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PTERIDINS AND STEROID HYDROXYLATION – I.

1–THE EFFECT OF FOLIC ACID AND AMINOPTERIN ON THE STEROID 11β- AND 21-HYDROXYLATION BY DOMESTIC DUCK (*ANAS PLATYRHYNCHOS*) ADRENOCORTICAL TISSUE PREPARATIONS

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SUMMARY

The effects of folic acid and aminopterin were studied on the steroid 11β and 21-hydroxylases of domestic duck adrenals. Aminopterin partially inhibited the transformation of labelled, exogenous progesterone to 11β-, 18- and 21-hydroxylated metabolites by a duck adrenal gland whole homogenate, while the presence of folic acid resulted in a slight stimulation of the sequential hydroxylation of progesterone. When added to duck adrenal mitochondrial or microsomal preparations aminopterin effected a concentration dependent inhibition of the 11β- or 21-hydroxylase activity while the presence of folic acid resulted in a concentration dependent stimulation. Aminopterin inhibition of mitochondrial 11β-hydroxylase was competitive, while that of the microsomal 21-hydroxylation was non-competitive. Neither folic acid or aminopterin induced difference spectra of mitochondrial or microsomal cytochrome P-450.

INTRODUCTION

THE POSSIBLE involvement of pteridin co-factors in the enzymatic hydroxylation of steroids was suggested by the experiments of Hagerman[1] in which tetrahydrofolic acid was shown to effect 17α-hydroxylation of progesterone by rat testes. Subsequent experiments in our laboratories have demonstrated that folic acid stimulated and aminopterin inhibited the 18-oxygenation of exogenous corticosterone to 18-hydroxycorticosterone and aldosterone by duck adrenal gland mitochondria[2, 3].

The present report is concerned with the possible effects of these two pteridins on the steroid hydroxylation reactions by duck adrenal gland preparations. The following experiments were performed: (1) Metabolism of exogenous, radioactively labelled progesterone by duck adrenal gland whole homogenates to corticosteroids in the presence and absence of folic acid or aminopterin; (2) The effect of aminopterin and folic acid on the 11β-hydroxylation of exogenous deoxycorticosterone (DOC) by duck adrenal mitochondria and on the 21-hydroxylation of exogenous progesterone by duck adrenal microsomes; (3) Kinetic studies on the type of aminopterin inhibition.

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MATERIALS AND METHODS

Tissue preparations

Immature male domestic ducks (*Anas platyrhynchos*) weighing between 1·5 and 2·0 kg were used. The animals were killed and the adrenal glands removed and dissected immediately. The glands were homogenized in 0·25 M sucrose (10% w/v). Adrenal mitochondria and microsomes were obtained by differential centrifugation by the method of Schneider and Hogeboom[4] using an L2-65B model preparative ultracentrifuge (Spinco Division, Beckman Instruments, Palo Alto, California). Once the intracellular organelles were sedimented, they were resuspended in an appropriate medium (see below).

Substrates

The following radioactively labelled substrates were used: progesterone-[4-^{14}C] (SA: 53·2 mCi/mM), desoxycorticosterone-[4-^{14}C] (SA: 45·5 mCi/mM) and corticosterone-[4-^{14}C] (SA: 57·5 mCi/mM). The labelled steroids were obtained from New England Nuclear Corporation, Boston, Mass. Radioinert steroids were purchased from IKHAPHARM, Ramat-Gan, Israel. Homogeneity of both labelled and non-labelled commercial steroids was checked by paper partition (PPC) and thin-layer (TLC) chromatographic techniques and by crystallizations using isotopic dilution methods. Folic acid was purchased from Sigma Chemical Co., St. Louis, Mo. and aminopterin was a gracious gift of the Lederle Division, American Cyanamide Company, Pearl River, N.Y.

Medium

A Krebs–Ringer bicarbonate solution (pH: 7·4), containing 200 mg of glucose/100 ml medium was used as an incubation medium. As a source of reducing equivalents, exogenous NADPH or a NADPH generating system, consisting of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase was used. These substances were obtained from Boehringer Mannheim Corp., New York, N.Y. Amounts of co-factors used are specified in the description of individual experiments. Steroidal substrates were dissolved in 0·05 ml of propylene glycol prior to incubation.

Incubation

Incubations were performed in a metabolic shaking incubator at 39°C in an O_2–CO_2 (95–5%) atmosphere. The length of incubation is specified for each experiment in the "Results" section. Incubations were terminated by adding ethyl acetate and freezing.

Isolation, purification and identification of metabolic transformation products

The incubation media were extracted with a 1 : 1 mixture of chloroform–ethyl acetate. The isolation, purification and identification of radioactive biosynthetic compounds were done as described previously[5–8].

Calculation and presentation of results

To correct for losses during the isolation and purification procedures, authentic, tritium labelled carrier steroids were added to the incubation mixtures prior to extraction. The effect of aminopterin and folic acid upon the transformation of a given substrate to metabolites was expressed as a percentage value relative to

the control experiment not containing pteridins. The transformation values of the control experiments were taken as 100%. Thus any transformation value above 100% denotes stimulation, while those less than a 100% indicate inhibition. In experiments, where varying substrate concentrations were used, results were expressed in molarity.

RESULTS

Experiment 1: Incubation of progesterone-[4-^{14}C] with duck adrenal gland whole homogenate in presence and absence of aminopterin or folic acid

In this experiment, three incubation vessels were prepared. Each contained a duck adrenal gland whole homogenate (10 %), equivalent to 330 mg of adrenal tissue, progesterone-[4-^{14}C] ($2{\cdot}45 \times 10^6$ dpm; 0·44 mM) and a NADPH generating system (0·44 mM). One vessel did not contain any pteridins, the second contained 0·36 mM of folic acid and the third 0·36 mM of aminopterin. Following 30 min incubation authentic tritiated DOC, corticosterone and aldosterone were added to each vessel.

In this experiment, the following carbon-14 labelled metabolites were isolated and identified: 11β-hydroxyprogesterone, DOC, corticosterone, 18-hydroxycorticosterone and aldosterone. The quantitative results obtained are shown in Table 1.

Table 1. Quantitative effect of folic acid and aminopterin on the transformation of progesterone-[4-^{14}C] to corticosteroids by a duck adrenal gland whole homogenate. (Experiment 1)

Metabolites formed	Yield of metabolites					
	No pteridins added		Aminopterin (0·36 mM) added		Folic acid (0·36 mM) added	
11β-hydroxy-progesterone	100*	(6·5)†	96·6*	(6·3)†	108·4*	(7·1)†
DOC	100	(0·7)	73·9	(0·5)	117·3	(0·8)
Corticosterone	100	(12·8)	68·0	(8·7)	110·3	(14·1)
18-OH B	100	(0·14)	71·0	(0·10)	135·7	(0·19)
Aldosterone	100	(0·08)	62·5	(0·05)	112·5	(0·09)

*Yield of metabolites relative to control (100%).

†Yield of metabolites as per cent of initial substrate activity. The balance of the carbon-14 radioactivity was recovered as non-transformed progesterone-[^{14}C].

Experiment 2: Incubation duck adrenal gland microsomes in the presence of a concentration of progesterone-[4-^{14}C] and increasing concentrations of aminopterin or folic acid

(a) Incubation in the presence of aminopterin: Five identical preparations of duck adrenal microsomes, equivalent to 0·8 mg of protein were incubated in the presence of progesterone-[4-^{14}C] ($8{\cdot}52 \times 10^5$ dpm; 5·62 μM) and NADPH (8·75 μM). One flask did not contain any aminopterin, while to the other four, aminopterin was added in increasing amounts (24, 60, 114 and 618 μM). The incubation lasted for 20 min.

(b) Incubation in the presence of folic acid. Incubations similar to those described in (a), were performed to study the effect of increasing concentrations of

folic acid upon the duck adrenal microsomal 21-hydroxylase system. Four vessels were prepared. One contained microsomes, substrate and co-factor as specified in (a), while the other three vessels additively had either 7·5, 75 or 375 μM of folic acid.

Results obtained in both series (a) and (b) are shown graphically in Fig. 1. In both series DOC-^{14}C was isolated as transformation product.

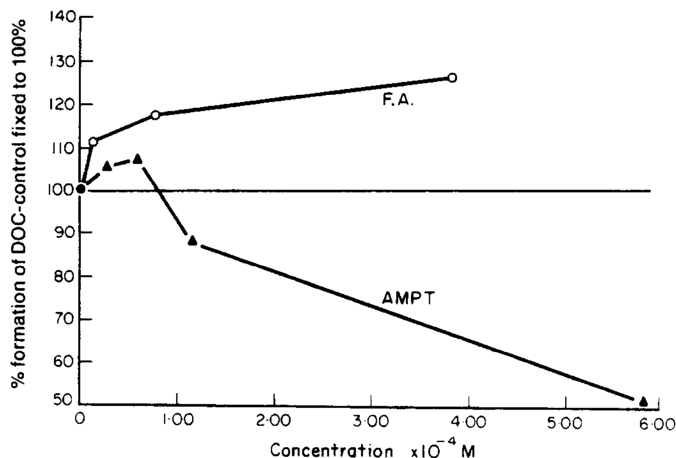

Fig. 1. Effect of increasing concentrations of aminopterin (AMPT) and folic acid (F.A.) upon the 21-hydroxylation of exogenous progesterone by duck adrenal gland microsomes. The abscissa represents the concentration of the pteridins in the incubation media, while the ordinate shows the formation of DOC-^{14}C in percentage relative to the control experiments not containing pteridins (Experiment 2).

Experiment 3: *Incubation of duck adrenal microsomes in the presence of increasing amounts of progesterone*-[4-^{14}C] *but with constant concentration of aminopterin or folic acid*

A duck adrenal microsomal preparation was divided into 12 aliquots, each corresponding to 0·42 mg of protein. The first series of 4 vessels contained 4·66, 21·75, 38·51 and 88·78 μM of progesterone-[4-^{14}C] (carbon-14 activity in each vessel was 0·5 μCi, 0·13 mM of aminopterin and NADPH as specified in Experiment 2.

The second series of four vessels were identical to the first series except that 0·13 mM of folic acid was substituted for aminopterin.

The third series was used as control and contained only microsomes, substrate and co-factor. All 12 samples were incubated for 30 min and DOC-^{14}C was isolated from each incubation as transformation product. Table 2 shows the effect of aminopterin and folic acid upon the amount of DOC formed. Calculation of Michaelis–Menten constant (K_m) gave identical values (2·5 μM/L) in absence or presence of aminopterin.

Experiment 4: *Incubation of duck adrenal mitochondria in the presence of deoxycorticosterone*-[4-^{14}C]: *the effects of increasing concentration of aminopterin or folic acid upon the* 11β-*hydroxylation of the substrate*

A duck adrenal mitochondrial preparation was divided into 10 identical samples, each containing mitochondria equivalent to 1·0 mg of protein.

Table 2. Effect of increasing concentrations of progesterone-[4-^{14}C] upon the formation of deoxycorticosterone-[4-^{14}C] in the absence or presence of fixed concentration (0·13 mM) of aminopterin or folic acid. Source of 21-hydroxylase: duck adrenal gland microsomes (Experiment 3)

Progesterone-[4-^{14}C] concentration × 10^{-6} M	Deoxycorticosterone-[4-^{14}C] formed Concentration × 10^{-6} M		
	Control	Folic Acid	Aminopterin
4·66	3·64	3·74	0·86
21·75	5·09	6·61	1·19
38·51	4·91	6·41	1·36
88·78	5·41	6·11	1·28

One series of five vessels contained deoxycorticosterone-[4-^{14}C] ($3{\cdot}6 \times 10^5$ dpm, 0·16 μM), 0·16 μM of NADPH and 25, 51, 102 or 560 μM of aminopterin added.

The second series of incubation vessels was set up similarly to the first one except that folic acid (25, 51, 102 or 560 μM) took the place of aminopterin. Again one vessel was kept as a control with no pteridin added. Incubation time was 20 min. In these experiments, corticosterone-^{14}C, 18-hydroxycorticosterone-^{14}C and aldosterone-^{14}C were isolated. The results of these experiments are shown in Fig. 2. Values appearing on this figure represent the sum of the three above mentioned 11β-hydroxylated metabolites of deoxycorticosterone.

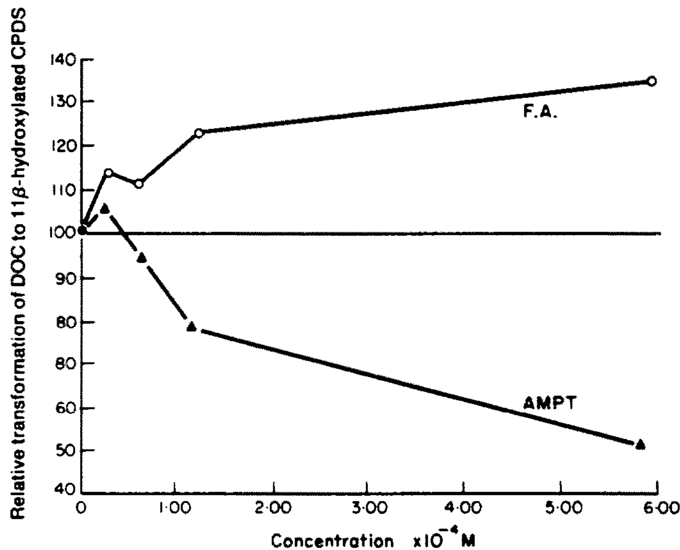

Fig. 2. Effect of increasing concentrations of aminopterin (AMPT) and folic acid (F.A.) upon the 11β-hydroxylation of exogenous deoxycorticosterone-[4-^{14}C] by duck adrenal gland mitochondria. The abscissa represents the molar concentration of the pteridins in the incubation media while the ordinate shows the formation of the sum of the hydroxylated derivatives isolated (carbon-14 labelled corticosterone, 18-OH B and aldosterone) in percentage relative to the control experiment not containing pteridins (Experiment 4).

Experiment 5: Incubation of duck adrenal mitochondria in the presence of increasing amounts of deoxycorticosterone-[4-^{14}C] *but with constant concentration of aminopterin or folic acid.*

A duck adrenal mitochondrial preparation was divided into 12 equal aliquots, corresponding to 1·0 mg of protein. The twelve vessels were divided into three groups of four. The first group contained deoxycorticosterone-[4-^{14}C] in the amount of 3·4, 24·0, 51·5 and 106·6 μM respectively (carbon-14 activity in each vessel was $5{\cdot}56 \times 10^5$ dpm) and to each vessel was added a NADPH generating system and 0·21 mM of aminopterin. The second group of four was similar except that 0·21 mM of folic acid replaced aminopterin. The third group were incubated without addition of pteridins. After 30 min of incubation, tritiated corticosterone and tritiated aldosterone were added as internal standards. As in Experiment 4, carbon-14 labelled corticosterone, 18-hydroxycorticosterone and aldosterone were isolated. Results of this experiment are shown in Table 3.

Table 3. Effect of increasing concentrations of deoxycorticosterone-[4-^{14}C] upon the formation of 11β-hydroxylated metabolites (corticosterone-^{14}C + 18-OH-B-[^{14}C] + aldosterone-[^{14}C]) in the absence or presence of aminopterin and folic acid in fixed concentration. Source of 11β-hydroxylase: duck adrenal gland mitochondria (Experiment 5)

Deoxycorticosterone-4-^{14}C concentration $\times 10^{-6}$ M	11β-hydroxylated metabolites formed concentration $\times 10^{-6}$ M		
	Control	Folic acid	Aminopterin
3·4	2·4	2·4	2·5
24·0	18·7	18·9	16·6
51·5	36·4	40·0	30·1
106·6	43·5	48·2	33·2

DISCUSSION

Data obtained in these experiments indicate that aminopterin has an inhibitory effect on both mitochondrial and microsomal steroid hydroxylating enzyme systems of duck adrenals. This inhibitory effect could also be demonstrated when an adrenal whole homogenate was incubated with labelled progesterone (Experiment 1). In addition, folic acid had a slight stimulatory action upon the transformation of progesterone to 21, 11β and 18-hydroxylated metabolites by this same preparation. These same effects could be reproduced by studying the mitochondrial 11β-hydroxylase and the microsomal 21-hydroxylase. The nature of aminopterin inhibition was somewhat different in adrenal mitochondria as compared to adrenal microsomes. In microsomes, the Michaelis–Menten constant in the absence or presence of aminopterin was the same. Similar non-competitive inhibition of adrenal microsomal 21-hydroxylase by aminopterin has been reported in the snake and the frog [9]. In regard of the mitochondrial 11β-hydroxylation, the K_m values obtained in these experiments in the absence and presence of aminopterin were slightly different, though duck adrenal mitochondrial 18-hydroxylation of corticosterone was shown to be inhibited non-competitively by aminopterin [2, 3].

It has been previously reported that reduced pteridins increased the 17α-hydroxylating capabilities of rat testicular preparations. In addition, pteridin

derivatives were shown to serve as electron carriers in the aromatic hydroxylation of phenylalanine to tyrosine[10]. In this latter case, tetrahydrobiopterin reacted with phenylalanine and molecular oxygen yielding tyrosine and 6,7-dihydrobiopterin.

The only chemical difference between folic acid and aminopterin is the substitution of an -OH group in the former by an $-NH_2$ group in the latter. It was recently reported that some primary amines interact with liver microsomal cytochrome P-450[11] and that the removal of the $-NH_2$ group from aminoglutethimide reduces is effectiveness in blocking cytochrome P-450 reduction in bovine adrenal mitochondria[12]. Difference spectra of mitochondrial and microsomal suspensions taken in the presence of folic acid and aminopterin gave only negative results. This rules out the direct action of these compound on cytochrome P-450.

As neither the folic acid activation, nor the aminopterin inhibition could be satisfactorily explained by these experiments, a second series of studies have been initiated on the possible role of reduced pteridins as source of reducing equivalents in steroid hydroxylation.

These studies have shown that (1) in the presence of NADPH dihydrofolic acid or tetrahydrofolic acid caused up to a 2-fold increase in 11β hydroxylation of DOC by duck adrenal mitochondria. (2) in the absence of NADPH, the above mentioned pteridins maintained 11β hydroxylation though to a much lesser degree than did NADPH. These will be reported in a subsequent paper[13] and form part II of this series of studies.

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