Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 865–869. Pergamon Press. Printed in Great Britain

PRODUCT-PRECURSOR RELATIONSHIP DURING INCUBATION OF DUCK ADRENAL SLICES*

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(*Received* 18 *April* 1972)

SUMMARY

Domestic duck (*Anas platyrhynchos*) adrenal slices were incubated with progesterone-[4-^{14}C] for various time intervals. Using ^{3}H labelled acetic anhydride the total mass of various metabolites formed was measured. The yield-time curves for the labelled products elaborated from the added ^{14}C precursor confirmed the previous *in vitro* results obtained from the duck adrenal slices. Thus labelled cpd. B‡, 18-OH-B and aldosterone were the major steroids formed at the end of 3 h incubation. However, DOC was quantitatively the major metabolite formed from endogenous substrate(s) during the course of incubation, though presence of the three above mentioned steroids was also demonstrable. At any given incubation time, the specific activities of the isolated products and the added precursor remained constant with the exception of DOC which showed a marked decline. These observations seemingly suggest that the added precursor formed a homogeneous pool with the corresponding endogenous substrate. The biosynthesis of large amounts of radio-inert DOC might be explained by the operation of the 5-ene-pathway eventually taking place in a different metabolic compartment.

INTRODUCTION

DURING the past two decades interest has been focused on the *in vitro* biosynthesis of adrenocortical hormones. Thus the nature of the steroids produced by adrenocortical parenchyma of various vertebrates has been explored [1, 2]. In this respect, the use of the radioactive precursors greatly facilitated the investigators to explore the biosynthesis of corticosteroids [1, 2]. However, in recent years some investigators have questioned the validity of these *in vitro* studies. The problem arose whether *in vitro*, exogenous and endogeneous substrates form a homogeneous pool or not [3–5].

Thus the present investigation was undertaken in an attempt to clarify some of the above mentioned questions. Domestic duck (*Anas platyrhynchos*) adrenal slices were incubated for various time intervals, using progesterone-[4-^{14}C] as a model substrate. At each time point, both yield from the added substrate and the total amount of steroid synthesized were measured. For the measurement of the mass of steroids, the double isotope technique of Kliman and Peterson was adopted [6].

*Presented in part at 6th Conference of European Comparative Endocrinologists, Montpellier, France, August 1971.

†Holder of a Medical Research Council of Canada Associateship.

‡Abbreviations or trivial names used in this paper: 11β, 18, 21-trihydroxy-4-pregnene-3,20-dione, 18-hydroxycorticosterone or 18-OH-B; 11β, 21-dihydroxy-4-pregnene-3,20-dione, corticosterone or B; 11β-hydroxy-4-pregnene-3,20-dione, 11β-OH-progesterone or 11β-OH-P; 3β-hydroxy-5-pregnen-20-one, pregnenolone: 20β-dihydroprogesterone, 20β-OH-P.

MATERIALS AND METHODS

Tissue incubation procedure. Adrenal glands were obtained from male domestic ducks of Pekin white variety (*Anas platyrhynchos*) weighing 1·5–2 Kg, after the animals were killed by exanguination. The glands were cleaned free of surrounding renal parenchyma and the tissue was cut into 0·5 mm slices with a Stadie–Riggs hand microtome. Adrenal slices (100 ± 2 mg) were preincubated in Krebs–Ringer bicarbonate glucose (KRBG) pH 7·4 at 41°C under an atmosphere of 95% O_2 and 5% CO_2. After the preincubation period, the tissue was transferred to fresh KRBG medium (5 ml) containing progesterone-[4-^{14}C] (0·25 μCi; 0·318 n moles) and incubations were carried out at different time intervals. At the end of each time interval the enzymatic reaction was stopped by the addition of few drops of acetone. The medium was decanted and the tissue was rinsed several times with distilled water. The washings were added to the media.

Extraction and purification of steroids. Two aliquots (aliquots 1 and 2) were taken from each of the incubation media.

Aliquot 1 was used for the determination of percent conversion of the substrate into different metabolites. To this aliquot known amounts of purified tritiated reference steroids (18-OH-B, aldosterone, cpd. B, DOC and progesterone) were added and extracted repeatedly with chloroform and ethyl acetate as reported elsewhere[7]. For the resolution of the extract a previously described paper partition (PPC) chromatographic scheme was adopted[8]. Once separated, the individual steroids were further purified by thin-layer (TLC) chromatography to a constant ^{3}H/^{14}C ratio through formation of the acetates and their oxidation products with either CrO_3[6] or periodic acid[10] (for details see Fig. 1).

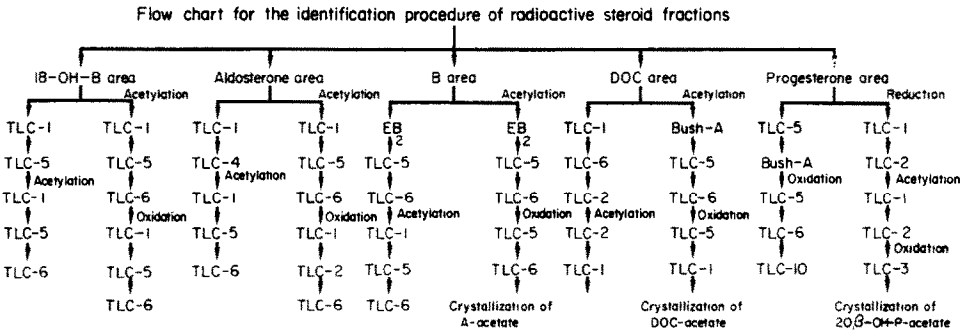

Fig. 1.

Thin layer systems:

TLC-1 benzene : ethyl acetate (1 : 10 v/v)
TLC-2 cyclohexane : ethyl acetate (1 : 10 v/v)
TLC-3 benzene : methanol (9 : 1 v/v)
TLC-4 methanol : ethyl acetate (1 : 20 v/v)
TLC-5 chloroform : acetone (95 : 5 v/v)
TLC-6 cyclohexane : ethyl acetate (50 : 50 v/v)
TLC-10 cyclohexane : ethyl acetate (70 : 30 v/v)

Paper partition systems:

E_2B iso-octane : t-butanol : water (100 : 50 : 90 by vol). W. R. Eberlein and A. M. Bongiovanni: *Arch. Biochem.* **59** (1955) 90.

Bush A, petroleum ether : methanol : water (100 : 80 : 20 by vol). I. E. Bush: *The Chromatography of Steroids.* Pergamon press, Appendix II (1962) 358.

Following extraction, aliquot 2 was processed for the determination of specific activity of different metabolies. It was directly subjected to sequential PPC technique [8]. Individually isolated steroids were acetylated with ^{3}H-acetic anhydride (50 mCi/mmole). The progesterone fraction was enzymatically reduced to 20β-dihydroprogesterone [9], before acetylation with the labelled acetic anhydride. Prior to further chromatography, whenever possible, authentic radio-inert (100 μg) steroid acetates were added to the corresponding double labelled steroid acetate.

The following substances were identified (for details see Fig. 1): 20β-dihydroprogesterone acetate: successive chromatography in various TLC systems followed by dilution with mg amounts of non-radioactive carrier and serial crystallization to constant ^{3}H/^{14}C ratios and specific activity: 21-acetoxy DOC: similar procedure was adopted as in the case of 20β-dihydroprogesterone acetate; 21-acetoxy B: oxidation to 21-acetoxy A followed by dilution with carrier and serial crystallization to constant ^{3}H/^{14}C ratios and specific activity: 18,21-diacetoxy-aldosterone: formation of 21-acetoxy-18-lactone-aldosterone followed by chromatography in various TLC systems and evaluated by constancy of ^{3}H/^{14}C ratios; 21-acetoxy-18-OH-B: homogeneity evaluated by constant ^{3}H/^{14}C ratios in various TLC systems. The specific activity and/or isotope ratios were considered to be constant, if 3 consecutive steps yielded values with a coefficient of variation of less than $\pm$ 5%.

Detection and assay of radioactive substances. Radioactive zones were detected on paper chromatograms and thin-layer plates with radio-autography and/or a radiochromatogram scanner (Model 7200, Packard Instrument Company, Downers Grove, Ill.). Quantitative measurement of radioactivity was done with a 3-channel liquid scintillation spectrometer, equipped with automatic external standardization for control of quenching (Tri-Carb, Model 3375, Packard Instrument Company). Counting error was kept at $\pm$ 1% (10^4 net cpm accumulated) and isotope contents were expressed as disintegrations per minute (dpm).

Calculations and presentation of results. Percentage conversion, specific activity, exogenous production from the precursor and endogenous production of each metabolite were calculated as described previously [3].

RESULTS AND DISCUSSION

The pattern of biosynthetic products of progesterone-[4-^{14}C] by incubating duck adrenal slices at various time intervals is shown in Fig. 2. During the first 30 min of incubation cpd. B, DOC and 18-OH-B were the major metabolites formed. However, at the end of the incubation cpd. B, 18-OH-B and aldosterone emerged as the major conversion products. The last observation is almost identical with the results previously obtained using duck adrenal slices [1].

Quantitative comparison of various products isolated from exogenous and endogenous sources during the course of the tissue incubation is shown in Table 1. The pattern of corticosteroids originating from the exogenous source is similar to that shown in Fig. 2. On the other hand, various products elaborated from the endogenous source(s) showed a continuous increase in output of all the corticosteroids, except progesterone. The most striking increase occurred in the case of DOC which showed an abundant increase.

The specific activities of the steroids isolated at various incubation time intervals are shown in Table 2. These specific activities are expressed in terms of the radioactivity associated with each of the steroid to the total mass of the

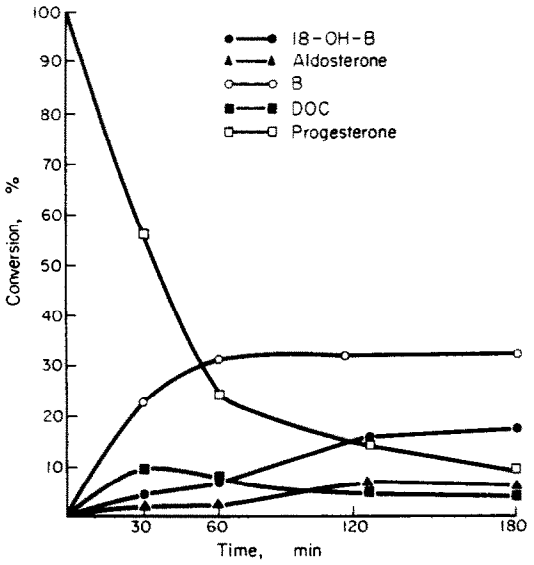

Fig. 2.

Table 1. Yield of various products isolated during incubation of duck adrenal slices with progesterone-[4-^{14}C]

Steroids	30*		60		120		180	
	Exog.	Endo.	Exog.	Endo.	Exog.	Endo.	Exog.	Endo.
18-OH-B	12·7	1·3	20·0	1·9	49·0	5·1	53·7	5·7
Aldosterone	4·5	0·6	5·1	0·6	21·0	2·5	18·8	1·9
cpd. B	70·6	7·3	96·3	9·9	99·5	10·2	101·8	10·5
DOC	28·6	49·6	21·3	47·7	14·3	74·4	10·2	57·6
Progesterone	177·1	18·4	75·0	8·0	44·2	4·5	27·0	2·9

*Incubation time in min. Exog. – Exogenous; Endo. – Endogenous.
Note: The values in the table are given in p moles per incubation flask.

Table 2. Specific activities of various products isolated at various time intervals incubations of duck adrenal slices with progesterone-[4-^{14}C]

Steroid	Specific activity dpm/p mole			
	30*	60	120	180
18-OH-B	117·5	117·4	117·0	117·1
Aldosterone	115·0	115·8	116·4	117·5
cpd. B	117·4	117·3	117·1	117·2
DOC	48·5	41·1	21·3	19·8
Progesterone	117·2	116·9	117·3	117·3

*Incubation time in min.

corresponding steroid. Total mass implies the amounts of steroid isolated from endogenous plus exogenous sources. It is interesting to note, that except in the case of DOC, the specific activities remained constant in all the cases including

the substrate. The fact, that specific activity of progesterone remained constant throughout the incubation period, does indicate that substrate indeed formed a homogenous pool with the endogenous precursor. Most of the steroids isolated had similar specific activity as progesterone, therefore suggesting that 18-OH-B, aldosterone and cpd. B were indeed derived from the same pool.

It is quite noteworthy that the production and specific activity of DOC was not similar to the rest of the compounds. Similar types of results have been reported by Vinson and Whitehouse using duck adrenal minces[5]. Previously it has been suggested by our group that in the duck adrenal 11β-OH-P could be an alternative intermediary route for the biosynthesis of corticosteroids from progesterone[1]. In the present investigation 11β-OH-P could not be demonstrated. However, the possibility still remains that this intermediary is formed and instantly converted to other metabolites.

The abundant endogenous production of DOC could be associated with the 5-ene-pathway leading to the biosynthesis of DOC from endogenous pregnenolone without its oxidation to progesterone. This phenomenon does occur in mammalian[11, 12] and duck[13] adrenocortical tissue.

The methodology employed in this study indeed provides insight into the primary importance of metabolic processes undergone by endogenous precursor, the nature of which is either not reflected or blurred when the metabolic products of the precursor are only assessed in terms of yield of radioactivity.

No doubt one needs more experimental proof to draw definite conclusions on the *in vitro* biosynthesis of corticosteroids, coming from endogenous and exogenous sources. However, we feel that the present study has given a certain degree of insight to the problem. Thus further studies based upon these experimental bases might lead us to draw definite conclusions regarding the studies of product-precursor relationship *in vitro*.

ACKNOWLEDGEMENTS

The present investigation was supported by a grant from the Medical Research Council of Canada (MT-1302) and by a grant from the Ministère de l'Education, Province de Québec.

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