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STUDIES ON THE BIOSYNTHESIS OF 18-OXYGENATED STEROIDS FROM EXOGENOUS CORTICOSTERONE BY DOMESTIC DUCK (*ANAS PLATYRHYNCHOS*) ADRENAL GLAND MITOCHONDRIA

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SUMMARY

The transformation of exogenous, isotopically labelled corticosterone to 18-hydroxycorticosterone and aldosterone by domestic duck (*Anas platyrhynchos*) adrenal gland mitochondria was studied. The mitochondrial 18-oxygenating system was NADPH dependent. K^+, Na^+, Ca^{2+} and Mg^{2+} were necessary for maximal enzymatic activity. The enzyme present in 1 mg of mitochondrial protein became saturated with 13·2 μM of substrate (13·8 μg). Under saturation conditions the maximal production of 18-hydroxycorticosterone was found as 1·43 nmole/min/mg protein and that of aldosterone 0·33 nmole/min/mg protein. The Michaelis constant for both 18-hydroxycorticosterone and aldosterone was $6·6 \times 10^{-6}$M. Q_{10} (average between 20°C and 40°C) was 2·16 for the reaction corticosterone → 18-hydroxycorticosterone and 1·34 for the reaction corticosterone → aldosterone. The mitochondrial enzyme system did not 18-oxygenate exogenous 11-deoxycorticosterone, 11-dehydrocorticosterone, 20β-dihydrocorticosterone. Exogenous 18-hydroxycorticosterone and aldosterone were not metabolized. The kinetics of the transformation of corticosterone to 18-oxygenated metabolites suggested a parallel pseudo-first order reaction rather than a series pseudo-first order reaction. 18-Oxygenation of corticosterone was strongly inhibited by d,l-18-hydroxycorticosterone, p-chloromercuribenzoate, carbon monoxide, metopirone (competitive inhibition; $K_i = 3·0 \times 10^{-6}$ M for 18-hydroxycorticosterone and $9·0 \times 10^{-6}$M for aldosterone) and by aminopterin (non-competitive inhibition; K_i = for both metabolites $2·0 \times 10^{-5}$M). Protein synthesis inhibitors did not have any effect. Cytochrome-P450 was shown to be present in mitochondria by spectrophotometric measurements. Addition of corticosterone, 11-deoxycorticosterone and metopirone produced type II difference spectra. The mitochondrial P450 became saturated with either corticosterone or metopirone at a concentration of 21·8 nmoles/mg protein. From these studies it was concluded that the duck adrenal mitochondrial 18-oxygenating system differed from the mammalian adrenal system previously described, especially in regard of substrate specificity. 18-Hydroxycorticosterone, either endogenous or exogenous, could not serve as substrate for aldosterone production with duck adrenal mitochondria. However, under ordinary circumstances, 18-hydroxycorticosterone synthesis was always associated with aldosterone

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Trivial names used: Metopirone, SU-4885: 2-methyl-1,2-bis(3-pyridyl)-1-propanone; folic acid: pteroylglutamic acid; aminopterine: 4-amino-N^{10}-methylpteroylglutamic acid; p-CMB: p-chloromercuribenzoic acid; dicoumarol: 3,3′-methylene-bis(4-hydroxycoumarin); cycloheximide: 3-(2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl)glutarimide; KRB: Krebs–Ringer bicarbonate buffer.

synthesis in rather fixed proportions. Circumstantial evidence suggested the role of cytochrome-P450 in 18-oxygenation. As to the mechanism of the corticosterone → 18-hydroxycorticosterone → aldosterone reaction, this sequence could not be proven. The possibility should not be discarded that atmospheric oxygen is introduced into a hitherto unknown intermediary substance, which by enzymatic action and/or chemical rearrangement gives rise simultaneously to 18-hydroxy-corticosterone and aldosterone.

INTRODUCTION

IN RECENT years, the steroid hydroxylating enzyme systems of the adrenal cortex have been investigated in detail. These studies resulted in the discovery of the now familiar electron transport sequence associated with the oxygen activator, cytochrome-P450 necessary for both mitochondrial and microsomal steroid hydroxylation reactions[1–5]. Most of these studies were concerned with steroid 11β- and 21-hydroxylation. Similar detailed studies on adrenal 18-oxygenation are much less numerous, though one of the products of the reaction, aldosterone, has relevance in vertebrate homeostasis.

According to our present knowledge, in all the vertebrate species investigated, corticosterone can be regarded as the most important precursor of aldosterone [6, 7]. In addition, in all aldosterone producing species, formation of this hormone has been accompanied by that of 18-hydroxycorticosterone. The view is widely held at present that the biosynthesis of aldosterone from corticosterone proceeds through 18-hydroxycorticosterone by a reaction sequence as yet not completely understood. While it has been suggested that 18-hydroxylation of corticosterone, through the participation of cytochrome-P450 was a pre-requisite of aldosterone formation[8], exogenous 18-hydroxycorticosterone yielded little[9–12] or no aldosterone[13–17] when incubated with adrenal gland preparations *in vitro*. In addition, all the reported transformations of 18-hydroxycorticosterone to aldosterone were achieved with mammalian tissue.

In the course of our studies on non-mammalian adrenocortical steroidogenesis, adrenals of the domestic duck (*Anas platyrhynchos*) were found to be efficient producers of 18-hydroxycorticosterone and aldosterone under *in vitro* conditions [15, 18–21]. The duck adrenal 18-oxygenating enzyme complex is localized in the mitochondrial sediment, similarly to those of other vertebrates[11, 17, 22–24]. This system was partially characterized[22, 23]. Thus it was felt that domestic duck adrenal gland mitochondria would be a useful model for the study in detail of the transformation *in vitro* of corticosterone to 18-hydroxycorticosterone and aldosterone.

It is understood that the mitochondrial system investigated did not have the homogeneity desirable for unequivocal interpretation of some of the kinetic data. However, all attempts to obtain a soluble and enzymatically active adrenal 18-oxygenating preparation were unsuccessful and the data presented in this paper and the conclusions drawn from these data have to be interpreted in this context.

MATERIALS AND METHODS

Tissue preparations

Male, sexually immature ducks of the Pekin white variety weighing between 1·5–2 kg were used. On the average, 6 animals yielded 1 g (wet weight) of cleaned adrenal tissue. The glands were homogenized for 2 min in 0·25M sucrose (9 parts of sucrose to 1 part of tissue; vol./wt.) with a Teflon-glass homogenizer. The mitochondrial sediment was obtained by the 0·25M sucrose method[25]

using 5300 *g* (average) after having separated the low-speed sediment (550 *g* average). The mitochondrial sediment was washed twice and finally compacted by centrifugation at 21,000 *g* (average) for 10 min. The differential ultracentrifugation was performed in a Model L2-65B centrifuge (Spinco Division, Beckman Instruments, Palo Alto, California). The mitochondrial pellet thus obtained was suspended in an appropriate medium. The purity of the mitochondrial sediment was assessed by measuring its cytochrome oxidase[26] and glucose-6-phosphate phosphohydrolase content[27]. Using these criteria, no gross microsomal contamination was found. Protein content of the mitochondrial sediment was measured by the method of Lowry *et al.*[28]. One g of adrenal tissue yielded 20·25 ± 4·72 mg of mitochondrial protein. Density gradient centrifugation of the presumed mitochondria, using a continuous sucrose gradient (0·25–2·0M) showed that 66% of the total mitochondrial protein was recovered in particles sedimented in sucrose of molarities between 1·3 and 1·6. Eighty six percent of the total 18-oxygenase activity was associated with these particulate fractions[29]. Acetone powder of mitochondria was prepared as described by Williamson and O'Donnell [30]. A semi-purified preparation of duck mitochondrial cytochrome-P450 particles was obtained from sonicated mitochondria (ultrasound generator: Biosonik III, Bronwill Scientific, Rochester, N.Y.) as described by Omura *et al.* [31].

Substrates and steroid markers. [4-^{14}C] Corticosterone (SA: 52·7 mCi/mmole) or [1,2-^{3}H] corticosterone (SA: 57·2 Ci/mmole) were used as substrates. [4-^{14}C] Aldosterone (SA: 55·0 mCi/mmole) or [1,2-^{3}H] aldosterone (SA: 34·0 Ci/mmole), used as internal standard, was obtained, together with the substrates from New England Nuclear Corporation, Boston, Mass. Radioactive, (^{14}C or ^{3}H labelled) 18-hydroxycorticosterone was biosynthesized from labelled corticosterone by incubation with duck adrenal mitochondria. 11β,20β,21-Trihydroxy-4-pregnen-3-one labelled with carbon-14 was prepared from [4-^{14}C] corticosterone with 20β-hydroxysteroid dehydrogenase by the method of Margraf *et al.*[32]. The radiochemical purity of substances was checked shortly before use by paper partition and thin-layer chromatographic systems. Non radioactive steroids were purchased from IKHAPHARM, Raman-Gat, Israel and were recrystallized prior to use. Non radioactive, synthetic d,l-18-hydroxycorticosterone was a gracious gift from Dr. J. G. Schmidlin, CIBA A. G., Basel, Switzerland to one of us (A.G.F.).

Medium. Mitochondrial pellets were taken up in a Krebs–Ringer bicarbonate buffer, containing 200 mg glucose/100 ml or in a Tris–HCl buffer, supplemented with metal ions, or in some instances in a 0·15 M glycyl-glycine buffer again supplemented with metal ions (K^+, Na^+, Mg^{2+}, Ca^{2+}). All three media were adjusted to pH: 7·45.

Cofactors, inhibitors and other additives were obtained from commercial sources. Metopirone was a generous gift of CIBA Company, Dorval, P.Q. and aminopterin that of the Lederle Division, American Cyanamide Company, Pearl River, N.Y.

Incubation

The activity of the mitochondrial 18-oxygenating system was measured by its ability to transform radioactive corticosterone into radioactive 18-hydroxycorticosterone and aldosterone. Incubations were performed in a metabolic shaking incubator at 40°C for 30 min in air unless otherwise specified. In general,

mitochondrial sediments equivalent to 1 mg of mitochondrial protein were used per incubation vessel, in a total volume of 3 ml. The labelled corticosterone was diluted with non active material to yield the desired concentration. Unless otherwise specified, NADPH or an NADPH generating system consisting of NADP, glucose-6-phosphate (disodium salt) and glucose-6-phosphate dehydrogenase was added to the incubation mixtures a few minutes prior to the start of the experiment. Final NADPH concentration was 0·5 mM. The amounts of substrates and the nature and amounts of any other additives will be indicated for each experiment.

Isolation, purification and identification of transformation products

In each experiment, the total radioactivity, representing the transformation products and the non metabolized portion of the substrate were recovered and quantitated. Recovery was in the range of 90–95% of the initial substrate radioactivity.

The incubation of corticosterone with duck adrenal mitochondria or with duck adrenal slices yielded 18-hydroxycorticosterone and aldosterone as major transformation products. The identity of these products has been established previously [18, 19, 21, 23, 24]. Occasionally, the presence of 11-dehydrocorticosterone was also noted[33], but the appearance of this metabolite was not regular or predictable and its quantity not significant as the reaction corticosterone → 11-dehydrocorticosterone is NADP supported. At the end of each incubation, authentic, radioactively labelled aldosterone and when available, 18-hydroxycorticosterone was added to the cold-deactivated reaction mixtures (markers were labelled with isotopes different from that of the substrate). The biosynthetic 18-hydroxycorticosterone, aldosterone and the non metabolized portion of the corticosterone substrate were isolated purified and identified as previously described[19, 21]. In incubations where labelled marker 18-hydroxycorticosterone was not available, the yield and recovery of the biosynthetic material was calculated with reference to the values obtained for aldosterone. The detection of radioactivity on chromatographic strips or plates and the quantitative measurement of the carbon-14 and/or tritium activity was done as described previously [21].

Spectrophotometric determinations

Difference spectra of mitochondrial preparations were taken manually in a UNICAM SP500 Series 2 spectrophotometer, using matched quartz cuvettes with 10 mm lightpass. Cuvettes utilized were either the 3 ml macro- or the 1·5 ml semimicro variety. The instrument was equipped with a temperature controlled cell holder. Spectra were taken at 18 ± 1°C.

RESULTS

Characterization of the duck adrenal mitochondrial system

Time course of the transformation of corticosterone. This study has been reported previously[23]. It was found that between 0 and 30 min the amount of both 18-hydroxycorticosterone and aldosterone formed increased in a linear fashion. The formation of 18-hydroxycorticosterone reached a plateau after 60 min while that of aldosterone after 40 min.

Transformation of corticosterone as a function of enzyme concentration. This study has been previously reported[23]. The transformation of corticoster-

one to 18-hydroxycorticosterone and aldosterone increased proportionally with the presence of increasing amounts of mitochondria.

Influence of metal ions. Corticosterone (16·6 μM) was incubated with mitochondria (1 mg protein equivalent) in a Tris-HCl buffer. Na^+, K^+, Mg^{2+} and Ca^{2+} were added, alone or in combination in chloride form in 4·0 mM concentration and the formation of 18-hydroxycorticosterone and aldosterone measured after 20 min of reaction time. Table 1 shows the results of these experiments. None

Table 1. Metal ion requirements of the mitochondrial synthesis of 18-hydroxycorticosterone and aldosterone from exogenous corticosterone

	Steroids formed as % of control	
Ions added†	18-hydroxy-B*	Aldosterone
Complete system (control) containing $K^+ + Na^+ + Ca^{2+} + Mg^{2+}$	100	100
Minus Mg^{2+}	41	41
Minus Ca^{2+}	15	22
Minus Na^+	48	37
Minus K^+	51	48
Only Ca^{2+}	60	42
Only Mg^{2+}	20	25
Only Na^+	14	20
Only K^+	11	23
No metal ions	11	18

*18-hydroxycorticosterone.

†All systems, including the control were made up with Tris-HCl buffer and ions were added in 4·0 mM concentration. For details, see text.

of the four ions tested could alone support the 18-oxygenation to the full extent. Presence or absence of Ca^{2+} showed the most dramatic effect. However, the simultaneous presence of all these ions was necessary for maximal enzymatic action. Thus the basic composition of the KRB buffer—as noted empirically earlier—could supply all the necessary metal ions. When mitochondria were incubated in a Tris-HCl buffer containing the four metal ions and EDTA, formation of the 18-oxygenated metabolites was not different from the incubation with no metal ions added.

Influence of substrate concentration. Mitochondria (1 mg protein equivalent) were incubated in the presence of increasing concentration of corticosterone (final concentration, 0·66, 1·33, 3·33, 13·33, 33·33 and 66·66 μM). The enzyme present in 1 mg of mitochondrial protein became saturated with 13·2 μM of substrate. At this concentration, both 18-hydroxycorticosterone and aldosterone production levelled off and on the average, 38% of the substrate appeared as 18-hydroxycorticosterone and 9% as aldosterone. Thus the maximal production of 18-hydroxycorticosterone was calculated as 1·43 nmole/min/mg mitochondrial protein and that of aldosterone as 0·33 nmole/min/mg mitochondrial protein. The rate of steroid synthesis was linear for both 18-hydroxycorticosterone and aldosterone when plotted according to Lineweaver and Burk[34] (Fig. 1). The concentration of corticosterone giving half maximal rate of synthesis was $6·6 \times 10^{-6}$M for both metabolites. This is very similar to the K_m values reported by Psychoyos *et al.*[16] for the transformation of exogenous corticosterone to 18-

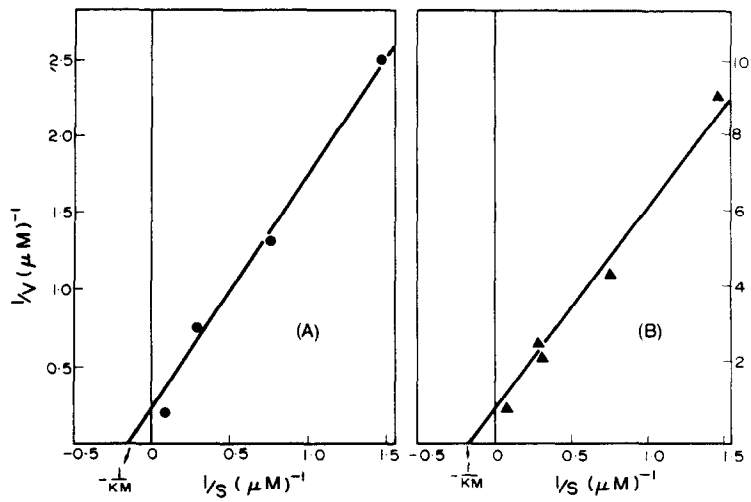

Fig. 1. Effect of corticosterone concentration upon the rate of 18-oxygenation by duck adrenal mitochondria. (A) Lineweaver–Burk plot of the rate of formation of 18-hydroxycorticosterone (1/v) (● – ●) as a function of corticosterone concentration (1/s). (B) Lineweaver–Burk plot of the rate of formation of aldosterone (1/v) (▲ – ▲) as a function of corticosterone concentration (1/s), in the same experiments as in (A). Formation rates for both 18-hydroxycorticosterone and aldosterone are expressed as μM/30 min.

hydroxycorticosterone and aldosterone by bullfrog adrenal mitochondria ($7{\cdot}1 \times 10^{-6}$M).

Cofactor requirements. Earlier experiments have shown that the 18-oxygenation of corticosterone required the presence of NADPH[22, 23]. The cofactor requirements of this reaction were investigated in more detail. Table 2 shows the experimental conditions and the results obtained. Our previous findings were confirmed. It has to be noted that an NADPH generating system—at the same

Table 2. Cofactor requirement for the 18-oxygenation of corticosterone by adrenal mitochondria

	Product formed (nmoles)	
Cofactor(s) added	18-hydroxy-B*	Aldosterone
None	0·06	0·11
NAD	0·08	0·07
NADH	0·07	0·08
NADP	0·06	0·00
NADPH	3·99	1·59
NADPH-G.S.†	5·32	1·47
NADPH-G.S. + ATP	4·40	1·84
NADPH-G.S. + ATP + K-fumarate	2·86	1·21
NADPH-G.S. + ATP + nicotinamide	3·14	1·72

Cofactors were added to a final concentration of 0·5 mM. Corticosterone was added at a concentration of 9·6 μM and the incubation medium (KRB) contained adrenal mitochondria equivalent to 3·4 mg protein. Incubation time was 60 min.

*18-hydroxycorticosterone.

†NADPH-G.S.: NADPH generating system (see: Materials and methods).

concentration—is a better source of reducing equivalents for the biosynthesis of 18-hydroxycorticosterone than is NADPH. Neither fumarate nor nicotinamide stimulated the reaction to any significant extent. In regard of the amount of NADPH necessary for maximal velocity, optimal 18-oxygenation of corticosterone by duck adrenal mitochondria required the presence of NADPH in a molar concentration 10 times that of the substrate.

The effect of temperature. To obtain an approximate value of the activation energies involved in the 18-oxygenation of corticosterone, the effect of temperature on the mitochondrial system has been studied. Table 3 shows the experi-

Table 3. The effect of temperature on the transformation of exogenous corticosterone to 18-oxygenated metabolites by duck adrenal gland mitochondria

Incubation time (min)	Temperature		
	20°C	30°C	40°C
	18-hydroxycorticosterone (μM)		
15	0·18	0·51	0·78
30	0·36	0·79	1·32
45	0·37	1·14	1·79
60	0·50	1·41	2·26
	Aldosterone (μM)		
15	0·10	0·15	0·17
30	0·18	0·26	0·29
45	0·21	0·35	0·45
60	0·29	0·46	0·48

Tritiated corticosterone (11·5 μM) was incubated with adrenal mitochondria (0·33 mg protein/ml) under the usual conditions.

mental conditions and results obtained. At each temperature (20, 30 and 40°C) the time course of the reaction was also studied (vessels analyzed after 15, 30, 45 and 60 min of reaction time). Calculation of the average Q_{10} between 20 and 40°C give the following values: corticosterone → 18-hydroxycorticosterone: 2·16; corticosterone → aldosterone: 1·34. These values correspond to Arrhenius activation energies of 13,600 and 5370 cal/mol respectively.

Substrate specificity. Duck adrenal mitochondria were incubated with the following substrates in addition to corticosterone: progesterone, 11-deoxycorticosterone, 11-dehydrocorticosterone, 20β-dihydrocorticosterone, 18-hydroxycorticosterone and aldosterone. All substrates were carbon-14 labelled. Progesterone gave rise to 11β-hydroxyprogesterone, 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and aldosterone, while 11-deoxycorticosterone was transformed to corticosterone, 18-hydroxycorticosterone and aldosterone [21. 23]. The presence of 18,21-dihydroxy-4-pregnene-3,20-dione could not be demonstrated. 11-Dehydrocorticosterone, 18-hydroxycorticosterone and aldosterone were not metabolized at all and all the radioactivity recovered in these experiments could be accounted for by the added substrates. 20β-Di-

hydrocorticosterone yielded small amounts of 18-oxygenated metabolites. However, the quantities of these two transformation products were only a fraction of those synthesized from labelled corticosterone utilizing the same mitochondrial suspension (18-hydroxycorticosterone: 33·8% and aldosterone: 14·2% of the amount yielded by corticosterone). 11-Dehydro-18-hydroxycorticosterone was not detected in any of the incubations.

Kinetic studies. In an attempt to study the mechanism of formation of 18-hydroxycorticosterone and aldosterone from corticosterone by duck adrenal tissue a detailed kinetic analysis of the transformation *in vitro* of corticosterone to 18-oxygenated products was undertaken. In these experiments, the possibility of two theoretical models was explored. One was the series first order transformation of the type

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where A would represent the concentration of corticosterone, B that of 18-hydroxycorticosterone and C that of aldosterone. The other model was the parallel first order transformation of the type

$$A \begin{cases} \xrightarrow{k_1} B \\ \xrightarrow[k_2]{} C \end{cases} (k_1 + k_2 = K)$$

where again A stands for the concentration of the added exogenous corticosterone. B represents the concentration of 18-hydroxycorticosterone and C the concentration of aldosterone. In the first expression (series first order) k_1 represents the first order disappearance rate constant of corticosterone. The same definition applies for K in the second model (parallel first order).

While exogenous, added 18-hydroxycorticosterone was never metabolized to aldosterone by the duck adrenal mitochondrial system the possibility of the formation of aldosterone through a series first order reaction could not be excluded. Even if we suppose that the intermediary compound between corticosterone and aldosterone is a tautomeric or enzyme-bound form of 18-hydroxycorticosterone the total amount of 18-hydroxycorticosterone appearing in the incubation medium must reflect the amount of 18-hydroxycorticosterone transformed to aldosterone in *statu nascendi.* Kinetically, the hypothetical enzyme-bound or tautomeric 18-hydroxycorticosterone and the 18-hydroxycorticosterone released in the incubation medium cannot be considered as originating from different compartments since they are produced by the same enzymatic step.

Before describing the experiments and results, the theoretical applicability of such models to the systems used in this study had to be established. It is understood that kinetic expressions hold for homogeneous solutions (such as soluble enzyme preparations). However, we could not at any time obtain a solubilized and active 18-oxygenating preparation. In the preparations used in this study (adrenal slices and adrenal mitochondria) the rate limiting step(s) might have been very well permeability and membrane saturation factors rather than the formation and dissociation of the enzyme-substrate complex. The kinetic analysis has been applied to the overall phenomena taking place in a given system and the results obtained are not necessarily valid for the purified and soluble enzyme complex.

As mentioned above, the models used implied that the transformation of exogenous corticosterone to 18-hydroxycorticosterone and aldosterone will occur under conditions of first order kinetics. It was found that using both adrenal gland slices and adrenal gland mitochondria the disappearance of corticosterone from the incubation media followed first order kinetics (see also Fig. 3). The effect of substrate concentration on the first order disappearance rate constant of corticosterone incubated with adrenal mitochondria is shown in Table 4.

Table 4. The influence of concentration on the first order disappearance rate constant (k) of corticosterone

Corticosterone concentration (M)	k(min^{-1})
$6{\cdot}6 \times 10^{-7}$	0·025
$1{\cdot}3 \times 10^{-6}$	0·025
$3{\cdot}3 \times 10^{-6}$	0·027
$1{\cdot}3 \times 10^{-5}$	0·030

[4-^{14}C]-Corticosterone was used as substrate. Mitochondria concentration was 0·33 mg protein/ml and the total incubation volume was 3·0 ml. The incubations were done for 20 min under the usual conditions. The rate constant was calculated by the equation $k = 2{\cdot}303/t \times \log_{10} A_0/A_0 - X$, where A_0 = initial concentration of corticosterone at time 0; $A_0 - X$ = corticosterone concentration at time t.

Using an incubation time of 20 min, the rate constant did not vary significantly between $6{\cdot}6 \times 10^{-7}$M and $1{\cdot}3 \times 10^{-5}$M (variation less than 10%). Thus the experiments were performed in this concentration range.

The next factor to be considered was the endogenous production of steroids. While no such studies were done with adrenal mitochondria, the endogenous production of corticosterone, 18-hydroxycorticosterone and aldosterone was studied with adrenal slices. It was found that the rate of production of corticosterone and 18-hydroxycorticosterone was in the 10^{-9}M and that of aldosterone in the 10^{-10}M range (100 mg tissue, 120 min). In addition, the endogenously formed corticosterone, 18-hydroxycorticosterone and aldosterone formed a homogeneous pool with the radioactive analogues derived from a radioactive precursor (^{14}C-progesterone[35]).

The details of the kinetic experiments were as follows: *Slices*. Adrenal slices (268 mg wet weight) were incubated with [4-^{14}C] corticosterone (6·84 nmoles, $2{\cdot}27 \times 10^{-6}$M) in a KRB medium at 40°C under a 95% O_2–5% CO_2 atmosphere. The incubation was sampled at 5, 10, 15, 30, 60 and 120 min after the start of the reaction and the amounts of corticosterone, 18-hydroxycorticosterone and aldosterone determined for each time point. *Mitochondria*. Sediment equivalent to 10·8 mg of protein was incubated in a KRB medium at 40°C with the same amount of [4-^{14}C] corticosterone as in the slice experiment. The mixture was sampled at 8, 10, 15, 30, 60 and 120 min after the start of the reaction. The concentration-time relationships for these experiments are shown in Figs. 2A and 2B.

These data were analyzed in the following manner: 1. Test for series first order reaction. This was done by comparing the experimentally obtained time-

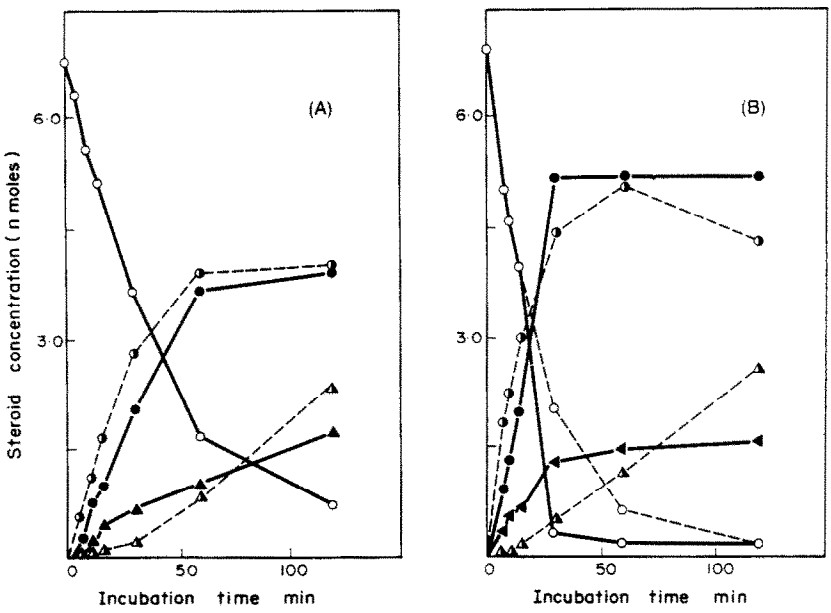

Fig. 2. The transformation *in vitro* of corticosterone to 18-hydroxycorticosterone and aldosterone as a function of the time of incubation. (A) Transformation of corticosterone by duck adrenal gland slices. (B) Transformation of corticosterone by a duck adrenal mitochondrial suspension. ○———○: corticosterone found; ○----○: corticosterone calculated; ●———●: 18-hydroxycorticosterone found; ◑----◑: 18-hydroxycorticosterone calculated; ▲———▲: aldosterone found; ▲----▲: aldosterone calculated. For further details, see text.

concentration curves with calculated ones. For calculation the following well known equations were used[36]:

$$A = A_0 e^{-k_1 t} \tag{1}$$

$$B = (A_0 k_1/k_2 - k_1)(e^{-k_1 t} - e^{-k_2 t}) \tag{2}$$

$$C = A_0(1 + 1/k_1 - k_2(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})) \tag{3}$$

where A_0 = initial concentration of corticosterone at time 0; A = corticosterone concentration at time t; B = concentration of 18-hydroxycorticosterone at time t: C = concentration of aldosterone at time t. The value of k_1 was calculated from the expression $k_1 = 2{\cdot}303/t$ ($\log_{10} A_0/A_0 - X$) in which A_0 was defined as above and $A_0 - X$ was the substrate concentration at time t. The values for k_1 were 0·019 min^{-1} for the slice experiment and 0·039 min^{-1} for the mitochondria experiment. Under ordinary circumstances, the values of k_2 could have been obtained experimentally by incubating radioactive 18-hydroxycorticosterone with adrenal gland preparations and from the time-yield values the first order disappearance rate of these steroids calculated. However, as mentioned earlier, exogenous 18-hydroxycorticosterone is not utilized by adrenal gland preparations *in vitro*. Thus, instead the rate constant of the appearance of aldosterone was calculated by the formula $k = 2{\cdot}303/t$ ($\log_{10} A_0/A_0 - X$). The value of A_0 in this instance represented the combined concentrations of corticosterone + 18-hydroxycorti-

costerone for every time point. The comparison of the experimental curves with the time-concentration curves obtained by calculation are shown in Figs. 1A and 1B. The calculated k_2 values were 0·005 min^{-1} for the slice experiment and 0·006 min^{-1} for the mitochondria experiment.

2. Test for parallel first order reaction. In a parallel first order reaction, one substrate undergoes two or more reactions independently and concurrently. The disappearance of the substrate is simple first order and the disappearance rate constant of the substrate is the sum of the constants of appearance of the products. All the concentrations vary exponentially with the same exponential constant K (the disappearance rate constant of the substrate). Thus, if $1-e^{-Kt}$ is plotted against the concentrations of substrate and products, a series of straight lines will be obtained as all reactants have the same half-life or half-growth time despite having different rate constants[36]. Such plots have been constructed for both slice and mitochondria experiments and are shown in Figs. 3A and B. The values

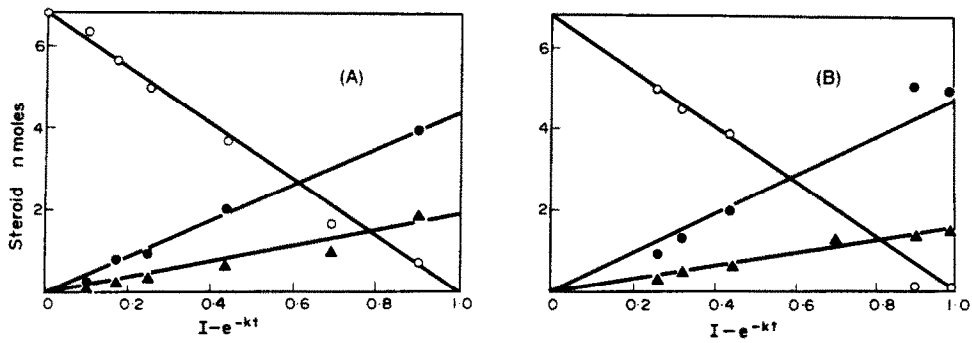

Fig. 3. Plot of concentration data shown in Fig. 1 against $1-e^{-Kt}$. K = first order disappearance rate constant of corticosterone. (A) Duck adrenal gland slices, K = 0·019 min^{-1}. (B) Duck adrenal gland mitochondria, K = 0·039 min^{-1}. ○———○: corticosterone; ●———●: 18-hydroxycorticosterone; ▲———▲: aldosterone.

of K are 0·019 min^{-1} for the slice experiment and 0·039 min^{-1} for the mitochondria experiment. Both plots yielded straight line relationships between $1-e^{-Kt}$ and steroid concentration.

On the basis of these kinetic experiments and the results shown in Figs. 2 and 3 the following conclusions could be drawn: The transformation of corticosterone to 18-hydroxycorticosterone and to aldosterone is kinetically pseudo-first order, both by slices and mitochondrial preparations. The concentration of 18-hydroxycorticosterone does not show in any of the experiments the characteristic decrease of intermediaries of series first order reactions. The biosynthesis of both 18-hydroxycorticosterone and aldosterone was dependent upon the availability of corticosterone. Once corticosterone was exhausted, aldosterone synthesis stopped even though large amounts of 18-hydroxycorticosterone were present in the system. The induction period, which should have been present in the formation of aldosterone at early time points and which is quite apparent on the calculated aldosterone time-concentration curves is absent in the experimental results. The concentration of aldosterone increased sharply right from the start of the reaction. This feature is again indicative of a parallel reaction. There is no essential difference between the course of the reaction whether slices or mitochondrial suspensions were used.

Inhibitor studies. Product inhibition. The transformation of corticosterone to 18-hydroxycorticosterone and aldosterone by mitochondria has been studied in the presence of increasing amounts of exogenous 18-hydroxycorticosterone and aldosterone. The following experiments were performed: (a) [4-^{14}C] corticosterone (4·7 nmoles) was incubated in with mitochondria (0·5 mg protein equivalent) in the absence and presence 55·0, 138·0 and 276·0 nmoles of synthetic, d,l-18-hydroxycorticosterone. (b) [4-^{14}C] Corticosterone as above was incubated with mitochondria (0·5 mg protein equivalent) in the presence and absence of 27·0, 69·0, 138·0 and 276·0 nmoles of d-aldosterone. Results are shown in Table 5.

Table 5. The transformation of corticosterone (4·7 nmoles) to 18-hydroxycorticosterone and aldosterone by adrenal mitochondria in the presence of exogenous 18-hydroxycorticosterone and aldosterone

Exogenous steroid added	Amount nmoles	Yield of metabolites (nmoles)	
		18-OH B*	Aldosterone
None	—	2·50	0·62
d,l-18-OH B	55·0	2·06	0·58
d,l-18-OH B	138·0	1·66	0·44
d,l-18-OH B	276·0	1·14	0·28
None	—	2·43	0·69
d-Aldosterone	27·0	2·36	0·52
d-Aldosterone	69·0	2·49	0·59
d-Aldosterone	138·0	2·38	0·53
d-Aldosterone	276·0	2·43	0·58

*18-Hydroxycorticosterone.

Exogenous 18-hydroxycorticosterone did not give a dilution effect on the production of radioactive aldosterone. However, there was a marked inhibitory effect on both 18-hydroxylation and aldosterone formation. Under the influence of exogenous 18-hydroxycorticosterone the decrease in the production of radioactive 18-hydroxycorticosterone and aldosterone was strictly parallel (mean 18-hydroxycorticosterone to aldosterone ratio: $3{\cdot}85 \pm 0{\cdot}24$). A plot of the exogenous 18-hydroxycorticosterone concentration against the reciprocal of either biosynthetic 18-hydroxycorticosterone or biosynthetic aldosterone concentration gave a straight line relationship. The inhibitor constant obtained from this plot gave a value of $2{\cdot}2 \times 10^{-4}$M. Exogenous d-aldosterone had no inhibitory effect on 18-hydroxycorticosterone, though it slightly inhibited aldosterone biosynthesis.

Enzyme inhibitors. Table 6 summarizes the effect of various inhibitors upon the formation of 18-hydroxycorticosterone and aldosterone from corticosterone by adrenal mitochondria. Most pronounced inhibitions were shown by the mercurial, p-chloromercuribenzoate, carbon monoxide, metopirone and the folic acid antagonist, aminopterin. Protein synthesis inhibitors did not have any effect upon mitochondrial 18-oxygenation.

Among the inhibitors listed in Table 6 the actions of p-chloromercuribenzoate (p-CMB), metopirone and aminopterine have been investigated in some detail.

p-CMB inhibition. The effect of organic mercurials upon steroid biosynthesis has been described earlier[37] and was found to be a powerful inhibitor especially

Table 6. Effect of various inhibitors upon the 18-oxygenation of corticosterone by duck adrenal mitochondria

Inhibitor	Concentration	Type of inhibition	Steroid produced as % of control	
			18-OH B*	Aldosterone
None (control)	—	—	100	100
p-CMB	0·05 mM	Sulfhydril group	22	50
Na-iodoacetate	1·0 mM	Sulfhydril group	86	83
Na-azide	1·0 mM	Cyt. "c"-Fe^{2+}-O_2	107	133
Na-cyanide	1·0 mM	Electron transport	92	94
Dicoumarol	1·0 mM	Uncoupling agent	145	183
Actinomycin-D	0·1 to 1·0 mM	RNA synthesis	100	100
Chloramphenicol	0·005 to 1·0 mM	Protein synthesis	100	100
Cycloheximide	0·6 to 12·0 mM	Protein synthesis	100	100
Carbon monoxide	5 min saturation	Cytochrome-P450	14	14
Metopirone	0·07 mM	Cytochrome-P450	7	19
Aminopterine	0·5 mM	Folic acid antagonist	49	43

*18-OH B: 18-hydroxycorticosterone.

In all experiments, mitochondria equivalent to 1 mg protein were incubated in the presence of an NADPH generating system (0·4 mM) and the additives for 10 min. This was followed by the addition of tritiated corticosterone (19·2 μM) and the incubation left to proceed for an additional 50 min period.

of microsomal 21-hydroxylation. The concentration dependent action of p-CMB upon 18-oxygenation of corticosterone by mitochondria is shown in Fig. 4. The following conclusions could be drawn: at low p-CMB concentrations, the mercurial stimulated rather than inhibited the formation of 18-hydroxycorticosterone and aldosterone. Inhibition of 18-hydroxycorticosterone formation started at a substrate : inhibitor ratio of 1·7 : 1. The inhibition of aldosterone formation became noticeable at a ratio of 3·8 : 1. At the point, where the molar concentrations of substrate and p-CMB were equal, 18-hydroxycorticosterone synthesis was inhibited by 62% and that of aldosterone by 35%. The increase in p-CMB concentration effected 18-hydroxycorticosterone synthesis to a much larger degree than it did aldosterone formation. When the inhibitor concentration reached about twice that of the substrate concentration, the initial 18-hydroxycorticosterone to aldosterone ratio decreased from the initial 7·9 (no p-CMB added) to 4·0.

Construction of a Dixon plot[38] did not give an unequivocal value for inhibitor constants due to the initial stimulation by low p-CMB concentrations. However, by extrapolation, the estimated K_i for aldosterone was $2{\cdot}5 \times 10^{-5}$M and $3{\cdot}0 \times 10^{-6}$M for 18-hydroxycorticosterone.

Increasing the p-CMB concentration to 10^{-3}M levels resulted in complete inhibition of the 18-oxygenation of corticosterone. However, it is doubtful, whether at these concentration levels one can speak about enzyme inhibition. Large amounts of p-CMB changed the macroscopic appearance of the mitochondrial suspension indicating possible general denaturation of proteins.

Metopirone inhibition. The inhibitory effect of metopirone upon adrenal 18-oxygenation was first described by Kahnt and Neher[39]. The concentration

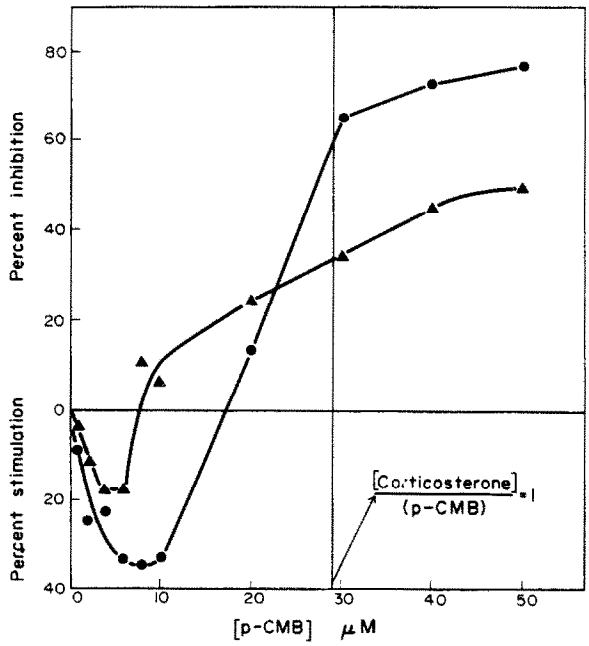

Fig. 4. Effect of increasing concentrations of p-CMB upon the transformation of corticosterone to 18-hydroxycorticosterone and aldosterone by duck adrenal gland mitochondria. Corticosterone (28·9 μM) was incubated with mitochondria equivalent to 1 mg of protein in a 0·15M glycylglycine buffer. For further details, see text. Code of compounds as in Fig. 3.

dependent metopirone inhibition of 18-hydroxycorticosterone and aldosterone formation from corticosterone by adrenal mitochondria is shown in Fig. 5A. Metopirone present in equimolar quantities with the substrate corticosterone inhibited 18-hydroxycorticosterone synthesis by 75% and aldosterone synthesis by 51%. Similarly to the effect of p-CMB, increasing metopirone concentrations effected more the formation of 18-hydroxycorticosterone than that of aldosterone. Thus the 18-hydroxycorticosterone to aldosterone molar ratio changed from the control value of 7·2 to 2·7. At this metoprione concentration (3·8 times that of corticosterone) the formation of 18-hydroxycorticosterone was inhibited by 92·8% and aldosterone by 80·4%.

Lineweaver–Burk plots[34] of 1/s (s = corticosterone concentration) vs. 1/v (v = amount of 18-hydroxycorticosterone or aldosterone produced/min) in the presence or absence of metopirone have shown that the inhibition was competitive for both 18-hydroxycorticosterone and aldosterone. The inhibitor constants were found by constructing Dixon plots. The value of K_i was $3{\cdot}0 \times 10^{-6}$M for 18-hydroxycorticosterone and $9{\cdot}0 \times 10^{-6}$M for aldosterone.

Inhibition by aminopterin. The inhibitory effect of aminopterin upon steroid hydroxylation has been noted previously in studies from our laboratories[40, 41]. The inhibitory effect of this substance upon 18-oxygenation of corticosterone was distinct but less efficient than that of metopirone. The inhibition as the function of aminopterin concentration is shown in Fig. 5B. Lineweaver–Burk plots (1/s against 1/v; see metopirone inhibition) in the absence and presence of aminopterin indicated that the inhibition of the formation of both 18-hydroxycorticosterone

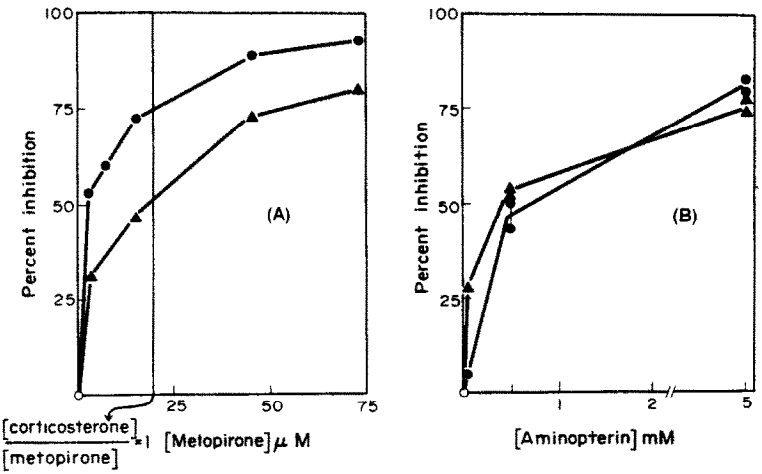

Fig. 5. Effect of inhibitors upon the transformation of corticosterone to 18-hydroxycorticosterone and aldosterone by duck adrenal gland mitochondria. (A) Effect of increasing concentrations of metopirone. (B) Effect of increasing concentrations of aminopterin. Corticosterone (19·2 μM) was incubated with mitochondria equivalent to 1 mg of protein in KRB. ●——●: 18-hydroxycorticosterone; ▲——▲: aldosterone.

and aldosterone is most probably of the noncompetitive type. Calculation of the inhibitor constant gave a value of $2{\cdot}5 \times 10^{-5}$M. Amethopterin was without effect.

*The presence of cytochrome-*P450 *in duck adrenal mitochondria*

To determine whether cytochrome-P450 participated in the 18-oxygenation of corticosterone by duck adrenal mitochondria, the following criteria were used: the presence of P450 in mitochondrial preparations as evidenced by the appearance of the characteristic Soret bands upon reduction and upon subsequent addition of CO; difference spectra induced by the addition of substrates and inhibitors to mitochondrial suspensions; the inhibitory action of CO and metopirone upon mitochondrial 18-oxygenation.

Figure 6 shows the difference spectra obtained with duck adrenal mitochondrial suspensions. Calculation of the amount of P450 present using the optical density differences between 450 and 490 nm and a $\Delta\varepsilon_{mM}$ of 91 $cm^{-1}mM^{-1}$ [42] gave the concentration of this cytochrome in the range of $0{\cdot}6 \times 10^{-9}$M per mg mitochondrial protein.

The presence of P450 could also be demonstrated in an acetone powder of mitochondria, prepared according to Williamson and O'Donnell[30]. In this preparation, the hemoprotein was present in its inactive form (P420) and the acetone powder was totally devoid of any 18-oxygenating capacity.

To confirm the presence of P450 in more purified preparations, sonicated duck adrenal mitochondria were fractionated according to the method of Omura *et al.*[31] developed for the resolution of mitochondrial 11β-hydroxylase system. This preparation after reconstitution was again devoid of 18-hydroxylase activity which was found to be lost by sonication of more than 1 min. However, in the fraction denoted by the authors as P_2 particles, difference spectra revealed the presence of a mixture of P450–P420. The difference spectra obtained on the mitochondrial acetone powder and on the mitochondrial P_2 particles is shown in Figs. 7A and B.

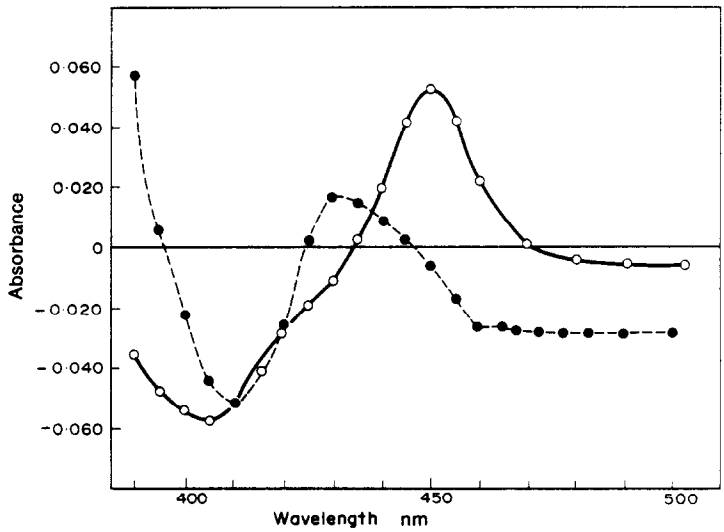

Fig. 6. Spectrophotometric determination of cytochrome-P450 of duck adrenal mitochondria. Mitochondrial suspensions (0·9 mg protein) were prepared in KRB and divided equally into two cuvettes. After establishing a baseline absorption, the contents of one of the cuvettes was reduced with a few crystalls of sodium dithionite and the difference in absorption between the reduced and oxidized pigment measured (●----●). This was followed by the reduction of the contents of the other cuvette and establishing a baseline absorption. Finally, the contents of one of the cuvettes was gassed with CO for 1 min and the difference spectrum between the reduced carbon monoxide complex and the reduced pigment measured (○———○.

Addition of corticosterone to mitochondrial suspensions produced difference spectra of type II (trough at 380–390 nm, peak at 420 nm). Though in the literature type II spectra were described as having additional peaks at 530 and 580 mμ, these last two spectral bands were not prominent in the corticosterone induced difference spectra. Similar spectra were obtained by the addition of 11-deoxycorticosterone to mitochondria. Representative difference spectra induced by these steroids are shown in Fig. 8.

Metopirone when added to mitochondrial suspensions gave spectra almost indistinguishable from that of corticosterone. Prior addition of corticosterone followed by metopirone or vice-versa did not result in any qualitative change in the spectra. Difference spectra obtained with metopirone and metopirone + corticosterone in mitochondrial suspensions are shown in Fig. 8.

Proportionality existed between the amount of corticosterone or metopirone added to mitochondrial suspensions and the intensity of the spectral band at 420 nm. Figure 9 shows the relationship between the amount of the added substance and the changes in absolute optical density as calculated by the difference between 420 and 390 nm. It appeared that the mitochondrial P450 of duck adrenals became saturated with either corticosterone or metopirone at a final concentration of 21·8 μM/mg protein. Addition of p-CMB resulted in a difference spectrum with a peak at 400 nm, while aminopterin failed to induce any difference spectra.

From these experiments it was concluded that cytochrome-P450 was present in duck adrenal mitochondria and was necessary for the introduction of the oxygen atom at C-18.

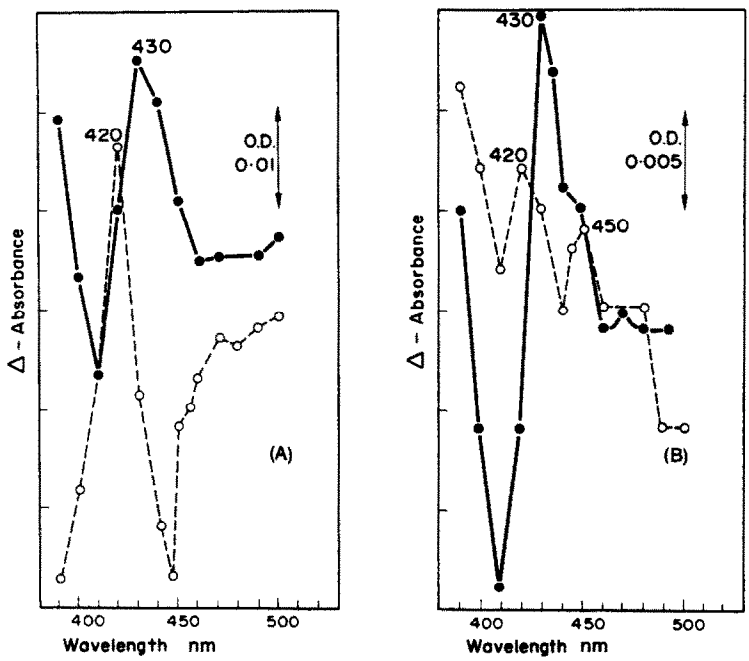

Fig. 7. Spectrophotometric determination of cytochrome-P450 of (A) Duck adrenal mitochondrial acetone powder (0·33 mg protein/ml) and (B) P450 particulate fraction of a duck adrenal mitochondrial sonicate (0·13 mg protein/ml). Both suspensions prepared in KRB. Solid lines: reduced minus oxidized pigment; dashed lines: CO complex of reduced pigment minus reduced pigment. In both preparations, the inactive form of the hemoprotein, cytochrome-P420 was much evident. Absorbance represented as absolute difference.

DISCUSSION

The aim of the present study was two-fold. Firstly it was attempted to characterize the duck adrenal mitochondrial 18-oxygenating system and secondly to compare this system with those of other vertebrate adrenals.

Results obtained in this study led us to believe that the overall kinetics of the transformation of exogenous corticosterone to 18-hydroxycorticosterone and aldosterone were more compatible with a parallel first order model than with a series first order model. In both slice and mitochondria experiments the formation of aldosterone depended entirely on the availability of the substrate corticosterone and not on the presence of 18-hydroxycorticosterone. Addition of exogenous 18-hydroxycorticosterone to the mitochondrial system resulted in the decrease of both 18-hydroxycorticosterone and aldosterone formation, but no dilution effect was observed in the resultant radioactive biosynthetic aldosterone. Such an inhibition was noted by Raman *et al.*[11] in the conversion of corticosterone to the two 18-oxygenated metabolites by sheep adrenal mitochondria and by Vecsei *et al.*[43] in the conversion of progesterone to 18-hydroxycorticosterone and aldosterone by quartered rat adrenals. However, sheep adrenal mitochondria were able to transform exogenous 18-hydroxycorticosterone into small but significant amounts of aldosterone. Similar transformations were noted with rabbit adrenal mitochondria[12] while bullfrog adrenal mitochondria, similarly

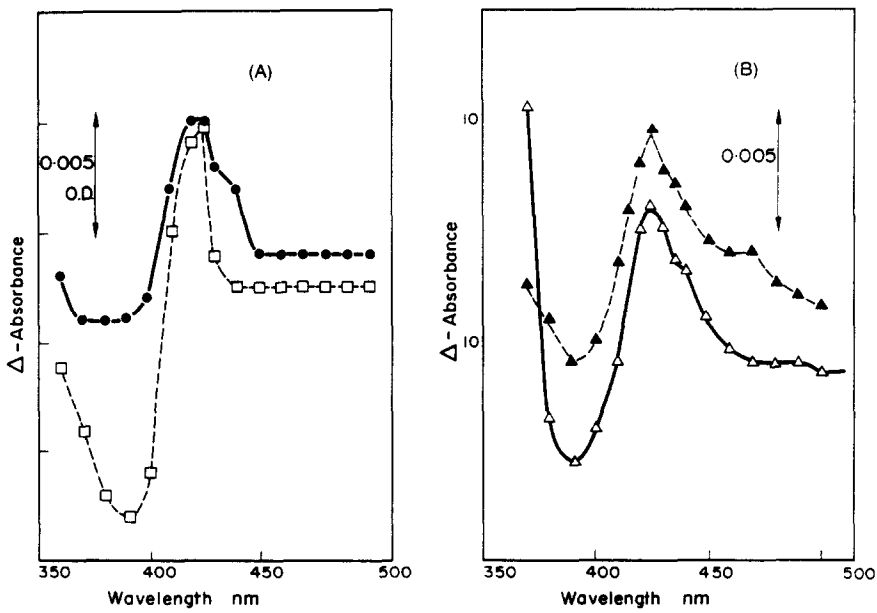

Fig. 8. Steroid and metopirone induced difference spectra of duck adrenal mitochondria. (A) Difference spectrum induced by the addition of corticosterone (173 μM) to a mitochondrial suspension in KRB containing 0·64 mg of protein/ml (●———●); difference spectrum induced by the addition of 11-deoxycorticosterone (50·0 μM) to a mitochondrial suspension in KRB containing 0·7 mg protein/ml (□----□). (B) Difference spectrum induced by the addition of metopirone (200 μM) to a mitochondrial suspension in KRB containing 0·66 mg protein/ml (△———△); difference spectrum induced by the addition of 200 μM of metopirone and +96·0 μM of corticosterone to the above suspension (▲----▲). In both (A) and (B) absolute absorbance differences were represented.

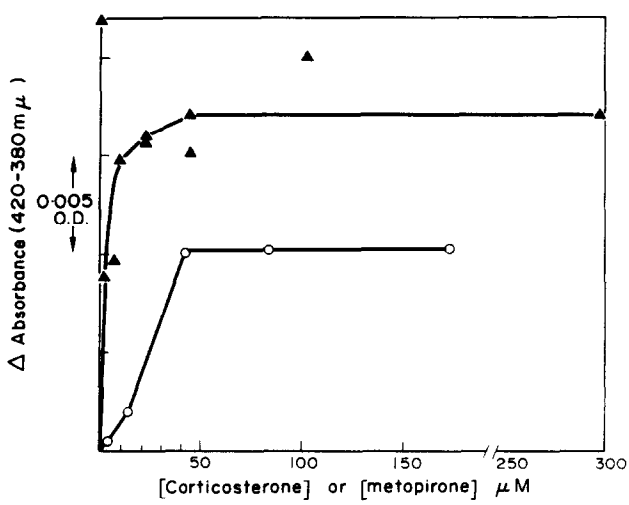

Fig. 9. Effect of corticosterone and metopirone concentration on the spectral changes of duck mitochondrial P450. ▲———▲: change in absolute absorbance (420–380 mμ) following the addition of increasing concentrations of corticosterone to a mitochondrial suspension (0·66 mg protein/ml in KRB); ○———○: change in absolute absorbance (420–380 mμ) following addition of increasing concentrations of metopirone to a mitochondrial suspension (as above).

to the duck preparations, did not utilize exogenous 18-hydroxycorticosterone as aldosterone substrate[17].

Similarly to all other vertebrate adrenal 18-oxygenating systems investigated, duck adrenal mitochondrial 18-oxygenation is supported by reduced NADP. The formation of 18-hydroxycorticosterone was somewhat higher in the presence of an NADPH generating system than following addition of NADPH. Similar but quantitatively more important differences were noted with sheep adrenal mitochondria[11] and with sheep adrenal gland homogenates[44]. However, the formation of 11-dehydro-18-hydroxycorticosterone from corticosterone, reported to occur with rabbit[12] and rhesus monkey[45] adrenals could not be shown with the duck adrenal mitochondrial system or with bullfrog adrenal preparations[46, 47]. Similarly, the pathway 11-dehydrocorticosterone → 11-dehydro-18-hydroxycorticosterone → 11-dehydroaldosterone[12] and the transformation 18-hydroxycorticosterone → 11-dehydro-18-hydroxycorticosterone [11, 13] are attributes of mammalian adrenal systems and were not detected with either frog or duck adrenal preparations.

In the frog, mitochondrial transformation of corticosterone to 18-hydroxycorticosterone required the presence of Ca^{2+} while Mg^{2+} was necessary for optimal aldosterone synthesis. With sheep mitochondria, the presence of Ca^{2+} was necessary for the effective 18-oxygenation of exogenous corticosterone [11, 17]. In our experiments with duck adrenal mitochondria, Ca^{2+} seemed a more important ion than Mg^{2+}. However, for optimal 18-oxygenation, Ca^{2+}, Mg^{2+}, K^+ and Na^+ were necessary.

The 18-oxygenation of corticosterone was effectively inhibited by p-CMB, metopirone, carbon monoxide and aminopterin. On the other hand dicoumarol, a known uncoupler of oxidative phosphorlyation[60] stimulated rather than inhibited corticosterone-18-oxygenation. The inhibition of 18-oxygenation by metopirone and CO, combined with the demonstration of the spectrophotometric presence of cytochrome-P450 in duck adrenal mitochondria and mitochondrial sonicates, implicated cytochrome-P450 as oxygen activator in the 18-oxygenation of corticosterone. The probable role of P450 was further confirmed by the corticosterone, 11-deoxycorticosterone, metopirone and p-CMB induced difference spectra. The difference spectra obtained with the reduced P450–CO complex of duck adrenal mitochondria were qualitatively similar to those obtained by adrenal mitochondria of other vertebrates, including mammals[48]. Corticosterone and 11-deoxycorticosterone induced difference spectra of the type II variety in duck contrary to the type I spectra obtained by these compounds with bovine adrenal mitochondria[49–52]. Mitanie and Horie[49] associated type I spectra with an increase in the high spin state and type II with an increase in the low spin state of the hemoprotein. Our results might indicate a species difference in the ligand state of this cytochrome.

Metopirone inhibition of steroid hydroxylation can be associated with the ability of this substance to compete for active sites with the steroidal substrates at the level of mitochondrial P450[53, 54]. It should be pointed out, that metopirone, at a given concentration, inhibits more the synthesis of 18-hydroxycorticosterone than that of aldosterone. This would in keeping with its presumed action upon the P450 mediated activation of atmospheric oxygen. A similar partial dissociation of 18-hydroxycorticosterone and aldosterone formation was noted in the presence of inhibitory amounts of p-CMB. The action of mercurials

on steroid hydroxylation has been connected with the transformation of P450 to its inactive form, P420, though recent work on hepatic microsomes seems to suggest that mercurials exert their inhibitory effect through limiting the supply of reducing equivalents along the electron transport chain required for P450 function[55]. In this connection mention should be made of the stimulatory effect of low p-CMB concentrations upon corticosterone 18-oxygenation by adrenal mitochondria (see Fig. 3). This seemingly anomalous effect could be best discussed in conjunction with the aminopterin inhibition of 18-oxygenation reported in this paper. It has been noted earlier that addition of exogenous folic acid to adrenal gland mitochondria or microsomes stimulated and aminopterin non-competitively inhibited mitochondrial 11β- and microsomal 21-hydroxylation [40]. These observations would be consistent with an NADPH-pteridine supported hydroxylation similar to the aromatic hydroxylation found in mammalian liver[56].

Such a hydroxylation mechanism would be inhibited by aminopterin and the dihydropteridine reductase would be stimulated by p-CMB[57]. Recent work in our laboratory has indeed shown that steroid hydroxylation by duck adrenal mitochondria can be partially supported by reduced folic acid derivatives[58]. Thus pteridines could function either as electron carriers in conjunction with the P450 system or again act as NADPH dependent oxygen activators not connected to the hemoprotein chain.

To summarize the action of enzyme inhibitors on the duck adrenal gland mitochondrial system: metopirone is the most efficient inhibitor (after carbon monoxide) and its inhibition acts through competition for active sites on the P450 system. For a given concentration of metopirone, the 18-hydroxylation of corticosterone is more strongly inhibited than the formation of aldosterone. By titrating mitochondrial P450 with corticosterone and metopirone, saturation of the hemoprotein was achieved with identical concentration for both substances. p-CMB at low (about 10 μM/mg mitochondrial protein) concentrations stimulated the 18-hydroxylation of corticosterone and to a lesser extent, the synthesis of aldosterone. It is quite conceivable, that at low concentration, p-CMB does not interfer with either P450 or the electron transport but stimulates hydroxylation through its action upon dihydropteridine reductase. Increasing p-CMB concentrations will result in decreasing oxygen activation, hence 18-oxygenation decreases as the inhibitory effect will override the dihydropteridine reductase activation.

p-CMB is a mercurial and it is customary to relate enzyme inhibition by mercurials with blockage of enzymatic sulhydril groups. It is interesting to note that another potent sulfhydril group inhibitor, Na-iodoacetate had a much less pronounced effect on the 18-oxygenation of corticosterone.

The inhibition of steroid hydroxylation by aminopterin is difficult to explain by its eventual action upon the hydroxylating electron chain or P450 itself. While both metopirone and p-CMB yielded difference spectra when added to mitochondrial suspensions, the presence of aminopterin did not result in any spectral changes. Aminopterin inhibition of 18-hydroxylation is non-competitive and the demonstration of a possible role of reduced pteridine derivatives in steroid hydroxylation in general supports our hypothesis that aminopterin interferes with the enzyme systems necessary to produce pteridines of different reduction levels. A more detailed study on the above mechanisms will be published[58].

Our findings that dicoumarol stimulates rather than inhibits 18-oxygenation of

corticosterone is at variance with the report of Williamson and O'Donnell[59] who have shown dicoumarol inhibition of 11β-hydroxylation by a bovine adrenal mitochondrial preparation. The divergences between their and our findings might be either due to differences in enzyme preparations or again might point to the fact that different hydroxylations might be performed by different species of P450.

Attempts to obtain a soluble duck adrenal mitochondrial 18-oxygenase system have not met with success. Ultrasonication or osmotic disruption of the mitochondria deactivated the 18-oxygenating enzyme complex though partly intact P450 particles could be still isolated. Though final characterization of this enzyme system must await solubilization and separation into its component parts, the following conclusions can be drawn on the basis of the available evidence:

(1) 18-oxygenation of corticosterone by duck adrenal mitochondria is accomplished by way of mixed function oxidase. The correct sequence of events is not known but the possibilities are as follows: (a) Corticosterone is first transformed to 18-hydroxycorticosterone which, *in statu nascendi* is oxidized to aldosterone or (b) Oxygen is introduced into corticosterone at position 18 to form a labile intermediary compound which, by enzymatic action and/or by chemical rearrangement yields simultaneously 18-hydroxycorticosterone and aldosterone. Kinetic data presented in this study argue in favour of this second possibility.

(2) Mitochondrial 18-oxygenating systems of vertebrate adrenals show distinct class and species differences.

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