoveries; and so on, alternately. The volume of Analar sulphuric acid used for colorimetry was reduced from 10 ml. to 5 ml. to increase the sensitivity of the method.

Table 1

Recovery of pregnanediol added to pooled 24 hr. specimens of urine from groups of 6 rats

Blank experiments		Recovery experiments			
Group	Apparent pregnanediol in urine from rats receiving olive oil (μg.)	Group	Pregnanediol added to urines before hydrolysis (μg.)	Pregnanediol recovered (corrected for blanks) (μg.)	(%)
4	4	1	50	43	86
3	6	2	50	47	94
2	7	3	25	22	88
1	8	4	25	25	100
4	4	1	25	20	80
3	8	2	25	21	84
2	4	3	12	9.5	79
1	5	4	12	11.6	97
4	7	1	12	10.0	83
3	8	2	12	10.5	88
2	7	3	12	9.2	77
1	7	4	12	10.2	85
4	5	1	12	12.2	102
3	6	2	12	12.2	102

Conclusions

(i) The results shown in Table 1 indicate that recoveries are satisfactory at these levels.

(ii) The apparent pregnanediol values in the blanks are fairly consistent and low.

2) After injection of progesterone solution

Exactly the same method of analysis was used as after injection of olive oil. Groups 1 and 2 of the rats were used as controls (injected with olive oil on three successive days) and groups 3 and 4 were used as 'metabolic' rats (injected with progesterone solution on the same days). Optical densities were read at 400 mμ. and 460 mμ. as well as at 430 mμ. in order to detect any selective absorption at 430 mμ.

Table 2

Measurement of apparent pregnanediol in rat urine

Day	Rat Group No.	Optical Densities 400 mμ.	430mμ.	460 mμ.
1	1	0.042	0.041	0.018
	2	0.040	0.039	0.015
	3	0.040	0.032	0.012
	4	0.041	0.032	0.012
2	1	0.038	0.030	0.020
	2	0.034	0.031	0.018
	3	0.032	0.028	0.017
	4	0.033	0.026	0.019
3	1	0.032	0.023	0.012
	2	0.034	0.022	0.009
	3	0.032	0.021	0.007
	4	0.035	0.021	0.010
4	1	0.028	0.025	0.020
	2	0.030	0.027	0.017
	3	0.029	0.026	0.016
	4	0.030	0.024	0.018

Conclusions

(i) The optical densities are not selective at 430 mμ.

(ii) The values for control rats (range 4 to 7 μg. apparent pregnanediol) and 'metabolic' rats (range 4 to 6 μg. apparent pregnanediol) are indistinguishable.

(iii) The values do nct show any change after cessation of injections.

(iv) 5β-pregnane-3α:20α-diol has not been detected in the urine of the male rat after intraperitoneal injection of three daily doses of 2 mg. progesterone in olive oil.

Table 3

Recovery of allopregnanediol added to pooled 24 hr. specimens of urine from groups of 6 rats

Blank experiments	Recovery experiments		
Apparent allopregnanediol in rat urine (μg.)	Allopregnanediol added to urine before hydrolysis (μg.)	Allopregnanediol recovered (corrected for blanks) (μg.)	(%)
6	25	19	76
7	25	20	80
6	25	21	84
6	25	24	96
9	12	9	75
9	12	9	75
10	12	9	75
10	12	10	83

D) Examination of Urine for allopregnanediol

Twelve male rats were used for this part of the work. They were divided as equally as possible as regards weight, into two groups of 6 and were allowed to become accustomed to the diet and the metabolism cages before urine collections were begun.

1) From normal rats

The urine from 6 rats, collected for 24 hr., was worked up in the same way as for pregnanediol determinations (p. 74) except for the chromatographic modifications described on p. 75 . For recovery experiments, allopregnanediol in ethanolic solution was added to the urine before addition of toluene.

On the first day, urine from group 1 was used for a blank determination and urine from group 2 for a recovery; on the second, urine from group 2 was used for a blank and urine from group 1 for a recovery; and so on, alternately.

Conclusions

(i) The results shown in Table 3 indicate that recoveries are satisfactory at these levels.

(ii) The apparent allopregnanediol values in the blanks are fairly consistent and low.

2) After injection of progesterone solution

Group 1 of the rats was used as a control group (injected with olive oil on three successive days) and group 2 was used as a 'metabolic' group (injected with progesterone solution on the same three days). Optical densities were read at 400 mμ. and 460 mμ. as well as at 430 mμ., in order to detect any selective absorption at 430 mμ.

Table 4

Measurement of apparent allopregnanediol in rat urine

		Optical Densities		
Day	Rat Group No.	400 mμ.	430 mμ.	460 mμ.
1	1	0.056	0.050	0.034
	2	0.034	0.028	0.020
2	1	0.064	0.057	0.046
	2	0.054	0.047	0.044
3	1	0.061	0.052	0.040
	2	0.041	0.033	0.023
4	1	0.064	0.058	0.044
	2	0.066	0.060	0.049

Conclusions

(i) The optical densities are not selective at 430 mμ.

(ii) The values for control rats (range 9 to 11 μg. apparent allopregnanediol) and 'metabolic' rats (range 5 to 11 μg. apparent allopregnanediol) are indistinguishable.

(iii) The values do not show any change after cessation of injections.

(iv) 5α-pregnane-3α:20α-diol has not been detected in the urine of the male rat after intraperitoneal injection of three daily doses of 2 mg. progesterone in olive oil.

E) Examination of Pregnancy Urine for Pregnanediol and allopregnanediol

Although these steroids had not been found in male rat urine after injection of progesterone, it was still possible that metabolism of endogenous progesterone in the female might yield these derivatives. Accordingly, two groups of 6 female rats were placed in metabolism cages and fed on the same diet as that given to the male rats. Urines were collected from both groups and determinations of apparent pregnanediol and apparent allopregnanediol carried out. One group was then kept as a control group, and the other group, together with two additional female rats were then mated. Of these eight rats, four became pregnant; as soon as pregnancy was detected, these four, together with two female rats, were placed in a metabolism cage (Group 2) and the six control rats in another metabolism cage (Group 1). Urines were collected for four days and examined for pregnanediol and allopregnanediol. Towards the end of the first and second 24 hr. periods, parturition occurred with two of the rats. These were removed from the cage and replaced with female rats.

Table 5

Measurement of apparent pregnanediol in female rat urine

Date	Rat Group No.	Optical densities 400 mμ.	430 mμ.	460 mμ.
6.7.56	1) non-pregnant	0.032	0.025	0.019
	2) non-pregnant	0.040	0.033	0.027
31.7.56	1 Control	0.048	0.043	0.032
	2 Pregnant	0.045	0.041	0.030
1.8.56	1 Control	0.047	0.042	0.031
	2 Pregnant	0.038	0.031	0.024

Measurement of apparent allopregnanediol in female rat urine

Date	Rat Group No.	Optical densities 400 mμ.	430 mμ.	460 mμ.
7.7.56	1) non-pregnant	0.064	0.057	0.046
	2) non-pregnant	0.060	0.052	0.037
2.8.56	1 Control	0.061	0.052	0.040
	2 Pregnant	0.051	0.043	0.032
3.8.56	1 Control	0.056	0.049	0.035
	2 Pregnant	0.054	0.047	0.039

Conclusions

(i) The apparent pregnanediol and allopregnanediol values in the non-pregnancy urines are fairly consistent and low.

(ii) The optical densities are not selective at 430 mμ.

(iii) The values for non-pregnancy and pregnancy urines are indistinguishable.

(iv) Neither 5β-pregnane-3α:20α-diolnor 5α-pregnane-3:20α-diol has been detected in rat pregnancy urine.

F) Examination of Urine for 5β-pregnane-3α:17α:20α-triol (pregnanetriol)

Cox and Marrian (1953) showed that this steroid occurs in male human urine. Klopper et al.(1955) found that, on boiling with dilute acids, pregnanetriol formed a number of compounds one of which, when acetylated, behaved chromatographically like pregnanediol diacetate and gave a chromogen with sulphuric acid. After oxidation with potassium permanganate, which does not affect pregnanediol, the oxidation products no longer contaminated the pregnanediol fraction.

Since, after injection of progesterone, no pregnanediol was detected in rat urine, it was decided to investigate the presence of any pregnanetriol by comparison of the chromogens produced after applying the normal pregnanediol method and after application of the same method, with the permanganate oxidation step omitted.

Two samples of urine from two groups of 6 control rats, which had been injected with olive oil, and two samples from two groups of 6 'metabolic' rats, which had been injected with progesterone, were examined for the pregnanediol fraction, omitting the permanganate oxidation.

Results

Rat Group No.		Optical Density at 430 mμ.
1)	Control groups	0.043
2)		0.046
3)	'Metabolic' groups	0.043
4)		0.046

Conclusions

(i) The values obtained are not appreciably higher than those obtained after including the permanganate oxidation (see p. 78).

(ii) The values from the control and the 'metabolic' groups are indistinguishable.

(iii) It appears unlikely that 5β-pregnane-3α:17α:20α-triol is excreted in the urine of the male rat after intraperitoneal injection of 2 mg. progesterone in olive oil.

G) Examination of Urine for Other Steroids

Urine samples, collected daily, from two groups of control rats, were combined, filtered and made up tp 200 ml. with distilled water. This diluted urine was hydrolysed with hydrochloric acid and toluene extracts made as in the pregnanediol method, except that three 40 ml. portions of toluene were used for extraction. The combined extracts were washed twice with 25 ml. 2.5% (w/v) NaCl in 0.1N-NaOH and twice with 25 ml. water; the toluene was distilled off *in vacuo* at 45° on a water bath. Similarly, extracts were made from urine samples from two groups of 'metabolic' rats.

Injections were continued for 8 successive days and collection of urine and faeces for a further two days; the combined urine extracts were dried *in vacuo* over P_2O_5.

In view of the fact that negative results were obtained, the Girard separation is given in some detail.

The extracts were dissolved in 10 ml. ethanol to which was added 0.2 g. Girard's Reagent T (Girard and Sandulesco, 1936) and 1 ml. glacial acetic acid. They were refluxed on a boiling water bath for 1 hr. and then were poured quickly into 55 ml. water, 30 g. crushed ice, a sufficient amount of NaOH to neutralize 9/10 of the acetic acid and 15 g. NaCl. This aqueous

material was extracted once with an equal volume and three times with half volumes of ether and the combined ether extracts washed once with water (this washing was added to the aqueous phase), twice with 5% $NaHCO_3$ and twice with water. The ether was evaporated and the residues (non-ketonic fractions) dried over P_2O_5. The aqueous phases were acidified with 10 ml. 18N-H_2SO_4, 100 ml. ether were added and the whole allowed to stand for $1\frac{1}{2}$ hr. at room temperature. The ether layers were separated and the aqueous phases extracted further with three 50 ml. portions of ether. The combined ether extracts were washed twice with $NaHCO_3$ solution and twice with water and the residues (ketonic fractions) dried over P_2O_5.

This Girard separation was repeated on the non-ketonic fractions and the resulting new ketonic fractions were combined with the original ones. The weights of the fractions so obtained are shown in Table 6.

Table 6

Weights of fractions from 'metabolic' and control urines on treatment with Girard's Reagent T

	Toluene extracts (alkali washed)	
'Metabolic'	65 mg.	
Control	59 mg.	
	Treated with Girard's Reagent T	
	Ketonic fraction	Non-ketonic fraction
'Metabolic'	15 mg.	18 mg.
Control	15 mg.	20 mg.

1) Examination of ketonic fractions with 2:4-dinitrophenylhydrazine

The 2:4-dinitrophenylhydrazones of allopregnanedione, pregnanedione and allopregnanolone were prepared by the method of Reich, Sanfilippo and Crane (1952). These steroids were chosen as possible ketonic progesterone metabolites in the rat, the first and third having been isolated by Taylor (1954) *in vitro*.

Alumina, activity III, was used to purify the products. Melting points and absorption maxima of these compounds were determined and are shown in Table 7.

Table 7

Physical properties of some 2:4-dinitrophenylhydrazones

2:4-dinitrophenylhydrazone of	Absorption Max. in $CHCl_3$ (mμ.)	Melting point
5α-pregnane-3:20-dione (bis DNP[1])	371	208-212° [2] and 228-232°
5β-pregnane-3:20-dione (bis DNP[1])	368-369 [3]	251-253° [4] and 265-268° [5]
3α-hydroxy-5α-pregnan-20-one	368-373	219-225°

[1] DNP = 2:4-dinitrophenylhydrazone.

[2] Lieberman, Dobriner, Hill, Fieser and Rhoads (1948) reported 207-211°.

[3] Reich, Crane and Sanfilippo (1953) reported 368-369.

[4] Lieberman *et al.* (1948) reported 251-253°.

[5] Reich *et al.* (1953) reported 265-267.5°.

A 12 mg. portion of the ketonic fraction of control urine was treated with 3.6 ml. of freshly prepared 2:4-dinitrophenylhydrazine reagent (Reich et al., 1952) and the purified products taken up in 50% benzene in hexane. This was placed on a 3 g. alumina column and 20 ml. fractions were collected, using the following solvent elution pattern:

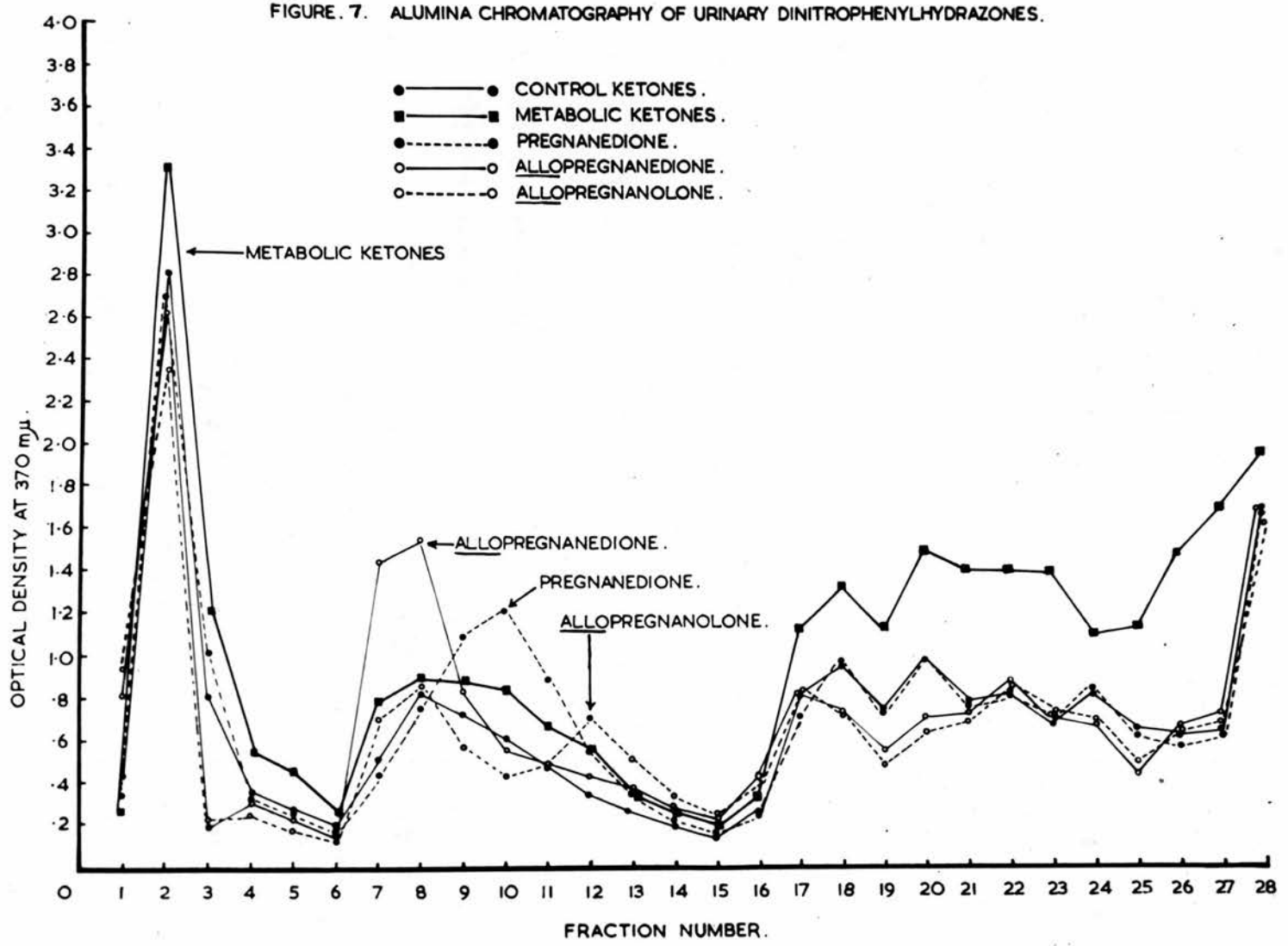
60 ml. 50% benzene in hexane
60 ml. 75% " " "
140 ml. benzene
40 ml. 2% chloroform in benzene
60 ml. 5% " " "
40 ml. 10% " " "
20 ml. 20% " " "
20 ml. 25% " " "
20 ml. 30% " " "
20 ml. 40% " " "
20 ml. 50% " " "
20 ml. 75% " " "
20 ml. chloroform
20 ml. methanol

This system was evolved in preliminary experiments to separate the above 2:4-dinitrophenylhydrazones (Table 7) in the presence of the ketonic fraction from toluene extracts made from normal male rat urine. The fractions were evaporated to dryness, desiccated in vacuo over $CaCl_2$, dissolved in 10 ml. chloroform and

their optical densities measured at 370 mμ. on a Unicam S.P. 600 spectrophotometer in 1 cm. cells. This wavelength was chosen since the 2:4-dinitrophenylhydrazones in Table 7 all have a peak in the region of 370 mμ.

This procedure was repeated with three other 12 mg. ketonic portions from extract of normal male rat urine. To these portions, before treatment with 2:4-dinitrophenylhydrazine, 100 μg. of allopregnanedione, 100 μg. of pregnanedione and 100 μg. of allopregnanolone, respectively, were added. The results of these four chromatograms are shown in Fig. 7 (opposite p. 90). It can be seen that the allopregnanedione was eluted in fractions 7 and 8. This was confirmed by combining these, dissolving in 50% benzene in hexane, placing on a 3 g. alumina column and eluting with 20 ml. portions of benzene in hexane of 50%, 60%, 70%, 80% and 90% benzene; 60 ml. of 95% benzene in hexane and 60 ml. of benzene. Absorption maxima were determined on the resulting fractions; the material eluted with 95% benzene in hexane was found to have a maximum at 371 mμ. and to have a double melt at 205-209° and 225-230°. Similar procedures were carried out with fractions 9, 10 and 11 (pregnanedione), material from which gave a maximum at 369 mμ. and a double melt at 252-255° and 260-270°. The same methods applied to fractions 12 and 13 (allopregnanolone) gave a maximum at 373 mμ. and a melting point range of 211-219° (cf. Table 7).

FIGURE. 7. ALUMINA CHROMATOGRAPHY OF URINARY DINITROPHENYLHYDRAZONES.



A 12 mg. portion of 'metabolic' ketonic fraction was treated in an identical manner to the control ketonic fraction. The resulting chromatogram is shown in Fig. 7. To investigate the nature of the substance in fraction 8 of the 'metabolic' ketonic material, it was rechromatographed in the same way as above. The resulting absorption maximum was 373 mμ, but the melting point was over the range 60-90° with a small residue melting at 90-130°.

Conclusions

(i) The 'metabolic' ketonic fraction from male rat urine contains less than 100 μg. of the three steroids investigated.

(ii) By the procedures carried out, it would appear that the male rat, after intraperitoneal injection of 8 daily doses of 2 mg. of progesterone in olive oil excretes less than 0.1% of 5α-pregnane-3:20-dione, 5β-pregnane-3:20-dione and 3α-hydroxy-5α-pregnan-20-one into the urine.

2) Examination of non-ketonic fractions

These were subjected to a digitonin separation (Butler and Marrian, 1938). The control fraction was transferred to a centrifuge tube with 5 ml. 90% v/v aqueous ethanol, heated to boiling, cooled, and the insoluble matter centrifuged off. To the supernatant solution, heated to boiling, was added a hot solution

of 200 mg. digitonin in 20 ml. 90% v/v aqueous ethanol and the mixture left for 40 hr. with occasional stirring and scratching with a glass rod. No insoluble digitonide was formed.

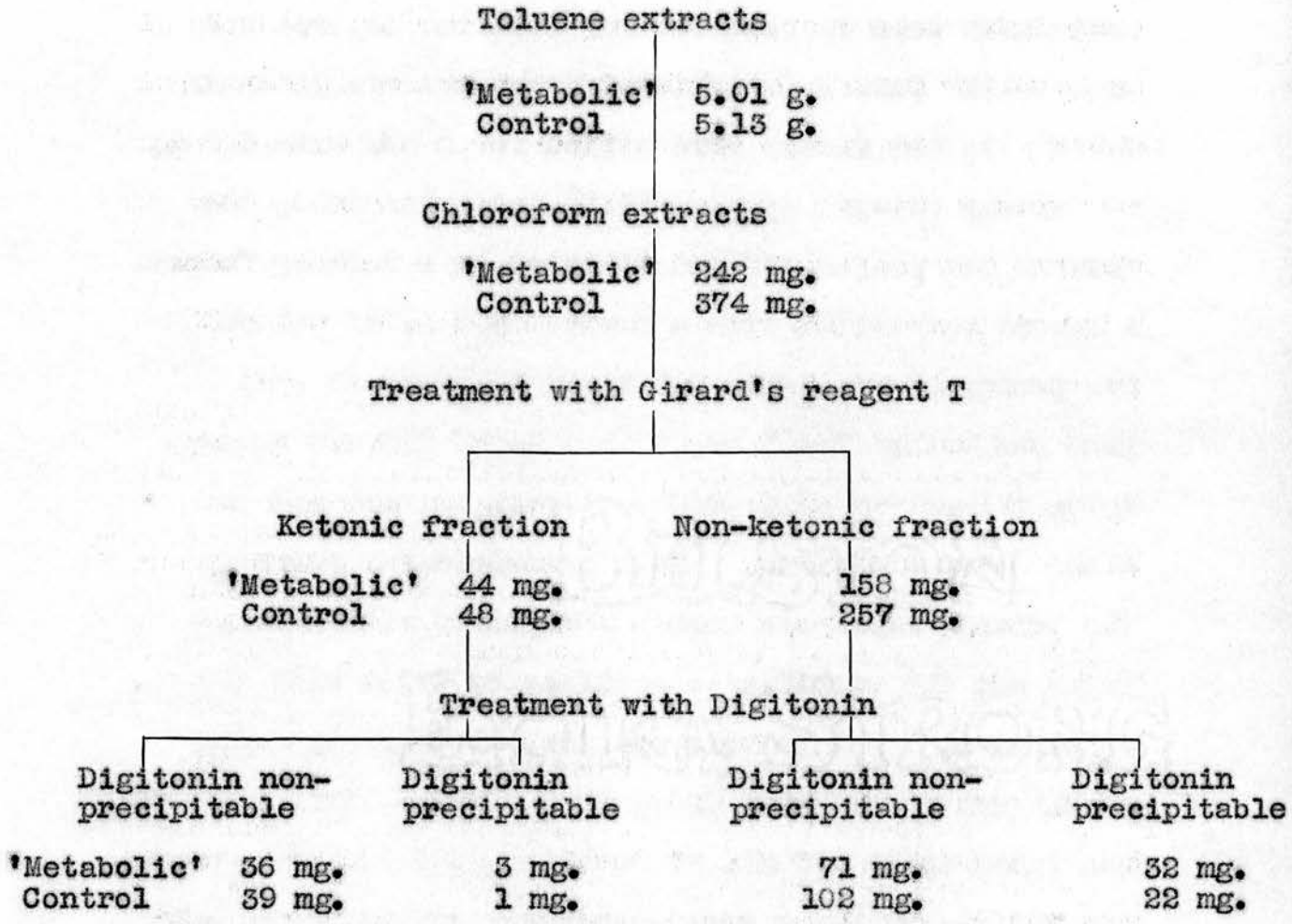
The solution was evaporated to dryness *in vacuo*, transferred to a 100 ml. centrifuge tube with 2 ml. methanol and a large volume of ether added. The digitonin precipitate was centrifuged, the supernatant solution removed, the residue washed with 90 ml. ether, centrifuged and the washing added to the first supernatant solution. The ether was evaporated off to leave the digitonin non-precipitable non-ketonic fraction.

The 'metabolic' non-ketonic fraction was treated in the same way; it also yielded no insoluble digitonides.

The two fractions were subjected to chromatography, using a gradient elution starting with 2% v/v ethanol in benzene (Taylor, 1955). Each was taken up in 5 ml. 2% v/v ethanol in benzene, put onto a 0.5 g. alumina (activity II) column and eluted by an ethanol-benzene mixture ranging from 2% v/v to 5% v/v ethanol by a linear gradient over 200 ml., followed by ethanol and chloroform. The main fraction, in each case, was eluted by 2% ethanol in benzene; unsuccessful attempts were made to crystallize this from acetone, ethanol and methanol.

Table 8

Weights of fractions from control and 'metabolic' faeces.



H) Examination of Faeces

After drying, the control and 'metabolic' faeces weighed respectively 512 g. and 520 g. The following procedures were carried out for both the control and 'metabolic' faeces (weights of fractions are given in Table 8); the faeces were boiled for 1 hr. with 2.5 l. of toluene (Riegel et al., 1950) and, when cold, the toluene was poured off and filtered on a Buchner funnel. A second extraction with a further 2.5 l. of toluene was performed, yielding 4.5 l. of filtrate in all; this was boiled for 1 hr. with 4 l. of 20% w/v aqueous K_2CO_3 (Riegel et al., 1950), allowed to cool and the lower layer discarded. This procedure was repeated; the toluene layer was washed four times with 1 l. of water and the washings back-extracted twice with 400 ml. of toluene. The toluene was distilled off in vacuo at 72° and the residue dried over $CaCl_2$. This extract was taken up in 600 ml. of methanol, 200 ml. of n-hexane and 200 ml. of water added, shaken vigorously and left for 2 hr. at room temperature (Taylor, 1954). The methanol layer was run off and the hexane back-extracted twice with 200 ml. of 75% v/v aqueous methanol. The combined methanol extracts were washed with 100 ml. of hexane.

The bulk of the methanol was removed by distillation in vacuo, 400 ml. of water added and the mixture extracted twice with 1 l. of chloroform. The combined chloroform extracts were washed with 200ml. of N-NaOH

and then with water until the washings were neutral. The extracts were dried over Na_2SO_4 and the chloroform removed by distillation *in vacuo*.

Separations were carried out using Girard's reagent T, as with the urine. The Girard separation was repeated on the non-ketonic fraction.

The control ketonic fraction was then subjected to a digitonin separation (Butler *et al.*, 1938), as described under the control urine non-ketonic fraction (p. 90) except that 300 mg. of digitonin were used. The white crystalline digitonide was centrifuged, the supernatant solution removed, the residue washed twice with small volumes of 90% v/v aqueous ethanol, once with ether and the washings combined with the supernatant solution. The residue was dried *in vacuo* over $CaCl_2$.

The dried digitonide residue was dissolved in 0.5 ml. of pyridine and 10 ml. of ether added. The digitonin was centrifuged off, the supernatant solution transferred to a separating funnel, the residue washed with ether, centrifuged and the washing added to the separating funnel. The ethereal solution was washed with 2N-H_2SO_4, twice with water and the ether evaporated to leave the digitonin precipitable control ketonic fraction.

The 'metabolic' ketonic fraction was subjected to a digitonin separation in the same way, as also were

both non-ketonic fractions, using 720 mg. of digitonin in 60 ml. of solvent for the latter two.

Investigation of fractions

1) Digitonin non-precipitable ketonic

The 'metabolic' fraction was dissolved in 10 ml. benzene, put on a 2 g. alumina (activity II) column and eluted as follows:

2 x 10 ml. benzene
10 ml. 1% ether in benzene
10 ml. 2% " " "
10 ml. 5% " " "
10 ml. 10% " " "
10 ml. 20% " " "
10 ml. 50% " " "
10 ml. ether
10 ml. chloroform
10 ml. methanol

The main fraction, 27 mg., was obtained on elution with benzene; various solvents, including acetone and ethanol, were used in attempts to obtain crystalline material, but without success.

The control fraction was treated in the same way and the main portion was eluted at the same point as in the 'metabolic' chromatogram, the weight being 28 mg.

2)Digitonin precipitable ketonic

Attempts were made to crystallize the control fraction (1 mg.) and the 'metabolic' fraction (3 mg.) from acetone, but contamination with yellow gum prevented this.

3) Digitonin non-precipitable non-ketonic

These were yellow gums; they were dissolved in 5 ml. 2% v/v ethanol in benzene, put on 3 g. alumina (activity II) columns and eluted by the same procedure as used for the corresponding urine fraction (p. 91). With the 'metabolic' material, two main fractions were obtained; the first (18 mg.) was eluted with 2% ethanol in benzene and after attempted crystallization from acetone and methanol, the semi-crystalline material obtained sublimed partially at 270° and melted at 310-320°. The other main fraction (21 mg.) was eluted with chloroform; it was still gummy and could not be purified further. The control material gave similar results, the corresponding fractions both weighing 20 mg.

4) Digitonin precipitable non-ketonic

These fractions were both white, but not crystalline. In the case of the control fraction, crystallization from ethanol yielded white crystals with a m.p. 101-104°. Mixed with authentic coprostan-3β-ol (m.p. 101-103°) the m.p. was 101-104°. Application

of the Liebermann-Burchard reaction (Kerr and Bauld, 1953) to residues obtained from the mother liquors gave a faint green colour.

Crystallization from ethanol and other solvents was attempted with the metabolic fraction, but was not achieved.

Conclusion

No known metabolites of progesterone have been found in the urine or faeces of the male rat after intraperitoneal injection of a solution of progesterone in olive oil.

SECTION IV

THE DESTRUCTION OF PROGESTERONE BY RAT LIVER HOMOGENATE

A) Introduction

Reaven (1955) has shown that steroids with an αβ-unsaturated ketonic group in Ring A lose their absorption maximum at 240 mμ. after incubation with human liver. As a possible research project, the author considered isolating the metabolic products resulting from the incubation of progesterone with human liver homogenate. With this end in view, experiments were done to find optimum conditions; rat liver was used for this purpose, since it was readily available and the assumption had to be made that these conditions would hold for human liver.

Since DPN is fairly expensive, it was decided to reduce the final incubation volume from 3 ml. to 2 ml. The following substances were present unless otherwise stated: 0.1M-nicotinamide; 0.04M-Tris buffer, pH 7.4; 0.01M-sodium citrate and 0.005M-$MnSO_4$. Homogenization was carried out in 0.25M-sucrose, or in 0.25M-sucrose containing nicotinamide to give a final molarity of 0.1 and, except where otherwise stated, a 25% homogenate was used. 1 ml. of homogenate was added to each flask.

B) The Effect of Storage Temperature

Since it was not known when human liver would be available, optimum conditions of storage had to be

ascertained; these would be required while incubation flasks were prepared prior to homogenization of the liver.

Equal portions of the same rat liver were:

1) homogenized immediately and incubations begun at once;
2) stored at 0° for 24 hr. and then homogenized;
3) stored at -20° for 24 hr. and then homogenized.

Results

After incubation	Progesterone recovered (µg.)	(%)
Fresh liver	25	5
	50	10
Liver stored at 0°	30	6
	60	12
Liver stored at -20°	144	29
	149	30
'Controls'	490	99
	495	100
Progesterone added	495	

Conclusions

(i) Storage of whole liver at 0° for 24 hr. does not affect its capacity to destroy progesterone.

(ii) Storage at -20° reduces the capacity to destroy progesterone.

C) Comparison of Homogenization in Sucrose and in Sucrose-Nicotinamide

1) In absence of added DPNH

Where homogenization was done in sucrose, nicotinamide was added to the incubation flasks to give the same final concentration (0.1M).

Results

After incubation	Progesterone recovered (µg.)	(%)
Prepared in sucrose	816	81
	858	84
Prepared in sucrose-nicotinamide	721	71
	745	73
'Controls'	1000	98
	1020	100
Progesterone added	1020	

2) In presence of added 0.0002M-DPNH

Results

After incubation	Progesterone recovered (µg.)	(%)
Prepared in sucrose	230	22
	272	26
Prepared in sucrose-nicotinamide	105	10
	115	11
'Controls'	986	94
	1018	97
Progesterone added	1050	

Conclusion

Nicotinamide aids the destruction of progesterone more effectively when present in the homogenizing medium than when added later, whether in the absence or presence of added DPNH.

D) The Effect of Varying Nicotinamide Concentration

1) Lower nicotinamide concentrations

Results

	Nicotinamide (M)	Progesterone recovered (µg.)	(%)
After incubation	-	770	75
		781	76
	0.02	412	40
		453	44
	0.06	206	20
		267	26
'Controls'	0.06	987	96
		1019	99
Progesterone added		1030	

2) Higher nicotinamide concentrations

a) 25% homogenate

Results

	Nicotinamide (M)	Progesterone recovered (µg.)	(%)
After incubation	0.06	157	15
		168	16
	0.10	95	9
		126	12
	0.20	179	17
		232	22
'Controls'	0.20	998	96
		1019	98
Progesterone added		1040	

b) 10% homogenate

Results

	Nicotinamide (M)	Progesterone recovered (µg.)	(%)
After incubation	0.06	113	11
		124	12
	0.10	226	22
		226	22
	0.20	565	55
		649	63
'Controls'	0.20	989	96
		1019	99
Progesterone added		1030	

Conclusion

With 25% homogenate, the optimum nicotinamide concentration is about 0.1M and with 10% homogenate it is somewhat lower than this.

E) The Effect of Varying Citrate Concentration

Results

	Citrate (M)	Progesterone recovered (µg.)	(%)
After incubation	0.01	105	10
		115	11
	0.02	105	10
		115	11
'Controls'	0.02	986	94
		1018	97
Progesterone added		1050	

Conclusion

Previous experiments (with supernatant) had shown that 0.01M-citrate was more effective than 0.002M. This experiment shows that with 25% homogenate, the effectiveness of citrate as an 'activator' of progesterone metabolism is not increased above a concentration of 0.01M.

F) The Mode of Addition of DPNH

Instead of the usual addition of coenzyme in bulk at the beginning of the incubation, the effect was tried here of adding coenzyme in six equal portions, every 10 min., starting with the commencement of the incubation.

Results

After incubation	Progesterone recovered (µg.)	(%)
DPNH added in bulk	724	71
	745	73
DPNH added in portions	640	63
	672	66
'Controls'	1000	98
	1020	100
Progesterone added	1020	

Conclusion

Metabolism is greater when DPNH is added in portions than when added in bulk before incubation.

G) The Effect of 0.01M-trans-aconitate

In order to investigate the role of citrate, the effect of trans-aconitate, which inhibits the conversion of cis-aconitate to isocitrate, was tried. A 10% homogenate was used with 0.06M-nicotinamide; 0.01M-citrate was added only where shown.

Results

After incubation	Progesterone recovered (µg.)	(%)
No additions	407	80
	412	81
With trans-aconitate	438	86
	449	88
With citrate	321	63
	332	65
With citrate and trans-aconitate	361	71
	377	74
'Control'	479	94
Progesterone added	510	

Conclusion

The destruction of progesterone is inhibited by trans-aconitate both in the absence and in the presence of added citrate.

H) Modification of the Klopper Method for the Determination of Pregnanediol and allopregnanediol in the presence of Liver Tissue

In view of the failure to detect pregnanediol or allopregnanediol in rat urine after the injection of progesterone (Section III) it was decided to search for these metabolites after the incubation of progesterone with rat liver homogenate. It was possible that these steroids, if produced, might be conjugated with glucuronic acid and so acid hydrolysis would be necessary to release the free steroids for extraction. It was felt that adaptation of the method of Klopper et al. (1955) for the determination of pregnanediol in urine (Section III) would yield the most promising results, and this was effected as described below.

Incubations were carried out in Quickfit & Quartz test tubes with B19 sockets, using 1 ml. of a 25% homogenate prepared in 0.25M-sucrose containing 0.3M-nicotinamide, in a final volume of 5 ml. The incubation medium consisted of 0.04M-Tris buffer, pH 7.4; 0.01M-sodium citrate and 0.005M-$MnSO_4$. 'Control' experiments were carried out by incubating rat liver homogenate with the medium described above and then adding 12 µg. of pregnanediol or allopregnanediol in ethanolic solution. Since Grant and Marrian (1950) showed that rat liver destroys pregnanediol, the mixture was boiled before addition of steroid. Five ml. of toluene were added, the tube attached to a reflux condenser and the mixture boiled again over a wire

gauze using a micro-Bunsen. For experiments other than 'controls' the preliminary boiling was omitted. The tube was detached from the condenser and 0.3 ml. of concentrated hydrochloric acid added. The tube was re-attached and the contents boiled for 10 min. By careful adjustment of the flame, it was found that 'bumping' was not excessive, but use of a wider and longer tube, such as the Quickfit & Quartz with B24 socket, would facilitate this operation. At the end of the hydrolysis the condenser was rinsed down with a few ml. of toluene. The tube was detached, its walls rinsed down with toluene, and cooled rapidly. The tube was centrifuged at 2,400 r.p.m. for 5 min. to clear the emulsion. The toluene layer was sucked over into another test tube of the same type, using the adaptor and U-tipped suction tube B described by Taylor (1954). The aqueous phase was re-extracted twice with 5 ml. volumes of toluene, shaking vigorously both times and again clearing emulsions by centrifugation. To the combined toluene extracts were added 10 ml. of a fresh 4% solution of $KMnO_4$ in N-NaOH, and the tube shaken by hand for 5 min. The mixture was transferred to a separating funnel, the aqueous phase discarded and the toluene layer washed with four or five volumes of 50 ml. water to remove permanganate. Direct transference of the toluene layers from the original incubation tube to a 100 ml. separating funnel fitted

Table 9

Recovery of pregnanediol and allopregnanediol added to rat liver homogenate

Blank Experiments		Recovery Experiments	
Apparent pregnanediol in rat liver homogenate	Pregnanediol added to rat liver homogenate before hydrolysis	Pregnanediol recovered (corrected for blank)	
(µg.)	(µg.)	(µg.)	(%)
9	12	9	75
10	12	10	83
	12 (As NaPG)	6	50
	12 (" ")	7	58
Using water in place of rat liver homogenate			
3	12 (As NaPG)	10	83
6	12 (" ")	12	100
Apparent allopregnanediol in rat liver homogenate	Allopregnanediol added to rat liver homogenate before hydrolysis	Allopregnanediol recovered (corrected for blank)	
(µg.)	(µg.)	(µg.)	(%)
9	12	8	67
9	12	8	67

with a B19 socket would eliminate the use of a second test tube. The toluene solution was filtered into a round-bottomed flask and distilled to a bulk of about 10 ml., thereby removing water and reducing the volume. The procedures thereafter, for pregnanediol and allo-pregnanediol, were the same as those described in Section III. Preliminary experiments with pregnanediol showed that this method gave recoveries of 67% or better.

The following experiment was carried out in one operation so that conditions would be identical throughout. To check the efficiency of acid hydrolysis in the presence of liver tissue, NaPG (in ethanolic solution) equivalent to 12 μg. of pregnanediol, was added both to the incubation medium plus rat liver homogenate and to 3 ml. of water. Blank incubations were carried out for pregnanediol with 3 ml. of water, and for pregnanediol and allopregnanediol with incubation medium plus homogenate. One mg. of progesterone was added to incubation vessels (except blanks and steroid 'controls'); the last six tubes were worked up for progesterone (two blanks, two 'controls' and two incubations) in order to measure the amount of progesterone metabolized.

The results are given in Tables 9 and 10.

Table 10

Measurement of apparent pregnanediol after incubation of progesterone with rat liver homogenate

	Optical Densities		
	400 mμ.	430 mμ.	460 mμ.
Blanks	0.059	0.042	0.039
	0.059	0.044	0.040
Incubations	0.051	0.041	0.036
	0.052	0.043	0.038

Measurement of allopregnanediol fraction after incubation of progesterone with rat liver homogenate

	Optical Densities		
	400 mμ.	430 mμ.	460 mμ.
Blanks	0.050	0.048	0.032
	0.055	0.051	0.034
Incubations	0.184	0.180	0.131
	0.153	0.142	0.104

Measurement of progesterone recovered after incubation with rat liver homogenate

	Progesterone recovered	
	(μg.)	(%)
Incubations	369	38
	388	40
'Controls'	931	96
	960	99
Progesterone added	970	

Conclusions

(i) The apparent pregnanediol and allopregnanediol values are consistent and low.

(ii) Recoveries of pregnanediol and allopregnanediol are satisfactory for a level of 12 µg.

(iii) Hydrolysis of NaPG in the presence of liver tissue does not appear to be complete, judging from the lower recoveries; hydrolysis in the absence of tissue appears to be complete in view of the higher recoveries.

(iv) By subtracting the mean value for the incubations from the mean value for the 'controls' it is found that about 570 µg. of progesterone have been metabolized.

(v) Assuming only 50% recovery of pregnanediol, if present as glucuronide, it appears that 5β-pregnane-3α:20α-diol constitutes less than 1%of the metabolic products obtained after incubation of progesterone with rat liver homogenate.

(vi) In view of the fact that the optical densities obtained in the case of the allopregnanediol fraction were not selective at 430 mµ, no definite conclusion can be drawn at this stage with regard to 5α-pregnane-3α:20α-diol.

SECTION V

GENERAL DISCUSSION

A) Interaction between Nicotinamide and Coenzymes

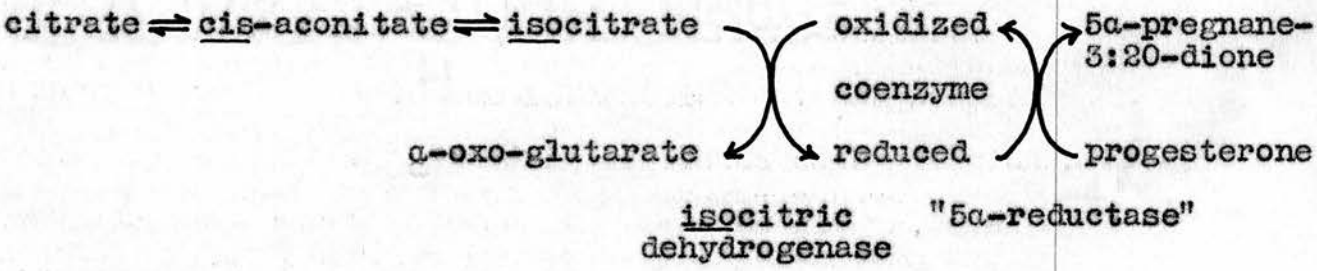
Tomkins et al.(1954) showed that the reduction of the double bond of cortisone by an ammonium sulphate precipitate prepared from a rat liver supernatant (78,000g) required only TPNH as a coenzyme. This preparation also catalysed the disappearance of absorption at 240 mμ. of progesterone and other αβ-unsaturated ketonic steroids, provided that TPNH was present. The present author has found (p. 57) that a dialysed supernatant (150,000g) also required TPNH for metabolism of progesterone and that the activity of the microsomal fraction was increased by DPN, or better, DPNH (p. 38), in the presence of which 150,000g supernatant was inactive (p. 39). The activity of 5,000g and 25,000g supernatants was increased by the addition of DPN and still more by DPNH or TPNH (p. 47) and the activity of a dialysed 5,000g supernatant was restored by DPN (p.44). Taylor (1954) showed that the activity of rat liver homogenate was increased by DPN. Hence it is surprising, at first sight, that Wiswell et al.(1953) failed to obtain any marked increase in activity upon addition of DPN to rat liver homogenate; however, this may be explained by the present studies with nicotinamide.

It was found that with a given tissue concentration there was an optimum nicotinamide concentration; if the tissue concentration were decreased, the optimum nicotinamide concentration was also decreased (p. 101).

The purpose of the nicotinamide is to inhibit destruction of coenzymes by a nucleosidase present in liver tissue (Mann and Quastel, 1941; Handler and Klein,1942). The former workers showed that if the nicotinamide concentration were too high, then partial inhibition of other enzymes occurred, for example, lactic dehydrogenase, and they attributed this effect to competition of the nicotinamide with coenzyme for the lactic dehydrogenase. The inhibiting effect on progesterone destruction caused by higher nicotinamide concentrations was possibly due to a similar effect, namely, competition between nicotinamide and the coenzyme for the steroid enzyme. Thus the optimum nicotinamide concentration may have been that which inhibited coenzyme destruction without undue inhibition of interaction between steroid enzyme and coenzyme. With higher tissue concentrations, the endogenous coenzyme concentration was higher, and a higher nicotinamide concentration was necessary for its protection. Wiswell *et al.*(1953) used a low tissue concentration (2%) and when 0.006mM-DPN was added to flasks, 40 mM-nicotinamide was also added; this was a relatively high concentration of nicotinamide to coenzyme (endogenous and added) and may explain the fact that only a slight increase in progesterone destruction was observed.

B) The Role of Citrate

With 25% homogenate, increase in the concentration of citrate above 0.01M did not increase its activating effect (p. 101). As mentioned above (p. 109) Tomkins et al.(1954) found that TPNH was the coenzyme required by the supernatant; they provided it by addition of TPN in presence of a reducing system, such as glucose-6-phosphate and glucose-6-phosphate dehydrogenase, or isocitrate and isocitric dehydrogenase. Since the latter enzyme occurs in the supernatant, it appeared to the author that the powerful activating effect of citrate might be due to the following series of reactions:



This mechanism was published first by Taylor (1955); it is substantiated by the fact that Wiswell et al. (1953) found that equimolar concentrations of citrate, cis-aconitate and isocitrate were equally effective for increasing progesterone metabolism by rabbit liver homogenate; Taylor (1955) has shown since that the enzyme systems responsible for metabolism of progesterone in rat and rabbit livers have similar properties with regard to coenzyme requirement.

Further support for the coenzyme-reducing role of citrate was given by the fact that whereas 25,000g supernatant reduced TPN rapidly, dialysed supernatant did so much more slowly (p. 51 and Fig. 1) but the addition of isocitrate to the latter brought about rapid reduction of TPN (p. 49).

Wiswell et al.(1953) proposed that citrate increased progesterone metabolism by chelation with an inhibiting metallic ion; this suggestion was supported by their own findings that two other metal-binding substances, cysteine and cyanide, increased metabolism also, in fact to a greater extent than did citrate. To investigate further this point, the effect of the well-known chelating agent, Versene, was tried (Altmann and Crook, 1953). It was found to have no appreciable influence and, unlike citrate, it did not increase the metabolism of progesterone (p. 46). Since the conversion of cis-aconitate to isocitrate by aconitase is inhibited by trans-aconitate, the effect of addition of this substance was tried (p. 103). It was found to cause inhibition of progesterone metabolism, thus supporting the coenzyme-reducing role of citrate. These findings cast doubt on the theory that citrate functions by chelation with inhibiting metallic ions since the presence of trans-aconitate should not diminish this process. It is a possibility that the fact that Wiswell et al.(1953) obtained increased

progesterone metabolism by addition of cysteine or cyanide, was due to the presence of an inhibiting metallic ion in the water or reagents used. As an example of this type of phenomenon Judah and Rees (see Altmann et al., 1953) found that histidine, which chelates heavy metals, no longer increased the activity of the succinic oxidase system if the mitochondrial preparation was made carefully with glass-distilled water and pure reagents were used.

C) Metallic Ions

Adler, von Euler, Gunther and Plass (1939) showed that isocitric dehydrogenase catalysed the reduction of TPN by isocitrate and that the system required Mg^{++} or Mn^{++}, the latter being more effective. It was found (p. 45) that metabolism of progesterone by 5,000g supernatant was greater with added Mn^{++} than with Mg^{++}.

In the case of a dialysed supernatant in which isocitrate was absent almost completely, this reduction of TPN by isocitric dehydrogenase could not take place and therefore the absence or presence of Mg^{++} or Mn^{++} should not have affected progesterone metabolism, as far as this aspect was concerned. Progesterone metabolism by dialysed supernatant was indeed found to be independent of Mg^{++} or Mn^{++} (p. 57); under these conditions, TPNH was acting stoichiometrically, there being no enzyme systems available for the reduction of TPN resulting from concurrent progesterone reduction and TPNH oxidation.

D) Coenzymes

Spectrophotometric studies (p. 51 and Fig. 1) showed that 25,000g supernatant reduced TPN. Thus it was difficult to understand why coenzymes were much more effective if they were reduced before addition to the incubation vessels (p. 47); this is possibly accounted for by the fact that although DPN and TPN are attacked by a nucleosidase found in mammalian central nervous system, DPNH and TPNH are not (McIlwain and Rodnight, 1949). However, it does not follow that liver nucleosidase has the same properties and, indeed, Axelrod (1955) reported that these reduced coenzymes disappeared rapidly in the presence of rabbit liver microsomes (and nicotinamide), even in the absence of substrate. This probably explains why DPNH added in portions at intervals, was more effective than when added in bulk at the commencement of incubation (p.102).

Although supernatant specifically requires TPNH for progesterone metabolism (Tomkins et al., 1954), homogenate (Taylor, 1954), 5,000g supernatant (p. 43), 25,000g supernatant (p. 47) and microsomes (p. 38) were able to utilize DPN or DPNH. There are two possible explanations for this, both of which may hold. Mehler, Kornberg, Grisolia and Ochoa (1948) showed that pigeon liver contains an enzyme which converts DPN to TPN and a similar enzyme may exist in rat liver. The progesterone metabolizing enzyme in the microsomes may be able to use DPNH as a coenzyme, in addition, possibly,

to TPNH; if this is the case, it could couple with a DPN-dependent isocitric dehydrogenase reported by Plaut and Sung (1954) to be present in rat liver. Glenn et al. (1956) found that absorption at 240 mμ, due to cortisone, was diminished by incubation with a washed microsomal fraction prepared from a rat liver homogenate and this destruction of cortisone was increased by addition of TPNH.

E) The Fractionation of Rat Liver Homogenate

It was found that the nuclear fraction did not metabolize progesterone to any degree, yet when this fraction was removed from homogenate the resulting supernatant was not as active as the original homogenate (p. 29). That this was not due solely to an ageing effect was shown when a 700g supernatant was found to be less active than the same homogenate stored during the preparation of the supernatant(p. 30). The results might be explained by the fact that the synthesis of DPN by the homogenate occurs mainly in the nuclei (Hogeboom and Schneider, 1952b). These workers found that the DPN-synthesizing enzyme, which is a water-soluble protein (Kornberg, 1950), was released into solution if the nuclei were disintegrated by sonic oscillations. Thus it would be possible to test the above explanation by adding the supernatant from disintegrated nuclei to the 700g supernatant, when the

original activity of the homogenate should be restored, at least to some degree.

The mitochondrial fraction alone was also found not to be active to any extent (p. 32) yet removal of it from a 700g supernatant, to yield a 5,000g supernatant, caused a drop in activity and this was partially restored when the mitochondrial fraction was resuspended in the 5,000g supernatant (p. 33). Again, as in the case of the nuclei, the effect might be explained by contribution of DPN from mitochondria. Hunter, Levy, Davis and Carlat (1956) reported that DPN can pass out through the mitochondrial membrane. The inactivity of mitochondria alone might be explained by the possibility that this membrane was acting as a barrier to the entry of progesterone, though this was unlikely since Brownie, Grant and Davidson (1954) achieved 11β-hydroxylation of progesterone with intact adrenal mitochondria; however, liver mitochondria still required investigation.

Berthet, Berthet, Appelmans and De Duve (1952) showed that acid phosphatase was released from rat liver mitochondria in soluble form when they were subjected to various treatments, including dilution with distilled water, freezing and thawing, and ageing at 0°. The results on p. 37 appear to indicate some metabolism of progesterone both by intact and damaged mitochondria; however, Schneider (1953) reported that mitochondria prepared by the above technique (p. 9) contain about

5% of the microsomes and these may be responsible, since they have been found to metabolize progesterone (pp. 38, 39 and 40).

When the nuclear and mitochondrial fractions were combined, fair activity was obtained (p. 35); this illustrates well the interdependence of cell fractions. Forchielli and Dorfman (1956) also obtained reduction of Ring A when they incubated 11-deoxycortisol (17α:21-dihydroxy-pregn-4-ene-3:20-dione) with a 6,000g residue from a rat liver homogenate.

The activity of the microsomal fraction was increased by DPN and still more by DPNH (p. 38); complete inhibition occurred with 0.001M-p-chloromercuribenzoate and this was reversed partially by 0.001M-reduced glutathione (p. 40). Tomkins et al.(1954) found that the reduction of dihydrocortisone (17α:21-dihydroxy-5β-pregnane-3:11:20:-trione) by their enzyme from rat liver supernatant was also inhibited completely by 0.0005M-p-chloromercuribenzoate and this inhibition was reversed by 0.005M-glutathione or cysteine.

These results were confirmed by Glenn et al.(1956) who found that their microsomal enzyme, from rat liver, which reduced the double bond of cortisone, was inhibited completely by 0.003M-p-chloromercuribenzoate. This does not, however, enable one to draw the conclusion that the steroid-reducing enzyme is of the sulphydryl type, since the above workers showed also that the same concentration of p-chloromercuribenzoate inhibited

strongly glucose-6-phosphate dehydrogenase, thus suggesting that the inhibition of the steroid enzyme was indirect, due to inhibition of the TPNH generating system.

In the experiment on p. 42 a 5,000g supernatant was divided into three equal portions, one being stored at 0° while the other two were separated into microsomal fractions and 25,000g supernatants. One of these supernatants was used as such but the microsomal pellet in the other tube was suspended back into its own supernatant. The activity of the latter preparation might have been expected to be equal to the original 5,000g supernatant. It was found, however, that only part of the activity was regained; it will be recalled that the same phenomenon was observed with the mitochondrial fraction (p. 33) and with the nuclear fraction (p. 30).

This effect is at least due in part to enzyme lability since a 5,000g supernatant prepared directly, without decantation of the intermediate 700g supernatant, was more active than a 5,000g supernatant prepared in the normal way (p. 35). When rat liver homogenate was centrifuged at 11,000g and the residue resuspended, Glenn and Recknagel (1955) observed enzyme lability with regard to removal of the side-chain of cortisone; they found also that homogenate lost activity while stored at 0° (cf. p. 33). This loss of activity may be explained by the fact that the concentration of DPN

and DPNH in liver tissue is reduced markedly by homogenization, even at 0° (Frunder and Richter, 1955). Unlike homogenate, whole liver (p. 98) and 5,000g supernatant (p. 41) were found to be stable when stored at 0°; however, when liver was cooled to -20° and allowed to thaw, some of the enzyme activity was lost (p.98).

The complex relationships between cellular structures are well shown by the findings that of the four main cell fractions, two (microsomal and supernatant) were involved directly in the metabolism of progesterone, while the other two (nuclear and mitochondrial) contributed only when combined together.

F) Inhibition of Progesterone Metabolism

GA has been found to inhibit the destruction of both progesterone and DOC by rat liver homogenate (pp. 64-66). This may mean that the reduction of Ring A of αβ-unsaturated ketonic steroids is inhibited in general by GA.

This inhibition could occur in two ways; GA could prevent the regeneration of TPNH (compare the inhibition by p-chloromercuribenzoate discussed above, p.117), or it could inhibit directly the reduction of progesterone. The former possibility is unlikely since GA did not affect the degree of reduction of TPN by the concurrent oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase (p. 70 and Fig. 5). To test the latter possibility, a 25,000g supernatant was

dialysed (to remove citrate, cis-aconitate and iso-citrate) and TPNH was added; thus the only reaction that should have occurred, as far as progesterone was concerned, was the reduction of progesterone by enzyme and preformed TPNH. GA was added to this system and was found to cause complete inhibition (p. 67). Thus it may be concluded that inhibition by GA is a direct effect on the steroid enzyme.

The fact that DGA inhibits to a small extent only (p. 69) demonstrates that the 11-keto group is required for inhibition. GA, administered to Addisonian patients maintained on DOC, was found to have a DOC-sparing effect in that it assisted in the maintenance of a positive sodium balance (Groen et al., 1951); Groen, Willebrands and Pelser (1956) found that analogues of GA, without the 11-keto group, when administered to patients did not maintain a positive sodium balance, characteristic of GA. This agreement between in vivo and in vitro results supports the postulate that GA acts in vivo by inhibiting steroid reduction.

The requirement for a keto group and the fact that methyl vinyl ketone and p-quinone, which both contain αβ-unsaturated ketonic groups, inhibited progesterone reduction (p. 69), though not proof, is suggestive that the αβ-unsaturated ketonic group of GA is the active part of the molecule. Simpler ketones such as acetophenone and acetone inhibited to a small extent only (p. 69). The structures of these compounds are

given in Fig.4 opposite p. 59.

To investigate whether methyl vinyl ketone is a specific or a general enzyme inhibitor, the effect of it on glucose-6-phosphate dehydrogenase was tried (p. 70 and Fig. 6). It was found not to affect the degree of oxidation of glucose-6-phosphate and hence the concurrent reduction of TPN; nor did it inhibit 11β-hydroxylation of DOC to any marked extent (p. 71). Thus it may be concluded that inhibition by methyl vinyl ketone is a direct effect on the steroid enzyme. It is possible that both GA and methyl vinyl ketone would inhibit also the reduction of 11-keto steroids to 11-hydroxy steroids.

G) In vivo Metabolism of Progesterone in the Rat

Neither pregnanediol (p. 78) nor allopregnanediol (p. 80) was detected in rat urine before or after intraperitoneal injection of progesterone, or during pregnancy (p. 82). The analytical methods used would have detected as little as 6 μg. of pregnanediol or allopregnanediol, an amount equivalent to 0.05% of the progesterone injected. Thus it may be concluded that these metabolites are not excreted in significant amounts in rat urine.

To recover allopregnanediol from rat urine, the method of Klopper et al.(1955) for pregnanediol was modified; the finding that pregnanediol was not detected in rat urine meant that it was not necessary to attempt

to separate these two stereoisomers, which facilitated greatly the modification for allopregnanediol. However, it might be possible, by adjustment of the nature and volume of eluting solvents to determine simultaneously both these steroids in one sample of urine.

The presence of pregnanediol or its glucuronide has been demonstrated in the urine of pregnant women (Marrian, 1929), non-pregnant women (Venning and Browne, 1937), men (Engel, Thorn and Lewis, 1941), pregnant mares (Marker, Kamm, Crooks, Oakwood, Lawson and Wittle, 1937), pregnant chimpanzees (Fish, Dorfman and Young, 1942), pregnant rhesus monkeys after injection of progesterone (Boscott, 1952), bulls (Marker, Wittle and Lawson, 1938) but not steers (Marker, 1939) and in the urine of male and female rabbits after subcutaneous injection of progesterone (Hoffman, 1942). However, it was not found in the urine of stallions (Marker et al., 1938), pregnant sows (Marker and Rohrmann, 1939), female goats after injection of progesterone (Grant, 1950; Boscott, 1952) or pregnant cows (Stevensen, 1947; Hill, Petersen and Cohen, 1954; Klyne and Wright, in the press). The fact that Marker et al. (1938) obtained crystalline pregnanediol from pregnant cow urine may be attributed to contamination, possibly with cow faeces. Pearlman and Cerceo (1948) obtained 5β-pregnane-3α:20β-diol and pregnanolone from the bile of pregnant cows, but pregnanediol was not detected.

The presence of allopregnanediol has been demonstrated in the urine of pregnant (Hartmann et al.,1935) and non-pregnant women, pregnant mares, bulls and pregnant cows (Marker et al., 1938), though this latter finding is suspect, as mentioned above for pregnanediol; also in the urine of men after intramuscular injection of progesterone (Kyle et al., 1951). Allopregnanediol was not found in the urine of pregnant chimpanzees (Fish et al., 1942), pregnant sows (Marker et al.,1939), steers (Marker, 1939) or stallions (Marker et al.,1938). Thus it is well established that there are differences amongst mammalian species for the urinary excretion of pregnanediol and allopregnanediol.

Dorfman (1954) has indicated that pregnanetriol may be a metabolite of 17α-hydroxyprogesterone but this has not been established experimentally yet. Ungar, Davis, Rosenkrantz and Dorfman (1954) isolated 10 mg. of pregnanetriol from the urine of a 66 years old woman with rheumatoid arthritis after oral administration of a total of 2.4 g. of 17α:21-dihydroxy-5β-pregnane-3:20-dione. Three mg. of pregnanetriol were isolated from the post-treatment urine, but none from the pre-treatment urine.

The permanganate oxidation step of Klopper et al. (1955) is designed to degrade any ethylenic compounds derived from pregnanetriol during hot acid treatment. If this step is omitted, chromogenic material derived from the pregnanetriol is found in the pregnanediol

fraction (Klopper et al., 1955). Thus, in the absence of pregnanediol, this could be used as a test for the presence of pregnanetriol. Since pregnanediol had indeed not been found (p. 78), it was decided to examine the urine for pregnanetriol although this may not be a progesterone metabolite; none was detected (p. 84).

Examination of the urine for allopregnanedione, pregnanedione and allopregnanolone, using the dinitrophenylhydrazine technique, failed to reveal the presence of these steroids (p. 90). This does not constitute proof, however, that they are not excreted in rat urine, since adequate controls were not carried out. These steroids, in ethanolic solution, should have been added to rat urine instead of to the ketonic fraction from rat urine.

Examination of the faeces and of the non-ketonic fraction of the urine, did not result in the isolation of any crystalline material, except for coprostan-3β-ol, which is known to occur in faeces and so was only identified tentatively by m.p. and mixed m.p. (p. 95).

Riegel et al.(1950) who injected progesterone-21-C^{14} into rats, showed that some radioactivity, which was not due to progesterone was recovered in the ketonic, non-alcoholic fraction from the faeces. In view of the metabolites isolated by Taylor (1954) this might have been pregnanedione or allopregnanedione. Only the latter forms an insoluble digitonide (Butenandt and

Mamoli, 1935) and the corresponding ketonic, digitonin precipitable metabolic fraction, isolated in the present work, weighed only 3 mg. and yielded no identifiable products (the corresponding control fraction weighed 1 mg.).

This non-alcoholic ketone, found by Riegel et al. (1950) in rat faeces, might be the same substance as the Compound X which was an αβ-unsaturated, non-alcoholic ketone isolated by Pearlman et al.(1948) from the bile of pregnant cows. Grady et al.(1952), after injection of progesterone-21-C^{14} into rats, found that about 75% of the radioactivity was recovered in the faeces, but did not identify any compounds therein. Shen et al. (1954) using also progesterone-21-C^{14}, showed that oxidative fission of the side-chain must have occurred since part of the radioactivity was recovered as CO_2 (15%) and urinary urea (0.5%) in the rat.

H) The Possible Conversion of Progesterone to Androgens and Other Steroids

It is considered by some workers that progesterone exerts androgenic effects, for example, when injected subcutaneously into immature castrated rats (Greene, Burrill and Thomson, 1940). Marrian (1938) suggested that allopregnanes might be degraded in vivo to androstanes. Longwell and Gassner (1947) found that the pregnant cow, unlike the bull, steer or non-pregnant cow, excretes considerable quantities of androgens in

the faeces. This became evident shortly after gestation and reached a maximum during the last two months of pregnancy. The activity was demonstrated by feeding the dry faeces to 10 days old chicks when, after three weeks, the comb growth was markedly greater than that of control chicks; progesterone itself was found to be inactive. The active substance was shown to be neither testosterone nor a 17-ketosteroid but it possessed, in ethanolic solution, maximum absorption at 240 mμ, characteristic of αβ-unsaturated ketonic steroids. Again, this could be the same substance as Pearlman's Compound X, isolated from pregnant cow bile. Miller and Turner (1955) reported a similar effect after injecting progesterone into male and non-pregnant bovines; the male and female faecal androgen content, as measured by comb response in cockerels, was increased significantly.

Miller, Turner, Fukushima and Salamon (1956) injected progesterone into a pregnant cow and examined the faeces. Androsta-1:4-diene-3:17-dione was isolated in crystalline form and androst-4-ene-3:17-dione, androstane-3:17-dione and 5β-androstane-3:17-dione were identified partially also. The value of this work would have been increased if the faeces of pregnant and non-pregnant cows had been analysed also. These substances could have arisen either by bacterial action in the gastrointestinal tract or by actual <u>in vivo</u> formation; androst-4-ene-3:17-dione could have been derived from

the adrenal gland (von Euw and Reichstein, 1941). It is interesting to note the work of Slaunwhite *et al.* (1956) who identified partially 17α-hydroxyprogesterone, androst-4-ene-3:17-dione and testosterone after incubation of isotopically labelled progesterone with rat testis; 17α-hydroxylation of progesterone in the female could occur in the adrenal gland (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker and Pincus, 1951). In this connection it has been found that whereas 17α-hydroxylation of progesterone was not achieved with adrenal glands of the steer, it was accomplished with adrenal glands of the cow (Knox, Auerbach and Lin, 1956). Werbin, Le Roy and Bergenstal (1955) injected a woman with progesterone-4-C^{14} and obtained radioactive crystalline pregnanediol from the urine. Cortisol (11β:17α:21-trihydroxy-pregn-4-ene-3:20-dione), tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one), tetrahydrocortisone (3α:17α:21-trihydroxy-5β-pregnane-11:20-dione) and 3α-hydroxy-5β-androstan-17-one were isolated also, but with very much lower specific activities than the pregnanediol indicating that only a small proportion of the progesterone was converted to these substances. This provides *in vivo* evidence for the scheme of Hechter and Pincus (1954) and for the removal of the side-chain of progesterone. It means also that besides the conversion of allopregnanes to androstanes, discussed above, pregnanes may be transformed to 5β-androstanes (ætiocholanes).

I) Biochemical Reduction Products of Progesterone

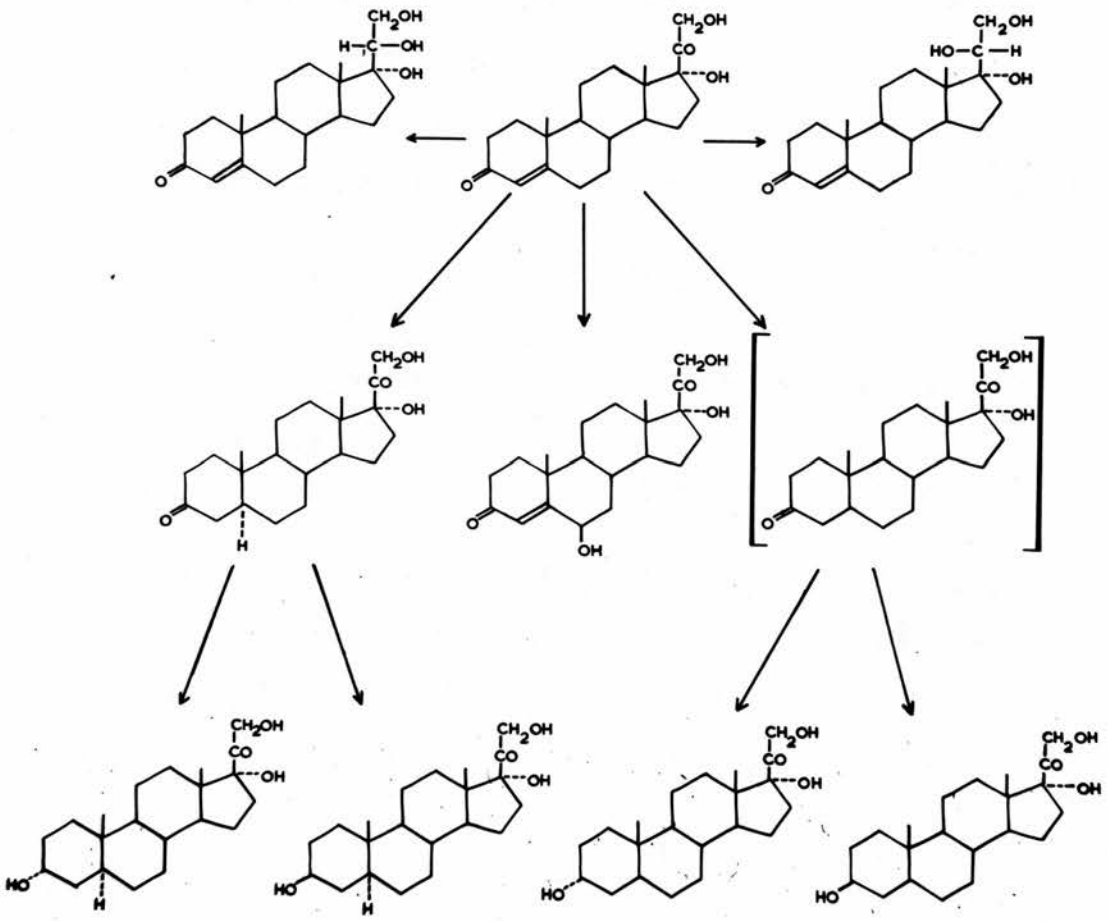
Taylor (1954) obtained allopregnanedione and allopregnanolone, but not pregnanediol, as metabolic products after incubation of progesterone with rat liver homogenate. The first metabolite was identified tentatively after incubation of progesterone with 5,000g and 25,000g supernatants (p. 56). The fact that pregnanediol is not a metabolite was confirmed by the present work, which showed that this steroid, if formed at all, constituted less than 1% of the metabolic products (p. 108). This method could possibly be applied to the determination of pregnanediol in the presence of other tissue homogenates or cell fractions. The solution obtained after addition of sulphuric acid to the allopregnanediol fraction (p. 107) had a clear yellow fluorescent appearance, typical of that given by some steroids and quite unlike the brown colour produced by the presence of impurities. The nature of this material has not been determined yet, but it is the author's intention to investigate it further.

After incubation of progesterone with rabbit liver homogenate Taylor (1955) isolated pregnanolone, 3β-hydroxy-5α-pregnan-20-one and pregnanediol as well as the two allopregnane derivatives obtained with rat liver homogenate, mentioned above. Ungar (see Forchielli, Rosenkrantz and Dorfman, 1955) incubated pregnanolone with rat liver homogenate and obtained 5β-pregnane-3α:20β-diol; since

the former has been shown to be a metabolite of progesterone (Dorfman et al.,1948) 5β-pregnane-3α:20β-diol may be considered an in vitro product of progesterone. Davis et al.(1956) observed the excretion of 5α-pregnane-3β:20α-diol in the urine of pregnant, but not non-pregnant, women; when progesterone was administered to pregnant women, Plotz et al.(1956) found increased urinary excretion of 5α-pregnane-3β:20α-diol in only one case out of three, that in which 1 g. of progesterone was administered orally. Thus 5α-pregnane-3β:20α-diol may be regarded tentatively as a progesterone metabolite.

Tomkins et al.(1954) using a purified 78,000g supernatant from a rat liver homogenate (Isselbacher and Tomkins, 1954) identified only pregnane derivatives after incubation with cortisone; these authors showed also that their preparation caused the loss of absorption at 240 mμ. of progesterone. After incubation of progesterone with 5,000g and 25,000g supernatants of rat liver homogenate, a 5α-pregnane was tentatively identified (p. 56). Taylor (1955) obtained both pregnanes and 5α-pregnanes from the same enzyme preparation, a rabbit liver homogenate. Thus it would appear that there are two enzymes in homogenate, one catalysing 5α reduction and the other 5β reduction. The former appears to be in the microsomes, while the latter must be in the supernatant. In confirmation of this, Forchielli and Dorfman (see Dorfman,

FIGURE 8. METABOLISM OF 11-DEOXYCORTISOL BY RAT LIVER PREPARATIONS.



1955b) incubated 11-deoxycortisol with both a residue (microsomes) separable between 6,000g and 78,000g from a rat liver homogenate and the 78,000g supernatant (microsome-free). With the microsomes, only a 5α-pregnane was obtained and with the supernatant, only pregnanes; incubation with the 6,000g supernatant yielded 5α-pregnanes only (Forchielli et al., 1956). The metabolism of 11-deoxycortisol (Hübener and Schmidt-Thomé, 1955; Forchielli et al., 1955, 1956) is shown in Fig. 8; one product not detected is bracketed and C_{19} derivatives are omitted.

Ungar, Dorfman, Stecher and Vignos (1951) administered pregnanedione orally to a woman and isolated pregnanediol from the urine; they also administered allopregnanedione orally to a man and obtained allopregnanediol and 5α-pregnane-3β:20α-diol from the urine. While this is supporting evidence that the two diones are intermediates in the reductive metabolism of progesterone, it is not conclusive since the amounts of the diols in the urines before administration were not determined. However, from the work of Tomkins et al. (1954) it would appear probable that, in rat liver at least, the normal metabolic pathway for the reduction of Ring A of progesterone is hydrogenation of the double bond to give the two diones, followed by reduction of the 3-keto group.

Liver tissue is capable of reducing the 20-keto

group of some steroids without affecting Ring A. Thus, Caspi et al. (1956) incubated DOC and 11-deoxycortisol with hog liver homogenate and isolated the corresponding 20α-hydroxy derivatives. Hübener et al. (1955) incubated 11-deoxycortisol with rat liver homogenate and obtained also mainly the 20α-hydroxy derivative with a small amount of the 20β-hydroxy compound; however, Forchielli et al.(1955) obtained only the latter substance after incubating 11-deoxycortisol with a 6,000g supernatant from rat liver homogenate. Cortisol, incubated with rat liver microsomes again yielded the 20β-hydroxy derivative (Glenn et al.,1956). As yet, however, reduction of progesterone to a 20-hydroxy derivative by liver tissue has not been reported, though reduction to the 20β-hydroxy compound by corpus luteum homogenate has been accomplished (Hayano et al., 1954).

J) Correlation of in vivo and in vitro Studies

One purpose of in vitro work is to attempt to isolate one small part of the metabolism of the whole organism and to carry out the reactions under conditions as near physiological as possible, consistent with the isolation of metabolic products. In so far as in vivo and in vitro results agree, this has been achieved successfully.

The rabbit has been shown to excrete pregnanediol

in the urine after injection of progesterone (Hoffman, 1942); this *in vivo* observation agrees with the *in vitro* finding of Taylor (1955) who obtained pregnanediol after incubation of progesterone with rabbit liver homogenate. Taylor (1954) did not find pregnanediol after incubation of progesterone with rat liver homogenate and the present work confirms this (p. 107); it agrees also with the finding that pregnanediol was not detected in the urine of the rat after progesterone injection (p. 78). Correlation between *in vivo* and *in vitro* results has been found also with the studies on the inhibition of steroid reduction by GA and DGA discussed on p. 120.

However, one difference between *in vivo* and *in vitro* studies is that intermediate metabolites, which might be removed from the cell and carried away in the blood *in vivo*, remain *in situ* and may be metabolized further, *in vitro*. Thus we may expect also some differences in results, as is indeed the case. For example, corticosterone (11β:21-dihydroxy-pregn-4-ene-3:20-dione) is the main steroid found in adrenal vein blood of the rat but Eisenstein (1956) showed that halved rat adrenals, incubated with ACTH (adrenocorticotrophic hormone), produced 11α:21-dihydroxy-pregn-4-ene-3:20-dione.

For reference, a Table of sixteen possible reduction products of progesterone has been included; abbreviated references have been used to provide a concise Table and, where no reference has been given,

Table 11

Compound	In vivo		In vitro	
	Source	Reference	Source	Reference
20α-hydroxy-pregn-4-en-3-one	Rat carcass	Wiest, 1956		
20β-hydroxy-pregn-4-en-3-one			Corpus luteum homogenate	Hayano et al., 1954
5α-pregnane-3:20-dione	Human pregnancy urine	Lieberman et al., 1948	Rat liver homogenate	Taylor, 1954
5β-pregnane-3:20-dione	Human urine	Lieberman et al., 1948	Blood albumin	Rongone et al., 1956
3α-hydroxy-5α-pregnan-20-one	Human urine	Dobriner et al., 1950	Rat liver homogenate	Taylor, 1954
3β-hydroxy-5α-pregnan-20-one	Human pregnancy urine	Pearlman et al., 1942	Rabbit liver homogenate	Taylor, 1955
3α-hydroxy-5β-pregnan-20-one	Human urine	Dorfman et al., 1948	Rabbit liver homogenate	Taylor, 1955
3β-hydroxy-5β-pregnan-20-one				
5α-pregnane-3α:20α-diol	Human pregnancy urine	Kyle et al., 1951		
5α-pregnane-3α:20β-diol				
5α-pregnane-3β:20α-diol	Human pregnancy urine	Plotz et al., 1956		
5α-pregnane-3β:20β-diol	Pregnant mare urine	Brooks et al., 1952		
5β-pregnane-3α:20α-diol	Human urine	Dorfman et al., 1948	Rabbit liver homogenate	Taylor, 1955
5β-pregnane-3α:20β-diol	Pregnant cow bile	Pearlman et al., 1948	Rat liver homogenate	Ungar, 1955
5β-pregnane-3β:20α-diol	Human urine (?)	Mason et al., 1945		
5β-pregnane-3β:20β-diol				

the detection of the substance has not been reported yet in the literature. Such compounds as 3:20-dihydroxy-pregn-4-enes, 20-hydroxy-5α(or β) -pregnan-3-ones and derivatives with oxygen functions in positions other than 3 or 20, or a double bond other than in position 4 have not been included. Where applicable, those references have been given to studies in which an attempt was made to demonstrate that the steroid reported was a progesterone metabolite. Otherwise, investigations on human pregnancy urine, for in vivo work, and on rat liver, for in vitro work, have been favoured.

K) Suggestions for Further Work

In view of the numerous metabolites of 11-deoxy-cortisol (Fig. 8, facing p. 130), it is possible that investigation of the metabolites of progesterone obtained with rat liver homogenate or cell fractions would repay further study. By means of partition chromatography, one could separate progesterone from its 20-hydroxy derivatives; their detection should be relatively simple by taking advantage of the strong absorption at 240 mμ. of αβ-unsaturated ketonic steroids. Any of the compounds listed in the Table opposite could be used as substrates and their metabolites investigated.

Advantage could be taken of the experience gained in this work to carry out an isolation study with human liver; Reaven (1955) has shown that αβ-unsaturated ketonic steroids lose their absorption at 240 mμ when incubated with human liver slices. In an experiment not reported here, the author obtained some destruction of progesterone with an homogenate prepared from human liver, obtained as long as 18 hr. after death.

L) Conclusion

It is apparent that the metabolism of progesterone is a subject of considerable complexity and therefore of great interest; in vitro studies and analyses of blood, bile and excreta of various species will all contribute towards its elucidation. Only when progesterone metabolism is fully understood, will it be possible to apply rational methods in the treatment of its disorders.

APPENDICES

APPENDIX I

Summary

It has been shown that when rat liver homogenate was separated into cell fractions, the major activity, with regard to the destruction of progesterone, resided in the 5,000g supernatant. The nuclear and mitochondrial fractions were inactive, but when combined they exhibited some activity. When the 5,000g supernatant was separated into its two components, the microsomal fraction was still active but the supernatant required the addition of TPNH. Citrate increased markedly the activity of 5,000g supernatant and of supernatant, and it is possible that this tricarboxylic acid functions by keeping TPN in the reduced state, since other workers have shown that a purified supernatant is not active in the presence of TPN, unless an enzyme system is added to keep the TPN in the reduced state. Isocitric dehydrogenase catalyses the reduction of TPN by isocitrate and requires the presence of Mg^{++} or Mn^{++}, the latter being more effective. Addition of Mg^{++} and Mn^{++} to a 5,000g supernatant showed that Mn^{++} was more effective as a co-factor for progesterone metabolism; further, addition of these metallic ions to a dialysed supernatant did not affect its activity. These observations are consistent with the TPN-reducing role of citrate, since dialysed supernatant contains no citrate or isocitrate.

GA inhibited progesterone and DOC metabolism; this

was not due to inhibition of TPN reduction since addition of GA to a TPN-reducing system did not affect the degree of reduction of TPN attained. Thus it may be concluded that GA inhibits the enzyme which reduces αβ-unsaturated ketonic steroids. DGA inhibited progesterone metabolism only to a small extent; since the ketonic group is lacking in this compound, it may be deduced that the αβ-unsaturated ketonic group of GA is necessary for its inhibitory activity. Confirmation of this was obtained when it was found that methyl vinyl ketone inhibited progesterone metabolism but did not inhibit TPN reduction.

Progesterone was injected intraperitoneally into male rats and the urine and faeces, and also the urine of pregnant rats, were examined for possible progesterone metabolites. Neither pregnanediol nor allopregnanediol was detected in the urine, though the analytical methods used would have detected amounts equivalent to 0.05% of the progesterone injected. By use of 2:4-dinitrophenylhydrazine followed by alumina chromatography, it was shown that allopregnanedione, pregnanedione and allopregnanolone, if present at all in male urine, constituted less than 0.1% of the progesterone injected. No progesterone metabolites were obtained from the faeces, but coprostan-3β-ol was isolated.

Rat liver, when subsequently homogenized and tested

for activity with regard to progesterone metabolism, was found to be stable for 24 hr. at 0°, whereas liver cooled to -20° for 24 hr. lost some activity; homogenate stored at 0° lost activity, but 5,000g supernatant did not. A method was evolved for the determination of pregnanediol in the presence of liver tissue; using this method it was shown that after incubation of progesterone with homogenate, pregnanediol, if formed at all, constituted less than 1% of the metabolic products.

APPENDIX II

Addendum

Recently, work has been published in the literature with a bearing on aspects of progesterone metabolism discussed in this thesis.

The probable pathway for reductive metabolism of Ring A of progesterone, mentioned on p. 130, has been supported by the work of Harold, Abraham and Chaikoff (1956) who incubated cholest-4-en-3-one with rat liver homogenate and obtained cholestan-3-one and cholestan-3β-ol; incubation of cholestan-3-one yielded cholestan-3β-ol.

Berliner and Wiest (1956) injected progesterone-4-C^{14} intravenously into male rats and found that after 5 hr. the highest concentration of radioactivity was in the liver, with the adrenals showing the next highest concentration; the concentration in the pituitary was not determined. Intravenous injection into eviscerated rats resulted in a concentration in the pituitary no greater than that in the blood; these results may be compared with those of Riegel et al. (1950) described on p. 3, bearing in mind that these investigators used progesterone-21-C^{14} with intact animals.

This work was followed up by Wiest (1956) who injected progesterone intravenously into eviscerated, adrenalectomized female rats, killed the animals 2 hr. later, extracted the carcasses and obtained 20α-hydroxy-pregn-4-en-3-one; this is the first reported instance of this

compound having been obtained by methods other than synthesis. Although, on chemical grounds, it seems probable that this steroid is a progesterone metabolite, this conclusion would have been strengthened if eviscerated, adrenalectomized female rats had been injected with the progesterone solvent and their carcasses examined.

This work demonstrated that it is possible to separate 20α-hydroxy-pregn-4-en-3-one and progesterone (see p. 133), since progesterone was recovered unchanged. It confirms also that extrahepatic tissues can metabolize progesterone (see p. 4). It is pointed out in this paper that progesterone metabolites in the normal rat seem to be different from those in the human, thus supporting the negative findings reported in Section III.

APPENDIX III

Terms, Abbreviations and Trivial Names used in this Thesis

Throughout this Thesis, the practice has been followed in general of giving the full description at the first mention, and also where it has been considered desirable.

ACTH: adrenocorticotrophic hormone

allopregnane: 5α-pregnane

allopregnanediol: 5α-pregnane-3α:20α-diol

allopregnanedione: 5α-pregnane-3:20-dione

allopregnanolone: 3α-hydroxy-5α-pregnan-20-one

A.R.: Analytical Reagent

ATP: adenosine triphosphate

Corticosterone: 11β:21-dihydroxy-pregn-4-ene-3:20-dione

Cortisol: 11β:17α:21-trihydroxy-pregn-4-ene-3:20-dione

Cortisone: 17α:21-dihydroxy-pregn-4-ene-3:11:20-trione

11-deoxycortisol: 17α:21-dihydroxy-pregn-4-ene-3:20-dione

DGA: 11-desoxoglycyrrhetinic acid (3β-hydroxy-olean-12-en-30-oic acid)

Dihydrocortisone: 17α:21-dihydroxy-5β-pregnane-3:11:20-trione

DNP: 2:4-dinitrophenylhydrazone

DOC: 11-deoxycorticosterone (21-hydroxy-pregn-4-ene-3:20-dione

DPN: diphosphopyridine nucleotide

DPNH: reduced diphosphopyridine nucleotide

GA: glycyrrhetinic acid (3β-hydroxy-11-oxo-olean-12-en-30-oic acid)

M.S.E.: Measuring & Scientific Equipment, Ltd.

NaPG: sodium 5β-pregnane-3α:20α-diol 3-glucuronidate

Pregnane: 5β-pregnane

Pregnanediol: 5β-pregnane-3α:20α-diol

Pregnanedione: 5β-pregnane-3:20-dione

Pregnanetriol: 5β-pregnane-3α:17α:20α-triol

Pregnanolone: 3α-hydroxy-5β-pregnan-20-one

Progesterone: pregn-4-ene-3:20-dione

Propylene glycol: propane-1:2-diol

Supernatant: supernatant fluid; where this term is used without qualification, it implies a microsome-free supernatant

Testosterone: 17β-hydroxy-androst-4-en-3-one

Tetrahydrocortisol: 3α:11β:17α-21-tetrahydroxy-5β-pregnan-20-one

Tetrahydrocortisone: 3α:17α:21-trihydroxy-5β-pregnane-11:20-dione

TPN: triphosphopyridine nucleotide

TPNH: reduced triphosphopyridine nucleotide

Tris: 2-amino-2-(hydroxy-methyl)-propane-1:3-diol

Versene: ethylene diamine tetra-acetic acid

APPENDIX IV

Purification of Organic Solvents

In general, the methods described by Weissberger and Proskauer (1935) were used. The fractionating column used was packed with Raschig rings and had dimensions of 40 x 3.5 cm.; final distillations were carried out in all-glass apparatus using a calcium chloride tube when solvents were required anhydrous. Usually an electric heating mantle was used for distillation flasks; solvents were stored in brown bottles in a dark cupboard.

Acetone: Acetone, B.P., the product of T. & H. Smith, Ltd., was refluxed for 2 hr. with potassium permanganate (10 g./l.) and NaOH (4 g./l.). It was then distilled, refluxed with freshly roasted potassium carbonate (50 g./l.) and distilled again under anhydrous conditions.

Benzene: National Coal Board Benzole No. 1 was distilled, the fraction boiling at 78-82° being collected; this was washed five times with water and twice with concentrated H_2SO_4, being shaken periodically with the latter during 24 hr. It was then washed with water till the washings were neutral, dried over $CaCl_2$ and distilled.

Chloroform: Chloroform, B.P., the product of T. & H. Smith, Ltd., was washed four times with water, dried over $CaCl_2$ and distilled. The fraction boiling at 59-63° was collected and redistilled, the first 50 ml. of distillate being rejected. This solvent was prepared freshly as required.

Diethyl Ether: Ether, A.R., the product of J.F. MacFarlan & Co., was dried over sodium wire and distilled under anhydrous conditions.

Ethanol: Absolute re-rectified alcohol, the product of the Distillers Co., Ltd., was refluxed with NaOH (4 g./l.) for 8 hr. and distilled twice under anhydrous conditions.

n- Hexane: n-Hexane, obtained from Carless, Capel, Leonard & Co., was allowed to stand in a large separating funnel with three batches of concentrated H_2SO_4 (100 ml./l.), with occasional shaking, for 12 hr. After washing with 2N-NaOH (100 ml./l.) and then with water until the washings were neutral, it was dried over $CaCl_2$ and distilled, the first 50 ml. of the distillate being rejected.

Methanol: I.C.I. methanol was treated in the same way as ethanol.

Petroleum Ether: Petroleum ether (b.p. range 40-60°), manufactured by W. & J. George & Becker, Ltd., was distilled, the fraction boiling at 40-60° being collected.

Pyridine: Commercial pyridine was dried over freshly roasted BaO, filtered, and distilled under anhydrous conditions, the fraction boiling at 112-117° being collected and stored over a few lumps of BaO.

Toluene: 'Caterex' brand toluene (Petrochemicals, Ltd.) was distilled, the fraction boiling at 107-113° being collected.

APPENDIX V

Numerical Details of Figures

1. The reduction of TPN by 25,000g supernatant and by dialysed supernatant

Time in min.	Optical Density at 340 mμ.	
	25,000g supernatant	Dialysed supernatant
0.5	0.235	0.157
1.0	0.307	0.159
1.5	0.401	0.162
2.0	0.484	0.165
2.5	0.560	0.166
3.0	0.630	0.170
3.5	0.680	0.172
4.0	0.740	0.174
4.5	0.780	0.176
5.0	0.810	0.180
6.0	0.880	0.184
9.0	0.900	0.198

2. The oxidation of TPNH by dialysed 25,000g supernatant

Time in min.	Optical Density at 340 mμ.	
	TPNH + dialysed supernatant	TPNH + water
0.5	0.340	0.438
1.0	0.322	0.438
1.5	0.313	0.438
2.0	0.300	0.438
2.5	0.298	0.438
3.0	0.288	0.438
3.5	0.278	
4.0	0.272	
4.5	0.267	
5.0	0.261	
5.5	0.254	
6.0	0.252	
6.5	0.241	
7.0	0.241	
7.5	0.240	
8.0	0.238	
8.5	0.233	
9.0	0.230	
9.5	0.228	
10.0	0.225	0.438

3. Metabolism of progesterone with time by dialysed 25,000g supernatant

Time in min.	Progesterone metabolized (%)
0	0
15	6
30	17
45	28
60	38
75	39

5. TPN reduction by concurrent oxidation of glucose-6-phosphate in the absence and in the presence of GA

Time in min.	Optical Density at 340 mμ.	
	GA absent	GA present
1	0.329	0.184
2	0.382	0.284
3	0.406	0.368
4	0.418	0.407
5	0.427	0.425
6	0.431	0.433
7	0.436	0.438
8	0.439	0.440
9	0.441	0.442
10	0.444	0.444
20	0.458	0.457

6. TPN reduction by concurrent oxidation of glucose-6-phosphate in the absence and in the presence of methyl vinyl ketone

Time in min.	Optical Density at 340 mμ.	
	methyl vinyl ketone absent	methyl vinyl ketone present
1	0.251	0.196
2	0.350	0.296
3	0.390	0.363
4	0.412	0.391
5	0.424	0.412
6	0.434	0.427
7	0.437	0.433
8	0.442	0.441
9	0.445	0.448
10	0.450	0.451
20	0.470	0.470

7. Alumina chromatography of urinary dinitrophenylhydrazones

Fraction Number	Optical Density at 370 mμ. C	M	P	ad	a
1	0.422	0.269	0.350	0.800	0.936
2	2.816	3.360	2.700	2.620	2.352
3	0.800	1.205	1.030	0.180	0.204
4	0.354	0.551	0.346	0.301	0.246
5	0.274	0.457	0.253	0.224	0.189
6	0.185	0.251	0.178	0.178	0.122
7	0.556	0.782	0.425	1.450	0.700
8	0.808	0.864	0.738	1.550	0.858
9	0.726	0.860	1.099	0.819	0.571
10	0.608	0.821	1.200	0.560	0.429
11	0.459	0.649	0.881	0.478	0.474
12	0.336	0.552	0.536	0.431	0.695
13	0.252	0.364	0.328	0.370	0.500
14	0.186	0.268	0.223	0.294	0.325
15	0.139	0.196	0.154	0.226	0.244
16	0.269	0.342	0.233	0.425	0.416
17	0.802	1.150	0.698	0.800	0.804
18	0.953	1.330	0.978	0.739	0.712
19	0.739	1.130	0.729	0.548	0.469
20	0.981	1.510	0.980	0.700	0.622
21	0.778	1.410	0.769	0.719	0.669
22	0.829	1.400	0.809	0.878	0.851
23	0.678	1.400	0.700	0.701	0.729
24	0.812	1.110	0.818	0.670	0.679
25	0.639	1.140	0.629	0.441	0.485
26	0.619	1.480	0.576	0.675	0.629
27	0.640	1.700	0.621	0.729	0.681
28	1.700	1.970	1.690	1.700	1.650

C = Control Ketones

M = Metabolic Ketones

P = Pregnanedione

ad = allopregnanedione

a = allopregnanolone

APPENDIX VI

References

Adler, E., von Euler, H., Gunther, G. & Plass, M. (1939). Biochem. J. 33, 1028.

Altmann, S.M. & Crook, E.M. (1953). Nature, Lond. 171, 76.

Axelrod, J. (1955). J. Pharmacol. 115, 259.

Beaton, J.M. & Spring, F.S. (1955). J. chem. Soc. 3126.

Berliner, D.L. & Salhanick, H.A. (1956). J. clin. Endocrin. 16, 903.

Berliner, D.L. & Wiest, W.G. (1956). J. biol. Chem. 221, 449.

Bernstein, S. & Lenhard, R.H. (1953). J. org. Chem. 18, 1146.

Berthet, J., Berthet, L., Appelmans, F. & De Duve, C. (1952). Biochem. J. 50, 182.

Boscott, R.J. (1952). Ciba Foundation Colloquia on Endocrinology II, 327.

Bradbury, J.T., Brown, W.E. & Gray, L.A. (1950). Recent Progr. Hormone Res. V, 151.

Brendler, H. (1951). Science 114, 61.

Brockmann, H. & Schodder, H. (1941). Ber.dtsch. chem. Ges. 74, 73.

Brodie, B.B., Axelrod, J., Cooper, J.R., Gaudette, L., La Du, B.N., Mitoma, C. & Udenfriend, S. (1955). Science 121, 603.

Brooks, R.V., Klyne, W., Miller, E. & Paterson, J.Y.F. (1952). Biochem. J. 51, 694.

Brownie, A.C. (1955) Ph.D. Thesis: University of Edinburgh.

Brownie, A.C. & Grant, J.K. (1954). Biochem. J. 57, 255.

Brownie, A.C., Grant, J.K. & Davidson, D.W. (1954). Biochem. J. 58, 218.

Bucher, N.L.R. & McGarrahan, K. (1955). Fed. Proc. 14, 187.

Bush, I.E. (1952). Biochem. J. 50, 370.

Butenandt, A. & Mamoli, L. (1935). Ber. dtsch. chem. Ges. 68, 1847.

Butler, G.C. & Marrian, G.F. (1938). J. biol. Chem. 124, 237.

Callow, N.H., Callow, R.K. & Emmens, C.W. (1938). Biochem. J. 32, 1312.

Caspi, E., Lindberg, M.C., Hayano, M., Cohen, J.L., Matsuba, M., Rosenkrantz, H. & Dorfman, R.I. (1956). Arch. Biochem. 61, 271.

Comandon, J. & de Fonbrunne, P. (1939). C.R.Soc. Biol., Paris 130, 740.

Cox, R.I. & Marrian, G.F. (1953). Biochem. J. 54, 353.

Davis, M.E. & Plotz, E.J. (1956). Acta endocr., Copenhagen 21, 245.

De Duve, C. & Berthet, J. (1954). Int. Rev. Cytol. III, 225.

Dobriner, K. & Lieberman, S. (1950). In "A Symposium on Steroid Hormones" p. 46. Ed. by Gordon, E.S. Madison: The University of Wisconsin Press.

Dobriner, K., Lieberman, S., Rhoads, C.P. & Taylor, H.C., Jr. (1948). Obstet. Surv. Baltim. 3, 677.

Doisy, E.A., Jr., Bocklage, B.C., Shen, N.H., & Valcourt, A.F. (1952). Science 116, 520.

Dorfman, R.I. (1954). Recent Progr. Hormone Res. IX,5.

Dorfman, R.I. (1955a). In "The Hormones" III, 589. Ed. by Pincus, G. & Thimann, K.V. Academic Press, Inc., New York.

Dorfman, R.I. (1955b). Ann. N.Y. Acad. Sci. 61, 291.

Dorfman, R.I., Ross, E. & Shipley, R.A. (1948). Endocrinology 42, 77.

Duryee, W.R. (1950). Ann. N.Y. Acad. Sci. 50, 926.

Eisenstein, A.B. (1956) Proc. Soc. exp. Biol., N.Y. 91, 657.

Engel, L.L., Thorn, G.W. & Lewis, R.A. (1941). J. biol. Chem. 137, 205.

Fish, W.R., Dorfman, R.I. & Young, W.C. (1942). J. biol. Chem. 143, 715.

Forchielli, E. & Dorfman, R.I. (1956). J. clin. Endocrin. 16, 947.

Forchielli, E., Rosenkrantz, H. & Dorfman, R.I. (1955). J. biol. Chem. 215, 713.

Frunder, H. & Richter, G. (1955). Hoppe-Seyl. Z. 302, 1.

Fukushima, D.K. & Rosenfeld, R.S. (1954). In "Chemical Pathways of Metabolism" I, 349. Ed. by Greenberg, D.M. Academic Press Inc., New York.

Girard, A. & Sandulesco, G. (1936). Helv. chim. acta 19, 1095.

Glenn, E.M. & Recknagel, R.O. (1955). Proc. Soc. exp. Biol. N.Y. 89, 153.

Glenn, E.M., Siu, P.M.L. & Recknagel, R.O. (1956). Proc. Soc. exp. Biol. N.Y. 91, 332.

Goldstein, L. & Plaut, W. (1955). Proc. nat. Acad. Sci., Wash. 41, 874.

Grady, H.J., Elliott, W.H., Doisy, E.A., Jr., Bocklage, B.C. & Doisy, E.A. (1952). J. biol. Chem. 195, 755.

Grant, J.K. (1950). Ph.D. Thesis: University of Edinburgh.

Grant, J.K. (1955). Chem. and Ind., 942.

Grant, J.K. & Brownie, A.C. (1955). Biochim. biophys. Acta 18, 433.

Grant, J.K. & Marrian, G.F. (1950). Biochem. J. 47, 497.

Green, D.E. (1952). J. cell. comp. Physiol. 39, Supplement 2, 75.

Green, D.E. (1954). Tex. Rep. Biol. Med. 12, 284.

Green, D.E., Loomis, W.F. & Auerbach, V.H. (1948). J. biol. Chem. 172, 389.

Greene, R.R., Burrill, M.W. & Thomson, D.M. (1940). Endocrinology 27, 469.

Groen, J., Pelser, H., Willebrands, A.F. & Kamminga, C.E. (1951). New Eng. J. Med. 244, 471.

Groen, J., Pelser, H.E., Willebrands, A.F., Veer, W.L.C. & Van der Kerk, G.J.M. (1953). Ned. Tijdschr. Geneesk 97, 3290.

Groen, J., Willebrands, A.F. & Pelser, H.E. (1956). Acta med. scand. Supplementum 312, 745.

Hagopian, M., Pincus, G., Carlo, J. & Romanoff, E.B. (1956). Endocrinology 58, 387.

Handler, P. & Klein, J.R. (1942). J. biol. Chem. 143, 49.

Harold, F.M., Abraham, S. & Chaikoff, I.L. (1956). J. biol. Chem. 221, 435.

Hartmann, M. & Locher, F. (1935). Helv. chim. acta 18, 160.

Haskins, A.L., Jr. (1950). Proc. Soc. exp. Biol. N.Y. 73, 439.

Hayano, M., Lindberg, M.C., Wiener, M., Rosenkrantz, H. & Dorfman, R.I. (1954). Endocrinology 55, 326.

Heard, R.D.H., Jacobs, R., O'Donnell, V., Peron, F.G., Saffran, J.C., Solomon, S.S., Thompson, L.M., Willoughby, H. & Yates, C.H. (1954). Recent Progr. Hormone Res. IX, 383.

Hechter, O. & Pincus, G. (1954). Physiol. Rev. 34, 459.

Hechter, O., Zaffaroni, A., Jacobsen, R.P., Levy, H., Jeanloz, R.W., Schenker, V. & Pincus, G. (1951). Recent Progr. Hormone Res. VI, 215.

Hill, D.L., Petersen, W.E. & Cohen, S.H. (1954). J. Dairy Sci. XXXVII, 355.

Hinsberg, K., Pelzer, H. & Seuken, A. (1956). Biochem. Z. 328, 117.

Hoffman, M.M. (1942). Canad. med. Ass. J. 47, 424.

Hogeboom, G.H. & Barry, G.T. (1948). J. biol. Chem. 176, 935.

Hogeboom, G.H. & Schneider, W.C. (1950). J. nat. Cancer Inst. 10, 983.

Hogeboom, G.H. & Schneider, W.C. (1952a). Nature, Lond. 170, 374.

Hogeboom, G.H. & Schneider, W.C. (1952b). J. biol. Chem. 197, 611.

Hooker, C.W. & Forbes, T.R. (1949). Endocrinology 44, 61.

Horecker, B.L. & Kornberg, A. (1948). J. biol. Chem. 175, 385.

Hübener, H.J. & Schmidt-Thomé, J. (1955). Hoppe-Seyl. Z. 299, 240.

Hunter, F.E., Jr., Levy, J., Davis, J. & Carlat, L. (1956). Fed. Proc. 15, 278.

Isselbacher, K.J. & Tomkins, G. (1954). Fed. Proc. 13, 236.

Jeener, R. & Szafarz, D. (1950). Arch. Biochem. 26, 54.

Kerr, L.M.H. & Bauld, W.S. (1953). Biochem. J. 55, 872.

Klopper, A., Michie, E.A. & Brown, J.B. (1955) J. Endocrin. 12, 209.

Klyne, W. & Rankeillor, J. (1947). Chem. and Ind., 224.

Klyne, W. & Wright, A.A. (in the press). J. Endocrin.

Knox, W.E., Auerbach, V.H. & Lin, E.C.C. (1956). Physiol. Rev. 36, 164.

Kochakian, C.D., Haskins, A.L., Jr., & Bruce, R.A. (1944). Amer. J. Physiol. 142, 326.

Kornberg, A. (1950). J. biol. Chem. 182, 779.

Kyle, T.I. & Marrian, G.F. (1951). Biochem. J. 49, 162.

Laird, A.K., Nijgaard, O., Ris, H. & Barton, A.D. (1953). Exp. Cell Res. 5, 147.

Le Page, G.A. (1949a). Biochem. Prep. 1, 28.

Le Page, G.A. (1949b). In "Manometric Techniques and Tissue Metabolism" p. 215. Ed. by Umbreit, W.W., Burris, R.H. & Stauffer, J.F. Burgess Publishing Co., Minneapolis.

Le Page, G.A. & Mueller, G.C. (1949). J. biol. Chem. 180, 975.

Le Page, G.A. & Schneider, W.C. (1948). J. biol. Chem. 176, 1021.

Levy, H., Jeanloz, R.W., Jacobsen, R.P., Hechter, O., Schenker, V. & Pincus, G. (1954). J. biol. Chem. 211, 867.

Levy, H. & Kushinsky, S. (1955). Arch. Biochem. 55, 290.

Lieberman, S., Dobriner, K., Hill, B.R., Fieser, L.F. & Rhoads, C.P. (1948). J. biol. Chem. 172, 263.

Lieberman, S. & Teich, S. (1953). Pharmacol. Rev. 5, 285.

Littlefield, J.W., Keller, E.B., Gross, J. & Zamecnik, P.C. (1955). J. biol. Chem. 217, 111.

Longwell, B.B. & Gassner, F.X. (1947). Fed. Proc. 6, 272.

Lorch, I.J. & Danielli, J.F. (1950). Nature, Lond. 166, 329.

Lynn, W.S., Jr. (1956). Fed. Proc. 15, 305.

Lythgoe, B. & Trippett, S. (1950). J. chem. Soc. 1983.

McIlwain, H. & Rodnight, R. (1949). Biochem. J. 45, 337.

Mann, P.J.G. & Quastel, J.H. (1941). Biochem. J. 35, 502.

Marker, R.E. (1939). J. Amer. chem. Soc. 61, 1287.

Marker, R.E., Kamm, O., Crooks, H.M., Jr., Oakwood, T.S., Lawson, E.J. & Wittle, E.L. (1937). J. Amer. chem. Soc. 59, 2297.

Marker, R.E. & Rohrmann, E. (1939). J. Amer. chem. Soc. 61, 3476.

Marker, R.E., Wittle, E.L. & Lawson, E.J. (1938). J. Amer. chem. Soc. 60, 2931.

Marrian, G.F. (1929). Biochem. J. 23, 1090.

Marrian, G.F. (1938). Harvey Lect. XXXIV, 37.

Marrian, G.F. (1949). Recent Progr. Hormone Res. IV, 3.

Mason, H.L. & Kepler, E.J. (1945). J. biol. Chem. 161, 235.

Masson, G. & Hoffman, M.M. (1945). Endocrinology 37, 111.

Mazia, D. (1952). In "Modern Trends in Physiology and Biochemistry" p. 77. Ed. by Barron, E.S.G. Academic Press, Inc., New York.

Mazia, D. (1956). In "Enzymes: Units of Biological Structure and Function" p. 261. Ed. by Gaebler, O.H. Academic Press, Inc., New York.

Mehler, A.H., Kornberg, A., Grisolia, S. & Ochoa, S. (1948). J. biol. Chem. 174, 961.

Miller, W.R. & Turner, C.W. (1955). Proc. Soc. exp. Biol. N.Y. 90, 142.

Miller, W.R., Turner, C.W., Fukushima, D.K. & Salamon, I.I. (1956). J. biol. Chem. 220, 221.

Molhuysen, J.A., Gerbrandy, J., De Vries, L.A., De Jong, J.C., Lenstra, J.B., Turner, K.P. & Borst, J.G.G. (1950). Lancet 2, 381.

Paigen, K. (1954). J. biol. Chem. 206, 945.

Pearlman, W.H. (1948). In "The Hormones" I, 407. Ed. by Pincus, G. & Thimann, K.V. Academic Press, Inc., New York.

Pearlman, W.H. & Cerceo, E. (1948). J. biol. Chem. 176, 847.

Pearlman, W.H., Pincus, G. & Werthessen, N.T. (1942). J. biol. Chem. 142, 649.

Pelser, H.E., Willebrands, A.F., Frenkel, M., van der Heide, R.M. & Groen, J. (1953). Metabolism 2, 322.

Pelser, H.E., Willebrands, A.F. & Groen, J. (1953). Ned. Tijdschr. Geneesk 97, 1900.

Plaut, G.W.E. & Sung, S. (1954). J. biol. Chem. 207, 305.

Plotz, E.J. & Davis, M.E. (1956). Acta endocr., Copenhagen, 21, 259.

Quastel, J.H. (1956). In "Enzymes: Units of Biological Structure and Function" p. 523. Ed. by Gaebler, O.H. Academic Press, Inc., New York.

Reaven, G.M. (1955). Endocrinology 57, 580.

Reich, H., Crane, K.F. & Sanfilippo, S.J. (1953). J. org. Chem. 18, 822.

Reich, H., Sanfilippo, S.J. & Crane, K.F. (1952). J. biol. Chem. 198, 713.

Revers, F.E. (1946). Ned. Tijdschr. Geneesk. 90, 135.

Revers, F.E. (1948). Ned. Tijdschr. Geneesk 92, 2968, 3567.

Revers, F.E. (1951). Ned. Tijdschr. Geneesk 95, 120.

Revers, F.E. (1956). Acta med. scand. Supplementum 312, 749.

Riegel, B., Hartop, W.L., Jr., & Kittinger, G.W. (1950). Endocrinology 47, 311.

Roberts, S. & Szego, C.M. (1955). Annu. Rev. Biochem. 24, 543.

Rongone, E.L., Bocklage, B.C., Strength, D.R. & Doisy, E.A. (1956). Fed. Proc. 15, 339.

Runnström, J. (1952). In "Modern Trends in Physiology and Biochemistry" p. 47. Ed. by Barron, E.S.G. Academic Press, Inc., New York.

Ruzicka, L., Jeger, O. & Ingold, W. (1943). Helv. chim. acta 26, 2278.

Ryan, K.J. & Engel, L.L. (1956). J. Amer. chem. Soc. 78, 2654.

Samuels, L.T. & West, C.D. (1952). Vitam. & Horm. X, 251.

Savard, K. (1953). J. biol. Chem. 202, 457.

Schneider, J.J. (1952). J. biol. Chem. 199, 235.

Schneider, W.C. (1953). J. Histochem. 1, 212.

Schneider, W.C. & Hogeboom, G.H. (1950). J. biol. Chem. 183, 123.

Selye, H. (1941). J. Pharmacol. 71, 236.

Shen, F.N.H. & Doisy, E.A., Jr. (1953). Fed. Proc. 12, 267.

Shen, N.C., Elliott, W.H., Doisy, E.A., Jr. & Doisy, E.A. (1954). J. biol. Chem. 208, 133.

Slaunwhite, W.R., Jr. & Samuels, L.T. (1956). J. biol. Chem. 220, 341.

Stevensen, W.C. (1947). Amer. J. comp. Med. vet. Sci. 11, 25.

Straub, F.B. (1942). Hoppe-Seyl. Z. 275, 63.

Taylor, W. (1953). Ph.D. Thesis: University of Edinburgh.

Taylor, W. (1954). Biochem. J. 56, 463.

Taylor, W. (1955). Biochem. J. 60, 380.

Tomkins, G. & Isselbacher, K.J. (1954). J. Amer. chem. Soc. 76, 3100.

Ungar, F. (1955). See Forchielli *et al.* (1955).

Ungar, F., Davis, J.W., Rosenkrantz, H. & Dorfman, R.I. (1954). J. biol. Chem. 207, 375.

Ungar, F., Dorfman, R.I., Stecher, R.M. & Vignos, P.J., Jr. (1951). Endocrinology 49, 440.

van Katwijk, V.M. & Huis in't Veld, L.G. (1955). Rec. Trav. chim. Pays-Bas 74, 889. Cited from Analyt. Abstracts 3, 1819 (1956).

Venning, E.H. & Browne, J.S.L. (1937). Endocrinology 21, 711.

von Euw, J. & Reichstein, T. (1941). Helv. chim. acta 24, 879.

Weissberger, A. & Proskauer, E. (1935). "Organic Solvents", Oxford: Clarendon Press.

Werbin, H., Le Roy, G.V. & Bergenstal, D.M. (1955). J. Lab. clin. Med. 46, 964.

Wiest, W.G. (1956). J. biol. Chem. 221, 461.

Wiest, W.G., Berliner, D.L. & Woods, G. (1954). Fed. Proc. 13, 417.

Wilson, C.W.M. (1954). Ph.D. Thesis: University of Edinburgh.

Wiswell, J.G. & Samuels, L.T. (1953). J. biol. Chem. 201, 155.

Zollinger, H.U. (1950). Rev. Hemat. 5, 696.