bone in the human being is due rather to the relative size and contents of the spaces within the bones than to a true similarity in degree of calcification.

The variation in composition of the alveolar bone of the three species of mammal is particularly interesting in relation to function. This bone is probably subjected to a greater number of changes in stress and strain than any other bone in the body. Also, of the three species examined, the rabbit has continuously growing molars, the rat has continuously growing incisors only, and the cat has a permanent secondary dentition. The relation of composition of the bone to these functions cannot, of course, be interpreted from the present results.

SUMMARY

1. The mean calcium content of the cortical femoral bones taken from groups of adult cats,

rabbits and rats varies only from 27.3 to 27.9%. These values are higher than the value quoted for the comparable human bone.

2. The cancellous femoral bone from the three species of animal contains significantly less calcium than the cortical bone, ranging from 25.1 to 26.2%.

3. The nitrogen contents of both cortical and cancellous femoral bone from the cat are higher (4.13 and 5.00%, respectively) than those for the corresponding bones in the rabbit (3.36 and 4.43%) and rat (3.35 and 3.94%).

4. The mean calcium and nitrogen contents of the alveolar bone differ from species to species, ranging from 28.0 and 2.94%, respectively, for the rabbit to 25.3 and 4.83% for the cat.

We should like to acknowledge the help of the Nuffield Research Foundation who generously financed the Research Unit. One of us (S. M. W.) would also like to thank the Medical Research Council for a grant.

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Studies on the Metabolism of Progesterone and Related Steroids *in vitro*

1. THE METABOLISM OF PREGNANE-3 α :20 α -DIOL BY LIVER PREPARATIONS

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(Received 9 June 1950)

It has been established that when progesterone is administered to human subjects by injection or orally a considerable proportion remains unaccounted for by the known progesterone metabolites, principally pregnane-3 α :20 α -diol, which are subsequently excreted in the urine (see Marrian (1949) and Sommerville & Marrian (1950*a*, *b*) for reviews of previous work). Recently, Sommerville & Marrian (1950*a*) found that when pregnane-3 α :20 α -diol was administered orally to human subjects the proportion of administered steroid subsequently excreted in the urine as 'pregnanediol' was no higher than when progesterone was administered to the same subjects by the same route. This finding led the

authors to suggest speculatively 'that administered progesterone may be largely reduced to pregnanediol in the body, and that the low recoveries of urinary pregnanediol following administration of the hormone, may be due to excretion of the reduction product by other routes or further metabolism into products so far unidentified or to a combination of both processes'.

In order to explore the possibility of the further metabolism in the body of pregnanediol, the present authors decided to investigate the effect of tissue preparations *in vitro* upon pregnane-3 α :20 α -diol. In the first instance experiments were carried out with liver preparations, since previous work by many

authors has clearly shown that the liver is an important site of steroid hormone metabolism. (See Fieser & Fieser (1949) for references.)

EXPERIMENTAL

Use of pregnane-3 α :20 α -diol dihemisuccinate as substrate

Since pregnane-3 α :20 α -diol is only very sparingly soluble in aqueous media, it was necessary to employ some suitable water-soluble derivative. In these preliminary experiments the dihemisuccinate was used in preference to the naturally occurring glucuronide, since the preparation of the latter in a state of satisfactory purity is time-consuming and difficult (Sutherland & Marrian, 1947). Schneider & Mason (1948*a, b*) and Pearlman & De Meo (1949) have successfully employed the hemisuccinates of other steroids for *in vitro* metabolism experiments.

Preparation of pregnane-3 α :20 α -diol dihemisuccinate (PDHS). Pure pregnane-3 α :20 α -diol (m.p. 236–237.5° (corr.); 500 mg.) and succinic anhydride (1.5 g.) were dissolved in dry pyridine (12.5 ml.) and heated for 2 hr. at 100°. After cooling, the reaction mixture was diluted to about 200 ml. with ice and water and extracted with ether. The ethereal extract, after thorough washing with 2*N*-H₂SO₄ to remove pyridine, was extracted with 5% (w/v) Na₂CO₃. This extract, after washing once with ether, was acidified to litmus with H₂SO₄, and extracted with ether. The ethereal extract, after washing thoroughly with water, was dried over CaCl₂ and evaporated, yielding 480 mg. crude PDHS. After crystallization from ethanol-hexane, fine white needles, m.p. 146.5–147.5° (corr.), were obtained. (Found, after drying at 80° *in vacuo* over P₂O₅: C, 67.4; H, 8.5. C₃₃H₄₄O₈ requires C, 67.2; H, 8.5%.)

Solutions of PDHS containing about 0.6 mg./ml. were prepared as required by heating the solid with the Ca-free phosphate saline (pH 7.4) of Krebs & Eggleston (1940) (subsequently referred to as 'phosphate saline') to 75°, cooling the mixture to 40° and filtering. Since on standing at room temperature PDHS tended to separate from such filtered solutions the required volumes were measured while still warm into the reaction vessels placed in a bath at 37°. As prolonged heating at 75° was required to dissolve accurately weighed amounts of dried PDHS completely in saline, it was found to be more satisfactory to determine the pregnanediol contents of the PDHS solutions by analysis as described below.

Liver preparations. Experimental animals were female rats of the Wistar strain approximately 1 year old, and female Chinchilla rabbits approximately 6 months old. They were killed by dislocation of vertebrae in the neck. The livers were rapidly removed and placed in covered beakers standing in crushed ice.

Liver slices of about 0.3 mm. thickness were prepared by the method of Deutsch (1936), as modified by Cohen (1945). They were not immersed in any solution, but were placed when cut in a covered Petri dish standing on ice. A moist atmosphere was maintained in the dish by lining the bottom with hardened filter paper moistened with phosphate saline. The filter paper was marked off in sectors corresponding to the number of samples required, and in order to ensure uniform sampling, slices were piled serially in the sectors. Samples were weighed on a torsion balance.

The acetone-dried liver powder was prepared by homogenizing fresh liver for 3 min. with ten times its volume of dry acetone cooled to –5°, in the well chilled vessel of an Atomix blender (Measuring and Scientific Equipment, Ltd.). The homogenate was filtered with suction through a chilled Büchner funnel. The filter cake was washed twice in the blender with 5 vol. cold dry acetone, and twice with 5 vol. cold dry ether, and dried *in vacuo* over P₂O₅ for 1 hr. The powder was not stored, but was used immediately after drying.

Determination of pregnane-3 α :20 α -diol in the presence of liver slices. For the purpose of these experiments it was necessary to devise a method for the quantitative determination of about 0.5–1.0 mg. pregnane-3 α :20 α -diol as the dihemisuccinate in the presence of about 250 mg. liver slices incubated in 2.5 ml. phosphate saline. After many preliminary experiments, the details of which are not reported here, the following procedure was adopted.

The incubated liver-slice suspension was heated at 100° for 1 hr. after the addition of 2.5 ml. phosphate saline and 5 ml. 10% (w/v) KOH, in order to dissolve the tissue and to hydrolyse any dihemisuccinate which had escaped hydrolysis by the tissue esterase during the incubation. The solution was then transferred to a small continuous extraction apparatus with three 2 ml. volumes of ethanol. After the addition of 8 ml. water the mixture was extracted with ether for 3 hr. with the addition of two further 5 ml. quantities of ethanol at the end of the first and second hours. Preliminary experiments had shown the necessity for these further additions of ethanol, since without them pregnanediol separated in the aqueous phase and was then only very slowly extracted by the ether. The ethereal extract was washed twice with 15 ml. of water, evaporated to dryness and transferred to a 25 ml. conical centrifuge tube with ethanol.

The pregnanediol in the residue was finally determined by the method of Sommerville, Gough & Marrian (1948), the residue being treated as though it were a toluene-soluble neutral urine fraction. Three modifications were introduced into the procedure of Sommerville *et al.* (1) Two precipitations were found to be sufficient to purify the pregnanediol for colorimetric determination. (2) As recommended by Verly, Sommerville & Marrian (1950) the Whatman no. 1 filter papers used for filtering the ethanolic solutions of the finally purified pregnanediol, after treatment with charcoal, were thoroughly washed with three 2 ml. volumes of hot ethanol, before use. (3) The filtrate was made up to 25 ml. with ethanol and 5 ml. volumes were taken for the final pregnanediol determination.

As a final test of the accuracy and sensitivity of the procedure a series of 'recovery' experiments was carried out in which pure pregnane-3 α :20 α -diol was added to incubated suspensions of rat-liver slices. Incubated suspensions of rat-liver slices without added pregnanediol were used in 'blank' determinations. These experiments were carried out as follows.

Known amounts of pregnane-3 α :20 α -diol were added in ethanolic solution to 2.5 ml. portions of phosphate saline. These were then evaporated to about 1.25 ml. to remove most of the ethanol, and then made up to 2.5 ml. with water.

Samples of rat-liver slices (250 mg.) were incubated at 37.5° for 2 hr. in 2.5 ml. phosphate saline with continuous shaking in O₂. After incubation 5 ml. 10% (w/v) KOH were added to each sample, which was then poured into the pregnanediol suspensions prepared as described above. In

the case of 'blank' determinations the pregnanediol suspension was replaced by 2.5 ml. phosphate saline. The mixture was then treated as previously described. The results of these experiments shown in Table 1 indicate that the method is reasonably satisfactory for the determination of as little as about 0.5 mg. pregnanediol added to 250 mg. of incubated liver slices.

Recovery experiments were not carried out with acetone-dried liver powders. Experiments to be described later (see Fig. 2) showed that using amounts of acetone-dried liver powder equivalent to 1 g. fresh liver 'blank' values were low and the recoveries of pregnanediol from liver powder suspensions in 'control' experiments were satisfactory.

Table 1. *Recovery of pregnane-3 α :20 α -diol added to 250 mg. rat-liver slices after incubation*

(In four 'blank' experiments, the following quantities of apparent pregnanediol were recovered: 0.025, 0.025, 0.030 and 0.030 mg. The mean 'blank' value (0.028 mg.) was used to correct the values given in column 2.)

Pregnanediol added (mg.)	Pregnanediol recovered (corrected for 'blank') (mg.)	Recovery of added pregnanediol (%)
1.050	0.987	94
1.050	0.972	93
1.050	1.017	97
0.788	0.687	87
0.788	0.687	87
0.788	0.737	94
0.788	0.717	91
0.525	0.497	95
0.525	0.487	93
0.525	0.517	98
0.525	0.497	95

The incubation of PDHS with liver preparations

Each experiment was conducted in four parts.

(1) *Incubation experiments.* Duplicate 250 mg. samples of liver slices or amounts of acetone-dried powder equivalent to 1 g. fresh liver were added to 25 ml. conical flasks containing 2.5 ml. volumes of PDHS solution. After gassing with O_2 the flasks were shaken for 2 hr. (unless otherwise stated) in a water bath at 37.5°. After incubation pregnanediol was determined as already described.

(2) *'Control' experiments.* In these, duplicate 2.5 ml. volumes of PDHS solution were mixed with samples of liver preparations previously incubated in 2.5 ml. phosphate saline. Pregnanediol in the mixture was determined as before without the addition of 2.5 ml. phosphate saline.

(3) *'Blank' experiments.* These were introduced in order to check that incubation of liver preparations did not produce material chromogenic in the H_2SO_4 reaction, and which was not removed by the pregnanediol purification method employed. The procedure was exactly as described in (1) above with the exception that the PDHS solution was replaced by saline.

(4) *Determination of pregnanediol content of PDHS solution.* This determination was carried out on duplicate 2.5 ml. volumes of PDHS solutions exactly as described in (1) above without liver preparations, and omitting the incubation and the two precipitations.

Fig. 1. Incubation of rat-liver slices with PDHS. A, pregnanediol recovered from 'controls'; B, pregnanediol recovered after incubation; C, apparent pregnanediol recovered from 'blanks'. Double lines at the ends of bars indicate duplicate values.

Fig. 2. Incubation with PDHS of acetone-dried liver powder of rat (Exps. 1 and 2) and rabbit (Exp. 3). A, pregnanediol recovered from 'controls'; B, pregnanediol recovered after incubation; C, apparent pregnanediol recovered from 'blanks'. Double lines at the ends of bars indicate duplicate values.

It may be seen from the results of these experiments shown in Figs. 1 and 2 that pregnanediol disappears on incubation of PDHS with rat- and rabbit-liver preparations. This disappearance is evidently

due to an enzymic system, since the addition of rat-liver acetone powder to boiling PDHS solution, with further 10 min. heating at 100° before incubation, destroys the activity of the liver preparation (Fig. 3).

Fig. 3. Effect of heating upon the activity of rat acetone-dried liver powder in metabolizing pregnanediol. A, pregnanediol recovered from 'controls'; B, pregnanediol recovered after incubation; C, pregnanediol recovered after incubation with acetone-dried powder previously heated at 100°; D, apparent pregnanediol recovered from 'blanks'. Double lines at the ends of bars indicate duplicate values.

Fig. 4. Incubation of rat-liver slices with PDHS—influence of acid hydrolysis on amount of pregnanediol recoverable. A, pregnanediol recovered from 'controls'; B, pregnanediol recovered after incubation; C, as A but with acid hydrolysis; D, as B but with acid hydrolysis; E, apparent pregnanediol recovered from 'blanks'. Double lines at the ends of bars indicate duplicate values.

There is good evidence that borneol, phenol and *p*-aminophenol (Lipschitz & Bueding, 1939; Levvy

& Storey, 1949; Storey, 1950) become conjugated to a small extent with glucuronic acid on incubation with liver slices. There is also some indication that phenol may be conjugated with H_2SO_4 under similar conditions (De Meio & Arnolt, 1944; Bernheim & Bernheim, 1943). Accordingly, the possibility had to be considered that the observed disappearance of pregnanediol on incubation with liver preparations might have been due to such a conjugation. A series of experiments with rat-liver slices was therefore carried out in which the extraction procedure was modified so as to include an acid hydrolysis. The modified procedure employed was as follows.

After heating with KOH the incubated mixture was neutralized to litmus with conc. HCl and an excess of 1 ml. of this acid was added. The acid mixture was then heated for 10 min. at 100°, cooled, made alkaline to litmus with KOH and transferred to the extraction apparatus. In one experiment the adequacy of the 10 min. hydrolysis was checked by prolonging the heating for 1 hr. Since the 'controls' in this experiment were subjected to identical treatment, the destruction of pregnanediol resulting from the prolonged heating with acid should not vitiate the result.

The results of these experiments, shown in Fig. 4, make it clear that with the conditions of acid hydrolysis employed there was no increased recovery of pregnanediol after incubation with the liver slices. Had the pregnanediol become conjugated with glucuronic acid it is reasonably certain that the conjugate would have been hydrolysed under these conditions, and as far as can be judged from the properties of other non-phenolic steroid sulphates (Klyne, Schachter & Marrian, 1948; Klyne, 1948) it seems probable that any pregnanediol sulphate which might have been formed would also have been hydrolysed. The observed disappearance of pregnanediol in these experiments almost certainly could not have been entirely due to conjugation with glucuronic or sulphuric acids.

Incubation of PDHS with liver preparations in a nitrogen atmosphere—influence of methylene blue

In the following experiments samples of rat-liver slices and acetone powders were incubated with PDHS in a N_2 atmosphere. The N_2 was obtained in cylinders from the British Oxygen Co. Ltd., and was used without any purification. Other samples were incubated in O_2 for comparison. 'Controls' and 'blanks' were incubated in N_2 . The results given in Fig. 5 show that the metabolism of pregnanediol by liver preparations is almost completely inhibited in the absence of O_2 . Some activity was restored to the anaerobic system by the addition of methylene blue (0.5 mM) (Fig. 6).

Incubation of PDHS with liver preparations in presence of cyanide

Fig. 7 shows the results of incubating rat-liver preparations with PDHS in oxygen in the presence of cyanide (0.01 M). It can be seen that the enzyme

Fig. 5. Incubation of rat-liver slices (Exps. 1 and 2) and acetone-dried liver powder (Exp. 3) with PDHS in O_2 and N_2 . A, pregnanediol recovered from 'controls'; B, pregnanediol recovered after incubation in O_2 ; C, pregnanediol recovered after incubation in N_2 ; D, apparent pregnanediol recovered from 'blanks'. Double lines at the ends of bars indicate duplicate values.

Fig. 6. Incubation of acetone-dried rat-liver powder with PDHS in N_2 and O_2 —influence of methylene blue. A, pregnanediol recovered from 'controls'; B, pregnanediol recovered after incubation in O_2 ; C, pregnanediol recovered after incubation in N_2 ; D, pregnanediol recovered after incubation in N_2 + methylene blue (0.5 mM); E, apparent pregnanediol recovered from 'blanks'. Double lines at the ends of bars indicate duplicate results.

system involved in the *in vitro* metabolism of pregnanediol by these liver preparations is not cyanide-sensitive.

Fig. 7. Incubation of rat-liver slices (Exps. 1 and 2) and acetone-dried rat-liver powder (Exp. 3) with PDHS— influence of added cyanide. A, pregnanediol recovered from 'controls'; B, pregnanediol recovered after incubation without cyanide; C, pregnanediol recovered after incubation in presence of cyanide (0.01 M); D, apparent pregnanediol recovered from blanks. Double lines at the ends of bars indicate duplicate values.

DISCUSSION

From the evidence presented it would appear that rat and rabbit livers contain an enzyme or enzymes which will metabolize pregnane-3 α :20 α -diol *in vitro*. The intact liver cell is not necessary to produce this metabolic effect. An oxidative mechanism appears to be involved since this metabolism will not proceed in absence of oxygen without the addition of methylene blue. The fact that no inhibition was observed in the presence of cyanide indicates that the cytochrome—cytochrome-oxidase system is not required as the terminal oxidation mechanism. It has not yet been found possible to obtain definite evidence regarding the nature of the products of metabolism, but experiments to this end will shortly be carried out.

At this stage of the work it would be unwise to discuss the possible biochemical significance of this enzyme system at length. It is possible that if the same system is present in human liver it might be partly responsible for the 'disappearance' of

administered pregnane-3 α :20 α -diol in *in vivo* experiments on human subjects. On the other hand, if the enzyme system is a dehydrogenase, then its *in vivo* function might rather be that of a 'reductase' playing some role in the formation of pregnane-3 α :20 α -diol and of other reduction products from progesterone.

SUMMARY

1. A method is described for the determination of small quantities of pregnanediol in incubated liver preparations.

2. The application of this method to the study of the *in vitro* metabolism of pregnanediol by rat and

rabbit liver has indicated that these tissues contain an enzyme or enzymes which metabolize this steroid to as yet unknown substances. This metabolism is inhibited in nitrogen but not by cyanide. The evidence presented indicates that processes of conjugation are not involved.

The authors are indebted to the Medical Research Council for a grant out of which the expenses of this work were largely defrayed; to Mr A. L. Bacharach of the Glaxo Laboratories and to Dr D. P. Cuthbertson of the Rowett Research Institute for many of the rats used in this work; to Mr T. I. Kyle who prepared the pregnane-3 α :20 α -diol; to Dr J. W. Minnis for the micro-analysis and to Mrs S. M. Atherden for skilled technical assistance.

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Studies in Dialysis

1. AN APPLICATION OF ION-EXCHANGE RESINS

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(Received 24 April 1950)

Ion-exchange resins have been used in many instances where it has been necessary to remove ions from solutions of uncharged molecules, and it was thought possible that the same methods might serve for charged molecules provided these were too large to penetrate the resin particles. The use of ion-exchange resins would then be an alternative to normal dialysis.

Experiments were carried out to see if proteins could be 'dialysed' in this way, and it was found that egg albumin could be freed from ions by passing it

through columns of cation- and anion-exchange resins without significant loss. The non-absorption of proteins by resins has been noted by other authors and applied to the fractionation of blood serum by Reid & Jones (1949). An upper limit of about 20 Å. has been suggested by Richardson (1949) for the pore size of certain commercial resins. The non-absorption of a protein by resins and its recovery in a soluble form is not a proof that minor changes have not occurred in it, and so more sensitive tests were carried out using enzymes. Urease, α - and