# The *in vitro* Enzymic Hydroxylation of Steroid Hormones

## 2. ENZYMIC 11β-HYDROXYLATION OF PROGESTERONE BY OX-ADRENOCORTICAL MITOCHONDRIA

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On the basis of experiments in which steroids were perfused through ox-adrenal glands, Hechter *et al.* (1951) were able to present a tentative scheme of adrenocortical hormone biogenesis. Progesterone is postulated as a key intermediate in the scheme, since perfusion of this steroid gave the following products: 17α-hydroxyprogesterone (3–7%), 17α-hydroxycorticosterone (10–12%), corticosterone (1·2–2·4%) and 11β-hydroxyprogesterone (less than 1%). The figures in parenthesis indicate the amounts of steroid recovered. Using ox-adrenal 'homogenates' prepared in potassium chloride solution Hayano & Dorfman (1952) confirmed the ability of the gland to form corticosterone from progesterone. In subsequent experiments using 'residue preparations of twice washed ox-adrenal "homogenates" obtained at 5000 *g*' the same workers (Hayano & Dorfman, 1953) failed to convert progesterone into 11β-hydroxyprogesterone under conditions which permitted the 11β-hydroxylation of 11-deoxycorticosterone (DOC) to take place. They attributed this failure to the absence of a hydroxyl group at the 21-position in the progesterone molecule. The 21-hydroxylating enzyme which Plager & Samuels (1953) have shown to be present in the 'supernatant fraction' of a 'homogenate' centrifuged at 20000 *g* would presumably have been removed on washing the sedimented particles used by Hayano & Dorfman.

In a previous paper (Brownie & Grant, 1954*a*) it was shown that the 11β-hydroxylation of DOC by ox-adrenocortical mitochondria requires concurrent oxidative phosphorylation, for which citric acid cycle intermediates are oxidizable substrates. The observation of Grant & Taylor (1952) that progesterone has a greater inhibitory action than DOC on certain reactions of the tricarboxylic acid cycle in rat-liver mitochondria suggested that similar effects in adrenocortical mitochondria might explain the failure of Hayano & Dorfman (1953) to obtain 11β-hydroxylation of progesterone. Consequently it was decided to study conditions for the 11β-hydroxylation of progesterone by ox-adrenocortical mitochondria with particular reference to the influence of progesterone on reactions contributing to or supporting the hydroxylation reaction. The results of this investigation are now reported.

A preliminary account of this work has been communicated to the Biochemical Society (Brownie & Grant, 1954*b*).

## EXPERIMENTAL

### *Materials and Methods*

Melting points were determined on a hot-stage type of apparatus and are corrected. Progesterone was purified by recrystallization from *n*-hexane and aqueous ethanol to give a product melting at 121–121·5°. DOC was prepared from the acetate as described by Brownie & Grant (1954*a*). 11β-Hydroxyprogesterone was a crystalline specimen, m.p. 187–189°. Reichstein & Fuchs (1940) reported m.p. 187–188° for this steroid.

A commercial preparation of the sodium salt of adenosine triphosphate (ATP) (L. Light and Co. Ltd., Slough) was used. Alumina (Peter Spence and Sons Ltd., Widnes) activity II (Brockmann & Schodder, 1941) was used for adsorption chromatography.

In the isolation of ox-adrenocortical mitochondria as described by Brownie & Grant (1954*a*), it has now been found more convenient to use a small Latapie mincer in the cold room at 0° for the preliminary disintegration of the tissue.

Celite 545 and solvents were purified as described by Taylor (1954).

For quantitative experiments, citric acid cycle intermediates and suspensions of mitochondria in the concentrations stated were added to a basal reaction mixture containing 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·0005 M potassium ATP and 0·04 M potassium phosphate (pH 7·4) in a total vol. of 3 ml. Steroids were added in solution in propylene glycol (propane-1:2-diol). Unless otherwise stated, incubations were in air, shaking for 1 hr. at 37°. Each experiment was conducted in four parts as already described (Brownie & Grant, 1954*a*). Incubations were terminated by addition of 15 ml. cold acetone, except when citrate was to be determined, in which case 3 ml. 30% (w/v) trichloroacetic acid was added. After centrifuging, citrate was determined in a portion of the supernatant solution by the method of Taylor (1953).

Oxygen consumption measurements were made by the usual Warburg technique (Umbreit, 1949).

### *Determination of DOC, progesterone and 11β-hydroxyprogesterone*

Steroids were determined by the method described by Taylor (1954) for progesterone with slight modifications. Acetone extracts were not chilled before filtering and benzene–$CHCl_3$ (6:1, v/v) was used for the extraction of

aqueous-acetone residues. The modifications introduced by Brownie & Grant (1954*a*) were used in the method for DOC. Progesterone and 11β-hydroxyprogesterone present together in incubated mixtures were determined in separate portions of the benzene–$CHCl_3$ extract. 11β-Hydroxyprogesterone was isolated by partition chromatography on a Celite column (5 g. Celite 545/4 ml. methanol–water (7:3, v/v)); the solvent systems were benzene–*n*-hexane (3:7, v/v), and methanol–water (7:3, v/v). 11β-Hydroxyprogesterone was eluted in the fraction from 20 to 40 ml. DOC if present would be eluted in the same fraction, but this steroid was not found among the products obtained on incubating washed adrenocortical mitochondria with progesterone.

Steroids eluted from chromatogram columns were determined by measuring their selective absorption at 240 mμ. in ethanol in a Unicam SP 500 spectrophotometer. Amounts of steroid present were found by reference to calibration curves.

Typical results for the recovery of progesterone and 11β-hydroxyprogesterone added to the same incubation mixture containing 200 mg. wet weight mitochondria are shown in Table 1.

'Apparent' steroid recovered in experiments in which no steroids were used was consistently very low (approx. 20 μg.). These values were subtracted in all steroid determinations.

## RESULTS

### *Influence of progesterone and DOC on reactions of the tricarboxylic acid cycle supporting 11β-hydroxylation*

The influence of progesterone and DOC on citrate metabolism by adrenocortical mitochondria was investigated by measuring the amount of citrate accumulating in reaction mixtures on incubation with pyruvate and L-malate. From the results of a typical experiment shown in Fig. 1 (*a*) it is seen that much less citrate accumulates in the presence of progesterone than with an equivalent amount of DOC. This might be due to a greater inhibition by progesterone of citrate-synthesizing reactions or a greater stimulation of citrate oxidation by progesterone. Direct measurement of the effect of progesterone and DOC on citrate oxidation showed that both steroids inhibited this reaction to about the same extent (Fig. 2). It would thus appear that progesterone exerts a more powerful inhibitory effect on the synthesis of citrate from pyruvate and L-malate than does DOC. The inhibitory action of DOC on citrate synthesis is eliminated and that of progesterone is somewhat decreased if increased amounts of enzyme are used (Fig. 1, *b*, *c*).

Another example of the greater inhibition by progesterone than by DOC is given in Fig. 3 (*a*), which shows the effect of these steroids on the oxygen consumption of mitochondria incubated with succinate.

Concurrent with these measurements of oxygen consumption, rates of metabolism of progesterone and DOC were determined. The results shown in Fig. 3 (*b*) indicate that the added DOC is almost completely metabolized in the first 10 min. of

Table 1. *Recovery of progesterone and 11β-hydroxyprogesterone added to reaction mixtures which had been incubated with adrenocortical mitochondria*

Values have been corrected by subtraction of 'apparent' steroid recovered in blank experiments in which no steroids were added.

| Added (μmoles) | Recovered (μmoles) | Recovery (%) |
|---|---|---|
| | Progesterone | |
| 1·83 | 1·75 | 96 |
| 1·65 | 1·56 | 95 |
| 0·744 | 0·716 | 96 |
| 0·744 | 0·716 | 96 |
| | 11β-Hydroxyprogesterone | |
| 1·23 | 1·18 | 96 |
| 1·23 | 1·17 | 95 |
| 0·712 | 0·636 | 89 |
| 0·712 | 0·621 | 87 |

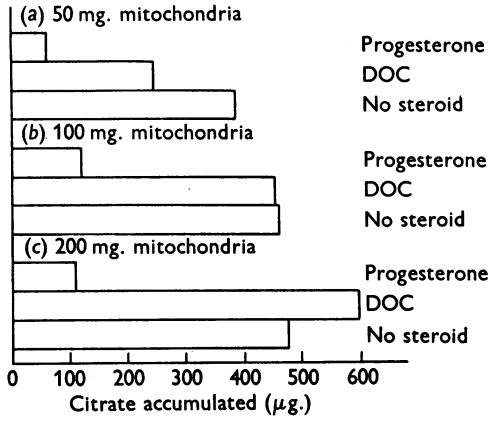


Fig. 1. Effect of progesterone and DOC on citrate accumulation by ox-adrenocortical mitochondria incubated in air for 1 hr. at 37°. Basal reaction mixture supplemented with 0·016M potassium pyruvate, 0·016M potassium L-malate and wet weights of mitochondria shown. 500 μg. steroid added in 0·04 ml. propylene glycol.

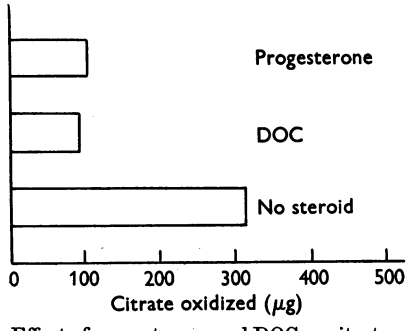


Fig. 2. Effect of progesterone and DOC on citrate oxidation by ox-adrenocortical mitochondria incubated in air for 1 hr. at 37°. Basal reaction mixture supplemented with 0·001M sodium citrate (576 μg. citric acid/flask) and 100 mg. wet weight mitochondria. 500 μg. steroid added in 0·04 ml. propylene glycol.

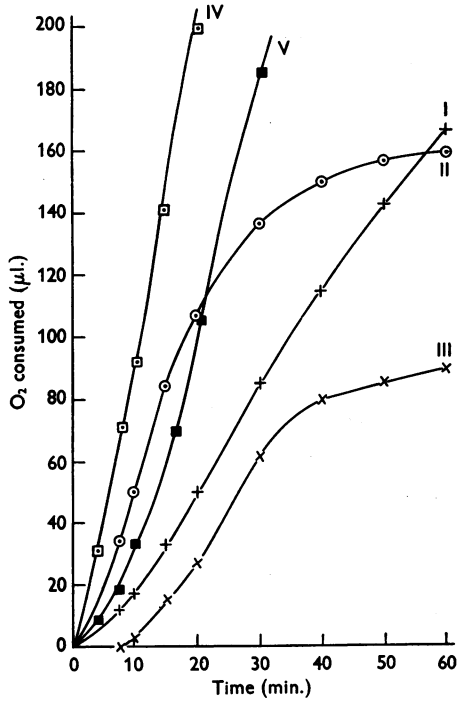


Fig. 3 (*a*). Effect of progesterone and DOC on succinate oxidation by ox-adrenocortical mitochondria. All flasks contained basal reaction mixture. 500 µg. steroids were added in 0·04 ml. propylene glycol. I. 100 mg. wet weight mitochondria, 0·002 M succinate. II. As I plus DOC. III. As I plus progesterone. IV. 200 mg. wet weight mitochondria, 0·01 M succinate. V. As IV plus progesterone.

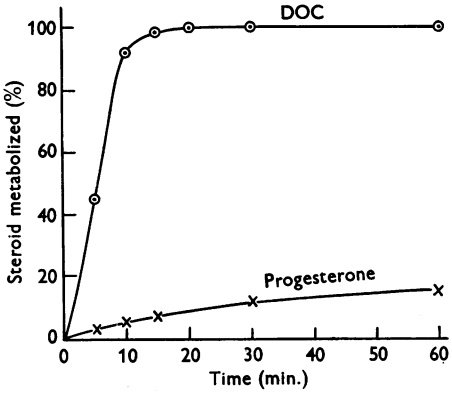


Fig. 3. (*b*). Rates of metabolism of progesterone and DOC by ox-adrenocortical mitochondria. Basal reaction mixture was supplemented with 0·02 M succinate and 100 mg. wet weight mitochondria. 500 µg. steroids were added in 0·04 ml. propylene glycol. Incubations in air at 37°.

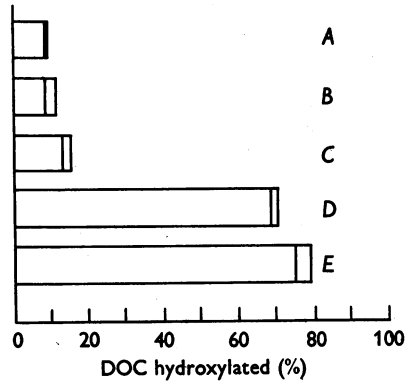


Fig. 4. Effect of pre-incubation of ox-adrenocortical mitochondria on DOC metabolism. 100 mg. wet weight mitochondria were added to the basal reaction mixture. 500 µg. of steroids were added in 0·04 ml. propylene glycol. Incubations were in air at 37°. (Double lines at ends of bars indicate duplicate values.) *A*. Progesterone, DOC and succinate (0·002 M) present from the start, 10 min. incubation. *B*. Progesterone and succinate (0·002 M) present from the start. After 20 min. pre-incubation DOC was added and incubation continued for further 10 min. *C*. After 20 min. pre-incubation, DOC and succinate (0·002 M) were added and incubation continued for further 10 min. *D*. Succinate (0·002 M) present from the start. After 20 min. pre-incubation DOC was added and incubation continued for further 10 min. *E*. DOC and succinate (0·002 M) present from the start, 10 min. incubation.

incubation, a period during which there is a marked stimulation of oxygen consumption. In contrast with this, added progesterone inhibits oxygen consumption and is not itself appreciably metabolized during the first 10 min. This suggests that the adrenocortical mitochondrial enzymes directly or indirectly involved in steroid 11β-hydroxylation may be 'inactivated' during the period in which the mitochondria are not actively respiring. In order to investigate this possibility, mitochondria were pre-incubated under the conditions shown in Fig. 4. The ability of the enzyme preparation to catalyse the 11β-hydroxylation of DOC was then measured. From the results shown in Fig. 4, it is clear that pre-incubation without oxidizable substrate (succinate) or with succinate oxidation inhibited by progesterone, greatly reduces the 11β-hydroxylation of DOC added subsequently. This loss of 11β-hydroxylating activity is not observed if succinate oxidation is uninhibited during the pre-incubation period (bar *D* of Fig. 4).

*Determination of conditions suitable for metabolism of progesterone by ox-adrenocortical mitochondria*

The results of experiments described above suggested that the inhibition of reactions supporting the 11β-hydroxylation of DOC in ox-adrenocortical

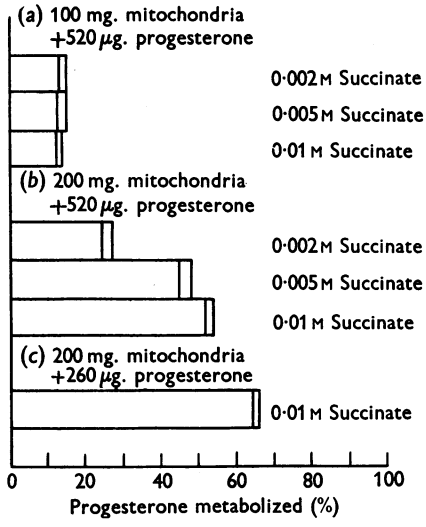


Fig. 5. Effect of varying concentrations of succinate, mitochondria and steroid on progesterone metabolism by ox-adrenocortical mitochondria. Basal reaction mixture supplemented with concentrations of succinate and mitochondria shown. Progesterone in propylene glycol added in the amounts shown. Incubation in air for 1 hr. at 37°. Double lines at ends of bars indicate duplicate values.

mitochondria by progesterone might account for the failure to hydroxylate progesterone itself under these conditions. Conditions were therefore sought which would decrease the inhibitory action of progesterone on the reactions supporting steroid 11β-hydroxylation.

From the results of experiments in which progesterone, succinate and enzyme concentrations were varied (Fig. 5) it appears that incubation of approximately 500 μg. progesterone with 0·01 M succinate and 200 mg. wet weight packed mitochondria (about 6 mg. total N) results in about 50 % metabolism of the progesterone. When the amount of progesterone is reduced to approximately 250 μg. about 65 % is metabolized.

*Investigation of the products of metabolism of progesterone by ox-adrenocortical mitochondria*

Consideration of the results described in the previous section indicated that the use of 0·01 M succinate and a progesterone:wet packed mitochondria weight ratio of 1:800 in incubation mixtures might be expected to result in 65 % metabolism of the steroid. The difficulty in preparing large amounts of mitochondria from the cortical tissue of fresh ox adrenals limited the amount of steroid which could be incubated at any one time. It appeared that incubation of 45 mg. of progesterone would be convenient for the isolation of the main products of metabolism. Consequently, separate 5 mg. samples of progesterone in 0·4 ml. propylene glycol were incubated on each of 9 days with 4 g. wet packed mitochondria in 60 ml. of the basal reaction mixture containing 0·01 M succinate. This volume was distributed in a number of conical flasks which were shaken in air for 1 hr. at 37°. Progesterone determinations on small portions of the mixture after incubation indicated that about 60 % of the steroid had been metabolized. A part of the residue from the benzene–$CHCl_3$ extract prepared for the progesterone determinations was chromatographed on a Celite column using the solvent system benzene–hexane (3:7, v/v), methanol–water (7:3, v/v) normally used in DOC determinations. Observation of the elution of material absorbing selectively at 240 mμ. indicated the presence of a substance slightly more polar than DOC. On oxidation with periodate this substance yielded no formaldehyde as determined by the method of Daughaday, Jaffe & Williams (1948). This indicated that it was not a steroid having an α-ketol side chain in the 17-position. The observed chromatographic behaviour of this substance determined the procedures employed for its subsequent isolation and purification.

Incubations were terminated by the addition of 5 vol. of chilled acetone and precipitated material was filtered off after standing 2 hr. at −20°. The precipitate was washed with cold acetone. The filtrate and washings were evaporated *in vacuo* to an aqueous residue of about 50 ml. This was diluted with an equal volume of water, saturated with NaCl, and extracted with 3 × 50 ml. ethyl acetate.

The pooled ethyl acetate extracts were washed with 50 ml. 0·2 N-$NaHCO_3$, 50 ml. 0·2 N-HCl and finally with water until the washings were neutral, dried ($Na_2SO_4$) and evaporated to dryness *in vacuo*. The residue obtained was partitioned in 100 ml. of a solvent system consisting of 20 ml. *n*-hexane and 80 ml. methanol–water (80:20, v/v). Much yellow material entered the less polar hexane phase. The methanol–water phase was evaporated *in vacuo* to an aqueous residue, which was diluted to 50 ml. with water and extracted with 3 × 50 ml. $CHCl_3$. The pooled $CHCl_3$ extract was dried ($Na_2SO_4$) and evaporated to dryness *in vacuo*.

At this stage the residues from extracts of individual incubation mixtures were combined and submitted to a seven-stage countercurrent distribution with double withdrawal (Craig & Craig, 1950). This was carried out in separating funnels with the solvent system methanol–water (80:20, v/v), benzene–*n*-hexane (80:20, v/v), using 50 ml. of each phase. The partition coefficient of progesterone in this system was 0·23 and that of 11β-hydroxyprogesterone 1·6. Fig. 6 shows the theoretical distribution of these two steroids in such an experiment and also the actual distribution of isolated

material absorbing selectively at 240 mμ. Residues obtained on evaporating the contents of funnels 0–6 to dryness *in vacuo* were semi-crystalline. Their combined weight was 12·5 mg. A solution of these combined residues in 3 ml. benzene–*n*-hexane (1:1, v/v) was poured on to a column of about 4 mm. diameter containing 400 mg. $Al_2O_3$. The chromatogram was developed as shown in Table 2.

The white crystalline material from fractions 7 to 18 was recrystallized from benzene–*n*-hexane (1:1, v/v) to give 8 mg. of crystals melting at 189–191°. On mixing with an authentic specimen of 11β-hydroxyprogesterone, m.p. 187–189°, the m.p. was 187–189°.

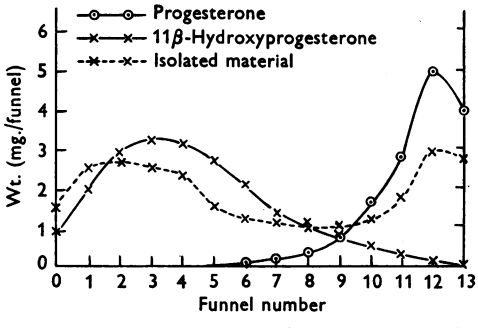


Fig. 6. Theoretical distribution of progesterone and 11β-hydroxyprogesterone and the distribution of isolated material absorbing selectively at 240 mμ. in a seven-stage countercurrent distribution with double withdrawal using the solvent system benzene–*n*-hexane (80:20, v/v) and methanol–water (80:20, v/v). Volume of each phase was 50 ml.

The isolated material and 11β-hydroxyprogesterone (70 μg. each) in stoppered tubes were separately treated with 5 ml. conc. $H_2SO_4$ for 2 hr. at 17°. Both solutions gave identical absorption spectra over the range 220–520 mμ. (Fig. 7).

The infrared spectra of the isolated material melting at 181–191° and of authentic 11β-hydroxyprogesterone were determined with mulls ground in paraffin in a double-beam Perkin-Elmer 21B spectrophotometer. The positions of all the bands and their relative intensity patterns within each spectrum were identical. From the evidence thus obtained there can be no doubt that the isolated material is 11β-hydroxyprogesterone.

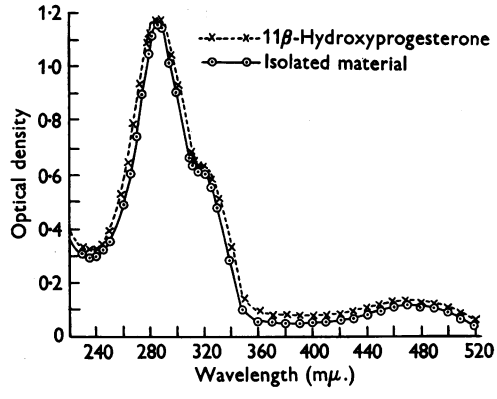


Fig. 7. Absorption spectra of solutions of 11β-hydroxyprogesterone and of isolated crystalline material in concentrated $H_2SO_4$.

Table 2. *Chromatography on $Al_2O_3$ of residues from funnels 0–6 in the countercurrent distribution of material obtained on incubation of progesterone with ox-adrenocortical mitochondria*

Each fraction, 3 ml. Solvent ratios, all v/v.

| Fraction no. | Solvent | Residue | |
|---|---|---|---|
| | | Wt. (mg.) | Description |
| 1–3 | Benzene–*n*-hexane (1:1) | — | — |
| 4–6 | Benzene–*n*-hexane (3:2) | — | — |
| 7–9 | Benzene–*n*-hexane (4:1) | 0·5 | White crystals |
| 10–12 | Benzene | 1·4 | White crystals |
| 13–15 | Benzene–ether (9:1) | 6·0 | White crystals |
| 16–18 | Benzene–ether (4:1) | 0·5 | White crystals |
| 19–21 | Benzene–ether (3:2) | 0·5 | Amorphous solid |

Table 3. *Recovery of progesterone and 11β-hydroxyprogesterone following incubation of progesterone with ox-adrenocortical mitochondria*

Figures in parenthesis indicate the number of results obtained.

| Expt. | Progesterone added (μmoles) | Progesterone recovered (μmoles) | 11β-Hydroxyprogesterone recovered (μmole) | Total recovery | |
|---|---|---|---|---|---|
| | | | | (μmoles) | (%) |
| 1 | 1·60 | 1·1±0·05 (8) | 0·37±0·04 (8) | 1·47±0·1 | 92±6 |
| 2 | 1·54 | 0·9±0·06 (8) | 0·42±0·07 (8) | 1·32±0·13 | 85±8 |

The results of quantitative experiments, shown in Table 3, indicate that the 11β-hydroxyprogesterone found may account for the greater part of the progesterone metabolized by ox-adrenocortical mitochondria. Attempts were made to obtain information on the nature of other metabolic products by paper chromatography of the benzene–$CHCl_3$ extract obtained in quantitative experiments. Portions of extract containing about 150 μg. material absorbing selectively at 240 mμ. were employed. For enzyme blanks, similar portions were taken from extracts obtained after incubation of mitochondria without added steroids. For standards, about 150 μg. each of progesterone, 11β-hydroxyprogesterone and DOC were added to separate portions of blank extract. The chromatograms were run in duplicate simultaneously for 2 hr. at 18° using the solvent system $B_1$ of Bush (1952). Dried papers were sprayed with (*a*) methanolic-NaOH reagent (Bush, 1952) which detects steroids having an αβ-unsaturated 3-ketone structure in concentrations of 2 μg./cm.$^2$ and (*b*) blue tetrazolium reagent (Mader & Buck, 1952) which detects steroids having an α-ketol side chain in concentrations of 1–2 μg./cm.$^2$. Spots corresponding in position to 11β-hydroxyprogesterone and progesterone were observed on chromatograms of extracts from progesterone incubation experiments. No trace of DOC was observed on these chromatograms, but the blue tetrazolium reagent showed the presence of a trace of material more polar than DOC. This was not observed on chromatograms of progesterone or with the mitochondrial blanks.

## DISCUSSION

Hechter (1953*a*) cautiously describes the scheme of adrenocortical-steroid biogenesis which he previously advanced (Hechter *et al.* 1951) as 'a series of deductions based primarily upon the ability of substances to react', and admits the possibility that such *in vitro* reactions may be relatively unimportant in normal metabolic pathways. When evaluating the physiological significance of *in vitro* reactions of adrenocortical steroids and their possible precursors, it is important to bear certain points in mind. The state of enzymes employed and the influence of steroids on enzyme reactions are factors which appear to the present authors to merit special attention.

The unsatisfactory nature of the 'homogenates' and 'washed sediments' used as sources of enzymes for the study of adrenocortical steroid metabolism has already been discussed (Brownie & Grant, 1954*a*). The skilful adaptation of the perfusion technique by Hechter *et al.* (1953) might be expected to preserve adrenocortical enzymes in a natural state, but the results obtained may have been influenced by exposing the perfused steroids to deliberately damaged adrenal tissue.

It is well known that mitochondria, carefully isolated under suitable conditions, contain enzymes and coenzymes which catalyse a wide variety of important reactions including the transfer of hydrogen and electrons to molecular oxygen. In particular, Kielley & Kielley (1951) have shown that such mitochondria catalyse high rates of phosphorylation coupled with oxidation of metabolites of the citric acid cycle. This sequence of reactions has been shown to be necessary for the 11β-hydroxylation of DOC by adrenocortical mitochondria (Brownie & Grant, 1954*a*).

While it can be argued that isolated mitochondria may be rapidly altered from their natural state by incubation in media which inadequately represent the intracellular fluid, it is notable that only very brief incubations at 37° are necessary in order to obtain extensive 11β-hydroxylation of steroids. These observations encourage the belief that carefully prepared mitochondria are suitable sources of enzymes for the study of certain reactions of adrenocortical-steroid biogenesis *in vitro*.

Little attention appears to have been paid to the influence of other steroids on enzyme reactions involved in the biogenesis of adrenocortical steroids. Hechter (1953*b*) has referred briefly to the possible influence of sex hormones on the pattern of adrenocortical steroids synthesized and released from the gland.

Sourkes & Heneage (1952) have observed a stimulation by DOC of succinate oxidation by rat-adrenal halves. Brummel, Halkerston & Reiss (1954) have made the same observation with ox-adrenal slices. This stimulation has now been observed using ox-adrenocortical mitochondria. At the same time the DOC is extensively converted into corticosterone. The oxygen required for this reaction does not however account for the increased oxygen consumption observed.

In contrast with the effect of DOC it has now been found that progesterone inhibits succinate oxidation by adrenocortical mitochondria, and that under such circumstances the 11β-hydroxylation of the steroid is inhibited. It has also been observed in the present work that progesterone has a greater inhibitory action than DOC on reactions involved in the synthesis of citrate from pyruvate and L-malate. It is possible that the inhibitory action of progesterone restricts 'high-energy' phosphate production with resultant inhibition of 11β-hydroxylation of the steroid.

The inhibition by progesterone is most marked during the first 10 min. of incubation. Thereafter oxygen consumption is almost parallel with that in control experiments without added steroid (Fig. 3*a*). No explanation can be offered for this observation.

It appears, however, that enzymes indirectly or directly concerned with 11$\beta$-hydroxylation of steroids by adrenocortical mitochondria suffer permanent damage during the first 10 min. of incubation if tricarboxylic acid cycle intermediates are absent, or if their oxidation is inhibited (Fig. 4). This may be explained by the well-known instability under these conditions of enzymes involved in oxidative phosphorylation.

The limited increase in steroid hydroxylation observed on increasing the concentration of enzyme in the case of progesterone as compared with DOC (Fig. 5) suggests that the progesterone effect may not be restricted to an inhibition of respiration. The increased rate of ATP breakdown by rat tissues in the presence of progesterone observed by Jones & Wade (1953) offers an additional explanation for the influence of progesterone on reactions concerned with 11$\beta$-hydroxylation.

By suitable adjustment of the concentrations of progesterone, succinate and mitochondria it has now been found possible to decrease the effect of the progesterone on reactions of the tricarboxylic acid cycle sufficiently to permit appreciable 11$\beta$-hydroxylation of this steroid.

In the scheme of adrenocortical-steroid biogenesis advanced by Hechter *et al.* (1951), 11$\beta$-hydroxyprogesterone is shown as a possible intermediary in the formation of corticosterone or 17$\alpha$-hydroxycorticosterone from progesterone. The status of 11$\beta$-hydroxyprogesterone as such as intermediary has been diminished by the following facts. This steroid has hitherto only been isolated in yields of less than 1 % after perfusion of progesterone through adrenal glands. It has not been found among the products obtained on incubation of adrenocortical tissue preparations with progesterone (Hayano & Dorfman, 1952, 1953). Finally, 11$\beta$-hydroxyprogesterone has not been converted into 17$\alpha$-hydroxycorticosterone or corticosterone in significant amounts (Hechter, 1953*a*). Since it has now been shown to be possible to convert progesterone into 11$\beta$-hydroxyprogesterone in good yield by adrenocortical mitochondria it would appear that the status of the latter steroid as an intermediary in the biogenesis of adrenocortical steroids should be reconsidered. The conditions under which attempts have been made to convert 11$\beta$-hydroxyprogesterone into corticosterone or 17$\alpha$-hydroxycorticosterone have not been described by Hechter (1953*a*). It is therefore not at present possible to decide whether these reactions may or may not occur.

The elucidation of the mechanism of adrenocortical-steroid biogenesis is further limited by lack of knowledge of the influence of steroids other than progesterone on the metabolism of their precursors and of the relative affinities of the enzymes concerned for the various steroid substrates which may be present. These problems must form the subjects for future investigations.

## SUMMARY

1. Progesterone has been found to be a more powerful inhibitor than 11-deoxycorticosterone (DOC) of certain reactions of the tricarboxylic acid cycle in ox-adrenocortical mitochondria.

2. These inhibitions offer some explanation of the failure of steroid 11$\beta$-hydroxylation in presence of progesterone, since this reaction in adrenocortical mitochondria appears to depend upon oxidative phosphorylation for which members of the tricarboxylic acid cycle are oxidizable substrates.

3. Conditions are described under which progesterone may be converted by ox-adrenocortical mitochondria into 11$\beta$-hydroxyprogesterone as the main product, and to traces of a more polar product which reduces blue tetrazolium.

4. The choice of suitable enzyme preparations and the influence of steroids on enzymes are discussed in relation to the physiological significance of steroid reactions observed *in vitro*.

It is a pleasure to record our appreciation of the interest which Professor G. F. Marrian, F.R.S., has shown in this work, and to thank him for his encouragement. The expenses of the work were defrayed in part by a grant to Professor Marrian from the Medical Research Council. The DOC acetate was a generous gift from Dr C. L. Hewett, Organon Laboratories Ltd., Newhouse, Lanarkshire. The 11$\beta$-hydroxyprogesterone was kindly given by Dr R. Levine, The Upjohn Company, Kalamazoo, Mich., U.S.A. We are indebted to Dr L. J. Bellamy, Chemical Inspectorate, Ministry of Supply, London, for the determination of the infrared spectra. The co-operation of the manager and staff of the Offals Department, Wholesale Meat Marketing Association, Edinburgh, in the collection of fresh adrenal glands was of the greatest value. Mr R. Bayne purified the Celite and the solvents used.

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# The Non-Protein Nitrogen of Extracts of *Pisum sativum*

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A number of methods have been used in the past for obtaining non-protein nitrogenous compounds from plant tissues, but little information about their relative efficiencies is available. The present study concerns the extraction of soluble nitrogen-containing compounds from green seed peas by cold and hot saline solutions and by cold and hot water. In these extracts the non-protein nitrogen (NPN) was determined after precipitation by trichloroacetic acid (TCA), ethanol, tungstic acid, colloidal iron and after dialysis. The NPN fractions were analysed for total N and carboxyl N (ninhydrin–carbon dioxide). The amino acids of the extracts were investigated by paper-partition chromatography.

## MATERIAL AND METHODS

Green seed peas freshly removed from pod were used, the variety Onwards being studied. The green pods, collected approximately 20 days after flowering, were fully swollen and corresponded to stage 12 (Hyde, 1954).

*Extraction.* Approx. 10 g. of peas were ground in a glass mortar with 20 ml. of saline buffer (pH 7) of composition 0·2 M-NaCl, 0·3 M-$Na_2HPO_4$, 0·011 M-$NaH_2PO_4$. The supernatant liquid was decanted and the process repeated with seven successive lots of saline buffer to give a total volume of 140 ml. The extracts were centrifuged at 1500 *g* for 30 min. and the supernatant fluid was decanted. The extraction with cold distilled water was made in a similar manner. With the hot-saline and hot-water extractions, solutions at 98° were used.

*Precipitation.* (*a*) Trichloroacetic acid. To the pea extract (20 ml.) TCA (1 g.) was added, the mixture was allowed to stand for 45 min., the volume was measured and the mixture filtered.

(*b*) Ethanol. The pea extract (20 ml.) was mixed with ethanol (80 ml.) and the solution allowed to stand for 24 hr. at 2°. It was then filtered.

(*c*) Tungstic acid. To the pea extract (20 ml.) were added 10% (w/v) sodium tungstate (20 ml.) and 0·66 N-$H_2SO_4$ (20 ml.). The mixture was allowed to stand at 2° for 24 hr. and then filtered.

(*d*) Colloidal iron. This method was successful only when saline buffer was used as an extracting agent. Pea extract (10 ml.) was mixed with dialysed iron (10 ml.) (British Drug Houses Ltd.) and the solution left for 12 hr. at 2°. Water (40 ml.) was then added and the mixture shaken and filtered.

*Dialysis.* This was carried out by placing 50 ml. of the pea extract in a cellophan sac and dialysing against 5–6 vol. of chloroform water for 96 hr. at 0–2° with occasional stirring (Synge, 1951). The diffusate was concentrated *in vacuo* for further study.

*Analytical methods.* Nitrogen was determined by the micro-Kjeldahl method of Hiller, Plazin & Van Slyke (1948). The distillation apparatus of Markham (1942) was employed. In determinations of extractable N and of NPN, samples containing 0·24–0·47 mg. N were used. Determinations were in triplicate, and agreed within ±0·5%.

The ninhydrin–$CO_2$ method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) with minor modifications was used. Pea extract (2 ml.) containing approx. 0·7 mg. carboxyl N was added to each tube. 2 ml. water were added in the case of the blank. It was found unnecessary to do blank determinations ($CO_2$ without ninhydrin) since these proved to be the same as water blanks. The determinations were made with trioxohydrindene hydrate (50 mg.) and 50 mg. citrate buffer (pH 2·5) using Thunberg vacuum tubes sealed with stopcock grease as reaction vessels. The various extracts were adjusted to a pH value suitable for the estimation. TCA was removed from the extract by means of ether, which raised the pH from 1 to 6. The ether dissolved in the extract caused an increase in pressure when the tube was evacuated but could be expelled after the $CO_2$ had been collected in the 0·5 N-NaOH. The ethanol extract was evaporated under reduced pressure at room temperature. The pH of the tungstic acid filtrate was adjusted to 2·5 by the addition of citrate buffer, excess $CO_2$ being removed by boiling the extract for 3 min. before the addition of