STEROID BIOSYNTHESIS BY FROG AND TURTLE INTERRENALS

A. Z. MEHDI

'IN VITRO' CORTICOSTEROID BIOSYNTHESIS BY FROG AND TURTLE INTERRENALS

by

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ABSTRACT

The pathways of steroid biosynthesis have been explored in interrenals from the American bullfrog (Rana catesbeiana) and the painted turtle (Chrysemys picta picta). In in vitro studies by both interrenals: 1) acetate-1-^{14}C is not incorporated into cholesterol or corticosteroids, 2) cholesterol-4-^{14}C is metabolized into C_{21} steroids. The cleavage mechanism is NADPH-dependent and mitochondrial-bound, 3) Δ^5-pregnenolone-4-^{14}C is converted into Δ^4-3keto metabolites. This reaction is effected by all subcellular fractions. The metabolism of this substrate differs in presence of NAD or NADPH generating systems, 4) progesterone-4-^{14}C is hydroxylated at carbons 11β-, 18 and 21 but not at 17α, 5) the compounds characterized are: 18-OH-B, aldosterone, B, A (frog), 11β-OH-P, DOC, Δ^5-pregnenolone (turtle) and progesterone, 6) G-6-P- and 6-phosphogluconate dehydrogenases are demonstrable (histochemistry) explaining the generation of NADPH from NADP plus G-6-P, 7) the process of steroid biosynthesis is similar to that in adrenals from aves and non-cortisol producing mammals.

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D E D I C A T E D T O

DR. ANDRES CARBALLEIRA

WHO INTRODUCED ME TO ENDOCRINOLOGY.

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NOMENCLATURE OF STEROIDS

Trivial or abbreviated names used	Systematic names
18-OH-Corticosterone (18-OH-B)*	11β, 18, 21-Trihydroxy-pregn-4-ene-3,20-dione
19-OH-Corticosterone (19-OH-B)	11β, 19, 21-Trihydroxy-pregn-4-ene-3,20-dione
Cortisol (F)	11β, 17a, 21-Trihydroxy-pregn-4-ene-3,20-dione
Aldosterone*	11β, 21-Dihydroxy-pregn-4-ene-3,20-dione-18-al
Cortisone (E)	17a, 21-Dihydroxy-pregn-4-ene-3,11,20-trione
19-OH-11-Deoxycorticosterone (19-OH-DOC)	19, 21-Dihydroxy-pregn-4-ene-3, 20-dione
18-OH-11-Deoxycorticosterone (18-OH-DOC)	18, 21-Dihydroxy-pregn-4-ene-3, 20-dione
11-Dehydrocorticosterone (A)	21-Hydroxy-pregn-4-ene-3, 11, 20-trione
Corticosterone (B)	11β-21-Dihydroxy-pregn-4-ene-3, 20-dione
11-Deoxycortisol (S)	17a-21-Dihydroxy-pregn-4-ene-3, 20-dione
11β-OH-Progesterone (11β-OH-P)	11β-Hydroxy-pregn-4-ene-3, 20-dione
17a-OH-Progesterone (17a-OH-P)	17a-Hydroxy-pregn-4-ene-3, 20-dione
11-Deoxycorticosterone (DOC)	21-Hydroxy-pregn-4-ene-3, 20-dione
20β-OH-Progesterone (20β-OH-P)[1]	20β-Hydroxy-pregn-4-ene-3, 20-dione

NOMENCLATURE OF STEROIDS (continued)

Trivial or abbreviated names used	Systematic names
11-Keto-progesterone (11-Keto-P)	Pregn-4-ene-3, 11, 20-trione
Δ^5-Pregnenolone (Δ^5-P)	3β-Hydroxy-pregn-5-ene-20-one
Progesterone (Δ^4-P)	Pregn-4-ene-3, 20-dione
Cholesterol (C_{27} sterol)	Cholest-5-ene-3β-Ol

*Systematic name of open form only.
1. This trivial name (or its abbreviation) is not correct. Its common appearance in the literature and the lack of a more precise final term justify its usage in this thesis.

ABBREVIATIONS OR TRIVIAL NAMES USED

ACTH	Adrenocorticotropic hormone
3', 5' AMP	Adenosine-3', 5' cyclic monophosphate
3', 5' AMP D.B.	2'-Dibutyryl 3', 5' cyclic adenosine-monophosphate-sodium
µc	Microcurie
mc	Millicurie
cpm	Counts per minute
g	Gram
mg	Milligram
µg	Microgram
hr.	Hour
min.	Minute
M	Molar
m-mole	Millimole
mµ mole	Millimicromole
G-6-P	Glucose-6-phosphate
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
T.C.	Total conversion
TLC	Thin-layer chromatography

I PURPOSE OF THE INVESTIGATION

Since the pioneer studies by Hechter and other workers of the Worcester Foundation, it has been firmly established that the process of steroid biosynthesis proceeds in the mammalian adrenal gland through the sequence acetate ⟶ cholesterol ⟶ Δ^5-pregnenolone ⟶ progesterone ⟶ corticosteroids (90, 97, 233). Considerable insight has been gained in the following years on the intimate nature of the reactions involved in various steps of this sequence. Thus, the localization of some of these enzymatic processes at the subcellular level, their coenzymatic requirements, the absolute need of molecular oxygen, the effects of agents like ACTH or 3', 5' AMP have fairly well elucidated (95, 96, 101, 106, 112, 130, 148-151, 218).

In sharp contrast with this vast information regarding the physiological chemistry of the mammalian adrenal, our present-day knowledge of corticosteroidogenesis is quite fragmentary in other classes of vertebrates.

If we make the exception of the avian adrenal, where the overall reaction acetate ⟶ corticosteroids has also been studied in all the above mentioned intermediate stages (48, 72, 74), the studies with other classes of vertebrates have only explored partial aspects of this overall reaction.

As far as amphibians and reptilians are concerned, for

example, most of the investigations have been mainly concerned with the reactions involved from the metabolism of Δ^5-pregnenolone onwards (46-48, 57, 59-61).

The present investigation was undertaken as an attempt to define the roles of acetate and cholesterol in the process of corticosteroidogenesis in frog and turtle adrenal glands and to explore in greater depth the metabolism of Δ^5-pregnenolone and progesterone by these tissues.

For this purpose, these ^{14}C labelled steroid precursors have been incubated with different preparations of frog and turtle adrenal under the influence of a variety of agents known to promote certain aspects of corticosteroidogenesis in the mammalian adrenal.

Beside the efforts to elucidate whether or not the synthesis of cortical hormones evolves in the frog and turtle adrenals along the patterns confirmed for mammals and aves, we have tried to localize the subcellular compartments in which these reactions preferentially occur and which nucleotides intervene in the mediation of such reactions.

Emphasis has also been placed in characterizing each isolated metabolite with as rigorous criteria as those currently used for the identification of products from mammalian sources (78). These criteria, where possible, include constant

^{3}H:^{14}C ratios after the formation of doubly labelled derivatives as well as constant specific activities and ^{3}H:^{14}C ratios following successive microcrystallization (189).

Unfortunately, these time consuming experiments have limited our observations to a single species from the two classes of vertebrates surveyed. The American bullfrog (Rana catesbeiana) and the painted turtle (Chrysemys picta picta) have been selected as the representative species of Amphibia and Reptilia respectively.

It should be pointed out, however, that while we refer to 'representative' species, we are aware that conclusion cannot be drawn for a whole class of vertebrates with observations limited to a single species.

II. REVIEW OF THE LITERATURE

Introduction

During the last two decades, the steroid hormones elaborated by the mammalian adrenal gland have been studied extensively. The quality and quantities, the biosynthetic pathways and mechanism of control of these hormones have been relatively well established. It is only in recent years, however, that these studies have been extended to non-mammalian vertebrates, thanks to the pioneering work of the Sheffield University group headed by Professor Chester-Jones. Still we are only at the fringes of this vast new field. The vertebrate kingdom encompasses some 47,000 different living species. Of these, and this is a rather optimistic value, only about fifty have been subjected to any detailed investigation in regards to their corticosteroid production.

The present study deals with the process of corticosteroidogenesis in a species from the class Amphibia (Rana Catesbeiana) and a species from the class Reptilia (Chrysemys picta picta). The review of the literature, however, will encompass studies conducted with adrenal preparations from other classes of vertebrates. This inclusion has been made in an attempt to give our studies a proper background against which the results can be discussed from a comparative point of view.

Chapter 1 is devoted to the comparative morphology and the elaboration of steroid hormones by the adrenal cortex or its homologous structure (interrenal tissue) in various classes of vertebrates. Due to the nature of the present investigation, emphasis has been placed on *in vitro* studies carried out with or without steroid substrates in non-mammalian classes of vertebrates, since it would be beyond the scope of this thesis to review the enormous number of studies conducted with the mammalian adrenal cortex.

Chapter 2 deals with the biosynthetic pathways of corticosteroidogenesis. The major enzymatic steps and their *in vitro* requirements are reviewed, following of necessity, the scheme that in general has been so well established for the mammalian adrenal cortex. Special consideration is given to the biosynthesis of aldosterone. The salient features of the pituitary-adrenal axis are also briefly summarized.

Before all these topics are considered, it was thought that a simplified tabulation of the classes of living vertebrates with their division and some typical examples, might facilitate the reading of the review of the literature (Table I).

TABLE I

VARIOUS CLASSES AND DIVISION OF LIVING VERTEBRATES

FISH

Cyclostomata	(Lamprey)	
Holocephali		Chondrichthyes
Elasmobranchii	(Shark, dogfish)	Chondrichthyes
Chondrostei	(Sturgeon)	
Holostei	(Pike)	
Teleostei	(Eel)	Osteichthyes
Dipnoi	(Lungfish)	Osteichthyes
Crossopterygii	(Lungfish)	Osteichthyes

AMPHIBIA

Apoda	(South American amphibian)	
Urodela	(Salamander)	
Anura	(Frog)	RANA CATESBEIANA

REPTILIA

Chelonia	(Turtle)	CHRYSEMYS PICTA PICTA
Lacertilia	(Lizard)	
Serpents	(Snake)	
Crocodilia	(Alligator)	
Sphenodon	(Tautara)	

AVES

Birds	(Duck, Chicken)

MAMMALIA

Prototheria	(Duckbill)
Metatheria	(Kangaroo)
Eutheria	(Rat, Bovine, Man)

CHAPTER 1

A. Comparative Morphology of Adrenal Gland

The review of this section is based on the descriptions by Chester-Jones (1), Gorbman and Bern (2), Phillips and Bellamy (3) and Bourne (4).

The adrenal gland as a rule is a composite structure containing two parenchymas of different embryological origin. In the typical mammalian adrenal, these two morphological components are well organized into an external cortex surrounding a central medulla; hence the terms 'cortical'and'medullary' tissues. This topographical arrangement, however, is not present in non-mammalian adrenals where the so-called 'cortical' and 'medullary' tissues are either contiguous, highly intermingled or even completely separated.

The 'cortical tissue' is a mesoderm derived structure, stemming from the mesonephros, keeping a close anatomic relationship with the kidney after its full development (thus the terms adrenal, suprarenal and interrenal). This is the tissue elaborating the cortical hormones or corticosteroids.

The 'medullary' tissue is formed by chromaffin elements and like other structures of chromaffin cells (i.e., sympathetic ganglia) it derives from the neuroectoderm and elaborates catechol amines.

While the terms 'cortical' and 'medullary' tissue apply well to typical mammalian adrenals, the terms 'interrenal tissue' and 'chromaffin tissue' seem more appropriate for other classes of vertebrates.

In the cyclostomata, interrenal and chromaffin tissues are separate, and the latter is metamerically arranged. They have been found associated with the posterior cardinal veins and their branches as isolated clusters of cells. Interrenal cell clusters may also occur similarly in the hagfish. In the elasmobranches, the interrenal tissue is variously concentrated into a discrete major mass and smaller masses in the region of the kidneys or occasionally into a single distinct organ consisting of cords of cells. In cases, two interrenal conglomerates are connected by an isthmus of interrenal tissue (horse-shaped interrenal).

In teleost, interrenal tissue is embedded in the anterior ends of the "head" kidneys, often organized as a pair of discrete structures. The "head" kidney, where it exists, is often truly in the head, with strands of tissue even projecting into the foramina of the skull. The "corpuscles of Stannius" are spherical bodies embedded in the kidney proper. They are found in ray-finned fish and were once considered homologous to the interrenals of other vertebrates, having been referred to as "posterior interrenals". In most teleosts there are two Stannius corpuscles, but in the holostean amia there are 40 to 50.

Lungfish appear to have small groups of interrenal cells embedded in so-called perirenal tissue which is composed largely of lipid-containing cells.

In the limbless amphibians, the interrenal and chromaffin tissues are contiguous; in other amphibians they are extensively intermingled. In salamanders, the adrenal masses generally appear as irregular accumulations on the ventral surface of the kidneys, between the kidneys, or embedded in the kidneys. In frogs and toads, they form a pair of organized elongate bodies on, or partly surrounded,by the ventral surface of the kidneys.

In reptiles, the adrenals are usually paired, but in many snakes, they are not symmetrically situated. The adrenal is generally a distinctly organized body, but in turtles it may be diffusely distributed on the ventral surface of the kidneys or fused into a single structure. There is considerable intermingling of interrenal and chromaffin tissues, but in many lizards the bulk of chromaffin tissue tends to form a kind of dorsal capsule on the interrenal without much interpenetration. Here, any tendency to cortex-medulla relation is exactly opposite from that seen typically in mammals. The changes which occur in the amphibian and the reptilian adrenal gland during the various seasons of the year have been well described by Chester-Jones (1).

In birds, the adrenals have a location similar to that in mammals, at the anterior ends of the multilobed kidneys. The chromaffin tissue is completely dispersed in the interrenal, and no cortex-medulla type of arrangement is observed.

In mammals, the paired adrenals are always located near the anterior poles of the kidneys. The typical adrenal picture of mammals is one of a distinct cortex consisting of columns of cells surrounding the medulla of chromaffin cells often arranged in lobules. The therian adrenal cortex is arranged into distinctive zones: the outer layer, where cells are arranged in a regular group, is called zona glomerulosa; the middle layer, where cells are comprised in a more radial fashion, is called zona fasciculata, and the innermost layer bordering the medulla is called zona reticularis.

B. Corticosteroids in Various Vertebrates

1. Fish

The lowest form of living vertebrate, where the presence of adrenocortical hormones was investigated, is the order cyclostomata. This order consisting of lampreys and hagfish show corticosterone and cortisol as predominant steroids in peripheral plasma (5). Phillips and coworkers (6) isolated and tentatively identified corticosterone and cortisol in the plasma of Myxine glutinosa (Atlantic hagfish). However, in vitro studies with progeste-

rone as substrate, no hydroxylation of the precursor occurred when post-cardinal veins strippings (this is where the interrenal cell clusters of cyclostomes are supposed to be) of Myxine glutinosa were incubated (7).

Chondrichthyes, the next class of fish, is a class which has almost all the vertebrate attributes, such as complete and separate vertebrae, movable jaws and paired appendages. Most of the studies of corticosteroid biosynthesis of this class of fish were carried out with species belonging to subclass elasmobranchii. Adrenocortical secretion in this subclass has been investigated only recently. In 1957, Phillips and Chester-Jones (8) established that in the plasma of both Raja eglanteria (ray) and Squalus acanthias (dogfish) steroid hormones exhibiting the chromatographic mobility of corticosterone and cortisol were present. A more detailed study, by Bern and coworkers (9) resulted in the tentative identification of aldosterone and cortisol from interrenal incubates of the Hydrolagus colliei (ratfish), while the in vitro biosynthesis of aldosterone and corticosterone was suggested in the Squalus acanthias (dogfish) and Raja rhina (skate). More recently, Idler and Truscott (10) re-examined the adrenocortical secretion pattern in Genus raja. They found that both the major plasma corticosteroid and the major product of in vitro incubation of the Raja rhina (skate)

interrenals was 1 α-hydroxycorticosterone, a new steroid hormone. The widespread occurrence of this steroid hormone in the interrenals of elasmobranchii has been demonstrated by the same investigators who showed *in vitro* production of 1 α-hydroxycorticosterone in eleven species of this subclass (11).

The next and the last class in the truly aquatic branch of the vertebrate family is that of osteichthyes or bony fish. This class is represented by the largest number of species amongst all the vertebrates. Most of the present knowledge of steroid hormones in this class of fish comes from the studies of teleost fish. There is no doubt that cortisol is the major corticosteroid secreted by the adrenocortical cells of the teleost fish. This hormone has been demonstrated in peripheral plasma of a variety of bony fish species (5, 8, 12-17). Incubation *in vitro* of adrenocortical tissue from several species of bony fish confirmed *in vivo* findings (17-22). Beside cortisol, variable amounts of cortisone could also be isolated and identified in most *in vivo* and *in vitro* studies. Evidence for the occurrence of aldosterone in bony fish is still inconclusive. Phillips and Murlow (23) incubated "head" kidney (containing the interrenal cells) of *Fundulus heteroclitus* (killifish) using tritiated progesterone and isolated labelled aldosterone. More recently, *in vitro* biosynthesis of aldosterone and 18-hydroxycorticosterone from

corticosterone by interrenal tissue of Claupea harengus harengus (Atlantic herring) has been reported (24). However, attempts to reproduce these results with the interrenals of other teleost species remained unsuccessful (18, 20-22). Isolation of 18-hydroxy-11-deoxycorticosterone from the incubation of the "head" kidney homogenates of Salmo gairdneri (Rainbow trout) using labelled 11-deoxycorticosterone has also been reported (25).

Few studies have been carried out with corpuscles of Stannius (another possible source of steroids in teleosts), and the results are quite controversial. Fontaine and Leloup-Hatey (26) have reported extractable corticosteroids from salmon corpuscles. However, Ford (27) and other workers (28, 29) failed to corroborate these findings using the corpuscles of Stannius from other species. Nandi and Bern (19), incubating trunk kidney (presumably containing some corpuscles of Stannius), also failed to detect measurable amounts of corticosteroids. In 1963 Ogawa (30) obtained evidence indicating steroid production in vitro by the corpuscles of Stannius of Carassius auratus (goldfish). Chieffe and Botte (31) also in 1963, found no $\Delta^{5-3}\beta$-hydroxysteroid dehydrogenase activity in histochemical studies with the corpuscles from Anguilla anguilla (eel) and Conger conger.

Few attempts have been made regarding the role of acetate,

cholesterol and Δ^5-pregnenolone as the precursors of corticosteroids of fish interrenal gland. Sandor et al (21) incubated fragments of the anterior part of the posterior cardinal veins plus "head" kidney (containing the interrenal cells) of Anguilla anguilla, with sodium acetate-1-^{14}C (200 μc) and failed to demonstrate any corticosteroid formation, either in the presence or absence of mammalian ACTH. The investigators claim small amount of ^{14}C-cholesterol formation, but did not report the criteria of identification of the sterol isolated. However, when cholesterol-7-^{3}H was employed as precursor, there was formation of labelled cortisol and Δ^5-pregnenolone (22). Homogenates of the interrenal tissue of the same species incubated with ^{3}H-Δ^5-pregnenolone and ^{14}C-progesterone in the same vessels, resulted in the isolation of doubly labelled cortisol and 11-deoxycortisol (22).

Before ending our survey of corticosteroids present in obligatory water dwellers, it is interesting to mention the lungfish, which is regarded as transitional between bony fish and amphibian. Janssens and coworkers (32) noted only formation of labelled corticosterone from the incubates of interrenal tissue of protopterus sp. (African lungfish) using progesterone-4-^{14}C as the precursor. No cortisol or aldosterone was identified.

The occurrence of corticosteroids in the group of obligatory

TABLE II

SURVEY OF 'IN VITRO' CORTICOSTEROID BIOSYNTHESIS OF VARIOUS FISH INTERRENALS

SPECIES	PRECURSOR USED	STEROIDS IDENTIFIED	REFERENCE
CYCLOSTOMATA			
Myxine glutinosa L	progesterone	none	(7)
CHONDRICHTHYES			
Squalus acanthias	none	B, Aldosterone	(9)
Raja rhina	none	F, B, Aldosterone	(9)
Raja radiata	none	1α-OH-B	(33)
	^{14}C-progesterone	1α-OH-B	(33)
Hydrolagus colliei	none	F, Aldosterone	(9)
OSTEICHTHYES			
Salmo salar	none	F, E, B	(26)
Salmo gairdnerii	none	F, E, B	(19)
	^{14}C-DOC	18-OH-DOC	(25)
Fundulus heteroclitus	^{3}H-progesterone	F, E, Aldosterone	(23)
Anguilla Anguilla	none	F, E, B	(34)
	^{3}H-progesterone	F	(7)
	^{14}C-progesterone	F, E	(21)
	^{3}H-Δ^{5}-pregnenolone	F, E, 17αOH-P, 17α-OH-pregnenolone, progesterone	(21)
	^{3}H-cholesterol	F, pregnenolone	(22)
	^{14}C-acetate	Cholesterol	(21)
Clupea harengus harengus	^{14}C-B	Aldosterone, 18-OH-B	(24)
Heteropneustes fossilis	none	F, DOC	(35)
Carassius auratus	none	E	(30)
Anoplopoma fimbria	none	F	(36)
Pneumatophorus diego	none	F	(37)
Mugil cephalus	none	F, E	(36)
Bodianus bilunulatus	none	F, B, S	(19)
Tilapia mossaambica	none	F, E, S, A	(19)
Conger conger	progesterone	F, B	(18)
Protopherus S.P.	^{14}C-progesterone	B	(32)

water dwellers is summarized on Table II. With the exception of chondrichthyes, 17 α-hydroxylated steroids predominate, while the so-called mineralocorticosteroids, such as aldosterone and 11-deoxycorticosterone are either absent or present at best in infinitesimally small amounts. This predominance of adrenal 17 α-hydroxylation, as we shall see later, will not reemerge again until the evolution of mammals.

2. Amphibia

It is believed that the Dipnoi, the lungfish, made the first tentative evolutionary steps towards terrestrial life and survival in a gaseous atmosphere. Keeping our survey of corticosteroid biosynthesis within evolutionary framework, we should cover the occurrence of corticosteroid in amphibian larval or other amphibian species where gills persist throughout adult life. Unfortunately, however, we do not have any data on these transitional forms.

After the proto-amphibians invaded the dry land, they acquired the mechanisms for the maintenance of their life in a gaseous atmosphere. They are jawed vertebrates without internal gills and usually without any gills in the adult stage. There is a metamorphosis from the fishlike larval stage to the adult stage; sometimes this is not a striking change (as in urodeles), but in others (anurans) the adult life is notably different.

Phillips and Chester-Jones (8), searching for steroid hormones in the renal blood from Xenopus laevis (African frog) isolated a steroid with the characteristics of cortisol. Also, in the blood of species Amphiuma tridactyla, the presence of cortisol as well as corticosterone has been reported (12). Crabbé (38), in an in vitro study of Bufo marinus (toad) adrenals incubated with progesterone-4-^{14}C as substrate, isolated labelled aldosterone and corticosterone. The same investigator in latter studies with the toad adrenals reported the conversion of labelled progesterone to 17 α-hydroxylated steroids, such as cortisol and 11-deoxycortisol (39). The previously mentioned corticosteroids, i.e. aldosterone and corticosterone were also isolated.

Most of the other investigations regarding the corticosteroid biosynthesis, both in vivo and in vitro with the amphibian class were carried out with Rana catesbeiana (American bullfrog). Johnston et al (40), using double isotope derivative techniques, demonstrated the presence of aldosterone and corticosterone in the peripheral plasma of the blood of the bullfrog. Also the same group reported the increase in aldosterone and corticosterone over the control, when mammalian ACTH (2 i.u.) was injected into the pithed bullfrog. This increase over control of corticosterone and aldosterone was three and ten folds respectively.

The earliest *in vitro* study with the bullfrog adrenals was carried out in 1956 by Macchi (41) who showed presence of blue tetrazolium (B.T.) reducing material in the methylene dichloride extracts of the bullfrog adrenal incubation. The increase in B.T. reducing material was observed when mammalian ACTH (10 i.u.) was added to the incubates. Later, using the same species, Carstensen et al (42, 43) in *in vitro* studies isolated aldosterone and corticosterone as the major adrenal steroid hormones. The ratio of aldosterone to corticosterone was about four to one, and the production of these corticosteroids, especially of aldosterone, was increased by the addition of bovine ACTH or frog anterior pituitary extract to the adrenal incubation. The production of aldosterone and corticosterone by the bullfrog adrenal has been reported by various other workers (44, 45).

In vitro studies by Ulick and Solomon (46) demonstrated that labelled progesterone is converted to labelled aldosterone by the adrenal tissue of the bullfrog. Later, Ulick and Kusch (47) in similar experiments isolated 18-hydroxycorticosterone which was characterized by using more sophisticated chemical techniques.

Studies with surviving interrenal sections of *Rana pipiens* (leopard frog) incubated simultaneously with Δ^5-pregnenolone-7-^{3}H and progesterone-4-^{14}C resulted in the isolation of doubly

labelled, 18-OH-corticosterone, aldosterone, corticosterone and 11-deoxycorticosterone (48). Tritiated progesterone was also isolated.

Since the biosynthesis and/or biosynthetic pathways leading to aldosterone are still controversial (see Chapter 2, B), and as has been reported that the frog adrenal produces aldosterone as the major corticosteroid (42, 43), many investigators used the frog adrenals to elucidate various biosynthetic pathways. Kraulis and Birmingham (49) incubated adrenal sections of Rana pipiens (leopard frog) using unlabelled progesterone, 11-deoxycorticosterone, 11β-hydroxyprogesterone and corticosterone as precursors. Aldosterone was isolated from all the substrates, but the greatest formation of it occurred when corticosterone was employed as the precursor. In a similar type of study, Nicolis and Ulick (50) incubated the bullfrog adrenals using labelled progesterone, 11-deoxycorticosterone, corticosterone and the corresponding 18-hydroxylated steroids. The degree of conversion to aldosterone was greater by the former group of substrates than the 18-hydroxylated ones. Also, corticosterone was transformed to the mineralocorticoid in greater amounts than any other precursor. Recently de Nicola and coworkers (51) incubated surviving adrenal sections of the bullfrog simultaneously with corticosterone-4-^{14}C and 11-dehydroxycorti-

costerone-^{3}H. Aldosterone and 18-hydroxycorticosterone were isolated as the conversion products of ^{14}C-labelled precursor and the tritiated substrate was mainly metabolized to tetrahydro-11-dehydrocorticosterone.

Psychoyos et al (52) using homogenates and other subcellular preparations of the bullfrog adrenals, showed that next to homogenates the mitochondria were most active in transforming corticosterone-4-^{14}C into labelled aldosterone and 18-hydroxycorticosterone. The authors pointed out that the presence of reduced NADP, fumarate and Mg^{++} was necessary for the biosynthesis of the two metabolites.

The seasonal variations of production of steroids by the bullfrog adrenals have been studied by Macchi and Phillips (44). Using the adrenal tissue from winter (December - March) and summer (July - August) bullfrogs, the incubation studies showed that both seasonal groups respond equally well to the mammalian ACTH. Nevertheless, the adrenals from summer bullfrogs exhibit lower average output rates both in the absence and presence of ACTH, although differencesbetween means of the two seasonal groups are not statistically significant.

From this survey (also summarized in Table III) one could tentatively suggest that the amphibian adrenal produces aldosterone and corticosterone. The presence of 17 α-hydroxylated

TABLE III

SURVEY OF 'IN VITRO' CORTICOSTEROIDS BIOSYNTHESIS OF AMPHIBIANS ADRENAL GLAND INCUBATIONS

SPECIES	PRECURSOR USED	STEROIDS IDENTIFIED	REFERENCE
ANURA			
Rana catesbeiana	none	B.T. reducing material	(41, 53)
(frog)	none	aldosterone	(42, 43)
	^{14}C-A	tetrahydro A	(51)
	^{3}H-DOC	aldosterone	(50)
	^{3}H-18-OH-DOC	aldosterone	(50)
	^{3}H-B	18-OH-B, aldosterone	(50)
	^{14}C-B	aldosterone, 18-OH-B	(52)
	^{3}H-18-OH-B	aldosterone	(50)
	^{3}H-18-OH-progesterone	aldosterone	(50)
	^{14}C-progesterone	18-OH-B, aldosterone, B, DOC	(46, 47, 50)
Rana pipiens	none	18-OH-B, aldosterone, B	(49)
(frog)	DOC	18-OH-B, aldosterone, B	(49)
	B	18-OH-B, aldosterone	(49)
	11β-OH-P	18-OH-B, aldosterone, B	(49)
	progesterone	18-OH-B, aldosterone, B	(49)
	^{14}C-progesterone	18-OH-B, aldosterone, B, DOC	(48)
	^{3}H-Δ5-pregnenolone	18-OH-B, aldosterone, B, DOC, progesterone	(48)
Bufo marinus	^{14}C-progesterone	aldosterone, B	(38)
(toad)	^{14}C-progesterone	18-OH-B, aldosterone, B, DOC, F, S	(39)

is reported only in few studies (8, 12, 39). However, in the main body of review the results indicate absence of this hydroxylase which was actively looked for,

3. Reptilia

Very few studies have been carried out to evaluate the adrenocortical steroid secretion in reptiles. However, from these scanty reports it appears that the adrenal steroid biosynthetic pattern is very similar to that of amphibians. Phillips and Chester-Jones analyzed the adrenal effluent blood of Natrix natrix (Grass Snake) and reported the presence of two steroids with the characteristics of corticosterone and cortisol (8). The presence of cortisol in the vena cava blood (taken at the level of adrenals) of Lepidochely kempi (turtle) has also been reported (12).

Macchi, 1963, reported the in vitro studies of adrenal sections of Pseudemyes sp. using progesterone-4-^{14}C in the presence or absence of mammalian ACTH (56). The investigator isolated labelled corticosterone as a major conversion product and aldosterone as a minor one. ACTH selectively increased the conversion of radioactive progesterone to labelled aldosterone. The production of aldosterone and corticosterone in the same species has been recently reported (55).

Sandor et al (57) investigated the adrenal steroid

pathways of two common North American turtles, pseudemys scripta elegans (the slider turtle) and Chrysemys picta picta (the painted turtle). Incubation studies, using surviving adrenal slices from the slider turtle with progesterone-4-^{14}C as substrate, resulted in the formation of labelled corticosterone, aldosterone, 18-hydroxycorticosterone and 11-deoxycorticosterone. Adrenal slices of the painted turtle incubated simultaneously with Δ^5-pregnenolone-7-^{3}H and progesterone-4-^{14}C gave rise to the four above mentioned corticosteroids, all of them containing both ^{3}H and ^{14}C. Tritiated progesterone was also isolated. The formation of cortisol and cortisone from these precursors, if any, was very low.

Ramasevami reported the presence of aldosterone, cortisol and corticosterone in the in vitro studies with uromastix (the spiny lizards) adrenals (58).

Phillips and coworkers in in vitro studies demonstrated that the adrenal slices of Lacerta viridis L. (green lizards) could hydroxylate tritiated progesterone (59). The two steroids isolated were corticosterone and aldosterone. Using the same precursor with the adrenals of Natrix natrix (grass snake), the investigators isolated the same corticosteroids (59). The addition of mammalian ACTH (1 i.u./100 mg of tissue) did not enhance the conversion of the exogenous precursor. These

results, in the same species have been corroborated by Macchi and Phillips, who noticed no increase in the production of corticosteroids when mammalian ACTH was added to the incubates (55). However, the authors claim the increase in Δ^4-3keto α-ketol corticosteroids when the mammalian ACTH was replaced by the grass snake's whole pituitary or pituitary extracts. Biotransformation of Δ^5-pregnenolone-7-^{3}H by the adrenals of two species of snake Natrix sipedon pictiventris and Coluber c-constrictor was reported by Callard and Leathem (60-61). Besides progesterone, other corticosteroids, i.e. aldosterone, corticosterone and 11-deoxycorticosterone, were isolated and characterized by the chromatographic behavior of free or derivative form in various solvent systems. The production of aldosterone, corticosterone, 18-hydroxycorticosterone and cortisone by in vitro studies of Naja naja (cobra) has also been demonstrated (62).

The studies carried out with alligator adrenals also demonstrated presence of aldosterone and corticosterone (63, 64). Gist and deRoos using Alligator mississipiensis adrenals isolated corticosterone and aldosterone, either as a metabolite of radioinert progesterone as precursor or from endogenous production. Mammalian ACTH (1 i.u./100 mg of the tissue) did not stimulate the production of corticosteroids. The occurrence of corticosteroids in the group of reptilia is summarized in

TABLE IV

SURVEY OF 'IN VITRO' CORTICOSTEROIDS BIOSYNTHESIS OF REPTILIAN ADRENAL GLAND INCUBATIONS

SPECIES	PRECURSOR USED	STEROIDS IDENTIFIED	REFERENCE
CHELONIA			
Emys orbicularis	none	B, aldosterone	(54)
	^{14}C-progesterone	B, aldosterone	(54)
Pseudemys sp.	none	B, aldosterone	(55)
	^{14}C-progesterone	B, aldosterone	(56)
Pseudemys scripta elegans	^{14}C-progesterone	B, aldosterone, 18-OH-B, DOC	(57)
Chrysemys picta picta	^{14}C-progesterone	B, aldosterone, 18-OH-B, DOC	(57)
	^{3}H-Δ^5-pregnenolone	B, aldosterone, 18-OH-B, DOC, progesterone	(57)
LACERTILIA			
Uromastix	none	B, aldosterone, F	(58)
Lacerta viridis	^{3}H-progesterone	B, aldosterone	(59)
SERPENTS			
Natrix natrix	^{3}H-progesterone	B, aldosterone	(59)
	^{14}C-progesterone	B, aldosterone	(55)
Natrix spideon pictiventris	^{3}H-Δ^5-pregnenolone	B, aldosterone, DOC, progesterone	(60, 61)
Clober C. constrictor	^{3}H-Δ^5-pregnenolone	B, aldosterone, DOC, progesterone	(60, 61)
Naja naja	none	B, aldosterone, 18-OH-B	(62)
CROCODILIA			
Alligator mississipiensis	none	B, aldosterone	(63)
	progesterone	B, aldosterone	(64)

Table IV.

4. Aves

This class of vertebrate belongs to the corticosterone, aldosterone and 18-hydroxycorticosterone secretors, such as the amphibia and reptilia mentioned earlier. Corticosterone is the principal glucocorticoid in the circulation of gallinaceous birds (8, 65, 66). Phillips and Chester-Jones (8), in 1957, found corticosterone as the major steroid and cortisol, cortisone and aldosterone in small quantities in the adrenal venous blood from capon. The positive identification of corticosterone from Anas platyrhynchos (duck) and cockerel plasma was achieved by Donaldson and Holmes (67) and Frankel et al (68). The in vivo studies carried out by Nagra et al (65), using gallinaceous birds, showed that the intravenous injection of mammalian ACTH (8 i.u.) increased the output of corticosterone from four to eight fold.

DeRoos examined the in vitro corticosteroid production of four different species of various orders of Aves (69). Adrenal tissue of Gallus domesticus (white leghorn cockerel) produced in vitro corticosterone as the principal steroid and aldosterone and 11-dehydrocorticosterone as the minor ones. This pattern of in vitro studies was common with the adrenals of Larus occidentalis (western gull), Columba livia (white king pigeon) and

Anas platyrhynchos (white pekin duck). The investigator also demonstrated that an addition of mammalian ACTH (8 i.u./100 mg of tissue) had no qualitative effect, though the production of corticosterone was increased significantly, especially in the case of the chicken and the duck. These results, in the duck, have been confirmed by Donaldson et al (70) and other workers (71). Comparative studies of the adrenal tissue of Gallus domesticus and Anas platyrhynchos were carried out by Sandor et al (72). Using Δ^5-pregnenolone-7-^{3}H and progesterone-4-^{14}C in the same incubation vessel, the adrenals from both species formed doubly labelled corticosterone, aldosterone, 18-hydroxycorticosterone, 11-deoxycorticosterone and probably also 11β-hydroxyprogesterone and cortisol. Analyzing the isotope content in each biosynthetic substance, the authors tentatively suggested that the utilization of the pregnenolone was greater than that of progesterone, thereby suggesting that Δ^4-3keto steroids could be formed directly from 3β-hydroxy-5-ene hydroxylated analogues. However, this claim is at variance with the report of Whitehouse and Vinson (73) who incubated the adrenals of the same species with pairs of differently labelled precursors. The workers showed that the Δ^4-3keto corticosteroids formed from pregnenolone via progesterone rather than through their 3β-hydroxy-5-ene hydroxylated analogues.

The biotransformation of labelled cholesterol by the adrenal sections of various avian species has also been investigated. Macchi and Brown failed to cleave radio-cholesterol by adrenal slices of the duck and the gull, even in the presence of mammalian ACTH. Similarly Sandor et al (74) failed to metabolize the labelled cholesterol or its esters by the duck adrenal slices. Recently, Hall and Koritz (75), using adrenal quarters of chicken adrenals demonstrated the conversion of cholesterol-7α-^{3}H to labelled corticosterone and further stimulation when mammalian ACTH (0.1 i.u./120 mg tissue) was added to the incubates. In these studies the radiocholesterol used was of very high specific activity and was emulsified in Tween 80. However, the incorporation of cholesterol-4-^{14}C into corticosterone, aldosterone and 18-OH-B by the duck adrenal homogenates has been reported (74).

A detailed study of steroid biosynthesis from acetate in adrenal sections of Anas platyrhynchos and Anser anser was reported by Sandor et al (74). Using sodium acetate-1-^{14}C (200 μc), these authors demonstrated the incorporation of the 2-carbon compound into molecules of cholesterol, corticosterone, aldosterone and 18-hydroxycorticosterone. Table V summarizes the in vitro corticosteroids biosynthesis of avian adrenal gland incubations.

TABLE V

SURVEY OF 'IN VITRO' CORTICOSTEROIDS BIOSYNTHESIS OF AVIAN ADRENAL GLAND INCUBATIONS

SPECIES	PRECURSOR USED	STEROIDS IDENTIFIED	REFERENCE
Anas platyrhynchos (domestic duck)	none	18-OH-B, aldosterone, B	(67)
	none	aldosterone, B	(67, 69)
	^{14}C-corticosterone	18-OH-B, aldosterone	(76)
	^{3}H-progesterone	aldosterone, B, F	(3)
	^{14}C-progesterone	18-OH-B, aldosterone, B, DOC, F	(77)
	^{14}C-progesterone	18-OH-B, aldosterone, B, DOC, 11β-OH-P	(72)
	^{3}H-Δ^{5}-pregnenolone	18-OH-B, aldosterone, B, DOC, 11β-OH-P, progesterone	(72)
	^{14}C-cholesterol	18-OH-B, aldosterone, B	(74)
	^{14}C-acetate	18-OH-B, aldosterone, B, cholesterol	(74)
Anser anser (goose)	^{14}C-progesterone	18-OH-B, aldosterone, A, B, 11β-OH-P, DOC	(48)
	^{3}H-Δ^{5}-pregnenolone	18-OH-B, aldosterone, A, B, 11β-OH-P, DOC, progesterone	(48)
	^{14}C-acetate	18-OH-B, aldosterone, B, cholesterol	(74)
Gallus domesticus (chicken)	none	aldosterone, B	(69)
	^{3}H-cholesterol	B	(75)
	^{14}C-progesterone	18-OH-B, aldosterone, B, 11β-OH-P, DOC	(73)
	^{3}H-Δ5-pregnenolone	18-OH-B, aldosterone, B, 11β-OH-P, DOC, progesterone	(73)
Larus occidentalis (gull)	none	aldosterone, B	(69)
Columbia livia (pigeon)	none	aldosterone, B	(69)

5. Mammals

The mammalian adrenocortical steroids have been studied and reviewed extensively (78-80). It is now well established that mammals fall largely into two groups from the point of view of adrenocortical hormone secretion (80). The one group which encompasses the larger portion of the class secretes cortisol as a primary corticosteroid, but in addition, corticosterone, aldosterone and 18-hydroxycorticosterone are also produced. Then we have the other group, exemplified by the order of Rodentia and Lagomorpha, secreting mainly corticosterone, aldosterone and 18-hydroxycorticosterone.

As previously mentioned, the mammalian adrenal cortex consists of three zones, namely, glomerulosa, fasciculata and reticularis. The morphological studies by Deane et al (81-83) and the biochemical observations of Ayres et al (84, 85) and Giroud et al (86, 87) have firmly established the specific functions of the glomerulosa on one hand and the fasciculata-reticularis on the other. The glomerulosa is mainly responsible for the production of mineralocorticoids (aldosterone as representative) and the inner zones for glucocorticoids (cortisol as representative). Corticosterone is the exception being elaborated in both outer and inner cortex. These studies have given ample support to the presently held idea that the inner cortex is under pituitary control through the action of ACTH, while it

appears that ACTH plays little role if any on zona glomerulosa, hence aldosterone secretion (88).

In our survey of occurrence and biosynthesis of adrenocortical steroids in vertebrates, we have seen that all evolutionary trend is absent and there does not seem to be any indication to correlate the pattern of adrenocortical secretion with the habitat of the animal. In non-mammalian vertebrates, with very little exception, there are two types of secretory patterns. Preponderance of 17 α-hydroxylation and abundance of 18-oxygenation. It seems that the two are separate functions and 17 α-hydroxylation inhibits 18-oxygenation. The biochemical characteristics of various zones of the mammalian adrenal cortex as mentioned earlier, did emerge already at the bottom of the vertebrate evolutionary ladder with the cortisol secreting Agnatha and osteichthyes and the aldosterone secreting Amphibia.

CHAPTER 2

A. Biosynthesis of Adrenocortical Steroids

The biosynthesis of the adrenal corticosteroids has been considered to start with acetate. One of the possible routes seems to involve cholesterol, pregnenolone and progesterone as key intermediates. This has been discussed by Heard et al (89) and Hayano et al (90) at the Laurentian Hormone Conference in 1955, basing their discussion on data obtained with preparations from mammalian adrenal cortices or whole adrenal glands.

Attempts to survey systematically the whole biosynthetic sequence acetate ⟶ cholesterol ⟶ Δ^5-pregnenolone ⟶ progesterone ⟶ corticosteroids in non-mammalian interrenals are few. Notably among these, are the complete studies by Sandor's group (74) on the corticosteroid biosynthetic pathways in avian (Anas platyrhynchos and Anser anser) interrenals starting from acetate.

Although there is controversy as to whether cholesterol is an obligatory intermediate in corticosteroids biosynthesis, much is still not known about the pathways between acetate and cholesterol in the mammalian adrenal cortex. For the sake of convenience, the steroidogenesis in the adrenal cortex is divided into the following classical sequences, namely, (1) from acetate to cholesterol, (2) side chain cleavage of choleste-

rol, (3) Δ^5-pregnenolone to progesterone, (4) steroid hydroxylations.

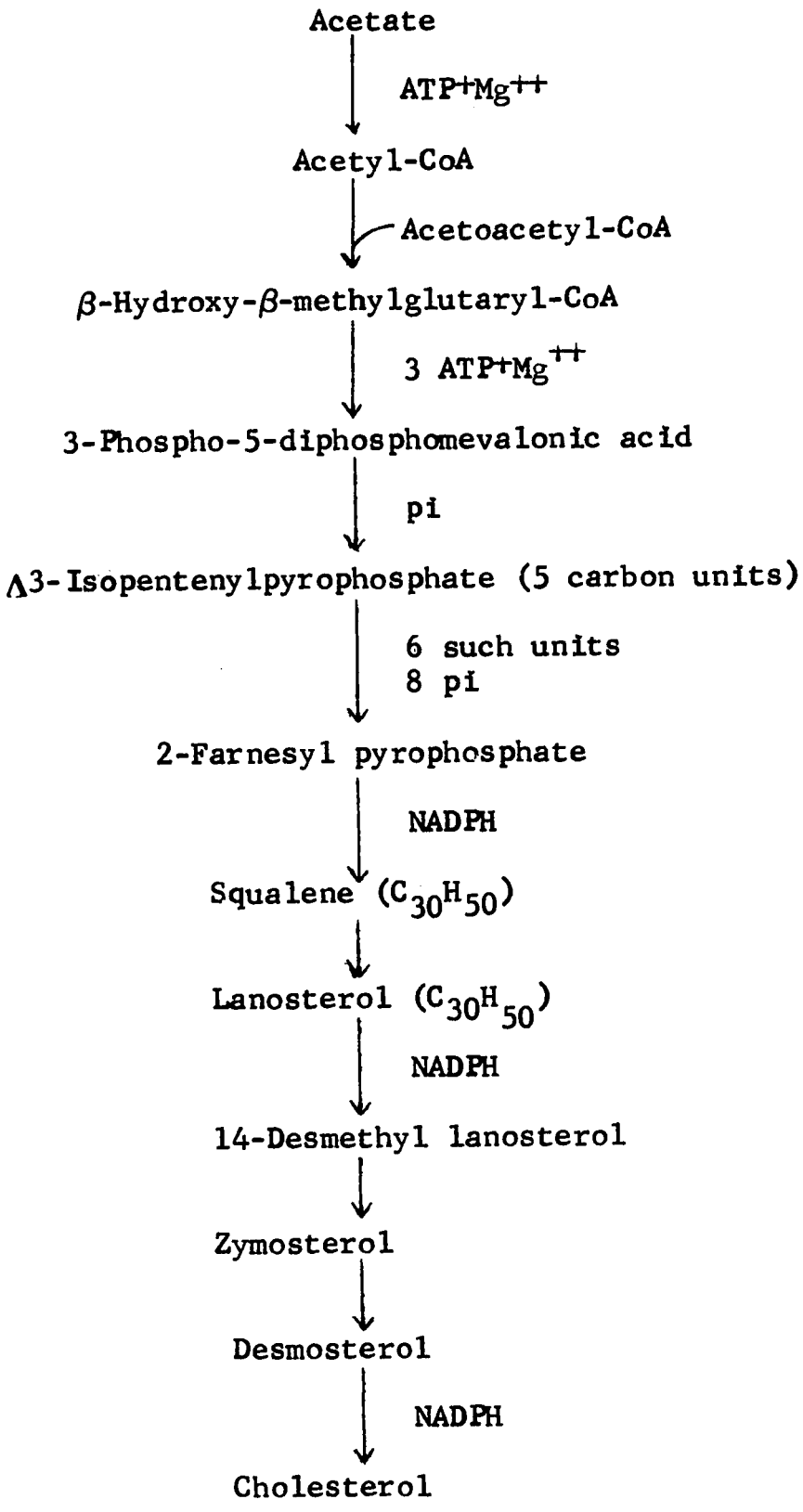
1. From Acetate to Cholesterol

Most of the sequences of the enzymatic reactions from acetate to cholesterol in the adrenal cortex remain obscure, although they are generally considered to be similar to the pathway elucidated in the liver, largely due to the efforts of Bloch (91). The scheme of such a pathway is illustrated in Figure 1.

Srere et al (92), as early as 1948, demonstrated the incorporation of ^{14}C-labelled sodium acetate into labelled cholesterol by beef adrenal slices. This finding was confirmed by Caspi et al (93), who isolated labelled cholesterol and cortisol from ^{14}C-acetate by perfusion of calf adrenals. The workers examined the distribution of radioactivity in some of the carbon atoms in ^{14}C-cholesterol and ^{14}C-cortisol and concluded that the individual carbon atoms of cortisol and cholesterol could be synthesized by the same route from acetate, without excluding the possibility of an alternative biosynthetic pathway, not involving cholesterol. Using ^{14}C-acetate, many other investigators isolated labelled corticosteroids but failed to demonstrate the presence of radiocholesterol (89, 94-96). This could possibly be due to the total transformation of the intermediate sterol to corticosteroids. However, perfusion studies carried

FIGURE 1

BIOSYNTHESIS OF CHOLESTEROL FROM ACETATE



out by Hechter et al (97) invariably showed a much higher specific activity in the isolated steroids than in the isolated cholesterol. This indicates that the incorporation of the 2-carbon fragments probably was partially achieved through another biosynthetic pathway. Moreover, Stone and Hechter (98) have also demonstrated that the effect of ACTH is at least ten times greater on ^{14}C-cholesterol than on ^{14}C-acetate. Thus it seems certain that the adrenal gland can synthesize cholesterol from acetate and that both compounds serve as precursors to corticosteroids. The results obtained by feeding ^{14}C-cholesterol to the rat and dog over a long period have indicated that plasma cholesterol might be directly utilized by the adrenal gland to synthesize corticosteroids (99, 100). This possibility was based upon the fact that specific activities of plasma free cholesterol in both species were essentially identical to those of adrenal free cholesterol.

Carballeira et al (96) using bovine adrenal slices, demonstrated the biotransformation of acetate-1-^{14}C to labelled cortisol. Five fold increase of ^{14}C-cortisol was observed when ACTH was added to the medium, however, addition of NADPH generating system did not enhance cortisol formation. Karaboyas and Koritz (101) in their <u>in vitro</u> studies reported that acetate-1-^{14}C is transformed to corticosterone by rat adrenal

slices and to cortisol by beef adrenal slices. Addition of 3', 5' AMP or ACTH in both types of adrenal incubations stimulated the conversion of acetate-1-^{14}C to labelled corticosteroids, accompanied by a drop of radioactivity of cholesterol.

2. Side Chain Cleavage of Cholesterol

Zaffaroni et al (102) first demonstrated conversion of cholesterol-^{14}C to corticosteroids in perfused bovine adrenal studies. In vitro studies conducted by Saba and Hechter (103) with cortical whole homogenates revealed the Mg^{++}, NAD and ATP were essential for the cleavage of cholesterol side chain. Lynn et al (104) confirmed the report by the previous authors. Later studies conducted by Constantopoulos and Tchen (105) and Halkerston et al (106) with adrenal mitochondria claimed that NADPH and oxygen were absolute exogenous requirements for the side chain scission of the cholesterol molecule. These findings are in accordance with earlier reports, since ATP and NAD in the presence of Mg^{++} can operate as a NADPH-generating system, especially in the presence of fumarate. The mechanism of the side chain cleavage of cholesterol is still not completely known. NADPH and oxygen are absolute requirements for the side chain scission of the sterol molecule, suggesting that some kind of hydroxylating mechanism might be involved. Solomon et al (107) isolated 20 α-hydroxycholesterol from bovine adrenal homogenates

incubated with labelled cholesterol. Later Shimizu et al (108) succeeded in isolating ^{14}C-isocaproic acid from 20 α-OH-cholesterol-22-^{14}C with the supernatant of bovine adrenal homogenates. Since this reaction required NADPH or an NADPH-generating system, it was suggested that another hydroxylation of side chain occurred, possibly at carbon 22. Based on their further experiments, Shimizu et al (109, 110) put forward this hypothesis, that another hydroxylation at C-22 is the next step followed by the acting of a "desmolase" between carbon 20-22. These findings have been confirmed by Constantopoulos et al (111).

Carballeira (78) in <u>in vitro</u> studies demonstrated the failure of bovine adrenal slices to catabolize labelled cholesterol under various experimental conditions. The conditions included the incubation of adrenal sections in diluted bovine plasma or blood, the use of mammalian ACTH and the emulsification of the substrate in Tween 80. Sandor et al (74) previously had encountered a similar difficulty with the duck adrenal slices. However, recently Hall and Koritz (75) using adrenal quarters of chicken adrenals, demonstrated the conversion of cholesterol-7 α-^{3}H (emulsified in Tween 80) to labelled corticosterone, thus suggesting that the entry of the sterol into the tissue is necessary for its catabolism.

3. <u>Δ^5-pregnenolone to progesterone</u>

At least two operations are required for the conversion

of Δ^5-pregnenolone to progesterone. These reactions are dehydrogenation of the 3β-hydroxyl group and shifting of Δ^5 to Δ^4. For these reactions at least two enzymes or enzymatic systems are involved, the Δ^5-3β-hydroxysteroid dehydrogenase (112) and $\Delta^5 \longrightarrow \Delta^4$ isomerase (113). Actually these two enzymes or enzymatic systems are difficult to separate from each other, and are studied together under the name of Δ^5-3β-hydroxysteroid dehydrogenase. It is now known that other steroid-producing tissues have this system, i.e. testis (112) and ovary (114).

Samuels et al (112) demonstrated that this enzymatic reaction is mediated by NAD which acts as a hydrogen acceptor. This finding has been confirmed by Byer and Samuels (115) and Halkerston et al (106) from microsomal fraction of beef adrenals. Also Halkerston et al (106) found that if this enzyme is dialyzed, it loses its activity, and this could be restored by addition of NAD. Kowal et al (116, 117) with acetone preparations of corpus luteum and adrenal cortex demonstrated NAD was the preferred cofactor as compared to NADP which had about 50% the activity of the former nucleotide. If NADH is added, it inhibits this reaction, and this inhibition could be overcome by the addition of excessive amounts of NAD. Moreover, it is interesting to note that their preparation could not utilize cholesterol or 20-deoxy-pregnenolone, suggesting that an oxygen function at

carbon 20 is necessary for the activity of Δ^5-3β-hydroxysteroid dehydrogenase.

Baille et al (118) in their histochemical studies reported that this enzyme seems to be mainly present in the zona glomerulosa. However, Levy and Rubin (114) have evidence that this is present in all zones of the adrenal cortex.

Krüskemper et al (120) using bovine and rat adrenals, showed that this enzymatic activity is present in all ultracentrifugal fractions, more specially in the particles.

4. Steroid Hydroxylations

Hydroxylation of steroid molecule at various sites requires specific enzymes. For the hydroxylation at carbons 11, 17, 21 and 18 beside specific enzymes, molecular oxygen and NADPH or NADPH generating systems are required as cofactors. A brief survey of these hydroxylating enzymes and various requirements is given below:

a) 11β-hydroxylation

This enzyme, known as the "trade mark" of the adrenal cortex, has been studied most extensively and appears to be present throughout the three zones of adrenal cortex (121). Sweat was the first who reported the location of this enzyme in mitochondrial fraction (19,000 x g 30 min.) (122). This was further confirmed by Hayano and Dorfman who also showed that

the pH optimum of this enzyme is at 7.4 (123). A soluble fraction of 11β-hydroxylase has been prepared (124-126). Tomkins et al (125, 127) obtained three protein fractions from the calf adrenal acetone powder, and all of them were required for 11β-hydroxylase activity, besides NADPH, oxygen and a heat stable co-factor obtained from the extracts of rabbit liver. Adrenal mitochondria can hydroxylase progesterone to 11β-hydroxyprogesterone (128-130).

b) 17 α-hydroxylation

17 α-hydroxylase activity is associated with the microsomal fraction of the adrenal cortex (131). Using bovine adrenals, Stachenko and Giroud in in vitro studies (132, 133) have reported that this enzyme is present only in zona fasciculata-reticularis.

c) 21-hydroxylation

Giroud and Stachenko (132, 133) in in vitro studies, using bovine adrenal gland, showed that this enzyme is present in all zones of the adrenal cortex. Ryan and Engle (134) showed that this enzyme is located in the "microsomal fraction" sedimented at 105,000 x g.

d) 18-hydroxylation

In 1956, Dorfman (135) suggested that the biosynthesis of aldosterone requires the hydroxylation of carbon 18, followed by oxidation of this group to aldehyde. This hypothesis was

based upon the studies by Kahnt et al (136), who demonstrated production of 18-hydroxy-11-deoxycorticosterone by adrenal homogenates incubation in the presence of 11-deoxycorticosterone. Since then many researchers have isolated 18-hydroxy-11-deoxycorticosterone (137-139) and 18-hydroxycorticosterone (47, 137, 140-143) from incubation studies with adrenal glands from various species. However, the possible role of 18-hydroxylation in aldosterone either via 18-OH-B or 18-OH-DOC is still controversial (see also Chapter 2, B). The 18-hydroxylating enzyme is located in the mitochondria of the mammalian adrenal cortex, being present in all zones (86, 144). This enzyme has also been found in the mitochondria of frog interrenals (52).

5) Oxygen and NADPH Requirement

Hayano and Dorfman (145, 146) first reported that oxygen is utilized by beef adrenal homogenates. Saffran and Bayliss (147) failed to produce corticosteroids if oxygen was replaced by nitrogen as a gas phase during the incubation.

Later investigations carried out by Hayano et al (148-151) using $^{18}O_2$, D_2O and $H_2{}^{18}O$ gave a direct evidence that molecular oxygen is involved in 11β, 17α and 21-hydroxylations.

Requirement of NADP for 11β-hydroxylation in adrenal gland homogenates was demonstrated by Hayano and Dorfman (146). However, it was later shown that NADPH was the actual cofactor

required for the hydroxylation (152). In the same study Sweat and Lipcomb pointed out that any Krebs cycle intermediates could stimulate the hydroxylation, by acting as substrate for the generation of NADPH, for example: isocitrate+NADP ⟶ oxalosuccinate+NADPH. This finding was confirmed by Grant (124).

Specific requirement of NADPH for hydroxylations at carbons 11, 17, 21 and of the side chain of cholesterol at carbons 20 and 22 prior to cleavage, has been demonstrated (105, 106, 131, 134). Also now it is well established, that NADPH or NADPH generating system stimulates endogenous steroid production in vitro (153-155).

6) Generation of NADPH in the Adrenal Gland

NADPH is known to be generated through two main pathways. These pathways, namely, the direct oxidative pathway and the Krebs cycle, generate NADPH through the action of dehydrogenases.

Biochemical studies carried out by Glock and McLean (156) and Kelly et al (157) have indicated a high level of G-6-P-dehydrogenase and 6-phosphogluconate dehydrogenase activities in mammalian adrenal gland, including the rat, the rabbit, and the ox. McKerns (158) in a systematic study of the dehydrogenases in the rat adrenal gland and beef adrenal cortex has found that in both species G-6-P-dehydrogenase has the highest activity, followed by 6-phosphogluconate, isocitrate, and malic dehydrogenases

in decreasing order of activity.

Histochemical studies by Cohen (159) and Greenberg and Glick (160) indicated presence of G-6-P-dehydrogenase and 6-phosphogluconate dehydrogenase activities in the rat adrenal gland. Both these activities were highest in the zona fasciculata-reticularis and low in zona glomerulosa. It has been also demonstrated that injection of ACTH, three hours prior to the killing of the animals, increased the activity of 6-phosphogluconate dehydrogenase in the whole cortex, whereas that of G-6-Pdehydrogenase was increased mainly in the fasciculata-reticularis (160).

B. Biosynthesis of Aldosterone

Ever since the first isolation and characterization of aldosterone by Simpson et al (161), studies have been undertaken on the biosynthesis of this hormone by various adrenal glands, especially mammalian. Morphologically speaking, the zona glomerulosa of the mammalian adrenal cortex is the site where this mineralocorticoid is produced (84, 86, 162). Since in lower vertebrates there is no functional zonation of adrenal gland, the type(s) of cells which produces aldosterone, is still unknown. However, it is relatively well established, that the adrenal mitochondrion (mammalian, avian and amphibian) is the organelle, where aldosterone is mainly synthesized (52, 163-166).

Direct evidence, that pregnenolone, progesterone, 11-deoxycorticosterone and corticosterone give rise to aldosterone, has come from tracer studies with the respective precursors (73, 167-171). While discrepancies occur as to which of these steroids above-mentioned is a better precursor, the bulk of evidence, particularly the detailed analysis of the works of Ayres (171) and Whitehouse (73) seems to indicate that the pathway from pregnenolone leading to progesterone, 11-deoxycorticosterone and corticosterone is likely a major pathway. However, this picture became more complicated by the isolation of 18-hydroxycorticosterone. This substance would logically fit as an intermediary between corticosterone and aldosterone. However, attempts to achieve the transformation of 18-hydroxycorticosterone to aldosterone by slices, or homogenates of mammalian, avian or amphibian adrenals were largely unsuccessful (50, 52, 77) with exceptions reported by Paqualini (172) and Kahnt and Neher (173).

To find a possible answer to this problem, experiments were carried out in various laboratories, to study the biosynthesis of aldosterone from corticosterone by adrenal tissue intracellular fractions of several vertebrate species. The three vertebrate species studied were the sheep, the bullfrog and the duck adrenals (163, 52, 164). In all these animal classes investigated, 18-oxygenation of corticosterone was

effected by adrenal mitochondria. Only mammalian adrenal mitochondria did transform 18-hydroxycorticosterone to aldosterone, while this was not achieved by either frog or duck adrenal mitochondria. However, Greengard et al (165) showed that in frog adrenal mitochondria, the biosynthesis of aldosterone from corticosterone proceeded via a hydroxylation step, placing 18-hydroxycorticosterone in the role of the immediate precursor of aldosterone. If 18-hydroxycorticosterone is indeed the immediate and obligatory precursor of aldosterone in vertebrate adrenals, the inability of these glands to utilize exogenous 18-hydroxylated substrates might be due to the 20-18 cyclic hemiketal structure of these substances (50).

Fazekas and coworkers (174-176) working with human and rabbit adrenals, described recently the transformation of 11-dehydrocorticosterone to 18-hydroxy-11-dehydrocorticosterone, aldosterone and 11-dehydroaldosterone. This new pathway seems to be important in rabbit adrenals. However, de Nicola et al (51, 177) showed in <u>in vitro</u> studies that 11-dehydrocorticosterone is not converted to aldosterone by mouse, rat and frog adrenals. It is possible that the pathways leading to aldosterone could be species-dependent.

C. <u>Adrenal Pituitary Axis</u>

The secretion of cortisol, corticosterone and other glucocorticoids from mammalian adrenal cortex is regulated

by pituitary corticotropin (ACTH). Experiments with other animals indicate that the interrenal is probably under pituitary regulation in most vertebrates. This has been well reviewed by Bern and Nandi (37). Hypophysectomy generally results in interrenal atrophy, but data are conflicting in some of the species of the vertebrates studied. Hypertrophy of interrenal or adrenal gland has been observed in almost all cases when mammalian ACTH was administered. However, the pituitary extracts derived from the experimental species were even more effective.

Aldosterone is not under obligatory pituitary control and is at least in part regulated by factors other than ACTH, but little is known of the possible existence of such factors in non-mammalian vertebrates. The addition of mammalian ACTH to the bullfrog adrenal incubation resulted in a large increase in aldosterone production (42, 43). Recently <u>in vitro</u> studies by Piper and deRoos (45) have further indicated, that in the bullfrog both corticosterone and aldosterone have a negative feed-back influence on the pituitary corticotropin.

III METHODS AND MATERIALS

A. Source of Adrenal Tissue

Adrenal glands were obtained from two different classes of lower vertebrates: Amphibia and Reptilia. The American bullfrog and the painted turtle were chosen as representative species for each class respectively.

1) Frog. American bullfrogs, (Rana catesbeiana: class: Amphibia; subclass: Anura) both males and females, weighing 100 - 300 g were used. These amphibians were kept at 10°C in shallow water at the McIntyre animal center, McGill University and brought to the laboratory just prior to the experiment. The frogs were decapitated with a guillotine, and their kidneys were removed through abdominal incision. The adrenal bodies, which were partly embedded in the ventral surface of each kidney, were dissected free of renal tissue. Each adrenal weighed between 12 - 18 mg.

2) Turtle. Painted turtles, (Chrysemys picta picta: class: Reptilia; order: Chelonia). Both males and females specimens, weighing 200-350 g were used. These reptiles were kept under similar conditions to the amphibians. The turtles were decapitated, the carapace and plastron were cut apart with the help of a saw, and kidneys were removed. The adrenal bodies, which were embedded in the ventral surface of each kidney,

were dissected free of renal tissue. Each adrenal weighed between 15-22 mg.

B. Types of 'in vitro' adrenal preparations

The adrenal tissue obtained was further prepared for *in vitro* studies. Three different kinds of preparations were used.

1) Surviving sections. The adrenal or interrenal tissue, which was usually an elongate body, was cut with the help of small scissors into four or five equal sections. These sections were kept in ice-cold KRBG buffer at pH 7.4. The buffer solution was decanted, and the tissue sections were rinsed twice with fresh ice-cold buffer. The tissue was gently but thoroughly blotted between layers of filter paper and weighed in aliquots of 100 mg. Variations of tissue weights in some experiments will be indicated in the appropriate places. These sections were thus ready for incubations.

2) Homogenates. Similar procedures of dissection, washing and weighing were followed as mentioned above. The homogenates were prepared in the cold room (approximately 4°C) with a tight-fitting all-glass apparatus. The homogenizing media was $KRPO_4$ buffer, supplemented with fumarate (0.04M) (unless otherwise mentioned) at pH 7.4. The homogenates, thus obtained, contained 100 mg of the tissue per 2.5 ml. These homogenates, prepared

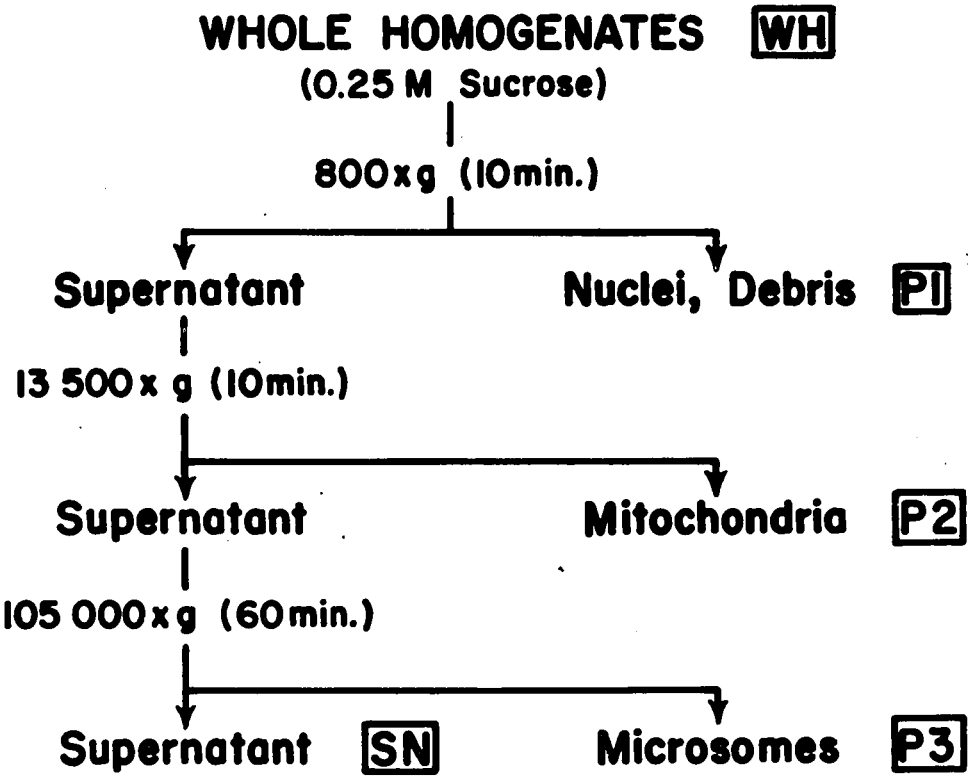
in $KRPO_4$ buffer, were ready for incubation and henceforth will be abbreviated as H.

3) Subcellular fractions. The subcellular fractions of frog and turtle adrenal were prepared in an essentially similar manner to that described by Psychoyos et al (52). The homogenates of whole adrenal glands were prepared in ice-cold 0.25M sucrose solution (previously adjusted to pH 7.4 with 1N KOH) in the cold room (approximately 4°C) with a tight-fitting, all-glass apparatus. The homogenates, thus obtained, contained 400 mg of the tissue per 10 ml. Two aliquots of 2.5 ml were fractionated in a refrigerated vacuum centrifuge, model BD-2 using rotor No. 969, (IEC) according to the scheme outlined in Figure 2. The remaining homogenates were stored, surrounded by ice, in the cold room, until the end of the centrifugation process. These sucrose-homogenates then were ready for incubation, and to differentiate them from the previously described $KRPO_4$ homogenates will be abbreviated as WH. The other abbreviations of various fractions are given in Figure 2.

All the sediments obtained during the centrifugation were resuspended in 2.5 ml of 0.25M sucrose and stored in ice until the spinning was over. The incubations of all various fractions and WH were carried out at the same time. The total time elapsed between the preparation of homogenates and the beginning

FIGURE 2

ULTRACENTRIFUGAL PROCEDURE FOR THE FRACTIONATION OF FROG AND TURTLE INTERRENAL HOMOGENATES INTO VARIOUS SUBCELLULAR PREPARATION.



of the incubations was approximately two hours.

C. Conditions of Incubations

Total incubation volume was 5 ml and was kept constant throughout all types of incubations; i.e. adrenal sections, homogenates or subcellular fractions.

Krebs-Ringer bicarbonate buffer (178) containing 200 mg % glucose at pH 7.4 was used (unless otherwise mentioned) for the incubation studies conducted with surviving adrenal sections.

Krebs-Ringer phosphate buffer (178), supplemented with fumarate (0.04M) at pH 7.4 was used for the studies conducted with adrenal homogenates H. Since Kahnt and Neher (173) pointed out that addition of nicotinamide to the incubation medium interferes with aldosterone formation, this agent was not employed.

The incubations with the homogenates WH and subcellular fractions (P1, P2, P3, SN) contained 0.13M sucrose in addition to $KRPO_4$ buffer. Variations in the composition of the buffer in special experimental designs will be indicated at appropriate places in 'Results'.

The incubation studies were carried out in a Dubnoff Metabolic apparatus using 95% O_2 and 5% CO_2 as gassing phases. The shaking speed was approximately 50 cycles/min. Incubation time was usually three hours, unless otherwise mentioned. In

selected studies with surviving sections, pre-incubation was carried out, as will be mentioned in 'Results'.

D. Labelled Precursors

All radioactive precursors were purchased from New England Nuclear Corporation. The following substrates were used:

Substrate	Specific Activity (μc/μg)
progesterone-4-^{14}C	0.17
Δ^5-pregnenolone-4-^{14}C	0.17
cholesterol-4-^{14}C	0.16
acetate-1-^{14}C	0.69

The chromatographic homogeneity of these labelled substrates was verified prior to their use.

In the case of C_{21} and C_{27} precursors, 2 μc of substrate dissolved in 0.2 ml of propylene glycol, were usually employed. In a series of observations, cholesterol-4-^{14}C was emulsified in Tween 80 according to the procedure described by Karaboyas and Koritz (101).

In the case of acetate-1-^{14}C, a total of 200 μc were used per incubation, either as a single addition or as equally divided aliquots added at regular intervals during incubation. Details of this stepwise incubating procedure will appear in 'Results'.

In the incubation studies with surviving interrenal

sections, all radioactive substrates were employed with the original specific activity of the commercial product without further dispersion with the corresponding unlabelled substances.

When homogenized (H or WH) and subcellular preparations (P1, P2, P3 and SN) were used, labelled C_{21} substrates were mixed with 100 µg of the corresponding radioinert substances to prevent complete utilization of the substrate.

E. Supplementation of 'in vitro' Systems

The cofactors added to the incubation medium were dissolved, either in KRBG or $KRPO_4$ at pH 7.4, according to the design of the experiment. They were prepared and added to the in vitro systems just prior to incubation. Unless stated otherwise, the following concentrations were used:

	µ moles/ml
NADPH	0.25
NADP	0.25
NAD	0.25
G-6-P	0.90
3', 5' AMP	18.00
3', 5' AMP D.B.	18.00

The first four substances were purchased from Sigma Chemical Company and latter two from Schwarz Bio Research.

Mammalian ACTH (Nordic Biochem Ltd.) was dissolved in a

few drops of 0.1N HCl and further diluted in KRBG. The concentration of ACTH in incubation medium was 1 i.u. per ml.

F. Extraction of Steroids

At the end of the incubation period, the incubation medium was separated from the sections. The sections were washed with 2x5 ml of KRBG and washings pooled with the original incubation medium in a beaker. Ten ml of ethyl acetate were added to each beaker to stop the enzymatic reaction. Contents of the beaker were poured into a separatory funnel and extracted three times; twice with 50 ml of ethyl acetate, once with 50 ml of chloroform. All the organic phases were pooled and washed with 20 ml of water. The total extract was dehydrated over anhydrous sodium sulphate and filtered. The sodium sulphate was washed twice with 20 ml of a chloroform: ethyl acetate (1:1) mixture, filtered and added to the previous filtrate. The total extract was evaporated to dryness under vacuum at 50°C.

In certain cases, pointed out in 'Results', the adrenal sections after the incubation period were homogenized in fresh KRBG. These homogenates were extracted similarly as described above (unless otherwise mentioned).

A similar extraction procedure was followed for the extraction of incubations with homogenates or cell-free preparations.

The dry residue in each case was dissolved in 10 ml of methanol, and a 0.1 or 0.2 ml aliquot was taken for the assay of radioactivity. The remainder was transferred quantitatively into a test tube and evaporated to dryness under nitrogen at 35°C. The crude dry extract was ready for application to paper for chromatography.

G. Chromatographic Resolution of Extracts

Extracts were resolved by a slight modification of a serial paper chromatographic scheme developed in this laboratory (144, 179). Figure 3 shows the various solvent systems, running time, overflows and running rates of various authentic steroids. Details of paper chromatography and these solvent systems will be given in section III, N, 1.

H. Detection of Steroids in Chromatograms

1) Radioactive materials. Radioactive metabolites and unconverted precursors were localized in the papergrams by

a) Radioautography

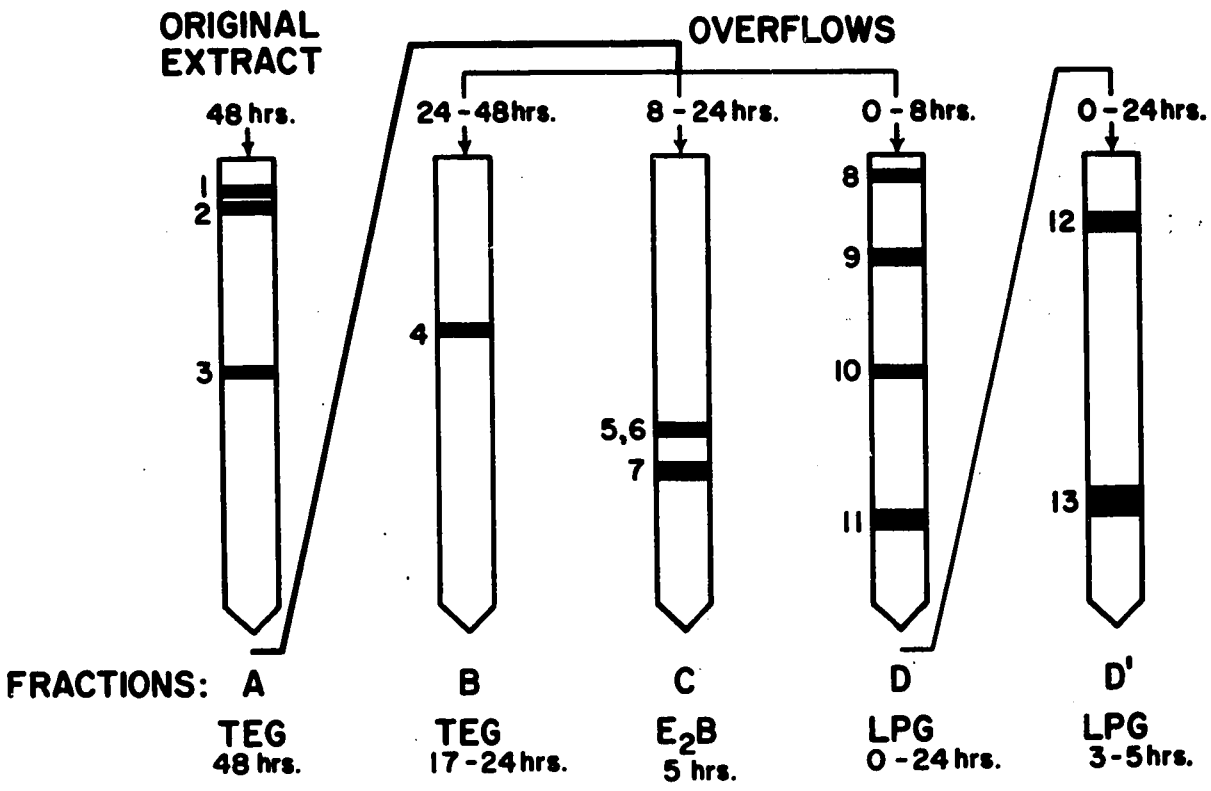
and/or

b) Radiochromatogram scanning.

a) Radioautography. Dry paper strips were exposed to a no-screen Kodak x-ray film in a standard x-ray cassette. After three days the film was developed by standard x-ray film developing techniques. Under these conditions 1500-3000 cpm of authentic

FIGURE 3

SERIAL PAPERCHROMATOGRAPHIC SCHEME FOR THE RESOLUTION OF STEROIDS IN CRUDE EXTRACTS FROM FROG AND TURTLE INTERRENAL INCUBATIONS.



1. 18-OH-B
2. 19-OH-B
3. Aldosterone
4. 19-OH-DOC
5. 18-OH-DOC
6. Cpd. A
7. Cpd. B
8. 11β-OH-P
9. DOC
10. 11keto-P
11. Δ^5-P
12. Δ^4-P
13. Cholesterol

^{14}C labelled substrates appear in the radioautograms as well defined dark areas. In certain studies indicated in 'Results' the time of exposure to x-ray films was prolonged to 10 days. With this prolonged exposure, 500 cpm of chromatographed ^{14}C-labelled substrates can be similarly detected in the radioautograms (180).

b) Radiochromatogram scanning. Dry paper strips were scanned in a radiochromatogram scanner (Packard Model 7200) at appropriate sensitivity and speed. This method was used for the detection of tritiated materials, since x-ray films are not sensitive to the β-rays emission of tritium under the above described standard techniques. This technique was also used for ^{14}C-labelled materials, when a rapid survey of the distribution of radioactivity on papergrams was desired.

2) Ultraviolet light absorbing materials. In certain experiments (see 'Results') in which the incorporation of radioactivity into metabolites was expected to be extremely low, even below the sensitivity of prolonged radioautography (10 days), 100 μg of authentic non-labelled materials corresponding to the major conversion products isolated in other studies were added to the extracts prior to chromatography. These paper strips were exposed to ultraviolet light using a Corning filter No. 9863. Steroids having a Δ^4-3keto group, absorb ultraviolet light

at 238-240 mμ and thus can be detected by this procedure. Kodak standard contact photographic paper was used in order to have a permanent record of these ultraviolet absorbing areas.

3) Other materials. Dry paper strips were sprayed with a saturated solution of antimony trichloride in chloroform to detect steroids having Δ^5-3β-ol structure such as Δ^5-pregnenolone or cholesterol. After heating the papergram at 90°C for five minutes, the areas containing the above mentioned steroids resulted in purple or violet colour.

I. Elution of Steroids

Thirty ml syringes were used for the elution. Areas which showed radioactivity or ultraviolet absorption in paper strips, were cut out and hung with the needle directly into a round bottom flask. The strips were eluted twice with 30 ml of methanol. At the end the solvent was evaporated to dryness under vacuum below 50°C.

In few cases, where radioactivity was spread over a wide area, the paper was cut into very small pieces and left overnight in methanol. The following day, the methanol was filtered, the pieces of paper washed three times with methanol, and the pooled solvents were evaporated to dryness as mentioned above.

J. Assay of Radioactivity

Radioactivity of labelled compounds was assayed in a

Packard liquid scintillation spectrometer, Model 3375.

Aliquots ranging from $\frac{1}{100}$ to $\frac{1}{2}$ of the total sample, were used for the radioactive assays. These aliquots were pipetted into the counting vials, dried completely under a stream of nitrogen and redissolved in 10 ml of scintillating solution. This solution contained 0.3% of 2:5 diphenyloxazole (P.P.O.) and 0.01% P-bis 1,2 (5-phenyloxazolyl) 1-benzene (POPOP). Efficiency of this machine for ^{14}C and ^{3}H was in the order of 88% and 50% respectively with 2% standard deviation.

Some of the samples containing both ^{3}H and ^{14}C were counted at a single voltage, 1580 volts (see section III, M). This model is equipped to discriminate ^{3}H and ^{14}C counts simultaneously. Under these dual labelled conditions, efficiencies for ^{3}H and ^{14}C were 22 and 55% respectively.

Samples were counted for 10 minutes or more, and two or more readings were usually procured. Background counts were not more than 10 cpm in the ^{3}H channel and 12 cpm in the ^{14}C channel.

K. Recovery of Steroids

To check the losses occurring during the whole experimental procedure, various amounts of cortisol-7α-^{3}H were added to different incubating beakers, right after the incubation. This tritiated steroid was chosen,because our preliminary studies

showed no cortisol formation by frog or turtle interrenal, and, therefore, it would not overlap with ^{14}C-labelled metabolites. After the usual procedure of extraction and chromatography, the radioactive zones corresponding to the cortisol standard were cut, eluted and counted. The counts added and recovered were as follows:

Total Counts Added ($x10^{-3}$)	Recovered ($x10^{-3}$)	Recovery (%)
24.7	18.2	73.8
	17.2	69.7
50.2	37.9	75.4
	37.8	75.3
76.8	59.0	76.8
	62.1	80.9
102.7	74.0	72.1
	76.1	74.1
127.4	98.0	76.9
	99.3	77.9

The recovery ranged between 70-80%.

L. Calculation of Results

The results have been usually expressed as percentage conversion/amount of tissue incubated/time of incubation and calculated by

$$\frac{\text{Radioactivity of isolated product}}{\text{Total radioactivity recovered}} \times 100$$

The figures have not been corrected for procedural losses which

were between 20-30% as shown above.

M. Identification of Steroids

The various metabolites isolated from different studies were further analyzed for their identity. The various criteria used were:

1) Successive chromatography of metabolites. The radioactive zone having the mobility of a given standard, after elution, was mixed with about 100 µg of the corresponding authentic steroid (unless otherwise mentioned). This mixture was chromatographed either on paper or TLC to ascertain the isopolarity of the metabolite and the authentic substance. Whenever possible, at this stage corresponding ^{3}H steroids were added to the ^{14}C isolated metabolites. The following ^{3}H steroids used were purchased from New England Nuclear Corporation and thoroughly purified prior to use:

	Specific Activity (µc/mg)
Cholesterol-1, 2-^{3}H	0.14
Δ^{5}-pregnenolone-7-^{3}H	0.17
progesterone-1, 2-^{3}H	0.17
11-deoxycorticosterone-1, 2-^{3}H	0.14
corticosterone-1, 2-^{3}H	0.17
11-dehydrocorticosterone-1, 2-^{3}H	0.17
D-aldosterone-1, 2-^{3}H	0.05

The amount of ^{3}H material added to the corresponding ^{14}C-steroids was in such an order as to give rise to a $^{3}H:^{14}C$ ratio of between 4 to 10. The mixture was applied on appropriate chromatographic solvent system either paper or TLC. After each chromatography, an aliquot was taken, and $^{3}H:^{14}C$ ratio was determined. Usually two or three different chromatographic systems were used. At this stage, the steroid was subjected to further analysis by using some of the following chemical means according to the nature of each steroid.

The method of detection of steroids in this successive chromatography and after the formation of derivates to be described below were exactly the same as those detailed in III, H.

2) Formation of derivatives of metabolites. Various metabolites after successive chromatography were further identified by formation of derivatives. This was achieved by adopting either a) chemical procedures or b) enzymatic reactions.

a) Chemical procedures.

i) Acetylation. Steroids were acetylated according to the method described by Bush (181). The dry steroid residue was dissolved in 0.1 ml dry pyridine and 0.4 ml of acetic anhydride was added. The mixture was maintained at 60°C for 15 minutes and then evaporated to dryness under a stream of nitrogen.

Twice 5 ml of methanol was added and evaporated to dryness. Finally 5 ml of dry benzene was added and again evaporated to dryness. The dry mixture was directly applied either on paper-grams or TLC plates.

Under these 'fast' conditions of acetylation, 21-hydroxyl group of an α-ketol steroid is completely esterified. Also 20β-hydroxy progesterone isesterified to about 80%. The 18-hydroxyl group of aldosterone (hemiacetal form) is only esterified to about 15%.

ii) Hydrolysis. Hydrolysis of steroid acetate was carried out by the modified method described by Bush (182).

Dry steroid acetate was dissolved in 0.5 ml of methanol and 0.5 ml of sodium carbonate (2.5% aqueous solution). The mixture was kept at room temperature for 30 minutes and extracted with 2x10 ml of ethyl acetate. The pooled ethyl acetate extract was washed with 2 ml of distilled water, dehydrated over anhydrous sodium sulphate, filtered and dried under a stream of nitrogen at 35°C. The dry residue was ready for chromatography.

iii) Oxidation with chromium trioxide (CrO_3). The method described by Bush (183) was employed for the transformation of an 11-hydroxyl group to an 11-keto group. Dry steroid residue was dissolved in 0.5 ml of glacial acetic acid and 0.6 ml of chromic acid (2% chromium trioxide) was added. The mixture was left in

the dark for 20 minutes at room temperature. Under these conditions, the 11 β-hydroxyl group of C_{19} and C_{21} steroids are oxidized completely. At the end of 20 minutes, 5 ml of distilled water was added and extracted with 2x10 ml of ethyl acetate. The organic phase was washed with 2 ml of saturated sodium bicarbonate, 2 ml of distilled water, dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was ready to be applied to the paper chromatogram or TLC plates.

iv) Oppenauer oxidation. Chemical conversion of Δ^5-pregnenolone to progesterone was achieved by Oppenauer oxidation (184). The steroid was kept in a small test tube and solvent evaporated. Two additions of absolutely dry ethanol followed by evaporation under reduced pressure were carried out to remove all traces of water. The residue was dissolved in 0.5 ml of absolutely dry benzene and 15 mg of aluminum t-butoxide was added, followed by 0.1 ml of dry benzene: acetone (1:1). The tube was sealed and heated at 100°C for 75 minutes. After cooling, the tube was opened and the solution was transferred with benzene and small volumes of 1N HCl into a small separating funnel. The benzene layer was retained and washed with further 1N HCl, sodium bicarbonate (2%) and water, dried over sodium sulphate and evaporated. Under these conditions about 80% of Δ^5-3β-ol was converted to Δ^4-3ketone.

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v) Oxidation with periodic acid for C-18 oxygenated corticosteroids. The oxidation of C-18 oxygenated steroids was carried out using the method described by Tait et al (185).

Dry residue of C-18 oxygenated steroid was dissolved in 0.2 ml of ethanol, to which 0.8 ml of 0.1M periodic acid was added,and contents were left overnight in the dark at room temperature. Thereafter, 1.0 ml of water was added, and the contents were extracted three times using 5 ml of methylene dichloride each time. The combined methylene dichloride extract was washed once with 1 ml of water, twice with 1 ml of 0.25N sodium bicarbonate and again twice with 1.0 ml of water. The combined aqueous washings were back extracted with 5 ml of methylene dichloride which was added to the original extracts and washed with 1 ml of 0.01N hydrochloric acid. The methylene dichloride extract was dried under nitrogen. It was redissolved in ethanol, an aliquot taken for counting,and remaining solution was dried under nitrogen. The residue was ready for application on paper chromatography.

vi) Formation and hydrolysis of digitonide. Cholesterol was subjected to digitonin precipitation by the method described by Srere et al (92).

The sterol residue was dissolved in minimum amounts of hot ethanol,an excess of 1% digitonin (in 80% ethanol) was added. The mixture was heated for another five minutes and

allowed to stand overnight at 5°C. The cholesterol-digitonide complex was filtered and washed successively with 85% ethanol, ether:acetone (1:2) mixture and finally with anhydrous ether and dried.

The cholesterol-digitonide complex was cleaved with pyridine using the method described by Schönheimer (186). The complex was dissolved in 0.5 ml of warm, water free, pyridine, and 10 ml of ether was added. After shaking the mixture, the etherial layer was removed. The pyridine layer was again extracted with 10 ml of ether and pooled with the previous etherial layer. These pooled ether extracts were dried under nitrogen, and free cholesterol was obtained in white crystalline form.

vii) Bromination, debromination. The pure crystalline cholesterol, obtained after digitonin treatment, was subjected to bromination and debromination cycles according to the method of Schwenk et al (187).

The crystalline sterol was dissolved in 1.5 ml of ether in a 10 ml of Erlenmyer flask, cooled in ice-cold water, and bromine water was added in small droplets from a fine pipette until the solution remained distinctly orange colored. The flask was kept in ice-cold water for another 30 minutes, and 1.5 ml of glacial acetic acid was added. Soon crystals of 5,6-dibromo-cholesterol began to appear, and the mixture congealed into a

mass of fine needles. After about 10 minutes, these were filtered by suction over a small filter paper. The mass of cholesterol-dibromide was washed with cold methanol and dried by sucking. Thus, the crystals obtained showed a white silky appearance.

For debromination, the crystals were transferred to a 10 ml beaker,and 1 gram of zinc dust was added, and the two substances were thoroughly mixed with a small glass rod,and care was taken to break the crystals thoroughly. About 1,5 ml of glacial acetic acid was added, and stirring was continued until the mixture became a magma, which was occasionally stirred during the next 10-30 minutes. Ten ml of ether was added, and the material was extracted. Similar extractions were repeated three times, and all the ether extracts were pooled. The extracts were washed twice with water, twice with 10% NaOH solution and finally again twice with water. The ether solution was dried under a stream of nitrogen. The white residue obtained was dissolved in methanol with the addition of small amounts of ethyl acetate, filtered and left in the cold overnight to crystallize. The crystals of cholesterol then obtained were washed with cold methanol and dried.

b) Enzymatic reduction. Progesterone, isolated as a metabolite, was reduced enzymatically to the 20 β-hydroxy derivative. The

modified method of Margraf et al was used (188).

Progesterone was incubated in a test tube with 0.1 mg of 20β-hydroxysteroid dehydrogenase and 0.2 mg of NADH in 0.5 ml phosphate buffer, pH 7.6, for two hours at room temperature.

The reduction product was quantitatively transferred to a separating funnel with 1.5 ml distilled water and extracted with 2x6 ml methylene dichloride. The methylene dichloride extract was dried in a centrifuge tube, and the residue was chromatographed.

c) Crystallization. Radioactive metabolites or their derivatives were crystallized to constant specific activity using the microcrystallization method of Axelrod et al (189).

The carrier (unlabelled) steroid was crystallized three times from ethanol-pentane mixtures. The crystals obtained were then dried in a vacuum oven at 60°C for 24 hours.

The purity of ^{14}C or a mixture of ^{3}H:^{14}C steroids, which was to be crystallized, was previously established by successive chromatography in various solvent systems. Since it was beyond our working possibilities to crystallize separately the same metabolite isolated from different precursors and each tissue preparation, pools from various experiments, were prepared (after the identity of each specimen was established by the previous criteria), and crystallizations were carried out.

About 25 mg of the carrier steroid was mixed with the corresponding radioactive steroid in a preweighed tube. The sample was brought into a solution in about 1 ml of boiling acetone. While boiling, the solution was constantly agitated with a glass rod to prevent boiling over or bumping. The volume was reduced to about half, and while the sample was still in solution, it was removed from the water bath and pentane was added, a few drops at a time, with agitation, until a cloud began to appear. As the mixture cooled, the cloud precipitated into crystals. When an adequate amount of crystals was formed, the tube was centrifuged for 3-5 minutes. The mother liquor was carefully decanted into a preweighed tube. Pentane-methanol (20:1) was added to the crystals to help remove any remaining mother liquor. The tube was again centrifuged and the supernatant added to the first mother liquor. Both the crystals and the mother liquor were separately brought to dryness under nitrogen. In order to insure a constant weight, the tubes were dried under vacuum overnight at 60°C. The tubes were weighed, and the residues, both in crystal and mother liquor tubes, were brought into solution with acetone. An appropriate aliquot was taken according to the amount of steroid present. The crystals in solution were again dried and subjected to further crystallization. Usually four to five successive crystalliza-

tions were carried out.

N. General Chromatographic Methodology

Two different kinds of methods were used for the chromatography of steroids.

1) Paper chromatography. For this purpose Whatman No. 1. chromatographic paper 57x46 cm was usually employed, the only exception being Bush T system forwhich Whatman No. 2 paper was preferred. Seventeen cm wide strips were washed in a Soxhlet apparatus with methanol:benzene (1:1) for three days. After three days, the paper was hung to dry and stored in a paper folder. Before chromatography, the paper was cut lengthwise, making 2-4 cm wide strips at 1/2 or 1 cm distance. All the strips were 44 cm long and were joined by a common head 13 cm long. The application line, where steroids residue was applied, was 2 cm from the common head.

The steroid dry residue, after dissolving in a few drops of methanol:chloroform (1:1), was applied on the application line with the help of a Wintrobe pipette. During the application, the solvent mixture was continuously evaporated under a stream of nitrogen. To make sure of complete transfer of the residue, the whole procedure was repeated twice.

Chromatographic solvent systems of Bush and of Zaffaroni and Burton (Table VI) were mainly employed. An equilibration

TABLE VI

PAPER CHROMATOGRAPHY SYSTEMS

SYSTEM	MOBILE PHASE/STATIONARY PHASE	EQUILIBRATION TIME	REFERENCE
	A[1]		
Bush A	Benzene/methanol:water 100 / 50 : 50	45 min.	(192)
Bush B5	Petroleum ether/methanol:water 100 / 80 : 20	45 min.	(192)
Bush C	Toluene:ethyl acetate/methanol:water 90 10 / 50 : 50	45 min.	(192)
D	Methylcyclohexane:ethyl acetate/methanol:water 100 : 25 / 80 : 20	45 min.	(193)
E_2B	Iso-octane/t-Butanol:water 100 / 50 : 90	12 hrs.	(194)
Bush T	Cyclohexane:benzene/methanol:water 75 : 45 / 75 : 15	45 min.	(185)
BL1	Petroleum ether: benzene/methanol:water 30 : 70 / 50 : 50	45 min.	(195)
	B[2]		
T.E.G.	Toluene/ethylene glycol		(196)
L.P.G.	Ligrion/propylene glycol		(197)
B.F.	Benzene/formamide		(198)

1. No impregnation of papergrams was required.
2. Papergrams were impregnated in stationary phase and methanol (50:50)

period is required before addition of the mobile phase in all Bush systems. In the latter systems, impregnation in methanol/ stationary phase (1:1) of the paper before application, is necessary. After application, the paper was developed in one of the chromatography jars in a constant temperature room at 25°C and humidity 50%. After the development, the paper was dried, and the areas showing radioactivity or ultraviolet absorption in paper strips, were eluted as described previously (see III, J).

2) Thin layer chromatography (TLC). Ready-to-use thin layer chromatography plates (20x20 cm) were purchased from E. Merck, A.G. These plates were evenly coated with 0.25 mm layer of silica gel F-254 and 1% of lamp phosphor to facilitate the detection of Δ^4-3-keto steroids under ultraviolet light (240 mμ). The plates were used either as such or activated in an oven at 100°C overnight. The application line was about 2 cm above the solvent meniscus. Similar procedure of application was adopted as mentioned for the paper chromatography. However, the steroid(s) was dissolved in methylene dichloride instead of methanol:chloroform mixture. The TLC system did not require any equilibration time. The plates were developed in a chromatography tank in a constant temperature room at 25°C and humidity 50% by ascending chromatography, until the

TABLE VII

THIN-LAYER CHROMATOGRAPHY SYSTEMS

SYSTEM	SOLVENT COMPOSITION	REFERENCE
TLC-1	Acetone:Benzene (1:4)	(199)
TLC-2	Chloroform:Methanol:Water (90:10:1)	(199)
TLC-3	Cyclohexane:Ethyl acetate (1:1)	(200)
TLC-4	Toluene:Ethyl acetate (9:1)	(201)
TLC-5*	Methylene dichloride:Methanol:water	
TLC-6	Chloroform:Ethanol (9:1)	(200)
TLC-7	Cyclohexane:Ethyl acetate:Ethanol (9:9:2)	(200)
TLC-8*	Chloroform:Acetone (9:1)	
TLC-9*	Benzene:Ethyl acetate (17:3)	
TLC-10*	Chloroform:Ethyl acetate (9:1)	

* Modifications of Lisboa's (200) solvent systems devised by the author.

solvent front was 15-16 cm from the origin. The time of development varied from 60-120 minutes, depending upon the system. A list of TLC systems used is given in Table VII. The areas of silica gel containing the steroids were detected similarly as described previously for the papergrams (see III, H) with the only difference, that TLC plates were exposed to x-ray films for a shorter duration of time, i.e. 16 hours only. This was done as a precaution to avoid deterioration of steroids in silica gel chromatoplates, as has been reported by some workers (190, 191).

The areas of silica gel containing steroids were extracted from the plates, using one or two drops of water. The steroids were eluted from the silica gel with 3x5 ml of methylene dichloride:methanol (9:1). The eluate was evaporated under a stream of nitrogen at 35°C. The recovery ranged between 85-95%.

O. Glassware and Reagents

1) Glassware, Clean glassware, free of radioactivity, was used throughout.

The glassware, in which radioactivity was used, was rinsed thoroughly with methanol and water. After that it was kept for two days in chromic acid solution and rinsed again (several hours) with tap water. Finally it was rinsed with distilled water.

Vials, (potassium free NO-SOL-VIT glass) in which aliquots of radioactivity was taken for counting, were used only once.

2) Reagents

Acetic acid glacial, reagent grade (Fisher).

Acetic anhydride, reagent grade (Fisher) distilled.

Acetone, reagent grade (Fisher) distilled once but always twice distilled for microcrystallization of steroids.

Aluminum t-butoxide (Alfa Inc.).

Antimony trichloride (crystals), reagent grade (Fisher).

Benzene, reagent grade (Fisher) distilled once over KOH pellets.

Bromine water (non-mailable) (Fisher).

Chloroform, reagent grade (Mallinckrodt) distilled.

Chromium trioxide (CrO_3) (Fisher).

Dichloromethane, reagent grade (Anachemia) distilled.

Digitonin, reagent grade (Fisher).

Ethanol (Fisher) purified with 2:4 dinitrophenylhydrazine and distilled three times.

Ether (diethyl ether), reagent grade (Fisher).

Hydrochloric acid. reagent grade (Fisher).

20 β -hydroxysteroid dehydrogenase (ammonium sulfate suspension) (Sigma Chemical Company).

Methanol, reagent grade (Fisher) distilled.

β-NADH (β-diphosphopyridine nucleotide, reduced form, disodium salt) (Sigma Chemical Company).

n-pentane, reagent grade (B.D.H.) twice distilled.

Periodic acid (H_5IO_6) reagent grade (Fisher).

Pyridine, reagent grade (Fisher) distilled.

Sodium carbonate ($Na_2C\Theta_3$) reagent grade (Fisher).

Sucrose, reagent grade (Fisher).

Tween 80 (polyoxyethylene sorbitan mono-oleate) (N.B.C.).

Zinc metal (dust) certified grade (Fisher).

The solvents used in paper or TLC chromatographic systems were treated exactly as in the given references (see Table VI and VII).

IV RESULTS

SECTION A: STUDIES WITH FROG ADRENAL TISSUE

1. Progesterone-4-^{14}C as substrate

a) Adrenal sections

Two aliquots of 100 mg of frog interrenal sections were incubated with 2 µc of progesterone-4-^{14}C in KRBG for three hours. One of the incubation studies was supplemented with NADP and G-6-P, while the other contained no additives.

Table VIII shows the total conversion obtained from each experiment, which was 55.8% in the case of the non-supplemented system and was further increased to 81.4% in the presence of NADP plus G-6-P. The table also records the pattern of conversion products and the percentage of radioactivity incorporated into each compound. Qualitatively, similar types of reaction products were present in both studies. In each case, radioactive compounds with the chromatographic mobility of 18-OH-corticosterone, aldosterone, 11-dehydrocorticosterone, corticosterone, 11β-OH-progesterone and 11-deoxycorticosterone were detectable. Various unidentified compounds were also present in the media. None of these unidentified metabolites showed the chromatographic behaviour of cortisol, 11-deoxycortisol or 17α OH-progesterone. The major conversion product either with or without added cofactors was corticosterone followed by aldosterone. Addition

TABLE VIII

TRANSFORMATION OF PROGESTERONE-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY ADRENAL SECTIONS OF THE FROG ADRENALS*

STEROIDS ISOLATED	PERCENTAGE CONVERSION/100 mg OF TISSUE	
	No cofactors	NADP+G-6-P
18-OH-B	4.0	12.0
Aldosterone	13.8	21.4
A	1.7	4.0
B	19.7	23.0
11β-OH-P	0.9	1.4
DOC	3.4	2.0
Others	12.3	17.6
Total	55.8	81.4

*Incubation media KRBG

of the NADPH-generating system also resulted in increase in conversion of all the metabolites with the exception of 11-deoxycorticosterone which showed a decrease. 11 β-OH-progesterone was also isolated but in small quantities. Some of these metabolites are illustrated in the radioautogram in Figure 4.

The degree of transformation of progesterone-4-^{14}C into corticosteroids at various time intervals of incubation was also investigated. For this purpose, five aliquots (100 mg each) of frog adrenal sections were incubated for 15, 30, 60, 120 and 180 minutes in 5 ml KRBG in presence of 2 μc of progesterone-4-^{14}C. No cofactors were added in these studies.

Table IX records the total conversion and the pattern of metabolites attained with these incubates. With the exception of 11 β-OH-progesterone which was not encountered in these studies, the pattern of metabolites at each interval investigated was identical with that previously detected in the three hours incubation. Corticosterone and aldosterone were once more the major reaction products in all instances.

The same table shows that the overall degree of conversion of progesterone-4-^{14}C increased proportionally to the time of incubation, from 7.7% at 15 minutes to 56.8% at 120 minutes. After 120 minutes, no further substantial increment in transformation was observed. These results are graphically depicted in Figure 5.

FIGURE 4

RADIOAUTOGRAMS FROM STUDIES OF SURVIVING SECTIONS (FROG INTERRENALS) INCUBATED WITH PROGESTERONE-4-^{14}C WITH OR WITHOUT EXOGENOUS NADP PLUS G-6-P.

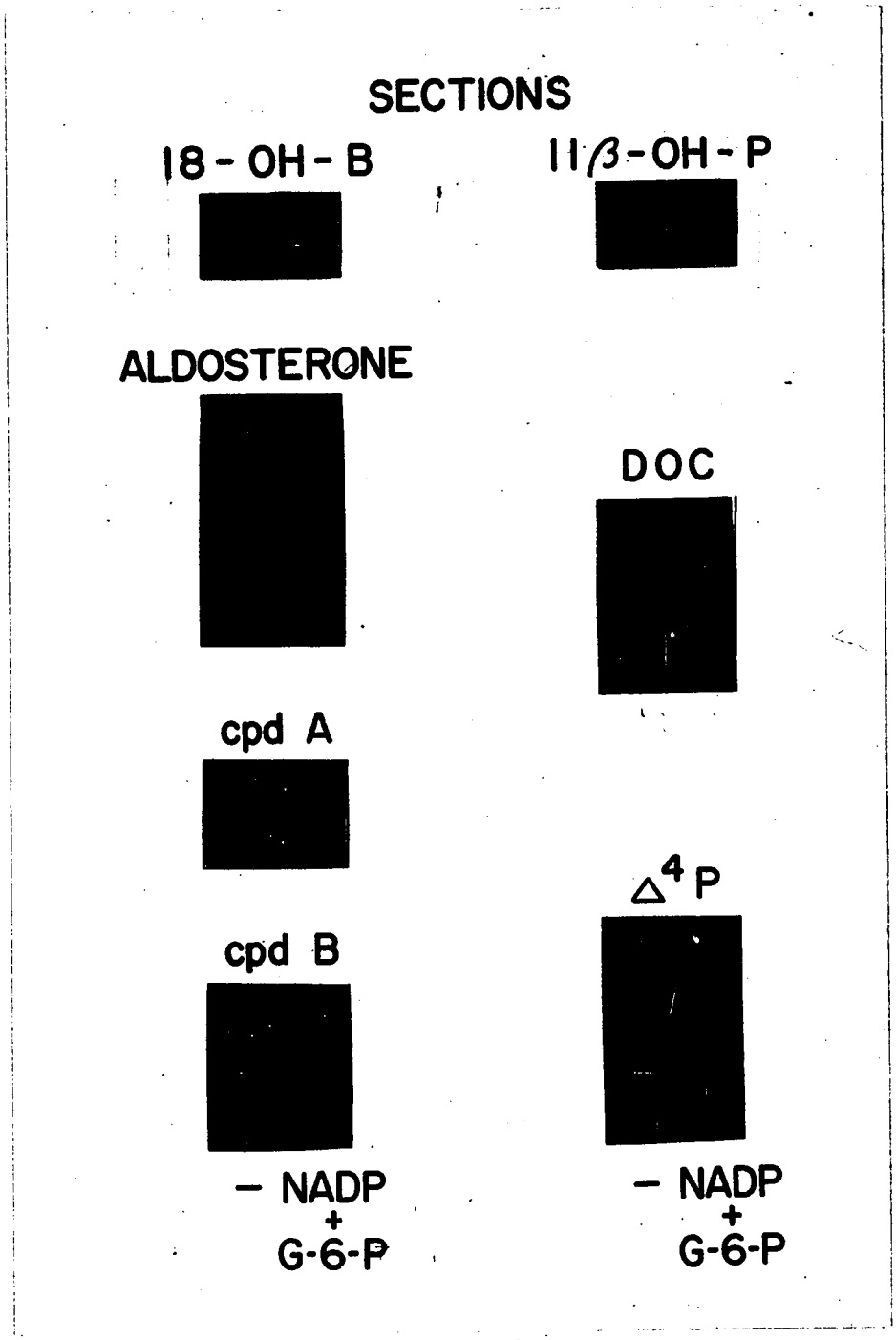


FIGURE 4

RADIOAUTOGRAMS FROM STUDIES OF SURVIVING SECTIONS (FROG INTERRENALS) INCUBATED WITH PROGESTERONE-4-^{14}C WITH OR WITHOUT EXOGENOUS NADP PLUS G-6-P.

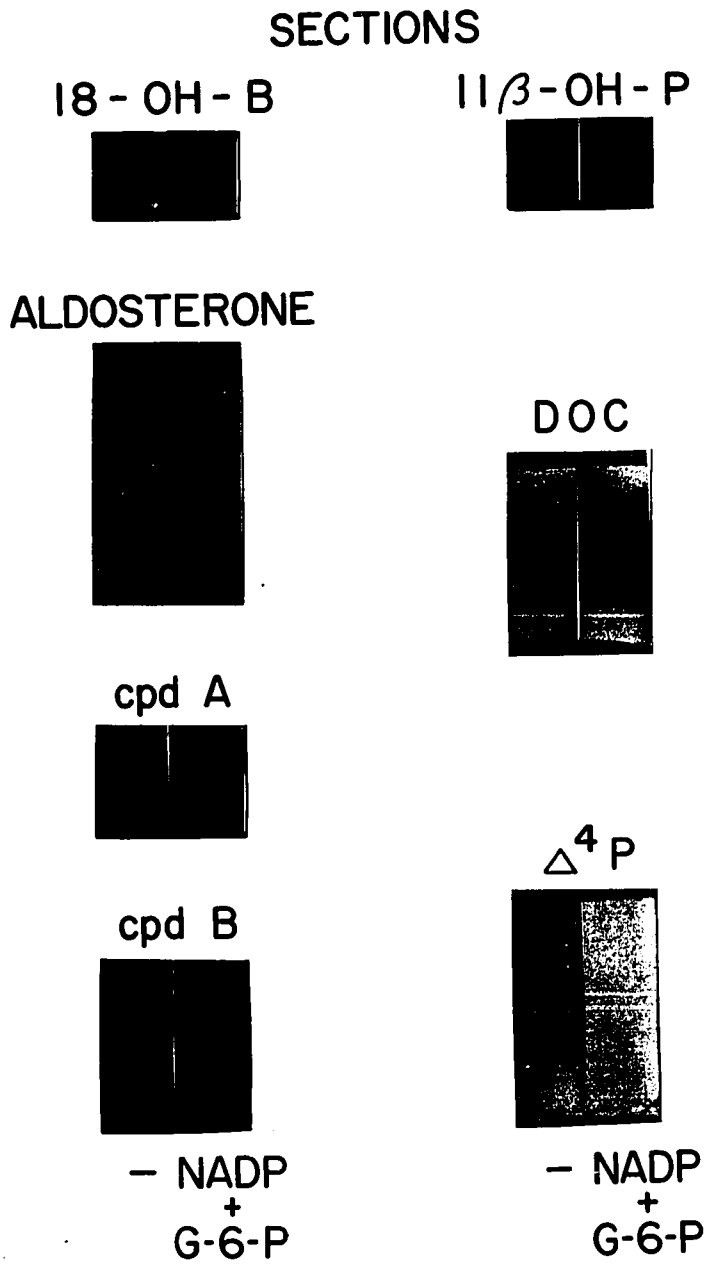


TABLE IX

TRANSFORMATION OF PROGESTERONE-4-^{14}C TO CORTICOSTEROIDS BY FROG ADRENAL SECTIONS INCUBATED AT DIFFERENT TIME INTERVALS*

STEROIDS ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE				
	15 min.	30 min.	60 min.	120 min.	180 min.
18-OH-B	0.3	1.4	2.5	4.3	4.9
Aldosterone	1.7	5.8	8.6	20.3	15.8
A	0.1	0.5	1.3	2.9	2.3
B	1.8	5.8	10.7	18.8	19.6
DOC	1.2	3.4	4.8	6.0	5.4
Others	2.6	1.1	5.9	4.5	14.3
Total	7.7	18.0	33.8	56.8	62.3

*Incubation media KRBG

FIGURE 5

CONVERSION OF PROGESTERONE-4-^{14}C BY SURVIVING SECTION (FROG INTERRENAL) AT DIFFERENT TIME INTERVALS OF INCUBATION.

FIGURE 6

CONCENTRATIONS OF UNCONVERTED PROGESTERONE-4-^{14}C FOUND IN TISSUES AND MEDIA AT DIFFERENT TIME INTERVALS OF INCUBATION.

In this particular set of experiments, the radioactivity present in the tissue sections was always analyzed. For this purpose, the tissues were extracted as indicated in 'Methods and Materials'. Radioautograms revealed varying amounts of unconverted progesterone-4-^{14}C as well as slight radioactive bands coinciding with the chromatographic mobility of the steroids isolated from the corresponding media.

Figure 6 contrasts the amounts of unconverted progesterone -4-^{14}C found in the tissue and in their corresponding media at the different times of incubations studied. The results are expressed as percentage of the total radioactivity extracted from both media and tissues. At 15 minutes the media contained 62% of the unconverted progesterone and on further incubation, it declined progressively thereafter. In the tissues, 12% of unconverted progesterone was found at 15 minutes, reaching a maxiumum of 18% at 30 minutes and declining steadily to 3% at 180 minutes.

The effects of 3' 5' AMP (unsubstituted or substituted) were also explored. For this purpose, five aliquots of 100 mg of frog interrenal sections were incubated with 2 μc of progesterone-4-^{14}C in 5 ml of KRBG for 30 minutes. These incubations were labelled as system I, no additives; system II, 3' 5' AMP (18 μ moles/ml); system III, 3' 5' AMP D.B. (18 μ moles/ml); system IV, 3' 5' AMP (36 μ moles/ml) and system V, 3' 5' AMP D.B.

TABLE X

TRANSFORMATION OF PROGESTERONE-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY FROG ADRENAL SECTIONS IN PRESENCE OF 3', 5' AMP (30-MIN. INCUBATIONS)[1]

STEROIDS ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE				
	System I[a]	System II[b]	System III[c]	System IV[d]	System V[e]
18-OH-B	0.9	1.1	0.6	1.2	0.8
Aldosterone	3.8	3.8	2.3	3.8	2.9
A	0.5	-	-	0.6	0.6
B	5.2	4.5	2.9	5.1	5.4
DOC	2.3	2.5	2.6	2.5	2.6
Others	5.7	6.3	5.2	6.7	5.2
Total	18.4	18.2	13.6	19.9	17.5

1. Incubation time 30 min. in KRBG
a. No additives
b. 3', 5' AMP (18 μ moles/ml)
c. 3', 5' AMP D.B. (18 μ moles/ml)
d. 3', 5' AMP (36 μ moles/ml)
e. 3', 5' AMP D.B. (36 μ moles/ml)

TABLE XI

TRANSFORMATION OF PROGESTERONE-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY FROG ADRENAL SECTIONS IN PRESENCE OF 3', 5' AMP (3-hr. INCUBATIONS)

STEROIDS ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE		
	System VI[a]	System VII[b]	System VIII[c]
18-OH-B	4.0	3.6	2.3
Aldosterone	13.8	10.6	5.2
A	1.7	3.2	0.9
B	19.7	15.0	11.0
11β-OH-P	0.9	2.8	5.1
DOC	3.4	7.0	7.3
Others	12.3	9.5	10.0
Total	55.8	51.7	41.8

1. Incubation time 3 hours KRBG
a. No additive
b. 3', 5' AMP (36 μ moles/ml)
c. 3', 5' AMP D.B. (36 μ moles/ml)

(36 μ moles/ml).

The total conversion of the labelled precursor as shown in Table X was of a similar order as observed in 30 minutes incubation previously mentioned. The total conversion in all the systems was between 17.5 to 19.9% except in system III, where 13.6% conversion was observed. Steroids with the chromatography mobility of 18-OH-corticosterone, aldosterone, 11-dehydrocorticosterone, corticosterone and 11-deoxycorticosterone were detected in all the studies. Corticosterone and aldosterone were the major conversion products in all the systems. 11-dehydrocorticosterone was isolated from system I, II and V only.

No increase in either total conversion or in the individual compounds was observed under the influence of 3' 5' AMP and 3' 5' AMP D.B. in 30 minutes incubation.

Another experiment under identical conditions as system I, IV and V was carried out but incubation time was lengthened to three hours. Once more, the cyclic nucleotide (substituted or not) failed to increase the overall degree of conversion attained in the control study (Table XI).

b) Adrenal homogenates

Frog adrenal homogenates were prepared in $KRPO_4$ buffer containing fumarate as described in 'Methods and Materials". Two aliquots, each containing 100 mg of the tissue, were poured

into beaker containing 2 μc of progesterone-4-^{14}C (previously mixed with additional 100 μg of radioinert progesterone). One aliquot contained no further additives and the other one was incubated with NADP and G-6-P. The final volume of 5 ml was made up with $KRPO_4$ and incubation was carried out for three hours.

As recorded in Table XII, the study without any cofactors exhibited about half of the capacity (T.C. 28.8%) of the supplemented system (T.C. 53.4%) to metabolize the substrate. Both studies on chromatographic analysis showed twelve different radioactive zones, among them, steroids with the chromatographic mobility of 18-OH-corticosterone, aldosterone, 11-dehydrocorticosterone, corticosterone, 11 β-OH-progesterone and 11-deoxycorticosterone were detectable. Analysis of individual reaction products showed increase in most of the above-mentioned steroids in the presence of the NADPH-generating system. 11-deoxycorticosterone was the major conversion product followed by corticosterone and 11β-OH-progesterone in the absence of the cofactors. However, the supplementation resulted in corticosterone as the major conversion product. A radioactive zone with the mobility of Δ^5-pregnenolone in fraction D (LPG system 24 hours) appeared in both studies. The amount of radioactivity associated with this zone was about 6% in each case. (see unidentified metabolite I

TABLE XII

TRANSFORMATION OF PROGESTERONE-4-^{14}C (2μc) TO CORTICOSTEROID BY FROG ADRENAL HOMOGENATES*

STEROIDS ISOLATED	PERCENTAGE CONVERSION/100 mg OF TISSUE	
	No cofactors	NADP+G-6-P
18-OH-B	0.6	3.0
Aldosterone	0.5	3.9
A	0.2	1.8
B	5.6	15.7
11β-OH-P	1.7	4.1
DOC	12.4	10.1
Others	7.8	14.8
Total	28.8	53.4

*Incubation media $KRPO_4$ + fumarate.

FIGURE 7

RADIOAUTOGRAMS FROM STUDIES OF HOMOGENATES (H) (FROG INTERRENALS) INCUBATED WITH PROGESTERONE-4-^{14}C WITH OR WITHOUT EXOGENOUS NADP PLUS G-6-P.

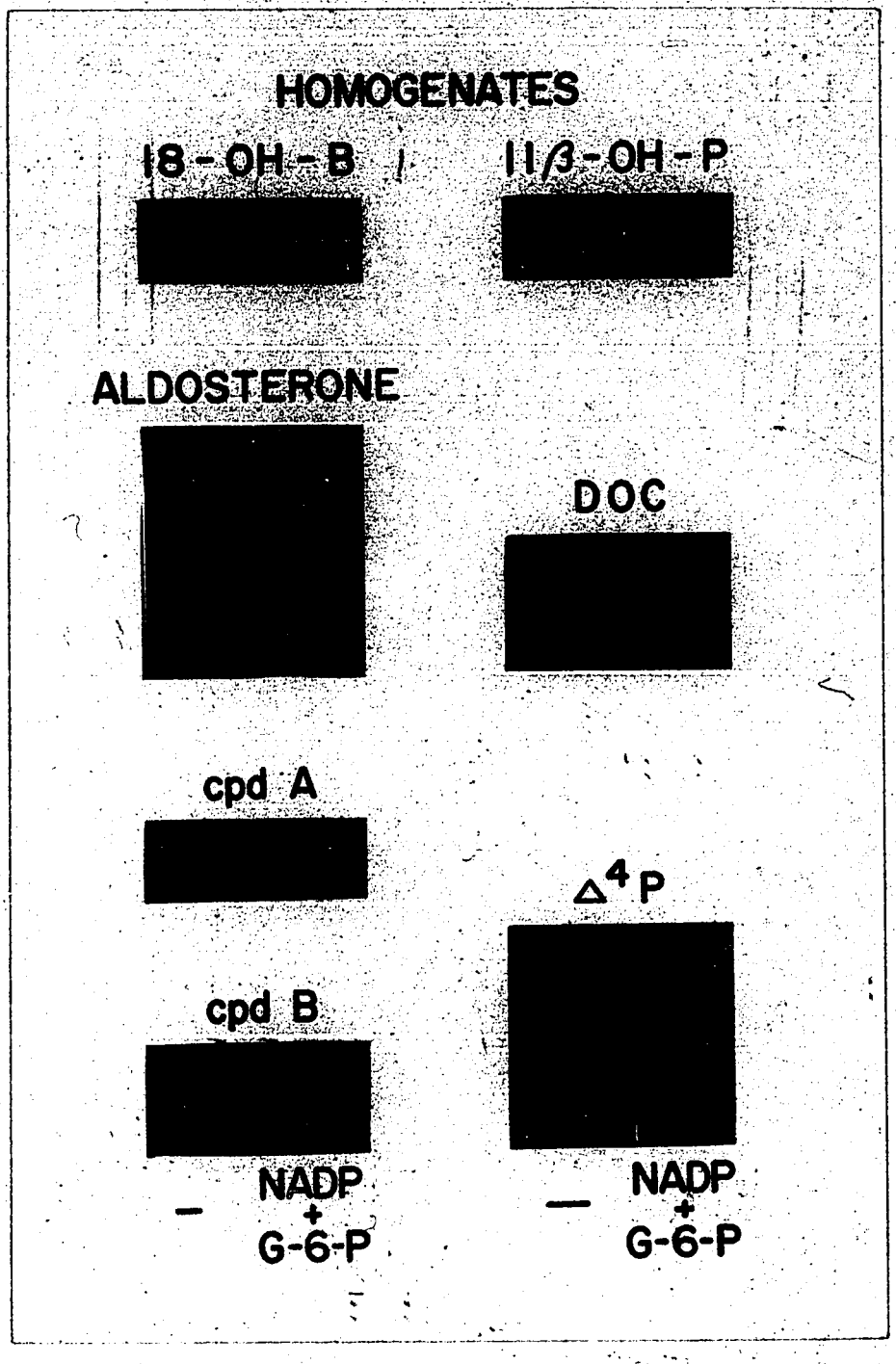
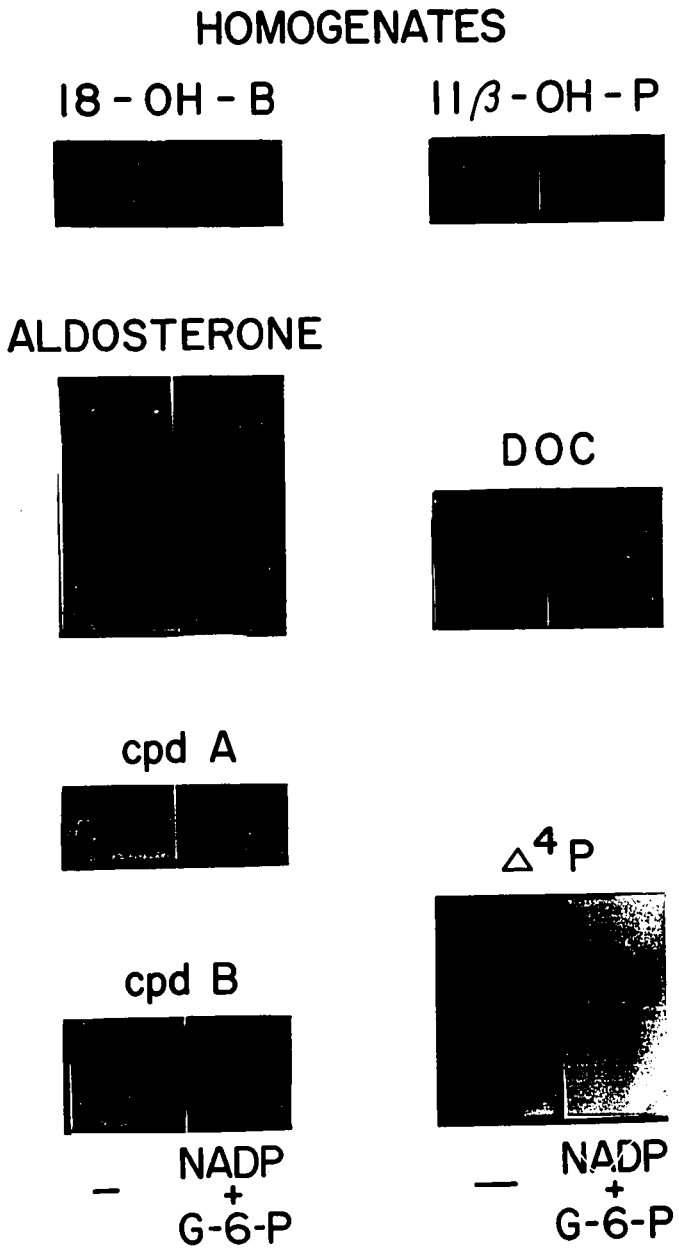


FIGURE 7

RADIOAUTOGRAMS FROM STUDIES OF HOMOGENATES (H) (FROG INTERRENALS) INCUBATED WITH PROGESTERONE-4-^{14}C WITH OR WITHOUT EXOGENOUS NADP PLUS G-6-P.



in 'Identification of Steroids').

Radioautograms in Figure 7 shows some of the reaction products of progesterone-4-^{14}C obtained with these homogenized preparations.

c) Subcellular fractions

Four hundred mg of frog interrenal tissue were homogenized in 10 ml of 0.25M sucrose. Two aliquots of 2.5 ml were taken out and centrifuged as described earlier in 'Methods and Materials'. The subcellular fractions P1, P2, P3 were resuspended in 2.5 ml of sucrose. The remaining 5 ml of homogenates WH were divided into two aliquots of 2.5 ml each. Thus two sets of subcellular fractions and homogenates WH were obtained. These preparations were poured into beakers containing 2 µc of progesterone-4-^{14}C (previously mixed with 100 µg of radioinert progesterone). One set of subcellular fraction, i.e. P1, P2, P3, SN and homogenates WH was supplemented with NADP and G-6-P and final volume of 5 ml was made up with $KRPO_4$ buffer containing fumarate. No cofactors were added in the other set and the final volume of 5 ml was made up similarly. The incubation was carried out for 3 hours.

Table XIII shows the total percentage conversion of the labelled progesterone by different subcellular fractions and homogenates WH. All the studies without exogenous cofactors

TABLE XIII

TRANSFORMATION OF PROGESTERONE-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES FROM AMERICAN BULLFROG ADRENAL

STEROID ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE									
	P1		P2		P3		SN		WH	
	A	B	A	B	A	B	A	B	A	B
18-OH-B	-	0.3	0.1	0.3	-	-	-	-	0.1	2.0
Aldosterone	-	0.2	0.1	0.2	-	-	-	-	0.1	1.9
Corticosterone	-	1.3	0.4	1.7	-	-	-	-	0.6	9.9
11β-OH-P	-	2.0	2.5	5.0	-	-	-	-	1.2	4.0
DOC	0.4	2.2	0.5	1.4	0.2	3.9	3.4	5.4	1.6	10.7
Others	-	-	-	0.2	-	-	-	-	-	0.9
Total	0.4	6.0	3.6	8.8	0.2	3.9	3.4	5.4	4.2	29.4

A = $KRPO_4$ + Fumarate + 0.13M Sucrose
B = $KRPO_4$ + Fumarate + 0.13M Sucrose + NADP+G-6-P

FIGURE 8

RADIOAUTOGRAMS FROM STUDIES OF HOMOGENATES (WH) AND SUBCELLULAR FRACTIONS (FROG INTERRENALS) INCUBATED WITH PROGESTERONE-4-^{14}C WITH OR WITHOUT EXOGENOUS NADP PLUS G-6-P.

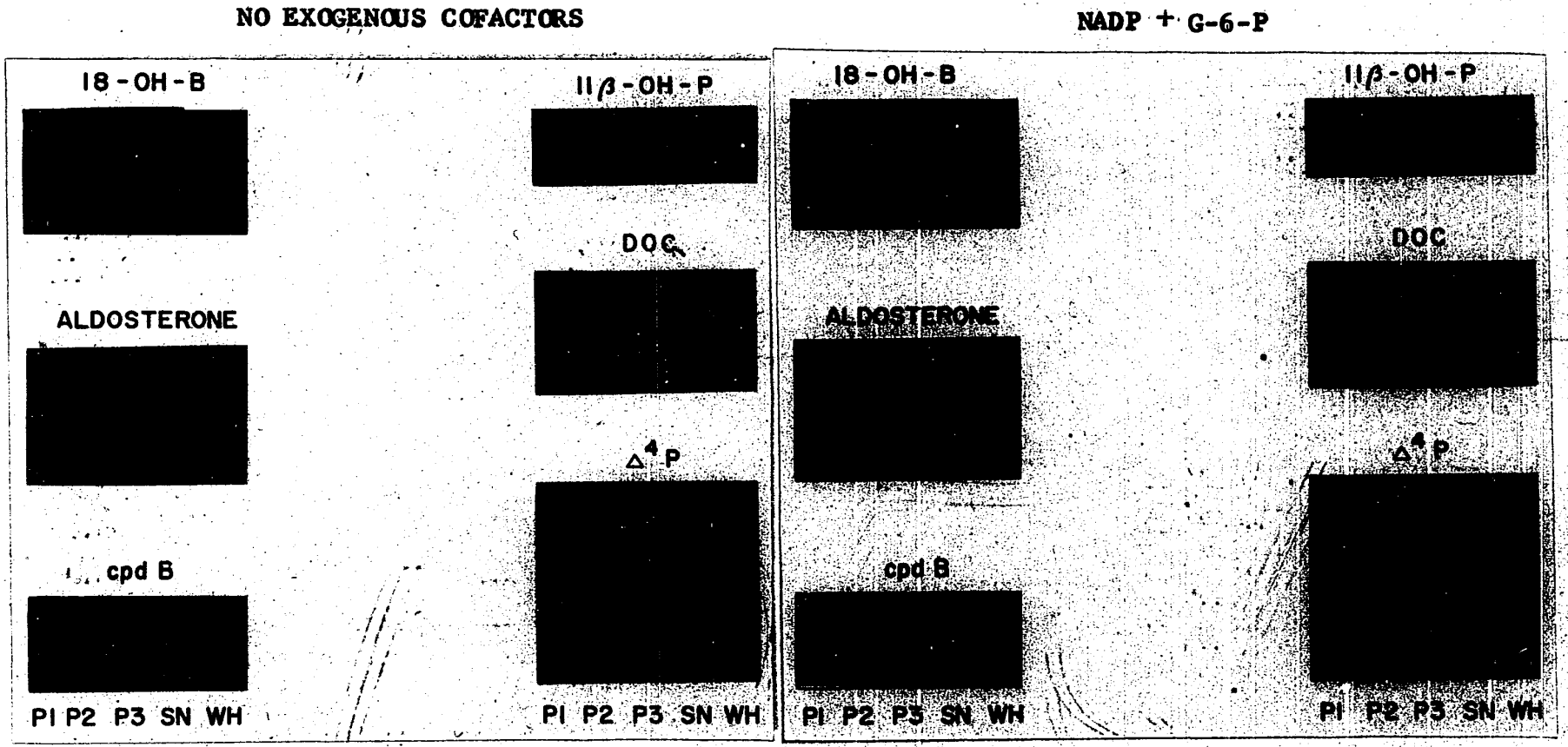
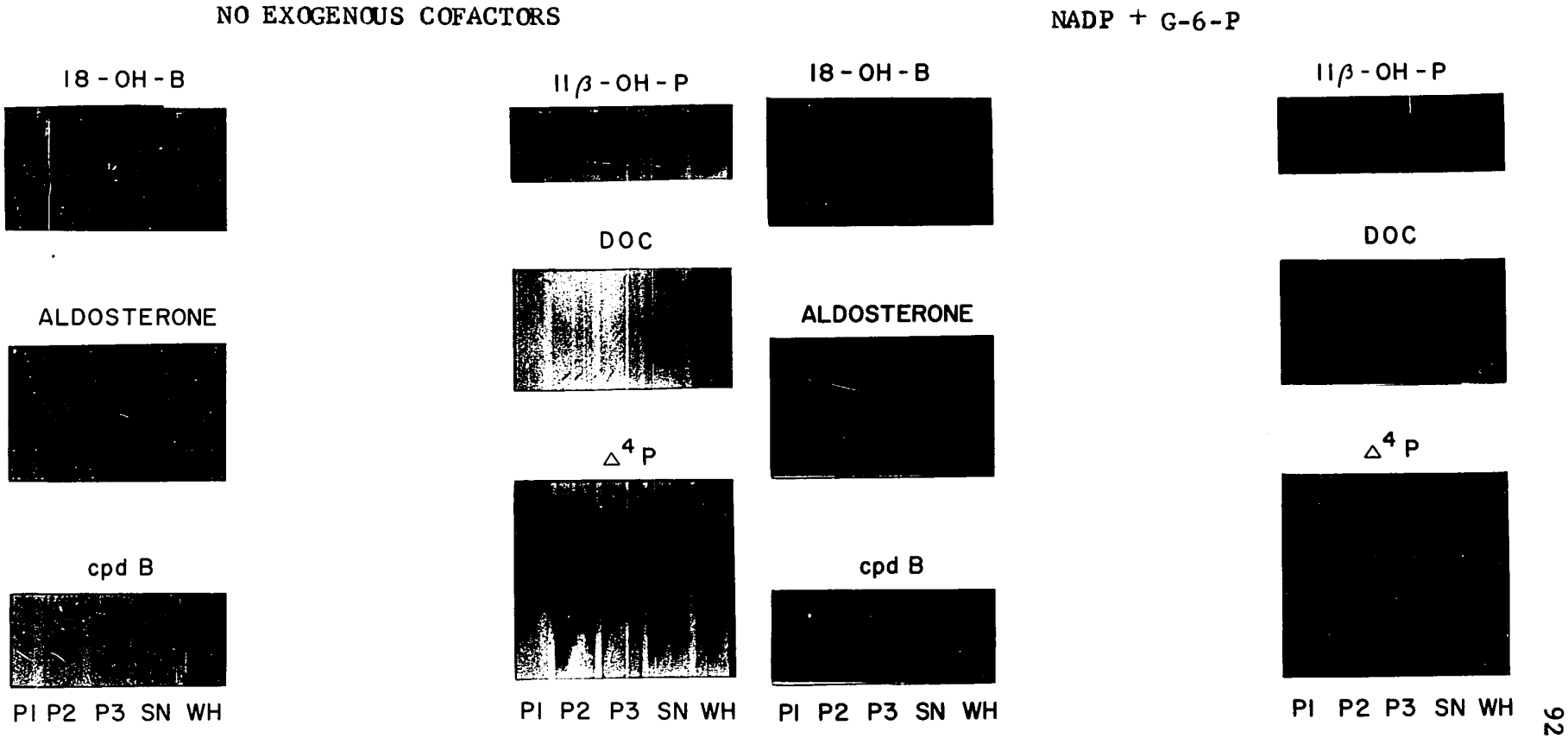


FIGURE 8

RADIOAUTOGRAMS FROM STUDIES OF HOMOGENATES (WH) AND SUBCELLULAR FRACTIONS (FROG INTERRENALS) INCUBATED WITH PROGESTERONE-4-^{14}C WITH OR WITHOUT EXOGENOUS NADP PLUS G-6-P.



showed a small degree of conversion. Among the subcellular fractions P2 showed the highest enzymatic activity to hydroxylate the precursor (T.C. 3.6%). Supplementation with the NADPH-generating system enhanced the conversion in all the cases, also resulting in the appearance of metabolites which were not present in absence of additives. The nuclei P1 transformed the precursor mainly into 11-deoxycorticosterone (2.2%) but 18-OH-corticosterone, aldosterone, corticosterone, 11 β-OH-progesterone and 11-deoxycorticosterone were also isolated. Mitochondrial preparation P2, with or without additives, hydroxylated the C_{21} precursor into similar steroids as mentioned above, 11 β-OH-progesterone being by far the major metabolite in either case (5 and 2.5%). Only 11-deoxycorticosterone was isolated as a conversion product from both microsomal P3 and supernatant SN preparations. The homogenates WH showed similar type of conversion products as the mitochondria P2. 11-deoxycorticosterone was the major conversion product by homogenates WH either without additives or supplemented with the NADPH-generating system.

Some of the metabolites isolated from these studies are shown by ratioautogram in Figure 8.

Discussion

Progesterone-4-^{14}C was the first of the series of the precursors incubated with frog interrenal sections, homogenates

and subcellular fractions.

The surviving sections exhibited the capacity to oxygenate the exogenous substrate at positions 11, 21 and 18, as demonstrated by the isolation of 18-OH-corticosterone, aldosterone, 11-dehydrocorticosterone, corticosterone, 11 β-OH-progesterone and 11-deoxycorticosterone. Of these, corticosterone, aldosterone and 18-OH-corticosterone appeared as the principal metabolic products. In this respect, the frog adrenal resembles functionally the zona glomerulosa of beef adrenals (202). Very similar patterns of metabolites have also been reported for the adrenals of the rat (144), duck (77), grass snake (59) and turtle (57). The data also confirms the previous studies carried out with bullfrogs interrenals by Ulick and Kusch (47) and are also in agreement with studies of leopard frog interrenals by Lamoureux (48). In all of these studies 17 α-hydroxylase activity, if at all present, was reported to occur in insignificant quantities. Our studies with bullfrog interrenals showed no radioactive zones with the mobility of any known 17-hydroxylated corticosteroids. Our studies also widen the spectrum of reaction products obtained with amphibian interrenal from radioprogesterone with the isolation of 11 β-OH-progesterone and 11-dehydrocorticosterone.

The addition of exogenous NADP plus G-6-P to the surviving interrenal sections resulted in a marked increase in their

hydroxylating capacity upon progesterone-4-^{14}C, exactly as it has been observed in mammalian adrenal sections (96). In view of the present-day knowledge of the adrenal hydroxylating mechanism, it is apparent that frog interrenal sections reduced 'in situ' NADP to NADPH which is the required nucleotide mediating steroid hydroxylations (124, 202). To our knowledge, the enzyme, responsible for the reduction of NADP which is G-6-P-dehydrogenase, has not been reported in frog interrenal tissue, as it has been for mammalian adrenal gland (156,-160). Our <u>in vitro</u> studies suggest by the indirect evidence of NADPH generation from exogenous NADP in the presence of G-6-P, that G-6-P-dehydrogenase is also present in this amphibian interrenal. Further evidence for the existence of this enzyme will be presented in the histochemical studies conducted with this tissue (see III, section A, 6.)

In connection with the capacity of frog interrenals to generate NADPH, some studies were conducted with 3', 5' AMP, in view of the theory postulated by Haynes et al (203), whereby this cyclic nucleotide, through a series of stimulating effects, increases NADPH formation responsible for steroid hydroxylations in mammalian adrenals. In our studies with frog interrenal, the addition of 3', 5' AMP, however, did not increase the overall transformation of labelled progesterone over that of the non-

supplemented systems in studies carried out for 30 minutes or three hours. This finding is in disagreement with the report by Roberts' group (130, 218) who showed increased rates of 11β- and 18-hydroxylation of labelled progesterone and 11-deoxycorticosterone by rat adrenal preparations when supplemented with 3', 5' AMP. It must be stated, however, that in our department this cyclic nucleotide failed to promote a significant stimulation upon the hydroxylation of progesterone-4-^{14}C by bovine cortical sections (204). In the present investigation, high concentrations of the nucleotide were used, since it is generally believed (205), that 3', 5' AMP does not penetrate the cell membrane easily. The failure of the frog interrenal sections to respond to 3', 5' AMP, however, cannot be explained on the basis of cell-membrane impermeability to this agent, since dibutyryl 3', 5' AMP also failed to increase the transformation of progesterone-4-^{14}C and it is known that this substituted nucleotide can penetrate the cell membrane easily (206).

The time incubation studies indicated that the utilization of progesterone-4-^{14}C by frog interrenal increases almost linearly with time up to the second hour of incubation leveling off thereafter. This is in contradiction to rather similar studies conducted with rat adrenal quarters in which maximal hydroxylating activity was reached within the first hour of

incubation (144).

Analyzing the separate data obtained in the media and their corresponding tissues, it appears that there is a continuous uptake of progesterone-4-^{14}C by frog interrenal sections from the media. Since at no given time of incubation the concentration of the precursor was higher in the tissue than in the corresponding medium, it seems that progesterone-4-^{14}C is rapidly metabolized as it enters the interrenal cells. The fact that the concentration of metabolites was always extremely low in the tissues is indicative that once formed the reaction products rapidly diffuse out from cells into the medium.

The results obtained with homogenates and cell free preparations cannot be quantitatively compared with those obtained with surviving sections due to the fact that the specific activity of the precursor was altered in the former preparations by the addition of 100 µg of radioinert material. We were forced to introduce this variation, since preliminary studies with homogenized or subcellular preparations revealed that precursors like progesterone or pregnenolone were totally utilized under basal conditions of incubation leaving no way of surveying the effects of various additives.

Halkerston et al (106) have shown in their elegant studies with radiocholesterol that NADPH-mediated hydroxylations (carbon

20, 22, 21, 17 and 11) can take place in cell-free bovine adrenal systems in absence of any exogenous nucleotide as long as the medium contains a tricarboxylic cycle intermediate such as fumarate. The authors suggested that this was due to the generation of NADPH from endogenous sources in the presence of fumarate.

In the present studies with frog adrenal homogenates this appeared also to be the case, since progesterone-4-^{14}C was hydroxylated at carbons 21, 11 and 18 in a fumarate containing medium without supplementation with exogenous nucleotides. Under these conditions, the overall degree of conversion was 28.8%. As in the case of bovine adrenal preparations, the addition of an NADPH-generating system increased the enzymatic capacity of frog interrenal homogenates, the overall hydroxylation of progesterone-4-^{14}C being 53.4% under these conditions.

The subcellular fraction studies revealed that both 11β and 18-hydroxylase are associated with mitochondrial fraction. These findings are in agreement with other studies carried out with mammalian adrenal glands (219, 220). They also agree with previous studies with bullfrog adrenal in which 18-hydroxylase activity was found associated with the mitochondrial fraction (52). Microsomal P3 and supernatant SN both showed high concentration of 21-hydroxylase, especially in P3. This is

in accordance with the studies of mammalian adrenal microsomes (134). The presence of 21-hydroxylated steroids in the studies with mitochondrial P2 indicate that these preparations were probably contaminated.

As in the case of homogenized preparations, a fumarate containing medium supported the different hydroxylations in each subcellular fractions. The enzymatic activities, however, were also greatly enhanced by further supplementation of the in vitro system by NADP plus G-6-P.

2. Δ^5-pregnenolone-4-^{14}C as substrate.

a) Adrenal sections

Four aliquots of 100 mg of interrenal sections were incubated with 2 μc of Δ^5-pregnenolone-4-^{14}C in 5 ml of KRBG for three hours. Three of these aliquots were supplemented with cofactors and one contained no additives, i.e. system I: no additives; system II: only NAD; system III: NADP and G-6-P; system IV: initially only NAD was added, and after one and a half hour the medium was further supplemented with NADP and G-6-P.

Table XIV shows the total conversion obtained from each experiment. It also shows the pattern of conversion products with the percentage of radioactivity incorporated into each compound.

Total conversion of the precursor by system I was 58.0% and was very little affected by the addition of NAD, which resulted in total conversion of 61.1%. The addition of NADP and G-6-P resulted in greater conversion as shown by systems III and IV. Qualitatively, similar type of reaction products were present in all studies. In each case radioactive compounds with the chromatographic mobility of 18-OH-corticosterone, aldosterone, corticosterone, 11-deoxycorticosterone and progesterone were detectable. Various unidentified compounds were also present in these media.

The study without any supplementation showed some accumulation of radioprogesterone (5.8%), but the major conversion products were corticosterone (14.1%) and aldosterone (11.9%). The addition of NAD to the medium resulted in marked accumulation of labelled progesterone (21.7%). NADP and G-6-P added to the incubation media resulted in transformation of the substrate mainly into hydroxylated Δ^4-3keto metabolites, and only insignificant amounts of progesterone (0.6%) were isolated. Similar results were obtained when NAD $\longrightarrow$ NADP and G-6-P was added as seen by system IV which showed only 1.1% of progesterone.

Corticosterone and aldosterone appeared to be the major conversion products in all different systems except the study with NAD only where progesterone was the main conversion product.

TABLE XIV

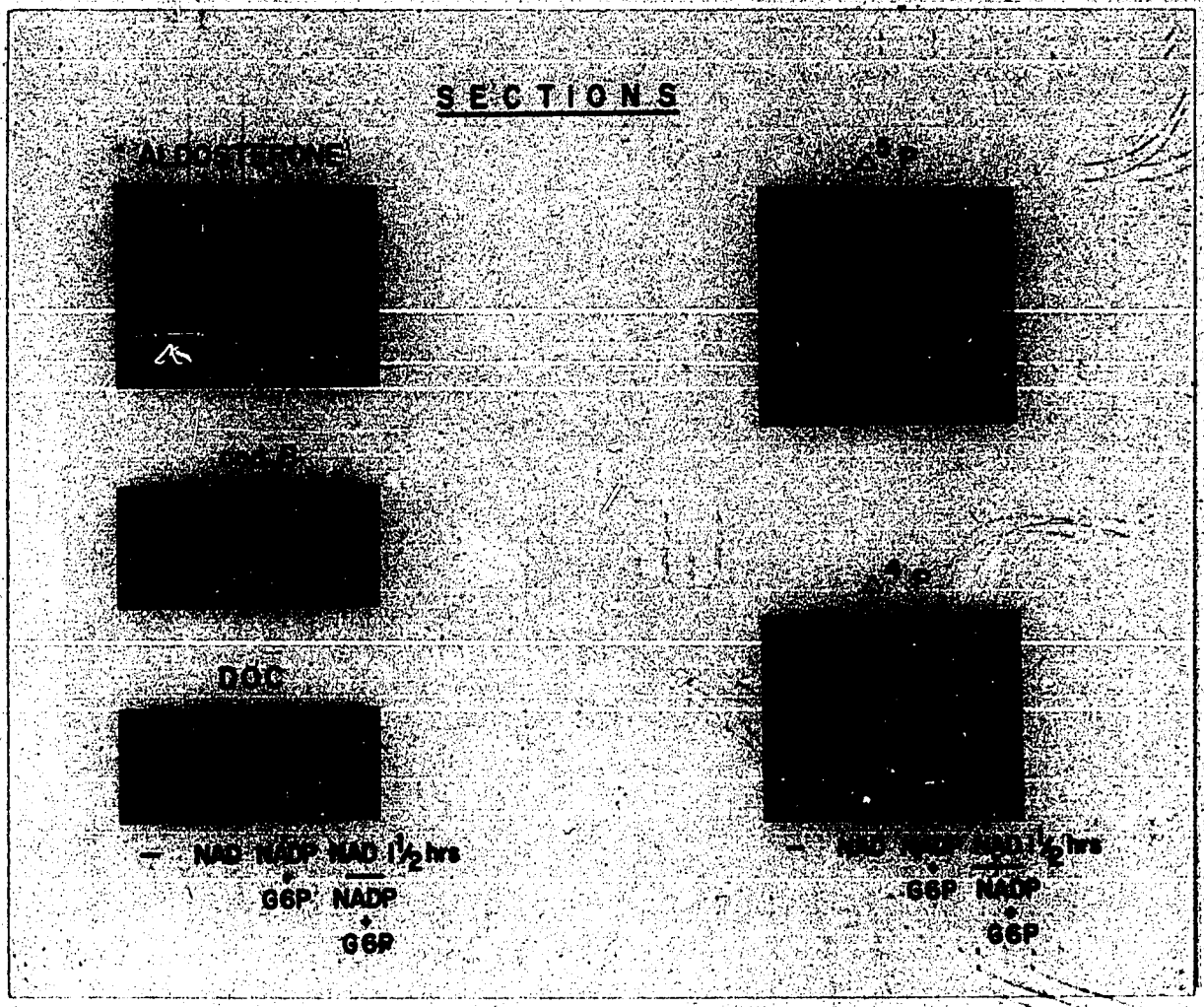
TRANSFORMATION OF Δ^5-PREGNENOLONE-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY FROG ADRENAL SECTIONS

STEROIDS ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE			
	System I[a]	System II[b]	System III[c]	System IV[d]
18-OH-B	3.5	3.1	6.6	9.5
Aldosterone	11.9	9.9	12.1	15.4
B	14.1	11.7	23.4	24.8
DOC	4.4	13.7	9.0	6.1
Progesterone	5.8	21.7	0.6	1.1
Others	18.3	10.0	13.3	22.9
Total	58.0	61.1	65.0	79.8

a = No additive
b = NAD
c = NADP + G-6-P
d = NAD 1 1/2 hours ——→ NADP + G-6-P 1 1/2 hours.

FIGURE 9

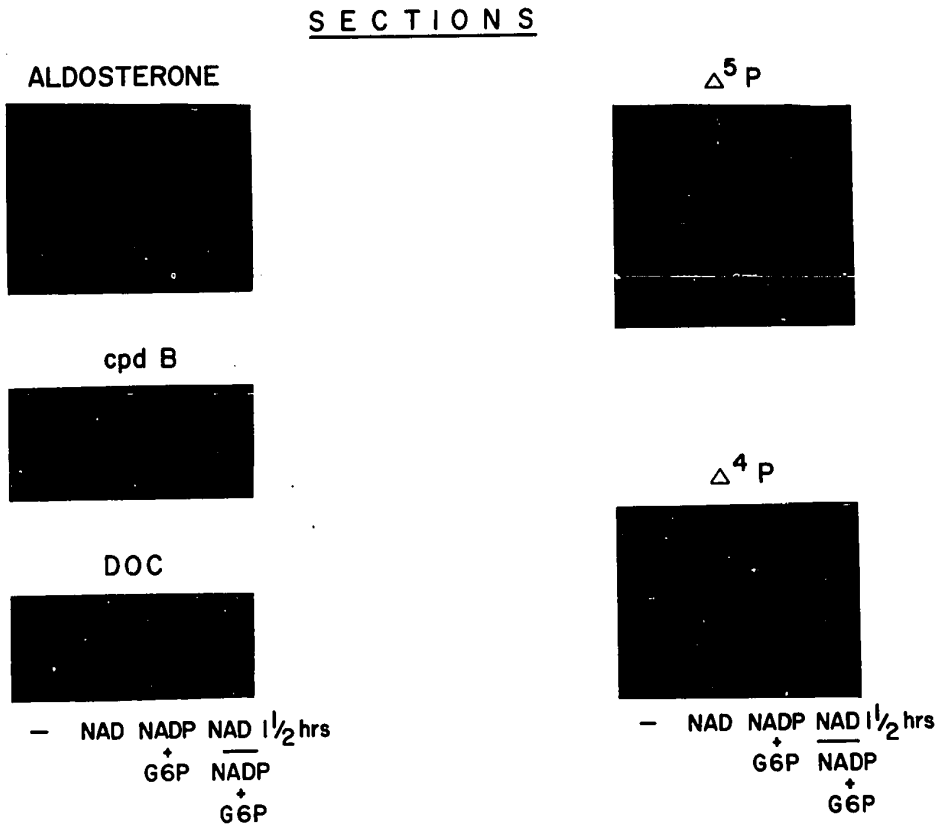
RADIOAUTOGRAMS FROM STUDIES OF SURVIVING SECTIONS (FROG INTERRENALS) INCUBATED WITH Δ^5-PREGNENOLONE-4-^{14}C UNDER VARIOUS EXPERIMENTAL CONDITIONS.



From left to right: no exogenous cofactor, NAD alone, NADP+G-6-P, and NAD for 1 1/2 hours followed by further addition of NADP+G-6-P.

FIGURE 9

RADIOAUTOGRAMS FROM STUDIES OF SURVIVING SECTIONS (FROG INTERRENALS) INCUBATED WITH Δ^5-PREGNENOLONE-4-^{14}C UNDER VARIOUS EXPERIMENTAL CONDITIONS.



From left to right: no exogenous cofactor, NAD alone, NADP+G-6-P, and NAD for 1 1/2 hours followed by further addition of NADP+G-6-P.

The radioautograms in Figure 9 shows some of the metabolites obtained.

The tissues from these incubates were extracted as stated in 'Methods and Materials'. Beside some unconverted Δ^5-pregnenolone, slight bands of radioactivity, coinciding in position with the major reaction products detected in the media, were found in the radioautograms from the tissue extracts. These studies with tissue extracts did not provide any further information.

b) Adrenal homogenates

Frog interrenals were homogenized in $KRPO_4$ buffer supplemented with fumarate as usual. Two aliquots, each containing 100 mg of the tissue were poured into the beakers containing 2 µc of Δ^5-pregnenolone-4-^{14}C (previously mixed with additional 100 µg of radioinert Δ^5-pregnenolone). One aliquot contained no further additives and the other was supplemented with NADP and G-6-P. The final volume of 5 ml was made up with $KRPO_4$ buffer and the incubation was carried out for three hours.

As recorded in Table XV, the study without any cofactors exhibited less than half of the capacity (T.C. 18.4%) of the supplemented system (T.C. 45.6%) to metabolize labelled Δ^5-pregnenolone to Δ^4-3keto compounds. Radioactive compounds with the chromatographic behaviour of 18-OH-corticosterone, aldosterone, 11-dehydrocorticosterone, corticosterone, 11β-OH-progesterone,

TABLE XV

TRANSFORMATION OF Δ^5-PREGNENOLONE-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY THE FROG ADRENAL HOMOGENATES*

STEROIDS ISOLATED	PERCENTAGE CONVERSION/100 mg OF TISSUE	
	No cofactors	NADP+G-6-P
18-OH-B	1.0	3.6
Aldosterone	1.5	6.7
A	0.3	1.5
B	6.1	14.1
11β-OH-P	1.1	3.8
DOC	1.3	6.8
Δ4-P	1.3	3.8
Others	5.8	5.3
Total	18.4	45.6

*Incubation media $KRPO_4$ + fumarate.

FIGURE 10

RADIOAUTOGRAMS FROM STUDIES WITH HOMOGENATES (H) (FROG INTERRENAL) INCUBATED WITH Δ^5-PREGNENOLONE-4-^{14}C WITH AND WITHOUT NADP PLUS G-6-P.

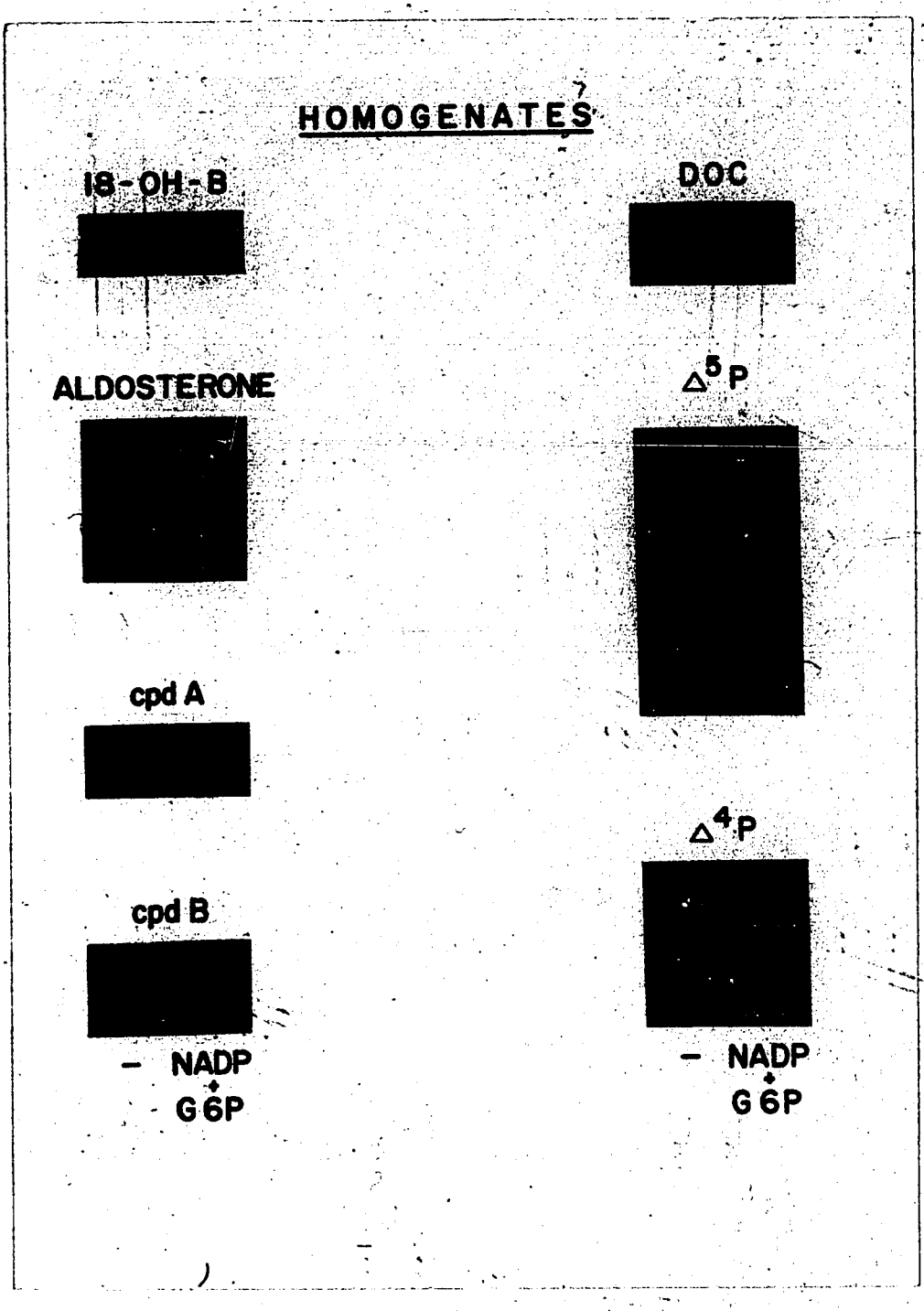
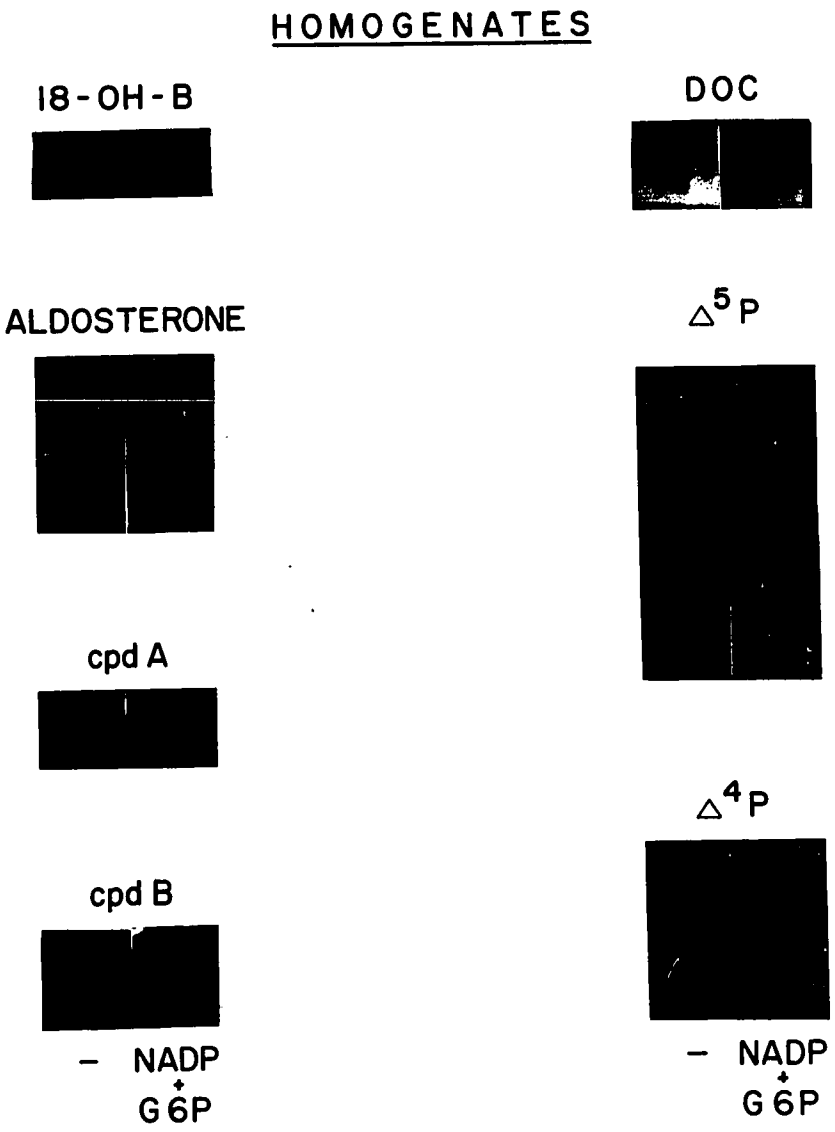


FIGURE 10

RADIOAUTOGRAMS FROM STUDIES WITH HOMOGENATES (H) (FROG INTERRENAL) INCUBATED WITH Δ^5-PREGNENOLONE-4-^{14}C WITH AND WITHOUT NADP PLUS G-6-P.



11-deoxycorticosterone and progesterone were detectable. Some unidentified compounds were also present. Analysis of individual reaction products showed an increase in all above mentioned steroids in the presence of the NADPH generating system. The major conversion products were similar to those obtained by the surviving sections. 11-dehycrocorticosterone and 11β-OH-progesterone were isolated in small quantities. There was only insignificant amount of progesterone present in both studies.

Radioautogram in Figure 10 shows some of the Δ^4-3keto metabolites of the Δ^5-pregnenolone.

c) Subcellular fractions

Homogenates of 400 mg of frog adrenal tissue were prepared in 10 ml of 0.25M sucrose. From these preparations, two sets of homogenates WH and subcellular fractions P1, P2, P3 and SN were obtained as described previously. Where indicated, the preparations were suspended in 2.5 ml of 0.25M sucrose. Each preparation was poured into a beaker containing 1 µc of Δ^5-pregnenolone-4-^{14}C (previously mixed with 100 µg of the authentic material). One set of preparations was supplemented with NADP and G-6-P and the other with NAD only. Final volume of 5 ml was made up with $KRPO_4$ buffer. Note that in these particular sets of observations the buffer contained no fumarate. The incubation was carried out for three hours as usual.

TABLE XVI

TRANSFORMATION OF Δ^5-PREGNENOLONE-4-^{14}C (1 μc) TO CORTICOSTEROIDS BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES FROM AMERICAN BULLFROG ADRENAL

STEROIDS ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE									
	P1		P2		P3		SN		WH	
	A	B	A	B	A	B	A	B	A	B
18-OH-B	-	-	0.3	-	-	-	-	-	0.9	-
Aldosterone	-	-	0.3	-	-	-	-	-	2.1	-
Corticosterone	-	-	0.5	-	-	-	-	-	2.8	-
11β-OH-P	-	-	0.9	-	-	-	-	-	2.9	-
DOC	1.3	-	2.9	-	8.3	-	8.1	-	10.5	-
Progesterone	1.0	6.4	3.6	9.5	2.0	12.3	1.9	12.4	3.8	18.7
Others	0.9	0.2	-	0.2	0.6	0.4	0.6	0.4	2.2	1.0
Total	3.2	6.6	8.5	9.7	10.9	12.7	10.6	12.8	25.2	19.7

A = $KRPO_4$ + 0.13M sucrose + NADP+G-6-P
B = $KRPO_4$ + 0.13M sucrose + NAD

FIGURE 11

RADIOAUTOGRAMS FROM STUDIES OF HOMOGENATES (WH) AND SUBCELLULAR FRACTIONS (FROG INTERRENAL) INCUBATED WITH Δ^5-PREGNENOLONE-4-^{14}C IN PRESENCE OF EXOGENOUS NAD OR NADP PLUS G-6-P.

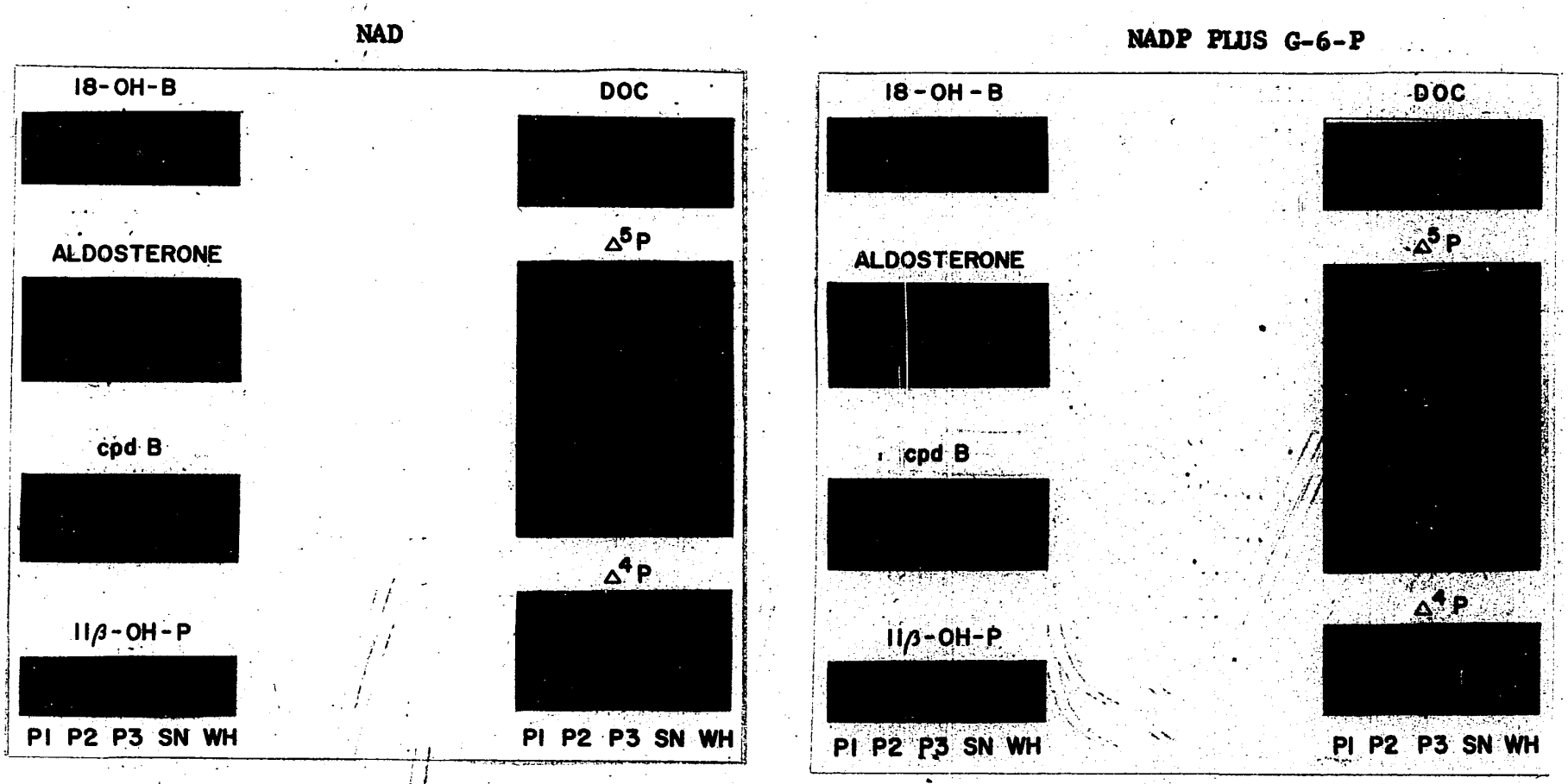
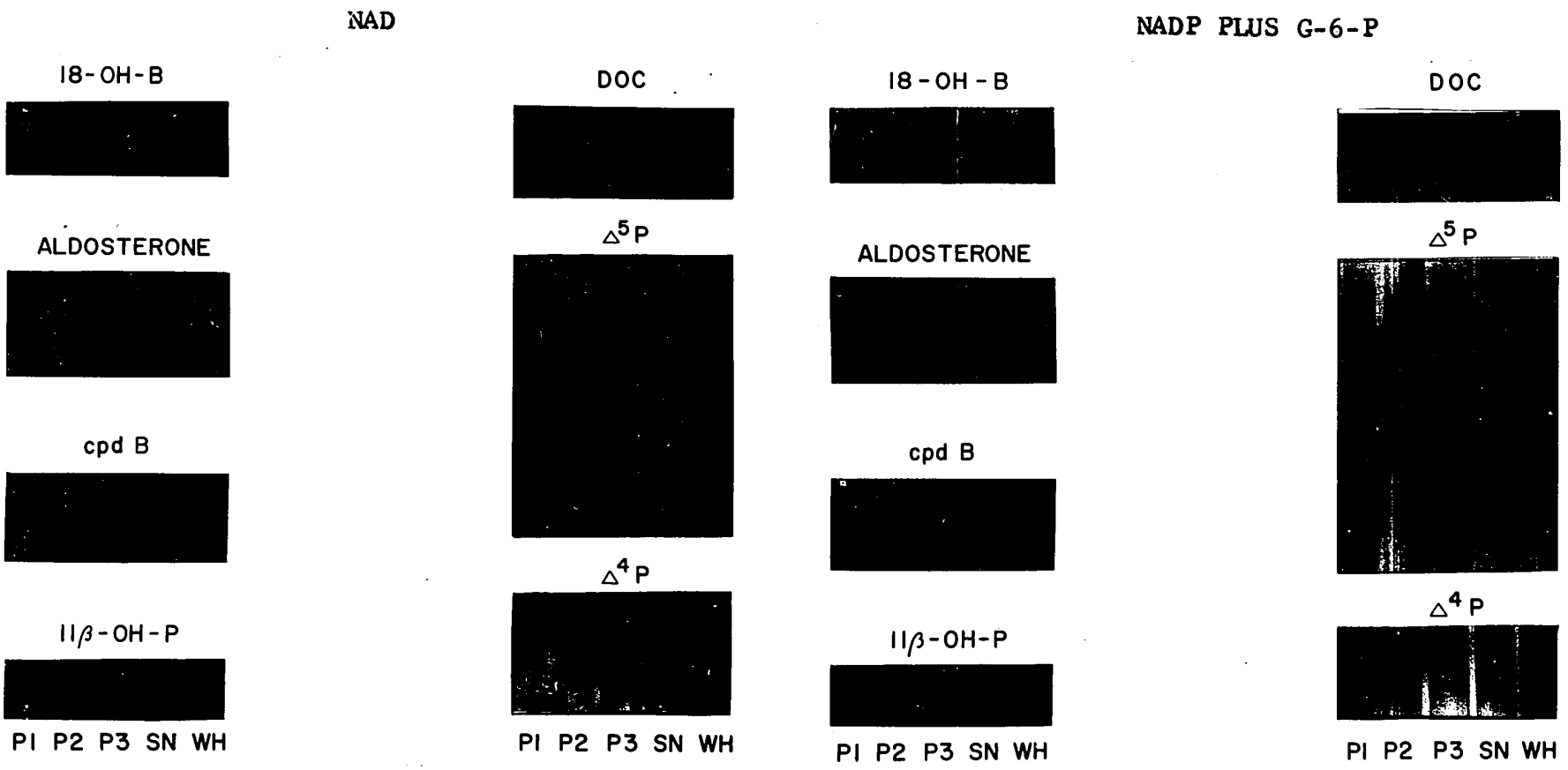


FIGURE 11

RADIOAUTOGRAMS FROM STUDIES OF HOMOGENATES (WH) AND SUBCELLULAR FRACTIONS (FROG INTERRENAL) INCUBATED WITH Δ^5-PREGNENOLONE-4-^{14}C IN PRESENCE OF EXOGENOUS NAD OR NADP PLUS G-6-P.



The results obtained from this study are recorded in Table XVI. The total conversion in the presence of the NADPH-generating system among subcellular fractions was highest in P3 (10.9%) followed by SN (10.6%), P2 (8.5%) and P1 (3.2%). The homogenates WH showed total conversion of 25.5%. Supplementation with NAD also resulted in similar degrees of overall conversion by the subcellular fractions and homogenates WH.

Labelled progesterone was isolated from all the studies irrespective of the cofactors used. However, studies with NAD resulted only in progesterone formation. Mitochondrial P2 incubation in the presence of NADPH-generating system transformed the precursor into 18-OH-corticosterone, aldosterone, corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone and progesterone. 11-deoxycorticosterone was the only other metabolite, beside progesterone, isolated from P3 and SN. The pattern of steroids isolated from the homogenates WH was similar to that obtained from the mitochondrial preparations.

The radioautograms in Figure 11 shows the main contrasts, both qualitatively and quantitatively, of Δ^5-pregnenolone-4-^{14}C metabolism under the influence of NADP+ G-6-P and NAD only.

Discussion

The present observations with Δ^5-pregnenolone-4-^{14}C

indicate the presence of Δ^5-3β-hydroxysteroid-dehydrogenase-isomerase in frog interrenal and substantiate the hydroxylating capacity of this tissue already manifested in the previous investigation with progesterone-4-^{14}C.

Δ^5-3β-hydroxysteroid-dehydrogenase-isomerase is the enzymatic complex responsible for the dehydrogenation at C-3 and the shift of double bond from Ring B to Ring A in the pregnenolone molecule (112, 113). The reaction Δ^5-pregnenolone $\longrightarrow$ progesterone is actually effected by two distinct enzymes: one, a true 3β-hydroxysteroid-dehydrogenase; the other a Δ^5-3keto isomerase (112, 113). Throughout the following discussion of our findings, we shall consider the Δ^5-3β-ol $\longrightarrow$ Δ^4-3keto transformation as a whole.

This Δ^5-3β-ol $\longrightarrow$ Δ^4-3keto is known to be mediated in mammalian adrenals by NAD (112), NADP (116, 117) and NADPH.

Our studies with surviving sections of bullfrog interrenals revealed a high capacity of this tissue to transform Δ^5-pregnenolone under basal <u>in vitro</u> conditions. The spectrum of Δ^4-3keto metabolites included 18-OH-corticosterone, aldosterone, corticosterone, 11-deoxycorticosterone and progesterone. Similar metabolites were isolated by Lamoureux (48) from incubation studies with leopard frog interrenals. Adrenal preparations from rat (207), duck (72) and turtle (57) also utilize

Δ^5-pregnenolone similarly.

The effects of above mentioned cofactors on the overall degree of substrate conversion was not striking with the exception of the enhancement brought about by the combined addition of NAD and NADP plus G-6-P. The marked tendency of NAD to mediate the substrate changes only as far as progesterone and the further hydroxylations induced by NADP plus G-6-P are perhaps more clearly illustrated by the results obtained with homogenates and subcellular preparations.

In previous incubations, fumarate alone supported the hydroxylation of progesterone-4-^{14}C by the bullfrog adrenal homogenates. The mediation of this reaction by fumarate probably occurred through the generation of NADPH from endogenous sources. In the studies under discussion fumarate alone also supported the dehydrogenation and isomerization of Δ^5-pregnenolone giving rise to a spectrum of hydroxylated Δ^4-3keto compounds without significant accumulation of progesterone in the system. This seems to indicate that the NADPH generated from endogenous sources by fumarate transformed Δ^5-pregnenolone into progesterone, and it further supported the hydroxylations of this intermediate at carbons 21, 11 and 18. The further addition of exogenous NADP plus G-6-P to another similar system only seemed to magnify this situation, increasing the overall degree of

transformation but yielding essentially the same pattern of hydroxylated Δ^4-3keto metabolites without significant accumulation of progesterone.

To avoid the probable endogenous generation of NADPH induced by fumarate, further studies were carried out in its absence with subcellular fractions and homogenates of frog interrenal, in an attempt to elucidate the effects of NAD alone and NADP plus G-6-P in the metabolism of Δ^5-pregnenolone.

This study revealed that all subcellular fractions regardless of type of exogenous supplementation, metabolized Δ^5-pregnenolone to Δ^4-3keto steroids. This is in agreement with the reports of Krüskemper et al (120) that Δ^5-3β-hydroxysteroid dehydrogenase-isomerase is associated with all the organelles from mammalian adrenal.

Of more interest is that all subcellular fractions or the homogenates supplemented with NAD only converted Δ^5-pregnenolone into progesterone as the only identifiable metabolite, while the same preparations supplemented with NADP plus G-6-P transformed the substrate mainly into hydroxylated Δ^4-3keto steroids. This was particularly noticeable in P2 and WH, preparations with a more complete array of steroid enzymes.

In the interpretation of our results, we have followed the classical view that the molecule of Δ^5-pregnenolone is converted

into progesterone before being attacked by steroid hydroxylations. However, recent evidence adduced by Weliky and Engel (208), Pasqualini et al (209) and Kowal et al (210) with mammalian adrenal preparations and Sandor et al (72) in avian adrenal preparations indicate that Δ^5-pregnenolone can be hydroxylated at various positions and ultimately transformed into a Δ^4-3keto structure.

No evidence for this alternate metabolic pathway of Δ^5-pregnenolone has been obtained in our studies, although it still remains a theoretical possibility. In this regard it should be pointed out that no attempts were made to investigate whether some of the unidentified metabolites in this study were Δ^5-analogues of the isolated corticosteroids.

3. Studies with cholesterol-4-^{14}C

a) Adrenal sections

Two aliquots of 100 mg of frog interrenal sections were incubated with 2 µc of cholesterol-4-^{14}C in KRBG for three hours. One of the incubation studies was supplemented with NADP plus G-6-P, and the other contained no additions.

The radioautograms developed after three days of exposure failed to show even the slightest radioactive metabolites, as found in the case of C_{21} precursors. Exposing the chromatograms for ten days failed also to reveal any conversion products. However, the bulk of unconverted cholesterol was present in

fraction D' (LPG 4 hrs.)

The tissues from these incubates were extracted separately as stated previously. The radioautograms after three days of exposure did not show the slightest radioactivity in the chromatogram from tissue extracts. The zone in fraction D' (LPG 4 hrs.) corresponding to the chromatographic position of radiocholesterol was also completely devoid of radioactivity.

Our previous experiments with labelled precursor of the C_{21} series have shown that these precursors (along with some conversion products) were always present in the tissue. The fact that cholesterol-4-^{14}C was not detectable in tissue extracts lead us to suspect that this precursor did not enter the tissues which would explain the failure of the surviving sections to degrade the substrate into corticosteroids.

Since Karaboyas and Koritz (101) reported that the emulsification of radiocholesterol in a surface agent like Tween 80 was necessary for its utilization by rat and bovine adrenal sections, another set of experiment was designed in which the substrate would be employed in an emulsified state.

Four μc of cholesterol-4-^{14}C in benzene: methanol (9:1) were pipetted out into a 15 ml glass-stoppered tube and evaporated to dryness; 2 mg of Tween 80 in 2 ml acetone were added to the tube, and the contents were thoroughly mixed by shaking.

Two equal amounts of the mixture were transferred into two beakers. Acetone was evaporated, 2.5 ml of KRBG was added to each beaker and thoroughly mixed with the emulsion of Tween 80 (1 mg) and cholesterol-4-^{14}C (2 µc). Two aliquots of 100 mg of frog interrenal sections were added to these beakers and similar type of incubation studies were carried out as mentioned above. Once more no conversion of radiocholesterol into corticosteroids was observed, in spite of the fact that 20% of the labelled precursor was found in separate analysis of the interrenal sections.

No further experiments were done with the surviving sections using cholesterol-4-^{14}C.

b) Adrenal homogenates

Two hundred mg of frog interrenals were homogenized in $KRPO_4$ buffer containing fumarate. Two aliquots each containing 100 mg of tissue were poured into the beakers containing 2 µc of cholesterol-4-^{14}C. Note here that no authentic cholesterol was added to the radiocholesterol. The addition was omitted since Toren et al (211) have demonstrated that dilution with radioinert cholesterol interferes with the cleavage of labelled cholesterol. One of the incubation was supplemented with NADP and G-6-P, and the other contained no additives. The final volume of 5 ml was made up with $KRPO_4$ buffer, and incubation was carried out for three hours.

Table XVII shows the total conversion obtained by these studies. No significant conversion occurred in the fumarate containing homogenates in absence of exogenous cofactors (T.C. 0.22%). However, addition of NADP plus G-6-P resulted in greater transformation of cholesterol-4-^{14}C (0.64%). A variety of corticosteroids were isolated, i.e. 18-OH-corticosterone, aldosterone, corticosterone and progesterone.

These results encouraged us to explore the effects of higher dosage of NADP and G-6-P or preformed NADPH on the metabolism of cholesterol-4-^{14}C by homogenized preparations. Another similar experiment was carried out as above, except that the concentration of the supplementation was changed. One of the incubation medium contained NADP (1.25 μ moles/ml) plus G-6-P (4.5 μ moles/ml), and the other was supplemented with preformed NADPH (1.25 μ moles/ml).

The results obtained are recorded in the same Table XVII. Total conversion by the former incubation was 2.28% as compared to 2.40% by the latter. In addition to 11-deoxycorticosterone, both types of studies resulted in similar type of conversion products as obtained from previous experiment.

c) Subcellular fractions

Two sets of homogenates and subcellular fractions were prepared from 400 mg of interrenal tissue as described previously.

TABLE XVII

TRANSFORMATION OF CHOLESTEROL-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY FROG ADRENAL HOMOGENATES

STEROIDS ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE			
	A[1]	B[2]	C[3]	D[4]
18-OH-B	-	0.19	0.23	0.28
Aldosterone	-	0.12	0.30	0.34
B	-	0.14	0.21	0.17
DOC	-	-	0.21	0.24
Δ^4-P	0.22	0.08	1.02	0.98
Others	-	0.11	0.35	0.39
Total conversion	0.22	0.64	2.28	2.40

1. No cofactors
2. NADP (0.25 μ moles/ml) + G-6-P (0.9 μ moles/ml)
3. NADP (1.25 μ moles/ml) + G-6-P (4.5 μ moles/ml)
4. NADPH (1.25 μ moles/ml)

TABLE XVIII

TRANSFORMATION OF CHOLESTEROL-4-^{14}C TO CORTICOSTEROIDS BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES FROM AMERICAN BULLFROG ADRENAL*

STEROID ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE			
	P2		WH	
	NADPH	NADP+G-6-P	NADPH	NADP+G-6-P
18-OH-B	0.19	0.33	0.17	0.21
Aldosterone	0.26	0.30	0.27	0.38
Corticosterone	0.15	0.37	0.13	0.13
Total	0.60	1.00	0.57	0.72

*Incubation media $KRPO_4$ + fumarate + 0.13M sucrose.

These preparations were poured into the beakers containing 2 μc of cholesterol-4-^{14}C. One set was supplemented with NADP (0.25 μ moles/ml) and the other with NADP (0.25 μ moles/ml) and G-6-P (0.9 μ moles/ml). $KRPO_4$ buffer containing fumarate was added to make a final volume of 5 ml and incubation was carried out for three hours.

No conversion of the labelled sterol occurred in the studies with P1, P3 and SN either in the presence of NADPH-generating system or when preformed NADPH was added. The mitochondrial P2 and homogenates WH transformed cholesterol-4-^{14}C with either of the additives as shown in Table XVIII. Overall transformation by P2 was 0.60% with preformed NADPH and 1.0% with the NADPH-generating system; the corresponding values for WH being 0.51% and 0.75

Corticosteroids with the chromatographic mobility of 18-OH-corticosterone, aldosterone and corticosterone were isolated from either preparation. There was no significant difference in transformation products in the presence of NADP and G-6-P or preformed NADPH.

Discussion

Surviving sections of frog interrenal failed to degrade cholesterol-4-^{14}C into corticosteroids under basal conditions of incubations with the substrate dissolved in propylene glycol. This finding was almost predictable since it is the experience in this

laboratory that radiocholesterol is not utilized by bovine adrenal cortical sections under the above mentioned and many other experimental conditions (78).

These results are also in agreement with previous findings by Oliver and Péron (212) with Mongolian gerbel adrenals, Ayres et al (171) with strippings from bovine zona-glomerulosa, Sandor et al (74, 213) with avian interrenals and Karaboyas and Koritz (101) with rat and bovine adrenal sections.

Exogenous NADP plus G-6-P in the system did not support the NADPH-mediated cleavage of (unemulsified) cholesterol-4-^{14}C by frog interrenal section. This is at variance with previous findings in this laboratory with bovine adrenal sections (78, 96) in which an NADPH-generating system promoted the scission of radiocholesterol. Our present observations, however, are in complete agreement with the failure reported by Sandor et al (74) for avian surviving interrenal sections to metabolize radiocholesterol under the influence of an NADPH-generating system.

Since the substrate did not prenetrate the interrenal cells as demonstrated by the radioautograms from tissue extracts, another set of experiments was carried out emulsifying the substrate which according to Karaboyas and Koritz (101) facilitates its entry into the adrenal cells. While this claim was corro-

borated in our investigation, the frog interrenal sections still failed in metabolizing cholesterol-4-^{14}C, opposite to the degradation of tritiated cholesterol reported by Karaboyas and Koritz (101) in rat and bovine adrenal studies and by Hall and Koritz (75) in avian interrenal sections under similar in vitro conditions. The present studies with emulsified cholesterol-4-^{14}C are in agreement with the inability of bovine adrenal sections to convert the emulsified substrate to corticosteroids as reported by Carballeira and Durnhofer (180).

Even when the substrate was emulsified, NADP plus G-6-P did not support its cleavage in the present studies in contradiction to the positive results reported for beef adrenal sections by Carballeira and Durnhofer (180).

That frog interrenal preparations possess the enzymatic complex required for the conversion of cholesterol to corticosteroids was demonstrated with homogenized preparations in the presence of either preformed NADPH or NADPH-generating system. Once the integrity of the interrenal was disrupted, cholesterol-4-^{14}C was metabolized into 18-OH-corticosterone, aldosterone, corticosterone, 11-deoxycorticosterone and progesterone. Δ^5-pregnenolone and the hydroxylated derivatives of cholesterol (20α, and 20α, 22) were actively searched for in these studies without success.

The scission of labelled cholesterol by homogenized adrenal preparations supplemented with NADPH-generating systems have been previously reported by other authors in several vertebrates: mammalian (96, 89, 103, 214), avian (48, 74, 75) and fish (25).

It should be noticed that while in previous experiments with substrates of the C_{21} series, fumarate alone (probably generating NADPH from endogenous sources) supported to a considerable extent several steroid enzymatic reactions, the tricarboxylic cycle intermediate was poorly effective in mediating by itself the NADPH-dependent side chain scission of cholesterol. This might be due, among other factors, to the relatively small degree of overall conversion of this C_{27} sterol even under optimal conditions of supplementations (T.C. 2.4%).

The studies with subcellular fractions demonstrate that the cleavage enzymatic complex is associated with mitochondrial fraction, corroborating the studies by Halkerston et al (106), Hall et al (215), Bryson and Sweat (216) Roberts et al (220) Cheng (217) in several mammalian species.

In summary, the metabolism of radiocholesterol in frog interrenal is similar to that reported by most authors in other classes of vertebrates in the sense that organized surviving tissues do not appear to metabolize the sterol for some hitherto unexplained reason; that after the disruption of the adrenal cell

membrane the metabolism of this important substrate takes place easily; that the cleavage reaction is NADPH-dependent and that the enzymatic complex responsible for it resides in the mitochondrion.

4. Acetate-1-^{14}C as substrate

The various experimental conditions under which the incorporation of acetate-1-^{14}C into corticosteroids of frog adrenal preparations was attempted are outlined in Table XIX. A total amount of 200 μc of radioacetate substrate (without further dispersion with radioinert material) was used in each study. In experiments 1-3,7 this total amount was introduced into the in vitro system as a single addition preceding the incubation. In experiments 4-6, the total amount was divided into aliquots which were added to the systems at regular intervals.

After the end of each incubation, the media and the tissue (after homogenization) were extracted separately. In each case (media or tissue homogenates), just prior to the extraction, 100 μg of authentic, non-radioactive aldosterone, corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone, Δ^5-pregnenolone, progesterone and cholesterol were added.

No detectable conversion of labelled acetate into corticosteroids was observed under any of the experimental conditions employed.

The radioautogram corresponding to the media extract of

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After the end of each incubation, the media and the tissue (after homogenization) were extracted separately. In each case (media or tissue homogenates), just prior to the extraction, 100 μg of authentic, non-radioactive aldosterone, corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone, Δ^5-pregnenolone, progesterone and cholesterol were added.

No detectable conversion of labelled acetate into corticosteroids was observed under any of the experimental conditions employed.

The radioautogram corresponding to the media extract of

TABLE XIX

CONDITIONS UNDER WHICH POSSIBLE TRANSFORMATION OF ACETATE-1-^{14}C TO CORTICOSTEROIDS BY FROG ADRENALS WERE STUDIED

EXPT. No.	TISSUE (mg) PREPARATION	INCUBATION MEDIA	INCUBATION TIME (hrs.)	ADDATIVES
1	Homogenates (150)	$KRPO_4$	3	- NADP+G-6-P
2	Sections (140)	KRBG	3	- NADP+G-6-P ACTH
3	Sections (150)	KRB	3	- ACTH
4(a)	Sections (490)	KRB	4	-
5(b)	Sections (370)	KRB	4	ACTH
6(c)	Sections (400)	KRB $KRPO_4$	5	NADP+G-6-P
7(d)	Sections (506)	KRBG	3	3', 5' AMP 3', 5' AMP D.B.

(a) 25 μc of substrate incubated every 1/2 hour.

(b) 33.3 μc of substrate incubated every 1/2 hour, for 3 hours followed by 1 hour incubation with ACTH.

(c) 33.3 μc of substrate incubated every 1/2 hour in KRB followed by homogenization of tissue with $KRPO_4$ and incubation for 2 hours with added cofactors.

(d) The surviving sections were pre-incubated for 1/2 hour, followed by 3 hours incubation with substrate and additives.

these experiments (exposure 3-10 days) showed no radioactive reaction products. The papergrams were analyzed (under ultraviolet light or by chemical tests) to detect the zones where the added C_{21} or C_{27} steroids were located. Radioassays of the eluates from each of these zones showed that total amount of the radioactivity present was almost equivalent to the blank values.

Chromatographic resolution of the corresponding tissue extract showed in all cases a single zone with substantial amount of radioactivity in the position of labelled cholesterol when papergrams were subjected to radioautography for the same length of time as the media. As it will be shown in the section of 'Identification of Steroids' we failed to identify this material as cholesterol.

Substantial amount of radioactivity were present in this region allowing for a plan of characterization with rigorous criteria. However, from the first steps, the non-identity of this radioactive material with authentic cholesterol was already demonstrated (see unidentified metabolite II in 'Identification of Steroids').

Discussion

The exact step by step sequence of events leading from acetate to corticosteroids in mammalian adrenal, either involving cholesterol as an obligatory intermediate or not, is still to be

established firmly. There is no question, however, that the mammalian adrenal elaborates *in vitro* both, cholesterol (92, 98, 102) and corticosteroids (95, 96, 89, 221) from the 2-carbon substrate. Similar conclusion could be drawn for the avian interrenal, another class of vertebrate in which the role of acetate in corticosteroidogenesis has been studied in detail (74).

In the results under discussion, the incorporation of acetate-1-^{14}C by frog interrenal preparations could not be demonstrated.

The negative results obtained with frog interrenal homogenized preparations were not surprising. Cellular integrity has been claimed to be indispensable for the utilization of acetate by adrenal cells (89), contrary to the activity displayed by liver cell free preparations (222). With the exception of the isolated reports by Heard et al (89) and Bryson and Sweat (223) which claimed utilization of acetate by hog or bovine adrenal homogenates, the bulk of studies showing incorporation of radioacetate in the adrenal has been conducted with surviving sections (74, 78, 95, 96, 98, 221) This discrepancy between the capacity displayed by surviving sections to incorporate acetate and the incapacity shown by homogenates has been fully studied and discussed in the case of bovine adrenal preparations (78).

However, in the present studies, frog adrenal surviving

sections also failed to incorporate radioacetate into either cholesterol or corticosteroids, under our standard *in vitro* conditions.

Since the results forecast a major metabolic difference between amphibian and mammalian (or avian) adrenal tissue, various efforts were made to elucidate this matter adopting in our further studies *in vitro* conditions which have yielded maximal incorporation of radioacetate.

Both ACTH (95, 96, 101) and 3', 5' AMP (101) have been reported to increase considerably the degree of acetate incorporation by mammalian adrenals. Either type of additive failed in our studies with the frog interrenal to bring about any detectable degree of conversion. The failure of ACTH (expt. 2, 3, 5) can hardly be attributed to the fact that the trophic hormone was of mammalian origin since Carstensen et al (42, 43) increased endogenous production of corticosteroids by the frog interrenal with mammalian ACTH. In the case of 3', 5' AMP (expt. 7) cell membrane impermeability cannot explain its lack of action, since the dibutyryl derivative of this cyclic nucleotide was equally ineffective and its passage across the cell membrane has been ascertained (206).

A reaction product of acetate-1-^{14}C with chromatographic behaviour of cholesterol was detected in all our tissue extracts.

Presuming that this substance was actually cholesterol, NADP and G-6-P were introduced in the system with the idea of facilitating its possible conversion into corticosteroids. This supplementation was ineffective in one-step incubation (expt. 2). It was equally without effects in the second stage of a two-step incubation (expt. 6) in which the tissues were homogenized after a three-hour incubation period with acetate-1-^{14}C. This experiment was designed in view of our previous finding that radiocholesterol is metabolized only after disruption of the adrenal cell membrane. It was later demonstrated that this metabolite of acetate-1-^{14}C was not identical with cholesterol (see 'Identification of Steroids' under i). However, the above discussed experiments still demonstrate that NADP plus G-6-P do not overcome the apparent inability of frog interrenals to utilize radioacetate.

Several successful investigations (224, 225) with acetate-1-^{14}C have omitted the glucose from the Krebs-Ringer-bicarbonate incubation medium. This was also tried in our studies without effects (expt. 3-6). (see 'General Discussion')

From our initial studies with acetate, the amount of tissues was increased to 140-150 mg instead of the usual 100 mg employed with other labelled substrates. Suspecting that this amount of tissue would be insufficient to metabolize acetate into

demonstrable conversion products, the tissue weights were increased up to 500 mg per incubation. Once more this did not produce positive results.

Similar failures resulted in lengthening the time of incubation up to five hours or administering the substrate in divided aliquots at regular intervals (expt. 4-6). The latter variation was tried suspecting that a very large amount of substrate could produce an inhibition in its metabolism. It should be pointed out that we have used 200 μc of substrate which was about the same amount used by Karaboyas and Koritz (101) in their successful studies with rat and bovine adrenal sections and by Sandor et al (74) with avian interrenal sections.

In summary, all our efforts to demonstrate the incorporation of radioacetate by frog interrenal have met with failure, since neither cholesterol nor corticosteroids could be isolated.

It appears that contrary to mammalian and avian adrenals the process of steroidogenesis in frog interrenal is not initiated at the 2-carbon fragment.

5. Identification of steroids

The sequential paper chromatographic scheme (Figure 3) employed in the resolution of crude adrenal extracts revealed a large number of conversion products from various labelled precursors. This procedure, while time consuming, offers the

distinct advantage of isolating metabolites in a relatively pure form from this initial stage.

Studies with different tissue preparations and precursors yielded metabolites with identical chromatographic mobility in the above mentioned scheme. A final characterization of these similar materials carried out individually seemed an excessive and impractical task. Therefore, the final identity of each compound was established in pools representative of various individual observations. The mixing of these similar metabolites, however, was only done after subjecting each separate material to at least two additional chromatographies in presence of 100 ug of radioinert authentic carrier.

The identification of the compounds is reported according to their decreasing polarity. The exact location of each compound within the fraction from which it was isolated is illustrated in Figure 3.

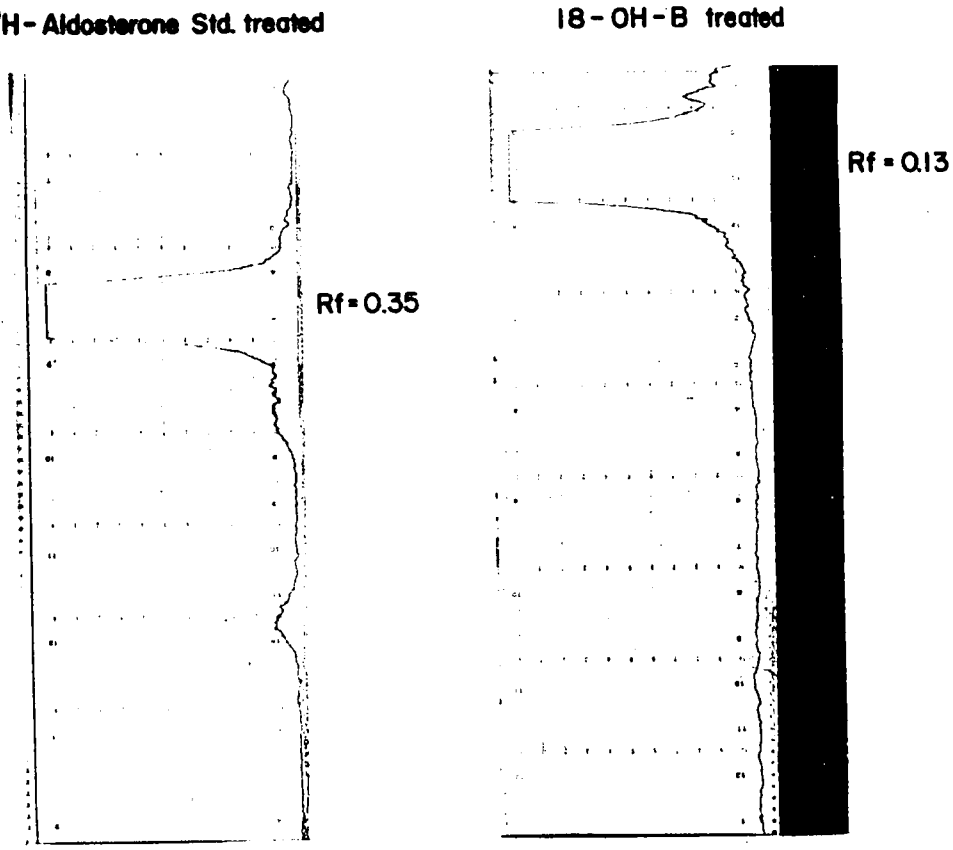
a) 18-OH-corticosterone.

In this study no authentic radioinert or tritiated materials were available. However, from earlier experience, the chromatographic mobility of this compound in fraction A (TEG system, 48 hrs.) was known. The radioactive zones, having similar mobility to authentic 18-OH-B, were pooled from various experiments. The pooled material was subjected to periodic acid oxidation

FIGURE 12

RUNNING RATES OF 18-HYDROXYCORTICOSTERONE LACTONE

(PERIODIC ACID OXIDATION PRODUCT OF 18-HYDROXYCORTICOSTERONE).



CHROMATOGRAPHIC SYSTEM BUSH T (4 1/2 hrs.)

To the left: scanning of authentic ^{3}H-aldosterone converted into 11β, 18-epoxy-3 oxoandrost-4-ene-17β-carboxylic acid-18 $\longrightarrow$ 20 lactone.
To the right: scanning and radioautogram of ^{14}C-labelled 18-hydroxycorticosterone converted into 11β, 18-dihydroxyandrost-4-ene-3-one-17β-carboxylic acid-18 $\longrightarrow$ 20 lactone.

as described earlier (185). The oxidized material was applied on Whatman paper #2 and run in the Bush T system (4 1/2 hrs.) as shown in the radioautogram in Figure 12. The Rf value of 0.13 of this compound was similar to that reported by Tait et al (185) for 11β, 18-dehydroxy-3-keto-4-etienic acid lactone (periodic acid oxidation product of 18-OH-B). To check the reproducibility of the whole procedure in our hands, authentic tritiated aldosterone was treated similarly, and the resultant oxidized product showed a similar Rf (0.35) to that reported by the above mentioned authors. (see Figure 12 also)

b) Aldosterone

The radioactive zone corresponding to authentic aldosterone in fraction A (TEG 48 hrs.) was cut and eluted. Different pools of this zone were made according to the type of the precursor used, i.e. the zone appearing in all the studies with progesterone-4-^{14}C, irrespective of the type of tissue preparation used, were pooled together. Similar pools of this zone, arising from cholesterol-4-^{14}C and Δ^5-pregnenolone-4-^{14}C were made. One hundred μg of synthetic d-aldosterone were added to each pool and chromatographed in the Bush B5 system. The radioautogram and ultra-violet-light absorption showed that the bulk of the radioactivity remained associated with the authentic material. After this step, purified tritiated aldosterone was added in

TABLE XX

CHARACTERIZATION OF ALDOSTERONE-4-^{14}C (FROG ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVE IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS			
	STARTING MATERIAL Aldosterone		DERIVATIVE (ACETYLATION) Aldosterone-21-monoacetate	
	Bush-C[1]	Bush-B5	Bush-C	E_2B
Cholesterol-4-^{14}C	4.6	4.7	4.7	-
Δ^5-Pregnenolone-4-^{14}C	4.9	4.8	4.7	4.7
Progesterone-4-^{14}C	4.0	3.8	3.9	3.9

*Isolated from various incubations of sections, homogenates and mitochondrial (P2) of the frog adrenals using above mentioned substrates.
1. Chromatographic solvent systems.

TABLE XXI

CHARACTERIZATION OF ALDOSTERONE-4-^{14}C (FROG ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF ALDOSTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	21989	2168	10.1
Mother Liquor	23089	2317	10.0
II			
Crystals	22351	2208	10.1
Mother Liquor	22008	2193	10.0
III			
Crystals	21906	2169	10.1
Mother Liquor	26420	2601	10.2

*Isolated from various incubations with surviving sections, homogenates and subcellular fractions (P2) of frog adrenals using progesterone-4-^{14}C, Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (homogenates and P2 incubations only) as substrates.

each case. The mixture was subjected to sequential paper chromatography in the systems of Bush C (4 hrs.) and Bush B5 (5 hrs.). As shown in Table XX, the $^{3}H:^{14}C$ ratio of aldosterone derived from different precursors, essentially remained constant. After the last chromatography, the material from all three different sources was acetylated as described earlier (181) and was re-chromatographed in the systems of Bush C and E_2B. Most of the acetylated material showed a similar mobility to authentic aldosterone-21-mono-acetate, and the $^{3}H:^{14}C$ ratio of the mono-acetate remained constant.

For the purpose of identification by microcrystallization, an aliquot was taken from each pool after initial chromatography in Bush B5 (5 hrs.). These aliquots were mixed together, and ^{3}H-aldosterone was added. This was further diluted with 25 mg of crystalline aldosterone. The $^{3}H:^{14}C$ ratios and specific activity in crystals and mother liquors after successive crystallization of the isolated metabolites are shown in Table XXI.

c) 11-dehydrocorticosterone.

This steroid was only detected in studies with progesterone-4-^{14}C (both sections and homogenates H) and Δ^{5}-pregnenolone-4-^{14}C (homogenates H only). It has[a] mobility identical to that of 11-dehydrocorticosterone in E_2B system (fraction C). After addition of the carrier compound A, it travelled concurrently

TABLE XXII

CHARACTERIZATION OF 11-DEHYDROCORTICOSTERONE-4-^{14}C (FROG ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVE IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS			
	STARTING MATERIAL 'A'		DERIVATIVE (ACETYLATION) 'A-acetate'	
	Bush B5[1]	E_2B	BLI	D
Δ^5-pregnenolone-4-^{14}C **	-	-	-	-
Progesterone-4-^{14}C	11.9	11.9	12.0	11.9

*Isolated from various incubations of sections and homogenates of the frog adrenals using progesterone-4-^{14}C as a precursor.
1. Chromatographic solvent systems.
** Samples were lost.

TABLE XXIII

CHARACTERIZATION OF 11-DEHYDROCORTICOSTERONE-4-^{14}C (FROG ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF 11-DEHYDROCORTICOSTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	2572	222	11.6
Mother Liquor	2464	207	11.9
II			
Crystals	2507	205	12.3
Mother Liquor	2019	164	12.2
III			
Crystals	2454	202	12.1
Mother Liquor	2515	200	12.6
IV			
Crystals	2531	206	12.3
Mother Liquor	2476	209	11.9

*Isolated from incubations of sections and homogenates of frog adrenals using progesterone-4-^{14}C and Δ^{5}-pregnenolone-4-^{14}C as substrates.

with it in the Bush B5 (5 hrs.) and E_2B (4 1/2 hrs.) systems. The material isolated from each source was treated separately.

11-dehydrocorticosterone-1, 2-^3H (recently purified) was added to the aliquots of the material and rechromatographed in the above mentioned systems. Acetylation by 'fast' micro-technique was carried out, and the resultant acetate in each case was chromatographed in the BLI (4 hrs.) and D (5 hrs.) systems. Pure compound A and its acetate served as standards. After each chromatography, samples were taken out and counted for ^3H and ^{14}C. These ratiosof free and acetylated compounds are shown in Table XXII.

A separate pool of ^{14}C-labelled 11-dehydrocorticosterone isolated from the C_{21} substrates was mixed with purified 11-dehydrocorticosterone, 1, 2-^3H and 25 mg of authentic compound A. The mixture was chromatographed on silica gel column. Four crystallizations were carried out using acetone and pentane (189). Table XXIII shows the specific activity of crystals and mother liquors and also ^3H:^{14}C ratios obtained in each case. The values permit claiming the radiochemical homogeneity of the ^{14}C-labelled material.

d) Corticosterone

This steroid has been detected as a major conversion product practically from all the incubations studies with

progesterone-4-^{14}C, Δ^5-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (only homogenates and P2). It has the mobility identical to that of authentic corticosterone in E_2B system (fraction C). After addition of carrier corticosterone and rechromatography, the radioactivity remained associated with the carrier in the Bush B5 (4 1/2 hrs.) and E_2B (4 1/2 hrs.) systems. Separate pools of this material were prepared according to the source of the substrate.

Purified authentic corticosterone-1, 2-^3H was added to aliquots from each pool. Rechromatography was carried out in the E_2B (4 1/2 hrs.) and D (5 hrs.) systems. Oxidation with CrO_3 (183) was carried out, and resultant compound 11-dehydrocorticosterone was rechromatographed in each case in E_2B (4 1/2 hrs.) and TEG (4 hrs.) systems. Pure corticosterone and 11-dehydrocorticosterone served as standards. The 11-dehydrocorticosterone thus obtained was acetylated by the 'fast' microtechnique (181). The resultant acetate in each case was chromatographed in the BLI (4 hrs.) and D (4 hrs.) systems. Authentic free 11-dehydrocorticosterone and its acetate served as standards. Samples were taken after each chromatography and counted for ^3H and ^{14}C. Table XXIV shows the ^3H:^{14}C ratios from these derivatives.

A separate pool of ^{14}C-labelled corticosterone isolated

TABLE XXIV

CHARACTERIZATION OF CORTICOSTERONE-4-^{14}C (FROG ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVES IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS					
	STARTING MATERIAL 'B'		DERIVATIVES CrO_3 OXIDATION 'A'		ACETYLATION OF OXIDATION PRODUCT 'A-Acetate'	
	E_2B[1]	D	E_2B	TEG	BLI	D
Cholesterol-4-^{14}C	6.6	6.9	6.9	6.5	6.8	6.3
Δ^5-pregnenolone-4-^{14}C	4.4	4.6	4.4	4.5	4.4	4.4
Progesterone-4-^{14}C	3.5	3.4	3.2	3.8	3.7	-

*Isolated from various incubations of sections, homogenates and subcellular fractions (P2) of the frog adrenals using above mentioned precursors, in the case of cholesterol-4-^{14}C homogenates and P2 only.

1. Chromatographic solvent systems.

TABLE XXV

CHARACTERIZATION OF CORTICOSTERONE-4-^{14}C (FROG ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF CORTICOSTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	19765	2688	7.4
Mother Liquor	33145	4592	7.2
II			
Crystals	20881	2807	7.4
Mother Liquor	25841	3751	7.3
III			
Crystals	20016	2720	7.4
Mother Liquor	22568	3040	7.4

*Isolated from incubations of sections and homogenates of frog adrenals using progesterone-4-^{14}C, Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (homogenate studies only) as substrates.

from different substrates was mixed with purified corticosterone-1, 2-^{3}H and 25 mg of authentic compound B. The mixture was chromatographed on silica gel column. Three crystallizations were carried out using acetone and pentane (189). Table XXV shows the specific activity of crystals and mother liquors and ^{3}H:^{14}C ratios in each case. The radiochemical homogeneity of corticosterone seems proven.

e) 11β-OH-progesterone.

A radioactive compound in the position of authentic 11β-OH-progesterone (fraction D, LPG 24 hrs.) in incubation studies with progesterone-4-^{14}C (sections, homogenates and P2) and Δ^{5}-pregnenolone-4-^{14}C (homogenates and P2 only) has been found. Upon rechromatography, after the addition of authentic 11β-OH-progesterone, the band from the above mentioned sources remained associated with the carrier in the Bush A (16 hrs.) and LPG (30 hrs.) systems. Two pools of the materials were made, one from homogenates and sections and the other one from the mitochondrial P2 studies. Due to non-availability of ^{3}H-labelled 11β-OH-progesterone, the constant ^{3}H:^{14}C ratios could not be established.

The pools were again rechromatographed in the Bush A and LPG as before. Aliquots from these pools were oxidized with CrO_3 (183) and the resultant derivative (11 keto-progesterone)

TABLE XXVI

CHARACTERIZATION OF 11β-OH-PROGESTERONE-4-^{14}C (FROG ADRENAL)*

a) S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF THE METABOLITE

CRYSTALLIZATION OF 11β-OH-PROGESTERONE	^{14}C cpm/mg
I	
Crystals	1790
Mother Liquor	2134
II	
Crystals	1755
Mother Liquor	1607
III	
Crystals	1812
Mother Liquor	1651
IV	
Crystals	1787
Mother Liquor	1838

*Isolated from sections and homogenates of frog adrenals using progesterone-4-^{14}C, Δ^5-pregnenolone-4-^{14}C as substrates.

TABLE XXVII

CHARACTERIZATION OF 11β-OH-PROGESTERONE-4-^{14}C (FROG ADRENALS)

b) S.A. AFTER SUCCESSIVE CRYSTALLIZATION, FOLLOWING CrO_3 TREATMENT OF METABOLITE

CRYSTALLIZATION OF 11-KETO-PROGESTERONE (CrO_3 oxidation product)	cpm/mg A[1]	cpm/mg B[2]
I		
Crystals	2420	488
Mother Liquor	2953	513
II		
Crystals	2432	488
Mother Liquor	2628	634
III		
Crystals	2450	498
Mother Liquor	1828	592
IV		
Crystals	2439	503
Mother Liquor	2379	467

1. Isolated from incubation of sections, homogenates of frog adrenals using progesterone-4-^{14}C and Δ^5-pregnenolone-4-^{14}C as substrates.
2. Isolated from incubation of mitochondrial (P2).

was chromatographed in each case in the Bush A (20 hrs.) and LPG (36 hrs.) systems. Pure 11β-OH- and 11-keto-progesterone served as standards.

Twenty-five mg of authentic 11β-OH-progesterone were added to a ^{14}C-labelled aliquot from the two pools. Four crystallizations were carried out from acetone and pentane (189). Table XXVI shows the specific activities of crystals and mother liquors.

Twenty-five mg of authentic 11 keto-progesterone were added to the ^{14}C labelled oxidized product from homogenates and sections and from mitochondrial P2. Four crystallizations were carried out using the above mentioned solvents. Table XXVII shows the specific activities of crystals and mother liquors corresponding to different pools.

f) 11-deoxycorticosterone

This band has been encountered (fraction D, LPG 24 hrs.) in practically all the studies with labelled cholesterol (homogenates only) Δ^5-pregnenolone and progesterone. It was the sole metabolite of labelled progesterone in incubations with microsomal P3 and supernatant SN preparations. After addition of authentic carrier, it travelled concurrently with it in the Bush A (6 hrs.) and LPG (24 hrs.) systems. Separate pools of this steroid

TABLE XXVIII

CHARACTERIZATION OF 11-DEOXYCORTICOSTERONE-4-^{14}C (FROG ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVE IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS			
	STARTING MATERIAL DOC	DERIVATIVES ACETYLATION DOCA		HYDROLYSIS OF DOCA DOC
	LPG[1]	BUA	D	LPG
Cholesterol-4-^{14}C	6.8	6.8	6.7	6.8
Δ^{5}-pregnenolone-4-^{14}C	5.8	5.7	5.9	5.9
Progesterone-4-^{14}C	5.8	5.8	5.8	5.9

*Isolated from various incubations of sections, homogenates and subcellular fractions (P3, SN) of the frog adrenal glands using above mentioned precursors, in the case of cholesterol-4-^{14}C from homogenates and subcellular fractions only.
1. Chromatographic solvent systems.

TABLE XXIX

CHARACTERIZATION OF 11-DEOXYCORTICOSTERONE-4-^{14}C (FROG ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF 11-DEOXYCORTICOSTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	1987	342	5.8
Mother Liquor	2009	332	6.1
II			
Crystals	1883	319	5.9
Mother Liquor	1925	322	6.0
III			
Crystals	1878	317	5.9
Mother Liquor	1793	311	5.8

*Isolated from the incubation of the frog adrenal sections, homogenates and subcellular fractions (P3, SN) using progesterone-4-^{14}C, Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (homogenates only) as the precursors.

were prepared according to the source of the substrate.

Aliquots were taken from each of these pools and purified 11-deoxycorticosterone-1, 2-^{3}H was added to each of them. After chromatography in the LPG system (24 hrs.), the material was acetylated with 'fast' microtechnique (181). The resultant acetate was chromatographed in the Bush A (5 hrs.) and D (4 hrs.) systems. Authentic free 11-deoxycorticosterone and its acetate were used as standards. The acetylated compound was hydrolized using a modified method of that recommended by Bush (182). The free compound was chromatographed on the LPG system (24 hrs.). Samples were taken before each chromatography to establish ^{3}H:^{14}C ratios. The values of these ratios are recorded in Table XXVIII.

A separate pool of ^{14}C-labelled deoxycorticosterone isolated from various precursors was mixed with purified 11-deoxycorticosterone-1, 2-^{3}H and 25 mg of authentic DOC. The mixture was chromatographed on silica gel column. Three crystallizations were carried out, using acetone and pentane (189) as solvent pair. The specific activity of crystals and mother liquor and the ^{3}H:^{14}C ratios in each case are recorded in Table XXIX.

g) Metabolite I

A radioactive zone coinciding with the chromatographic behaviour of authentic Δ^{5}-pregnenolone (fraction D, LPG, 24 hrs.) was detected in studies in which progesterone-4-^{14}C was incubated

with homogenates H or WH. Although it has been generally accepted for years that Δ^5-pregnenolone ——→progesterone is irreversible, recent lines of investigation have challenged this classical opinion with the demonstration that adrenal mammalian preparations may, under certain in vitro conditions, effect the reaction progesterone ——→Δ^5-pregnenolone (or other Δ^4, 3-keto ——→Δ^5, 3β-ol reactions) (226, 227).

Interested in elucidating whether this was also the case for frog interrenal preparations, the characterization of this metabolite I was undertaken tentatively considering that it might be Δ^5-pregnenolone on the basis of its polarity.

For this purpose, authentic Δ^5-pregnenolone-7-^{3}H was added to the isolated ^{14}C-labelled experimental material. Double-labelled radioassays were carried out subsequent to each chromatography in the following systems: TLC1, TLC2 and TLC8. The ^{3}H:^{14}C ratios encountered were 7.2, 7.9 and 6.8 respectively.

Twenty-five mg of recrystallized authentic Δ^5-pregnenolone were added to an ^{3}H:^{14}C mixture and three successive crystallizations carried out from pentane and acetone. Table XXX shows that the specific activities of the experimental ^{14}C-labelled material decreased tremendously in the crystals, being recovered in the corresponding mother liquors. The same table illustrates that on the other hand the specific activity of the authenticated

TABLE XXX

CRYSTALLIZATION OF ^{14}C-LABELLED METABOLITE I (FROG ADRENALS)*

^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF MIXTURE OF ^{14}C-METABOLITE I WITH AUTHENTIC Δ^5-PREGNENOLONE (tritiated and radioinert)	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	1296	41	31.5
Mother Liquor	1808	693	2.8
II			
Crystals	1292	9	144.0
Mother Liquor	1350	18	75.0
III			
Crystals	1290	7	185.0
Mother Liquor	1210	12	109.0

*Isolated from the incubation studies with frog adrenal homogenates H or WH using progesterone-4-^{14}C as substrate.

^{3}H-labelled compound remained constant in crystals and mother liquor within the limits of experimental error.

These findings clearly indicated that the ^{14}C-labelled compound in question was not identical with authentic Δ^{5}-pregnenolone. No further attempts to investigate the true identity of this metabolite were undertaken.

These results are a perfect illustration that isopolarity (even when established by ^{3}H:^{14}C ratios) as a single criterion in identification can be quite misleading and that isomorphism is a much more rigorous yardstick for establishing the true identity of steroid metabolites.

h) Progesterone

This steroid was isolated from the studies with cholesterol-4-^{14}C and Δ^{5}-pregnenolone-4-^{14}C. It has mobility identical to that of authentic progesterone in fraction D (LPG system, 4 hrs.). After addition of the carrier progesterone, it travelled concurrently with it in the LPG (6 hrs.), TLC1 and TLC2 systems. The material isolated from each subcellular fraction was treated separately, whereas a single pool was prepared from materials isolated from studies with homogenates (both from cholesterol-4-^{14}C and Δ^{5}-pregnenolone-4-^{14}C) and sections.

Recently purified progesterone-1, 2-^{3}H was added to the aliquots of the material and rechromatographed in the TLC3 and

TLC9 systems. Enzymatic reduction with 20β-hydroxy steroid dehydrogenase (188) was carried out and the resultant 20β-hydroxy derivative of progesterone was chromatographed in TLC2 and TLC10 systems. (The term 20β-hydroxyprogesterone is explained in the table of trivial and systematic nomenclature). Authentic progesterone and 20β-OH progesterone served as standards. The 20β-hydroxyprogesterone thus obtained was acetylated by the fast microtechnique (181). The acetate formed in each case was rechromatographed in the TLC4 and TLC3 systems. Authentic free 20β-OH-progesterone and its 20 acetate served as standards. Samples were taken after each chromatography and counted for ^{3}H and ^{14}C. Table XXXI shows the ^{3}H:^{14}C ratios of these derivatives.

An aliquot of doubly labelled progesterone was mixed with 25 mg of authentic progesterone. Three crystallizations were carried out from an acetone and pentane mixture (189). Table XXXII shows the specific activity of crystals and mother liquors and also ^{3}H:^{14}C ratios in each case.

Another aliquot of ^{3}H and ^{14}C containing 20β-OH-progesterone was mixed with 25mg of pure 20β-OH-progesterone and crystallized as mentioned above. Table XXXIII records the specific activities of mother liquors and crystals and ^{3}H:^{14}C ratios in four crystallizations. The radiochemical homogeneity of the ^{14}C materials

TABLE XXXI

CHARACTERIZATION OF PROGESTERONE-4-^{14}C (FROG ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVE IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS					
	STARTING MATERIAL Progesterone		ENZYMATIC REDUCTION[1] 20β-OH-Δ4P		ACETYLATION OF REDUCTION PRODUCT 20β-OH-Δ4P-Acetate	
	TLC3[2]	TLC9	TLC2	TLC10	TLC4	TLC3
P1	6.4	6.4	6.4	6.5	5.7	5.9
P2	7.5	7.4	7.6	7.2	6.2	6.4
P3	7.5	7.0	7.3	7.2	6.1	6.2
SN	7.0	6.9	7.1	7.1	6.2	6.2
Homogenates + Sections	5.4	5.5	5.8	5.9	4.9	4.9

*Isolated from incubations of various subcellular fractions (P1, P2, P3, SN) homogenates and sections of frog adrenals using Δ5-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (H only) as substrates.

1. With 20β-hydroxysteroid dehydrogenase (see Methods and Materials).
2. Chromatographic solvent systems.

TABLE XXXII

CHARACTERIZATION OF PROGESTERONE-4-^{14}C (FROG ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF PROGESTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	3273	430	7.6
Mother Liquor	4845	619	7.8
II			
Crystals	3443	446	7.7
Mother Liquor	4271	549	7.8
III			
Crystals	3560	477	7.4
Mother Liquor	3884	505	7.7

*Isolated from various incubations of sections and homogenates of frog adrenals using Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (H only) as substrates.

TABLE XXXIII

CHARACTERIZATION OF PROGESTERONE-4-^{14}C (FROG ADRENAL)*

c) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION FOLLOWING ENZYMATIC TREATMENT OF METABOLITE WITH 20β-HYDROXY-STEROID DEHYDROGENASE

CRYSTALLIZATION OF 20β-OH-PROGESTERONE (enzymatic derivative)	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	990	167	5.9
Mother Liquor	983	175	5.6
II			
Crystals	1014	176	5.7
Mother Liquor	1021	178	5.7
III			
Crystals	1026	181	5.5
Mother Liquor	1081	191	5.6
IV			
Crystals	1038	178	5.8
Mother Liquor	1081	182	5.7

*Isolated from incubations of homogenates and various subcellular fractions (P1, P2, P3, SN) of frog adrenals using Δ^{5}-pregnenolone-4-^{14}C as a substrate.

seems thus proven.

i) Metabolite II

Tissue extracts of incubates with acetate-1-^{14}C invariably showed a radioactive band in fraction D' (LPG system, 4 hrs.) coinciding with the chromatographic position of authentic cholesterol.

The material from these tissue extracts was pooled and mixed with 50 mg of recrystallized authentic cholesterol (radioinert). This mixture was subjected to digitonin treatment (92) followed by cleavage of the digitonide complex with pyridine (186). At this point recently purified authentic cholesterol-1, 2-^{3}H was added and the mixture subjected to repeated bromination and debromination in four successive cycles, following the procedure described by Schwenk et al (187). The specific activites after each of these chemical treatment are shown in Table XXXIV.

A significant decrease in specific activity (^{14}C cpm/mg) was observed after treatment of metabolite II with digitonin and cleavage with pyridine. Similar decrease in specific activities of ^{14}C-labelled material was observed after each brominationcycles, in contrast to the specific activities of authenticated ^{3}H-labelled compound which remained constant.

These findings indicated that metabolite II was not cholesterol as we had envisaged. No further attempts to investigate the

TABLE XXXIV

SPECIFIC ACTIVITIES OF ^{14}C-LABELLED METABOLITE II AFTER SUCCESSIVE CHEMICAL TREATMENT

MATERIAL	CHEMICAL TREATMENT	^{14}C cpm/mg	^{3}H cpm/mg
^{14}C-Metabolite	None	5229	
+ A	↓		
	Digitonide formation		
	↓		
	Pyridine cleavage	127	
	↓		
+ B	Bromination 1st cycle	103	644
	2nd cycle	25	500
	3rd cycle	19	535
	4th cycle	14	520

A = 50 mg of recrystallized radioinert authentic cholesterol
B = Authentic purified cholesterol-7-^{3}H

true identity of this metabolite were undertaken.

j) Other metabolites

Several other metabolites were detected scattered throughout the different fractions of the separating chromatographic scheme. They were especially distinct in studies using supplementation with different nucleotides. With the exception of one band, these zones did not coincide with the chromatographic position established previously in the above mentioned scheme for a large number of authentic steroids of the C_{21}, C_{19} and C_{18} series (228).

In the case where a perfect chromatographic coincidence was found, the standard compound was 19-OH-desoxycorticosterone (fraction B, TEG, 17-20 hrs.). However, lack of sufficient amounts of authentic material precluded the identification of this metabolite.

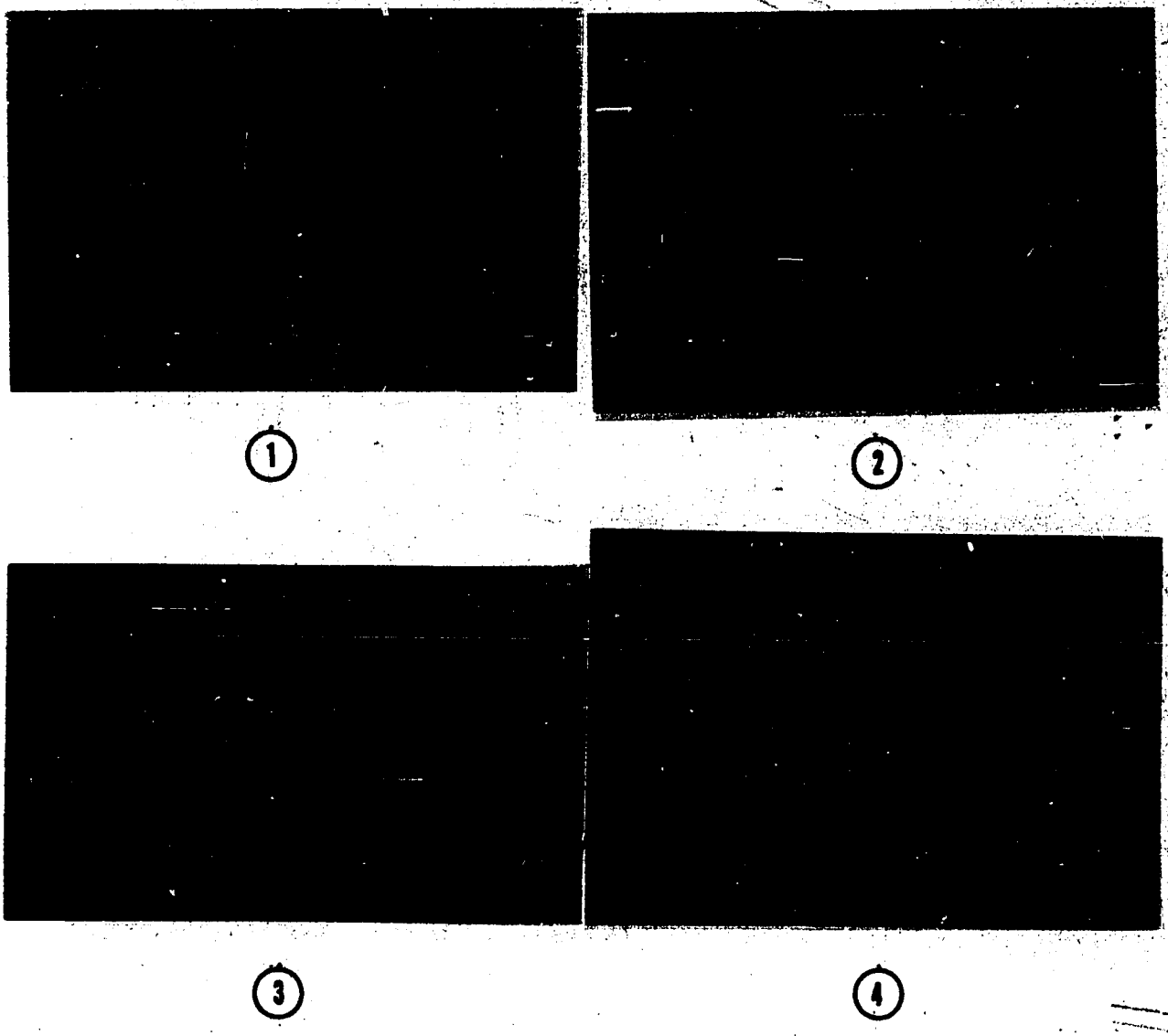
6. Histochemical studies

The enzymes of the hexose-monophosphate shunt (G-6-P dehydrogenase and 6-phosphogluconate dehydrogenase) involved in the reduction of NADP have been demonstrated to be present in the mammalian adrenal cortex by spectrophotometric (156-158) and histochemical (159, 160) methods.

To our knowledge, these enzymes have not been studied in the frog adrenal gland. Our previously described <u>in vitro</u>

FIGURE 13

HISTOCHEMICAL STUDIES WITH FROG INTERRENALS.



(1) Glucose-6-phosphate dehydrogenase

(2) 6-Phosphogluconate dehydrogenase

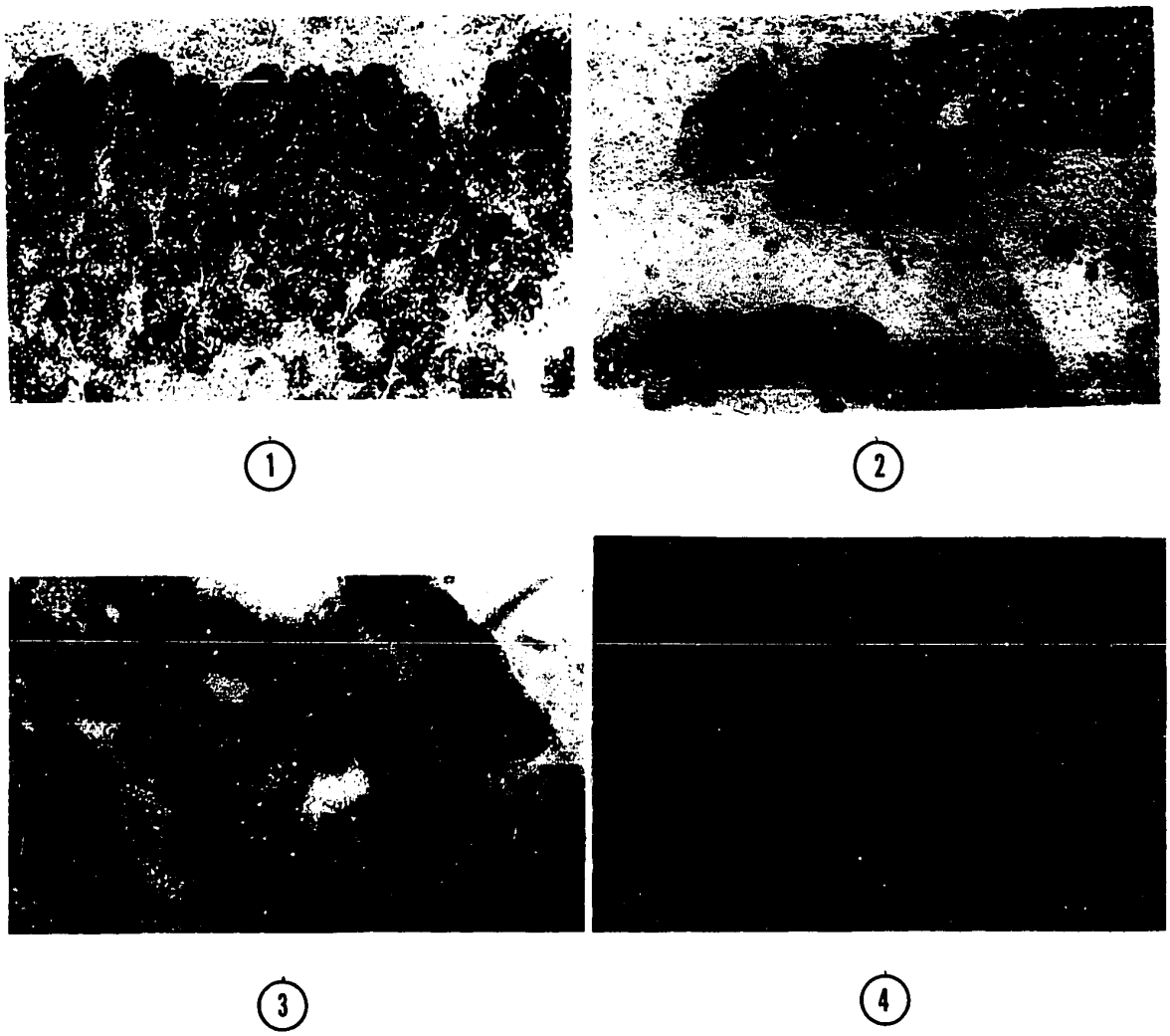
(3) NADPH-diaphorase

(4) NAD-diaphorase

In all cases magnification 6.3 x 15.

FIGURE 13

HISTOCHEMICAL STUDIES WITH FROG INTERRENALS.



(1) Glucose-6-phosphate dehydrogenase

(2) 6-Phosphogluconate dehydrogenase

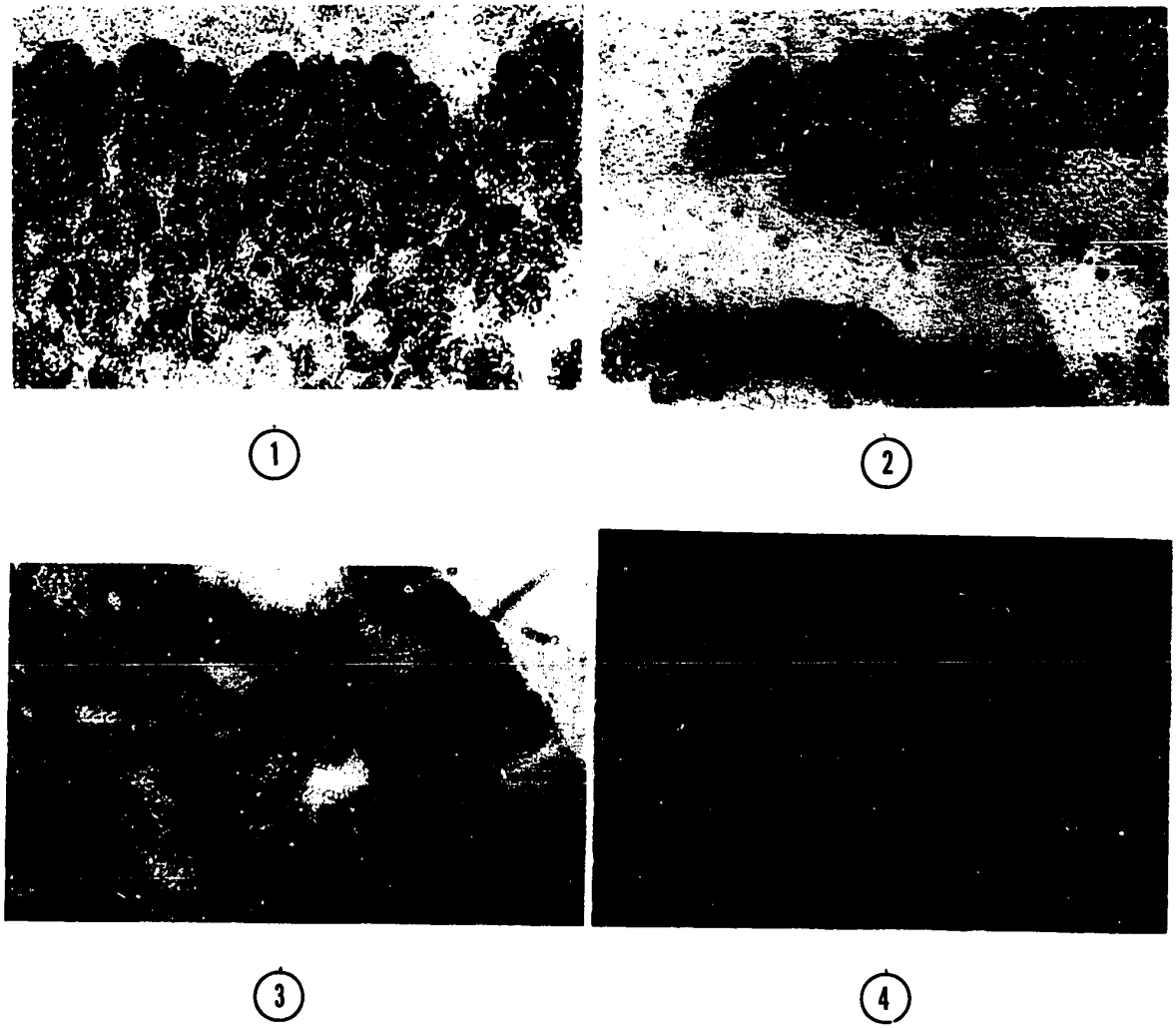
(3) NADPH-diaphorase

(4) NAD-diaphorase

In all cases magnification 6.3 x 15.

FIGURE 13

HISTOCHEMICAL STUDIES WITH FROG INTERRENALS.



(1) Glucose-6-phosphate dehydrogenase

(2) 6-Phosphogluconate dehydrogenase

(3) NADPH-diaphorase

(4) NAD-diaphorase

In all cases magnification 6.3 x 15.

studies with labelled substrate suggested their presence in this tissue, since exogenous NADP plus G-6-P stimulated various steroid enzymatic reactions mediated by NADPH.

In order to ascertain directly whether or not these dehydrogenases were available in frog interrenals, histochemical studies were carried out in collaboration with Dr. J.M. Rojo-Ortega, following the methodology described in detail in references (229) and (230).

The results of these studies are shown in the photomicrographs reproduced in Figure 13, which demonstrate the presence of both G-6-P dehydrogenase (1) and 6-phosphogluconate dehydrogenase (2) thereby explaining the capacity of frog interrenal tissue to reduce exogenous NADP in presence of added G-6-P.

The same figure illustrates that the frog interrenal also possesses NADPH-diaphorase (3) and NADH-diaphorase (4). These nucleotide tetrazolium reductases have also been demonstrated in mammalian adrenal glands (159, 231, 232).

SECTION B: STUDIES WITH TURTLE ADRENAL TISSUE

The biotransformation of labelled progesterone and Δ^5-pregnenolone by the interrenal sections of the Chrysemys picta picta (the painted turtle) has previously been studied by Sandor et al (57). Therefore, the present studies with the painted turtle were confined exclusively to interrenal subcellular fractions and homogenates WH, using labelled C_{21} steroids and cholessterol as precursors. However, surviving sections were used when acetate-1-^{14}C was employed as substrate.

1) Progesterone-4-^{14}C as substrate

Four hundred mg of turtle interrenals were homogenized in 10 ml of 0.25M sucrose. From this preparation, two sets of homogenates WH and subcellular fractions P1, P2, P3 and SN were obtained as described previously (see III, B, 3). These preparations were incubated with 2 µc of progesterone-4-^{14}C (previously mixed with 100 µg of authentic progesterone). All other experimental conditions were similar to those described for frog subcellular interrenal incubations with labelled progesterone.

Table XXXV records the total percentage conversion of progesterone-4-^{14}C by different subcellular fractions and homogenates WH. All studies without cofactors showed a very small degree of conversion. The highest enzymatic activity was shown by WH (T.C. 6.6%); in subcellular fractions total conversion ranged

TABLE XXXV

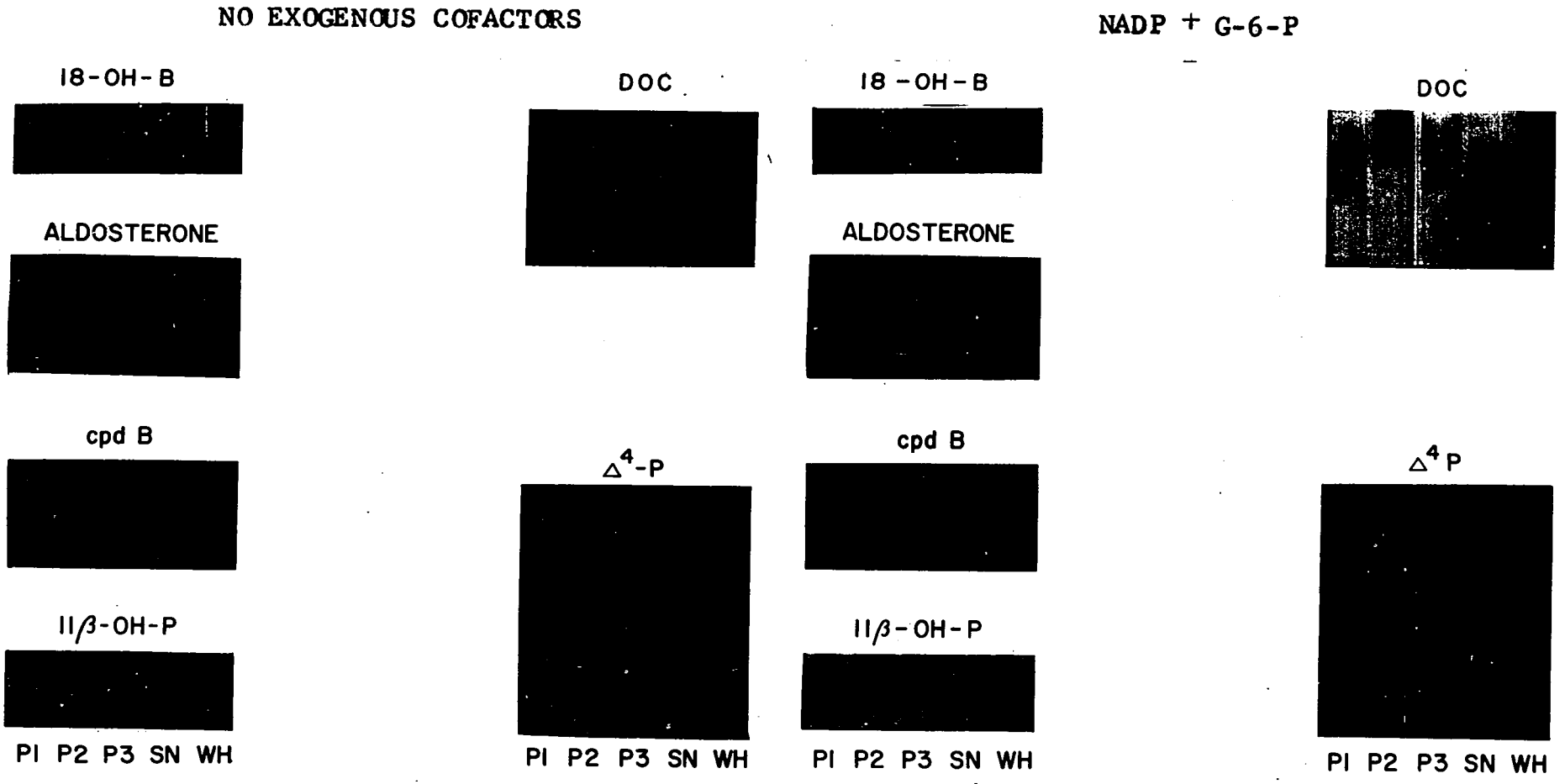
TRANSFORMATION OF PROGESTERONE-4-^{14}C (2 μc) TO CORTICOSTEROIDS BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES FROM TURTLE ADRENAL

STEROIDS ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE									
	P1		P2		P3		SN		WH	
	A	B	A	B	A	B	A	B	A	B
18-OH-B	-	-	-	-	-	-	-	-	-	1.5
Aldosterone	-	-	-	-	-	-	-	-	-	0.4
Corticosterone	-	0.2	-	0.6	-	-	-	-	1.0	13.1
11β-OH-P	-	1.8	-	3.5	-	-	-	-	0.7	7.6
DOC	-	0.9	-	1.3	-	1.5	-	8.3	3.4	21.1
Others	0.5	0.9	0.5	1.3	0.6	1.8	0.6	1.4	1.5	4.6
Total	0.5	3.8	0.5	6.7	0.6	3.3	0.6	9.7	6.6	52.3

A = $KRPO_4$ + fumarate + 0.13M sucrose
B = $KRPO_4$ + fumarate + 0.13M sucrose + NADP+G-6-P

FIGURE 14

RADIOAUTOGRAMS FROM STUDIES OF HOMOGENATES (WH) AND SUBCELLULAR FRACTIONS (TURTLE INTERRENALS) INCUBATED WITH PROGESTERONE-4-^{14}C WITH OR WITHOUT EXOGENOUS NADP PLUS G-6-P.



between 0.5 or 0.6%. It should be noticed that in these fumarate-containing preparations, conversion into well-identified steroids such as corticosterone, 11β-OH-progesterone and 11-deoxycorticosterone took place in homogenates WH only. In the case of subcellular fraction, the incorporation of radioactivity occurred into unknown metabolites. Supplementation with NADPH-generating system enhanced the conversion in all cases, also resulting in the appearance of metabolites which were not detectable in absence of exogenous cofactors.

The nuclei P1 transformed the precursor mainly into 11β-OH-progesterone (1.8%); corticosterone and 11-deoxycorticosterone were also isolated. Mitochondrial preparation P2 in the presence of additives hydroxylated progesterone at carbons 11 and 21, thus resulting in the formation of 11β-OH-progesterone (3.5%), 11-deoxycorticosterone (1.3%) and corticosterone (0.6%). 11-deoxycorticosterone was the only isolated conversion products from both microsomal P3 (1.5%) and supernatant SN (8.3%) preparations. The homogenates WH yielded in presence of the NADPH-generating system 18-hydroxylated compounds in addition to the metabolites isolated without supplementation. 11-deoxycorticosterone and corticosterone, however, were by and large the major conversion products.

Some of the metabolites isolated from these studies are shown in the radioautograms in Figure 14.

2) Δ^5-pregnenolone-4-^{14}C as substrate

Homogenates of 400 mg of the turtle adrenal tissue were prepared in 10 ml of 0.25M sucrose. From these preparations, two sets of homogenates WH and subcellular fractions P1, P2, P3 and SN were obtained as described previously (see III, B, 3). These preparations were poured into beakers containing 1 μc of Δ^5-pregnenolone-4-^{14}C (previously mixed with 100 μg of the authentic material). One set of incubations was supplemented with NADP and G-6-P and the other with NAD only. Final volume of 5 ml was made up with $KRPO_4$ buffer and incubation was carried out for three hours. The $KRPO_4$ used in this study did not contain fumarate.

The results obtained from this study are recorded in Table XXXVI. Total conversion in the presence of NADPH-generating system was highest among the subcellular fractions in SN (30.7%) followed by P3 (11.8%), P1 (10.9%) and P2 (7.0%). The homogenates WH transformed the labelled substrate to Δ^4-3-keto steroids to a greater extent, the total conversion being 71.9%.

Supplementation with NAD resulted in greater conversion of the Δ^5-3β-ol structure into Δ^4-3-keto steroids. Under these conditions, total conversion was highest amongst subcellular fraction in P3 (57.8%) followed by P1 (36.5%), SN (35.0%) and P2 (20.5%). Total conversion by homogenates WH was in the order of 81.8%.

TABLE XXXVI

TRANSFORMATION OF Δ^5-PREGNENOLONE-4-^{14}C (1 μc) TO CORTICOSTEROIDS BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES FROM TURTLE ADRENAL

STEROID ISOLATED	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE									
	P1		P2		P3		SN		WH	
	A	B	A	B	A	B	A	B	A	B
18-OH-B	-	-	-	-	-	-	-	-	1.0	-
Aldosterone	-	-	-	-	-	-	-	-	0.4	-
Corticosterone	0.7	-	-	-	-	-	-	-	15.0	0.5
11β-OH-P	0.8	0.4	0.6	0.7	-	-	-	-	1.8	-
DOC	3.6	0.6	1.1	-	0.6	1.0	11.5	1.1	26.7	29.0
Progesterone	4.2	35.5	4.0	18.0	8.9	54.5	13.1	32.8	17.4	50.0
Others	1.6	-	1.3	1.8	2.3	2.3	6.1	1.1	9.6	2.3
Total	10.9	36.5	7.0	20.5	11.8	57.8	30.7	35.0	71.9	81.8

A = $KRPO_4$ + 0.13M sucrose + NADP+G-6-P
B = $KRPO_4$ + 0.13M sucrose + NAD

Labelled progesterone was isolated as a major conversion product from all the studies irrespective of the cofactors used.

In the presence of NADPH-generating system, P1 transformed the precursor into corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone and progesterone, but mainly into the last mentioned metabolite (4.2%). Addition of NAD only resulted in a great accumulation of progesterone (35.5%). Mitochondrial P2 preparations behaved with either type of supplementation similarly to the corresponding P1 preparations. 11-deoxycorticosterone was the only other metabolite beside progesterone isolated from P3 and SN in the presence of either type of the supplementation. P3 preparation in the presence of NAD exhibited almost twice (57.8%) the capacity of SN (35.0%) to convert Δ^5-pregnenolone-4-^{14}C.

The homogenates WH in the presence of NADP and G-6-P resulted in a variety of conversion products including 18-OH-corticosterone, aldosterone, corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone and progesterone. 11-deoxycorticosterone was the major conversion product (26.7%) followed by progesterone (17.4%) and corticosterone (15.0%). When NAD was the sole additive, progesterone was once more the major metabolite although substantial amount of 11-deoxycorticosterone were also detected.

3) Cholesterol-4-^{14}C as substrate

Exploratory experiments with surviving sections incubated with cholesterol-4-^{14}C yielded negative results as those reported for frog interrenal sections (see IV, 3 a). In view of these findings detailed studies were only carried out with homogenates and subcellular fractions.

Two sets of homogenates and subcellular fractions were prepared from 400 mg of turtle interrenal tissue as described previously (see III, B, 3). These preparations were poured into the beakers containing 2 μc of cholesterol-4-^{14}C. One set, i.e. P1, P2, P3, SN and WH was supplemented with NADPH (0.25 μ moles/ml) and the other one with NADP (0.25 μ moles/ml) plus G-6-P (0.9 μ moles/ml). $KRPO_4$ buffer containing fumarate was added to make a final volume of 5 ml,and incubation was carried out for three hours.

No conversion of the labelled sterol occurred in incubation studies of P3 and SN either in the presence of NADPH-generating system or preformed NADPH. The other preparations, i.e. P1, P2 and WH transformed the labelled precursor in the presence of either type of supplementation as shown in Table XXXVII. Total conversion by all these preparations was of similar order of magnitude (about 2%).

The P1 fraction when supplemented with NADPH transformed

TABLE XXXVII

TRANSFORMATION OF CHOLESTEROL-4-^{14}C (2 μc) TO CHOLESTEROIDS BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES FROM TURTLE ADRENAL

STEROIDS	PERCENTAGE CONVERSION/ = 100 mg OF TISSUE					
	P1		P2		WH	
	A	B	A	B	A	B
18-OH-B	-	0.2	-	-	0.5	0.5
Aldosterone	-	0.1	-	0.1	0.2	0.2
Corticosterone	-	-	-	-	0.2	0.2
11β-OH-P	0.5	-	-	0.7	-	-
DOC	-	-	-	-	0.2	0.2
Progesterone	0.7	0.7	0.9	0.8	0.3	0.6
Δ^5-pregnenolone	0.7	0.9	0.7	0.5	-	-
Others	0.2	0.2	0.2	0.3	0.3	0.3
Total	2.1	2.1	1.8	2.4	1.7	2.0

A = $KRPO_4$ + fumarate + 0.13M sucrose + NADPH
B = $KRPO_4$ + fumarate + 0.13M sucrose + NADP+G-6-P

the precursor into 11β-OH-progesterone, progesterone and Δ^5-pregnenolone. However, in the presence of NADPH-generating system, two more polar steroids, i.e. 18-OH-corticosterone and aldosterone were also detected beside progesterone and Δ^5-pregnenolone. The last two mentioned steroids were also isolated from mitochondrial P2 incubation studies supplemented with either NADPH or NADP plus G-6-P. The homogenates WH with either type of supplementation, catabolize the labelled sterol into a variety of corticosteroids which ranged in polarity from 18-OH-corticosterone to progesterone.

4) Acetate-1-^{14}C as substrate

Homogenized preparations of turtle interrenal were not used in studies with acetate-1-^{14}C for the reason discussed previously (see IV, 4, under 'Discussion').

Six aliquots of 160 mg of turtle interrenal sections were preincubated in 5 ml KRBG for 15 minutes. After this period of preincubation, the aliquots were transferred into beakers containing 200 μc of acetate-1-^{14}C dissolved in KRBG. Additives, where indicated, were introduced into the systems and all incubates made up to 5 ml with KRBG. The following experimental vessels were set up: a) no supplementation, b) NADP plus G-6-P, c) NAD, d) 3', 5' AMP, e) 3', 5' AMP D.B. and f) mammalian ACTH. In all cases the final incubation lasted three hours.

Media and tissues (after homogenization) from all these incubates were analyzed separately after the addition of 100 µg of radioinert aldosterone, corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone, Δ^5-pregnenolone, progesterone and cholesterol.

Radioautograms from media extracts (exposure 10 days) showed no conversion products in any of the present studies. Radioassays conducted in eluates from zones containing the added radioinert authentic materials revealed that in each case the radioactivity was almost equivalent to blank values.

Radioautograms from tissue extracts showed a single radioactive zone in the position of authentic cholesterol. The non-identity of this metabolite with authentic cholesterol was established subsequently (see 'Identification of Steroids', 5, h).

Discussion

The biotransformation of radioactive substrates by turtle interrenal is in general strikingly analogous to that discussed for frog interrenals. The present results will be discussed collectively under this heading, pointing out the salient metabolic similarities and differences encountered between the reptilian and the amphibian interrenals.

As already indicated, emphasis has been placed in the

utilization of labelled substrates by homogenates (WH) or subcellular fraction from the turtle interrenals. Studies with surviving sections are already well documented by the previous studies published by Sandor and his group (48, 57) at least in regards to the metabolism of progesterone and Δ^5-pregnenolone.

Progesterone-4-^{14}C was incorporated by the turtle adrenal homogenates (WH) into 18-OH-corticosterone, aldosterone, 11β-OH-progesterone and 11-deoxycorticosterone. These results indicate the presence of 11β-, 21- and 18-hydroxylases in the adrenal of this reptilian as well as the absence of 17α-hydroxylase. With the exception of 11β-OH-progesterone, Sandor et al (57) found the same pattern of metabolites from incubation of this substrate with surviving section of the turtle interrenal.

An identical spectrum of reaction products was obtained in comparable experiments with frog interrenal homogenates (WH) reported in this thesis.

Like in the frog interrenal experiments, these observations also indicate the capacity of the turtle interrenal to generate NADPH from exogenous NADP and G-6-P, suggesting the presence of G-6-P-dehydrogenase in this tissue. This suggestion will be confirmed by the finding of this hexose monophosphate

shunt enzyme in histochemical studies to be reported later on (see IV, section B, 6).

The studies with subcellular fractions have essentially yielded identical results as in the case of frog interrenal. 11β-hydroxylase has been found to be mainly a mitochondrial bound enzyme and 21-hydroxylase mainly a microsomal bound enzyme, occurring also in the soluble fraction. One point of interest is, that in the present studies 18-hydroxylase activity could not be demonstrated in the mitochondrial fraction as in the case of frog adrenal studies.

Δ^5-pregnenolone-4-^{14}C was transformed by the turtle interrenal homogenates (WH) into 18-OH-corticosterone, aldosterone, corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone and progesterone, reproducing the same pattern of metabolites as the corresponding amphibian preparations. The results also duplicate studies by Sandor et al (57) with surviving sections of turtle interrenal with the exceptions of 11β-OH-progesterone which has also been isolated in our studies.

Δ^5-3 -hydroxysteroid-dehydrogenase-isomerase was supported by both NADPH (generated 'in situ' from NADP and G-6-P) and by NAD as in the frog interrenal. This enzyme also appears in all subcellular fractions in the reptilian adrenal tissue.

The main differences between the studies with the reptilian

and the amphibian interrenals was the higher enzymatic activity displayed by the former tissue in the metabolism of Δ^5-pregnenolone-4-^{14}C and the failure to demonstrate in it the mitochondrial bound 18-hydroxylase, already encountered in the studies with progesterone-4-^{14}C.

It should also be pointed out that in the turtle interrenal subcellular fractions and homogenates (WH) NAD did not promote the conversion of Δ^5-pregnenolone exclusively to progesterone (in terms of identifiable reactants) as it happened in frog interrenal. For some unexplained reasons, NAD stimulated in these fumarate-free turtle interrenal preparations 21-hydroxylase beside Δ^5-3β-hydroxysteroid-dehydrogenase-isomerase. This phenomenon was particularly striking in the case of homogenates (WH).

The transformation of cholesterol-4-^{14}C by homogenates (WH) and subcellular fractions of reptilian and amphibian interrenals presented a few differences in the appearance of some metabolites. In general, there was a tendency in turtle interrenal preparation for the appearance of early intermediates such as progesterone and/or Δ^5-pregnenolone. Reptilian adrenal tissue seemed also to possess a higher side chain cleavage activity than the amphibian one. In the turtle interrenal, this activity was detected in the nuclear fraction P1, in

addition to the mitochondrial P2 fraction.

Under the experimental conditions explored, turtle interrenal surviving sections failed to incorporate acetate-1-^{14}C into either cholesterol or corticosteroids reproducing the data obtained with frog interrenals.

5) Identification of Steroids

Resolution of extracts from turtle adrenal incubates was carried out in the same paper chromatographic scheme which was used for frog adrenal studies.

Here again, the final characterization was carried out on pools of similar reaction products obtained from the various experiments. This was done only after a preliminary screening of the behaviour of the compound in question in at least two additional solvent systems after addition of 100 µg of carrier. Usually, thin-layer chromatography solvent systems were used for the chromatography of the reactants isolated from turtle adrenal incubates. The identification of various steroids is described according to their decreasing polarity.

a) 18-OH-corticosterone

This steroid was detected in incubation studies with progesterone-4-^{14}C, Δ^5-pregnenolone-4-^{14}C and cholesterol-4-^{14}C. It appeared in fraction A (TEG systems 48 hrs.).

The radioactive zone after elution was quickly treated

with periodic acid as described earlier (185). The oxidized material was applied on Whatman paper #2 on Bush T system (4 1/2 hrs.). The Rf value (0.13) of this compound was similar to that reported by Tait et al (185) for 11β, 18-dehydroxy-3-keto-4-etienic acid lactone (periodic acid oxidations product of 18-OH-B).

b) Aldosterone

A compound in the position of authentic aldosterone (fractions A TEG 48 hrs.) has been consistently found mainly in incubates of homogenates WH of turtle interrenal using progesterone-4-^{14}C, Δ^5-pregnenolone-4-^{14}C and cholesterol-4-^{14}C. After addition of carrier authentic aldosterone, the radioactive material remained associated with the added mineralocorticoid in the TLC1 and TLC2 systems.

Recently purified aldosterone-1, 2-^{3}H was added to the aliquots of the material and rechromatographed in above mentioned systems. Acetylation by fast microtechnique was carried out, and the resultant 21-mono-acetate in each case was chromatographed in the TLC1 and TLC2 systems. Pure aldosterone and its 21-mono-acetate served as standards. After each chromatography, samples were taken and counted for ^{3}H and ^{14}C. These ratios of free and acetylated compound are shown in Table XXXVIII.

A separate pool of ^{14}C-labelled aldosterone isolated

TABLE XXXVIII

CHARACTERIZATION OF ALDOSTERONE-4-^{14}C (TURTLE ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVE IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITES	$^{3}H:^{14}C$ RATIOS			
	STARTING MATERIAL Aldosterone		DERIVATIVE (ACETYLATION) Aldosterone-21-acetate	
	TLC1[1]	TLC2	TLC1	TLC2
Cholesterol-4-^{14}C	7.0	7.0	6.9	7.0
Δ^{5}-pregnenolone-4-^{14}C	8.3	8.1	8.1	7.9
Progesterone-4-^{14}C	6.1	6.1	6.1	6.2

*Isolated from various incubations of homogenates WH and mitochondrial (P2) of turtle adrenals using above mentioned substrates.
1. Chromatographic solvent systems.

TABLE XXXIX

CHARACTERIZATION OF ALDOSTERONE-4-^{14}C (TURTLE ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF ALDOSTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	1285	215	6.0
Mother Liquor	1280	225	5.7
II			
Crystals	1300	222	5.9
Mother Liquor	1321	228	5.8
III			
Crystals	1310	225	5.9
Mother Liquor	1380	239	5.7

*Isolated from various incubations of homogenates and subcellular fractions (P2) of turtle adrenal using progesterone-4-^{14}C, Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C as substrates.

from the above mentioned substrate was made. It was mixed with purified aldosterone-1, 2-^{3}H and 25 mg of authentic material. Three microcrystallizations were carried out using acetone and pentane (189). Table XXXIX records the specific activities of crystals and mother liquor and also ^{3}H:^{14}C ratios, obtained in each case. The values permit claiming the radiochemical homogeneity of ^{14}C-labelled material.

c) Corticosterone

This band has been encountered (fraction C, E_2B 4 1/2 hrs.) in practically all the studies with labelled progesterone, Δ^5-pregnenolone and cholesterol. After addition of authentic carrier, it travelled concurrently with it in the TLC1 and TLC2 systems.

Aliquots were taken from these materials, and purified corticosterone-1, 2-^{3}H was added to them. After rechromatography in above mentioned systems, the material was oxidized with CrO_3 (183). The resultant compound A was chromatographed in the TLC1 and TLC2 systems. Authentic compound B and A served as standards. The 11-dehydrocorticosterone obtained was further acetylated by the fast microtechnique (181). The acetylated material was rechromatographed on the TLC1 and TLC2 systems. Stable compound A (both free and acetate) served as standard. Samples were taken after each chromatography and counted for

TABLE XL

CHARACTERIZATION OF CORTICOSTERONE-4-^{14}C (TURTLE ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVES IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS					
	STARTING MATERIAL 'B'		DERIVATIVES CrO_3 OXIDATION 'A'		ACETYLATION OF OXIDATION PRODUCT 'A-Acetate'	
	TLC1[1]	TLC2	TLC1	TLC2	TLC1	TLC2
Cholesterol-4-^{14}C	4.9	5.0	5.0	5.1	5.0	5.0
Δ^5-pregnenolone-4-^{14}C	8.9	8.8	8.4	8.6	8.8	8.7
Progesterone-4-^{14}C	7.4	7.4	7.3	7.5	7.5	7.5

*Isolated from various incubations of homogenates WH and mitochondrial (P2) of turtle adrenals using above mentioned substrates.
1. Chromatographic solvent systems.

TABLE XLI

CHARACTERIZATION OF CORTICOSTERONE-4-^{14}C (TURTLE ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF CORTICOSTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	4297	882	4.9
Mother Liquor	4319	872	5.0
II			
Crystals	4193	859	4.9
Mother Liquor	4235	862	4.9
III			
Crystals	4188	857	4.9
Mother Liquor	4103	851	4.9

*Isolated from various incubations of homogenates and various subcellular fractions (P2) of turtle adrenals using progesterone-4-^{14}C, Δ^{5}-pregnenolone-4-^{14}C and cholesterole-4-^{14}C as substrates.

^{3}H and ^{14}C. Table XL shows the ^{3}H:^{14}C ratios.

A separate pool of ^{14}C-labelled corticosterone isolated from different substrates was mixed with purified corticosterone-1, 2-^{3}H and 25 mg of authentic material. Three microcrystallizations were carried out using acetone and pentane (189). Specific activities of crystals and mother liquors and ^{3}H:^{14}C ratios in each case are recorded in Table XLI.

d) 11β-OH-progesterone.

A radioactive zone in the position of authentic 11β-OH-progesterone (fraction D, LPG 24 hrs.) in incubation studies of subcellular fractions or WH with progesterone-4-^{14}C, Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C has been found. Upon rechromatography, after addition of stable 11β-OH-progesterone, the radioactive band from the above mentioned sources remained associated with the carrier in the TLC1 and TLC2 systems.

Two separate pools of 11β-OH-progesterone were prepared, one from homogenates WH and the other from mitochondrial P2 studies., Aliquots from these pools were chromatographed in the Bush A (16 hrs.) and LPG (36 hrs.) systems. Oxidation with CrO_3 (183) was carried out, and the resultant oxidized product was chromatographed in the Bush A (20 hrs.) and LPG (36 hrs.) systems. Authentic 11β-OH- and 11-keto progesterone served as

TABLE XLII

CHARACTERIZATION OF 11β-OH-PROGESTERONE-4-^{14}C (TURTLE ADRENAL)*

a) S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF THE METABOLITE

CRYSTALLIZATION OF 11β-OH-PROGESTERONE	^{14}C cpm/mg
I	
Crystals	10137
Mother Liquor	12330
II	
Crystals	9867
Mother Liquor	11040
III	
Crystals	9932
Mother Liquor	9575

*Isolated from incubations of homogenates and mitochondrial (P2) of turtle adrenal using progesterone-4-^{14}C, Δ^5-pregnenolone-4-^{14}C and cholesterol-4-^{14}C.

TABLE XLIII

CHARACTERIZATION OF 11β-OH-PROGESTERONE-4-^{14}C (TURTLE ADRENAL)*

b) S.A. AFTER SUCCESSIVE CRYSTALLIZATION FOLLOWING CrO_3 TREATMENT OF METABOLITE

CRYSTALLIZATION OF 11-KETO-PROGESTERONE (CrO_3 oxidation product)	SOURCE OF ORIGINAL METABOLITE ^{14}C cpm/mg	
	SECTIONS & HOMOGENATES	MITOCHONDRIAL (P2)
I		
Crystals	674	603
Mother Liquor	713	853
II		
Crystals	677	603
Mother Liquor	722	662
III		
Crystals	695	613
Mother Liquor	750	606

*Isolated from incubations of homogenates and mitochondrial (P2) of turtle adrenals using progesterone-4-^{14}C, Δ^5-pregnenolone-4-^{14}C and cholesterol-4-^{14}C as precursors.

running mates.

The 11β-OH-progesterone and its CrO_3 oxidized product were crystallized independently. In each case 25 mg of the corresponding authentic material was mixed and microcrystallizations were carried out (189).

Tables XLII and XLIII show the specific activities of crystals and mother liquors.

e) 11-deoxycorticosterone

This steroid has been detected as a conversion product from most of the incubation studies with progesterone-4-^{14}C, Δ^5-pregnenolone-4-^{14}C and cholesterol-4-^{14}C. It has a mobility identical to that of 11-deoxycorticosterone in fraction D (LPG system, 24 hrs.). After addition of carrier 11-deoxycorticosterone, it travelled concurrently with it in the TLC2 systems.

Purified authentic 11-deoxycorticosterone-1, 2-^{3}H was added to aliquots of the compound isolated from different substrates. Rechromatography was carried out in the TLC1 and TLC2 systems. The material was acetylated with the fast microtechnique (181). The resultant acetate was chromatographed in the TLC1 and TLC2 systems. Authentic DOC and DOCA served as standards. The acetylated compound was hydrolized using a modified method of that recommended by Bush (182). The free

TABLE XLIV

CHARACTERIZATION OF 11-DEOXYCORTICOSTERONE-4-^{14}C (TURTLE ADRENALS)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVES IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS					
	STARTING MATERIAL DOC		DERIVATIVES ACETYLATION DOCA		HYDROLYSIS OF DOCA DOC	
	TLC1[1]	TLC2	TLC1	TLC8	TLC2	TLC8
Cholesterol-4-^{14}C	5.4	5.4	5.6	5.4	-	-
Δ^{5}-pregnenolone-4-^{14}C	4.8	4.8	4.8	4.9	4.7	4.7
Progesterone-4-^{14}C	6.3	6.6	6.4	6.4	6.6	6.6

*Isolated from various incubations of subcellular fractions (P3, SN) and homogenates WH of turtle adrenal glands using above mentioned substrates.

1. Chromatographic solvent systems.

TABLE XLV

CHARACTERIZATION OF 11-DEOXYCORTICOSTERONE-4-^{14}C (TURTLE ADRENAL)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF 11-DEOXYCORTICOSTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	2442	427	5.7
Mother Liquor	2464	417	5.9
II			
Crystals	2338	404	5.8
Mother Liquor	2380	407	5.8
III			
Crystals	2333	402	5.8
Mother Liquor	2248	396	5.7

*Isolated from various incubations of various subcellular fractions (P3, SN) and homogenates of turtle adrenal, using progesterone-4-^{14}C, Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (WH only) as substrates.

compound was rechromatographed on the TLC2 and TLC8 systems. Samples were taken after each chromatography to establish $^{3}H:^{14}C$ ratios. The values of these ratios are recorded in Table XLIV.

The free compound was mixed with 25 mg of authentic 11-deoxycorticosterone. Three crystallizations were carried out using acetone and pentane (189). Table XLV records the specific activities of crystals and mother liquor and $^{3}H:^{14}C$ ratios in each case.

f) Δ^{5}-pregnenolone.

This steroid was only detected in studies with cholesterole-4-^{14}C using nuclei P1 and mitochondrial P2 preparations. It has a mobility identical to that of authentic Δ^{5}-pregnenolone in fraction D (LPG system, 24 hrs.). After addition of authentic carrier, it travelled concurrently with it in the TLC1 and TLC2 systems.

Recently purified Δ^{5}-pregnenolone-7-^{3}H was added to aliquots of ^{14}C-material from each source. Rechromatography was carried out in the TLC1, TLC2 and TLC5 systems. The $^{3}H:^{14}C$ mixture was oxidized using Oppenauer oxidation method (184). The oxidized product, i.e. progesterone, was chromatographed on the TLC1 and TLC2 systems. Authentic Δ^{5}-pregnenolone and progesterone served as standards. Samples were taken after each chromatography and counted for ^{3}H and ^{14}C. Table XLVI

TABLE XLVI

CHARACTERIZATION OF Δ^5-PREGNENOLONE-4-^{14}C (TURTLE ADRENALS)*

a) ^{3}H:^{14}C RATIOS OF METABOLITE AND DERIVATIVE IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	^{3}H:^{14}C RATIOS			
	STARTING MATERIAL Δ^5-pregnenolone		DERIVATIVE OPPENAUER OXIDATION Progesterone	
	TLC1[1]	TLC2	TLC1	TLC2
P1	11.9	11.9	9.6	9.9
P2	12.3	13.1	10.2	9.3

*Isolated from incubations of various subcellular fractions (P1, P2) of turtle adrenals using cholesterol-4-^{14}C as substrate.

1. Chromatographic solvent systems.

TABLE XLVII

CHARACTERIZATION OF Δ^5-PREGNENOLONE-4-^{14}C (TURTLE ADRENALS)*

b) ^3H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

SOURCE OF METABOLITE CRYSTALLIZATION OF Δ^5-PREGNENOLONE	^3H cpm/mg	^{14}C cpm/mg	^3H:^{14}C
P1			
I			
Crystals	2129	179	11.9
Mother Liquor	3424	242	14.1
II			
Crystals	2198	185	11.9
Mother Liquor	2382	200	11.9
III			
Crystals	2105	183	11.5
Mother Liquor	1850	112	16.5
P2			
I			
Crystals	1274	98	13.0
Mother Liquor	1896	135	14.0
II			
Crystals	1282	99	13.0
Mother Liquor	1615	91	17.8
III			
Crystals	1244	96	13.0
Mother Liquor	1345	101	13.3

*Isolated from incubations of various subcellular fractions (P1, P2) of turtle adrenal gland using cholesterol-4-^{14}C as substrate.

shows the $^{3}H:^{14}C$ ratios from these derivatives.

Authentic 25 mg of Δ^5-pregnenolone was mixed with $^{3}H:^{14}C$ mixture from both sources (P1 and P2). Three crystallizations were carried out in each case. The specific activities of crystals and mother liquors and $^{3}H:^{14}C$ ratios in each case are recorded in Table XLVII.

g) Progesterone

This steroid was detected in studies with Δ^5-pregnenolone and cholesterol-4-^{14}C. It has a mobility identical to that of progesterone in fraction D (LPG system, 4 hrs.). After addition of the carrier, it travelled concurrently with it in the TLC3 and TLC9 systems. The material isolated from each subcellular fraction was treated separately, whereas a single pool was prepared from materials isolated from studies with homogenates WH (both from cholesterol-4-^{14}C and Δ^5-pregnenolone-4-^{14}C).

Purified progesterone-1, 2-^{3}H was added to the aliquots of the material and rechromatographed in the above mentioned TLC systems. Enzymatic reduction with 20β-hydroxysteroid-dehydrogenase (188) was carried out, and the resultant 20β-hydroxy derivative of progesterone was chromatographed in TLC2 and TLC10 systems. Pure progesterone and 20β-OH-progesterone served as standards. The 20β-OH-progesterone thus obtained was acetylated by the fast microtechnique (181). The resultant

TABLE XLVIII

CHARACTERIZATION OF PROGESTERONE-4-^{14}C (TURTLE ADRENAL)*

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE AND DERIVATIVES IN VARIOUS SOLVENT SYSTEMS

SOURCE OF METABOLITE	$^{3}H:^{14}C$ RATIOS					
	STARTING MATERIAL Progesterone		ENZYMATIC REDUCTION[1] 20β-OH-Δ4P		ACETYLATION OF REDUCTION PRODUCT 20β-OH-Δ4P-Acetate	
	TLC3[2]	TLC9	TLC2	TLC10	TLC4	TLC3
P1	8.4	8.3	8.4	7.9	7.6	7.6
P2	9.1	9.1	8.9	8.9	8.9	8.9
P3	6.1	6.1	6.1	6.1	6.2	6.1
SN	7.7	7.5	7.0	7.0	6.9	7.0
WH	8.1	8.1	7.5	7.5	7.1	7.2

*Isolated from incubations of various subcellular fractions (P1, P2, P3, SN) and homogenates (WH) of turtle adrenals using Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (P1, P2, WH only) as substrates.

1. With 20β-hydroxysteroid dehydrogenase (see Methods and Materials).
2. Chromatographic solvent systems.

TABLE XLIX

CHARACTERIZATION OF PROGESTERONE-4-^{14}C (TURTLE ADRENALS)*

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

CRYSTALLIZATION OF PROGESTERONE	^{3}H cpm/mg	^{14}C cpm/mg	^{3}H:^{14}C
I			
Crystals	3037	490	6.2
Mother Liquor	3000	482	6.2
II			
Crystals	2933	470	6.2
Mother Liquor	2975	472	6.3
III			
Crystals	2928	467	6.3
Mother Liquor	2798	445	6.3

*Isolated from incubates of homogenates (WH) and various subcellular fraction (P1, P2, P3, SN) of turtle adrenals using Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (P1, P2 and WH only).

TABLE L

CHARACTERIZATION OF PROGESTERONE-4-^{14}C (TURTLE ADRENAL)*

c) $^{3}H:^{14}C$ RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION FOLLOWING ENZYMATIC TREATMENT OF METABOLITE WITH 20β-HYDROXY-STEROID DEHYDROGENASE

CRYSTALLIZATION OF 20β-OH-PROGESTERONE (reduced derivative)	^{3}H cpm/mg	^{14}C cpm/mg	$^{3}H:^{14}C$
I			
Crystals	577	82	7.0
Mother Liquor	680	96	7.1
II			
Crystals	589	83	7.1
Mother Liquor	642	92	7.0
III			
Crystals	580	81	7.1
Mother Liquor	590	83	7.1

*Isolated from incubations of homogenates (WH) and various subcellular fractions (P1, P2, P3, SN) of turtle adrenals using Δ^{5}-pregnenolone-4-^{14}C and cholesterol-4-^{14}C (P1, P2 and WH only).

acetate in each case was rechromatographed in the TLC4 and TLC3 systems. Authentic free progesterone and 21-acetate served as running mates. Samples were taken after each chromatography and counted for ^{3}H and ^{14}C. Table XLVIII shows the ^{3}H:^{14}C ratios of these derivatives.

Progesterone and its 20β-hydroxy derivative were each crystallized independently. In each case 25 mg of the corresponding authentic material was mixed and microcrystallizations were carried out using acetone and pentane (189). Tables XLIX and L record the specific activities of crystals and mother liquors along with ^{3}H:^{14}C ratios in each case.

h) Metabolite II

Tissue extracts of incubates with acetate-1-^{14}C invariably showed a radioactive band in fraction D' (LPG system 4 hrs.) coinciding with the chromatographic position of authentic cholesterol.

This ^{14}C-labelled material was analyzed similarly as described previously (see IV, section A, i). However, we failed to establish the identity of metabolite II as cholesterol similar to our failure in the case of frog interrenal studies with acetate-1-^{14}C.

i) Other metabolites

Several other metabolites were detected scattered through-

out the different fractions of the separating chromatographic scheme. However, their further identification was not pursued for the similar reasons stated in the corresponding section of the studies with frog adrenal glands.

6. Histochemical studies

G-6-P dehydrogenase and 6-phosphogluconate dehydrogenase were also investigated in turtle interrenals for the same reasons stated in the corresponding section of the studies with frog adrenal glands. For this purpose, histochemical studies were also carried out in collaboration with Dr. J.M. Rojo-Ortega following the same methodology indicated previously (229, 230).

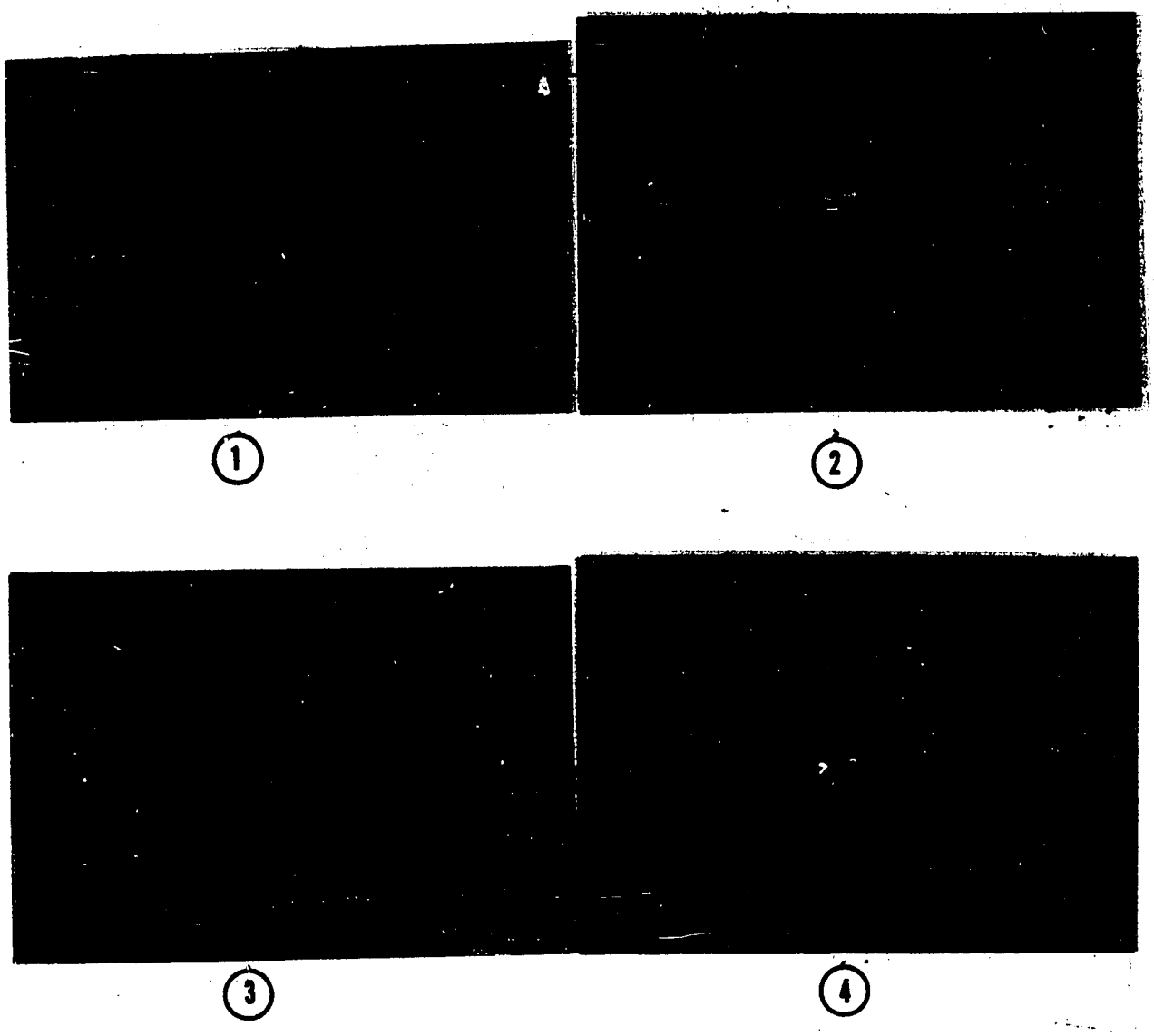
The photomicrographs in Figure 15 show that G-6-P dehydrogenase (1) and 6-phosphogluconate dehydrogenase (2) were also demonstrable in turtle interrenals.

As in the case of frog interrenals, these findings explain the effectiveness of exogenous NADP and G-6-P in our incubations with labelled precursors. These dehydrogenases by transforming NADP into NADPH, supply to the <u>in vitro</u> system the reduced nucleotide, mediating such steroid enzymatic reactions as hydroxylations and the cleavage of the cholesterol molecule.

The same figure shows the presence of NADPH-diaphorase (3) and NADH-diaphorase (4) in turtle adrenal tissue.

FIGURE 15

HISTOCHEMICAL STUDIES WITH TURTLE INTERRENALS.



(1) Glucose-6-phosphate dehydrogenase

(2) 6-Phosphogluconate dehydrogenase

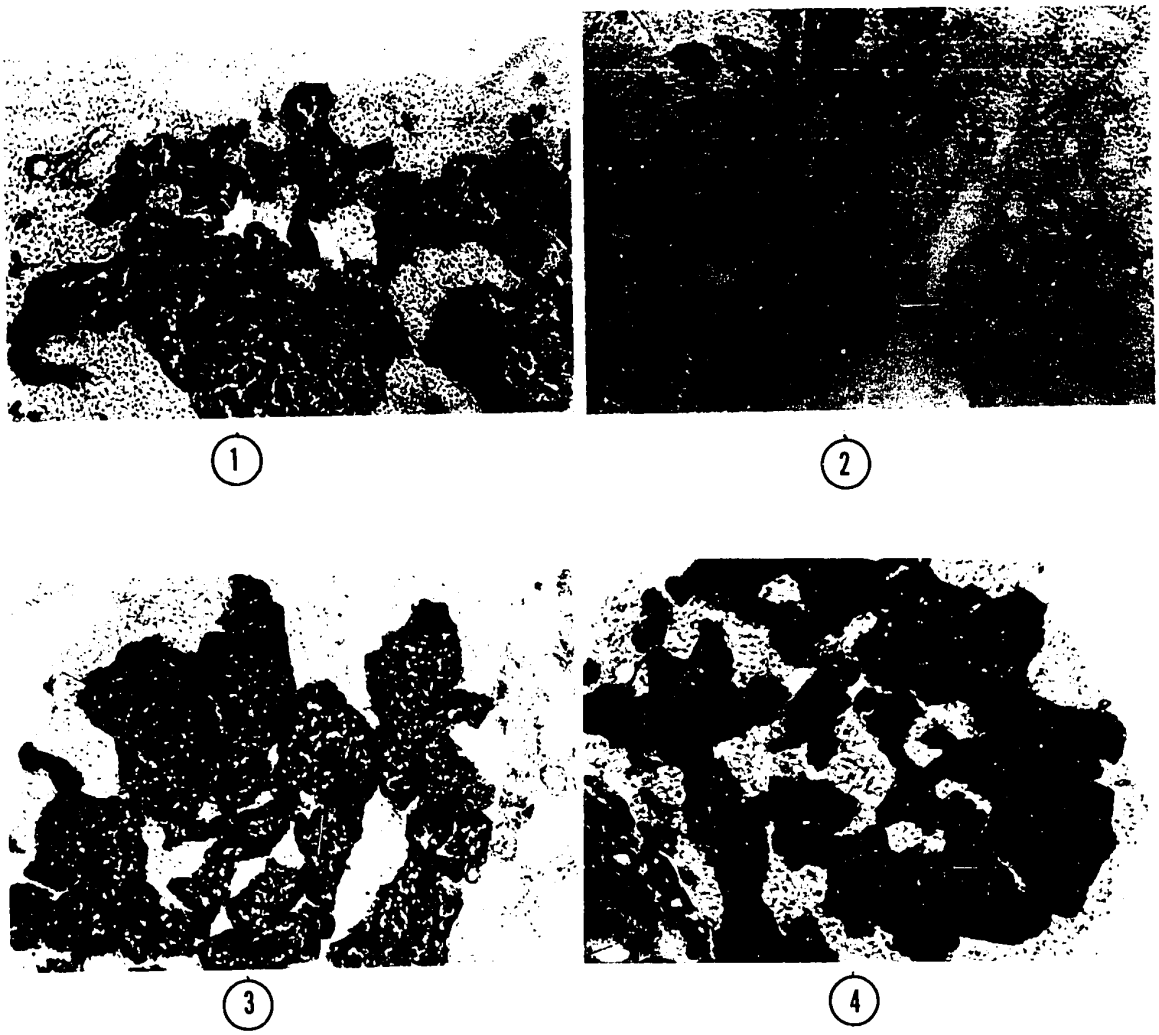
(3) NADPH-diaphorase

(4) NAD-diaphorase

In all cases magnification 6.3 x 13.

FIGURE 15

HISTOCHEMICAL STUDIES WITH TURTLE INTERRENALS.



(1) Glucose-6-phosphate dehydrogenase

(2) 6-Phosphogluconate dehydrogenase

(3) NADPH-diaphorase

(4) NAD-diaphorase

In all cases magnification 6.3 x 13.

V GENERAL DISCUSSION

As proposed in 'Purpose of the Investigation', we have attempted with the aid of labelled steroid precursors to elucidate whether or not the process of corticosteroid biosynthesis *in vitro* evolves in the frog and turtle interrenal glands through the sequence acetate ⟶ cholesterol ⟶ Δ^5-pregnenolone ⟶ progesterone ⟶ corticosteroids, as has been demonstrated for the mammalian (90, 97, 233) and avian (48, 72, 74) adrenals.

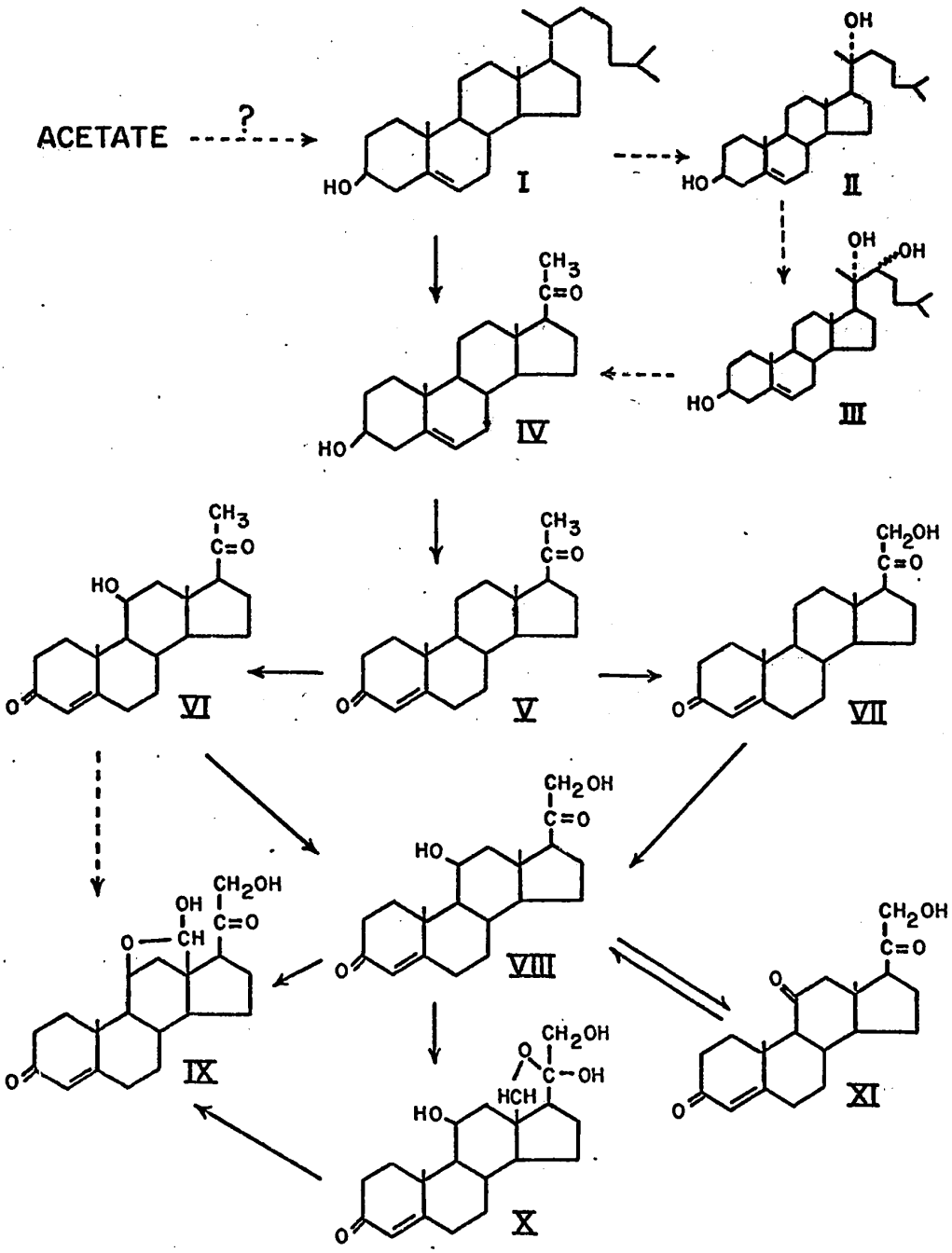
The results of our investigation permit us to propose the pathways of steroidogenesis depicted in Figure 16 for the frog and turtle interrenal glands. The scheme clearly indicates that from the cleavage of the molecule of cholesterol onwards, the synthesis of corticosteroids in frog and turtle interrenals is very similar to that encountered in aves (48, 72) and non-cortisol producing mammals (101, 214).

On the other hand, the results suggest that contrary to the situations postulated and proven for the mammalian (or avian) adrenals, the process of corticosteroidogenesis does not start from acetate in the two types of interrenal under study.

This suggestion is based in the repeated failure of frog and turtle interrenal preparations to incorporate radioacetate into cholesterol (and/or corticosteroids) under the multiple

FIGURE 16

PROPOSED SCHEME OF 'IN VITRO' BIOSYNTHETIC PATHWAYS IN FROG AND TURTLE INTERRENALS.



I	Cholesterol	IV	Δ^5-P	VIII	Cpd. B
II	20α-OH-Cholesterol	V	Δ^4-P	IX	Aldosterone
III	20α, 20ξ-Dihydroxy-cholesterol	VI	11β-OH-P	X	18-OH-B
		VII	DOC	XI	Cpd. A (frog only)

in vitro experimental conditions investigated. It should be recalled, that, with the exception of the temperature of incubation, these conditions were almost identical to those of *in vitro* mammalian or avian adrenal systems achieving the incorporation or the 2-carbon fragment into C_{27} sterol and/or cortical hormones. We have even carried out studies in absence of glucose in the medium after the successful studies by Savard's group (225) with the idea of not diluting our radioacetate with radioinert 2-carbon fragments that might result from the *in vitro* catabolism of exogenous glucose (234).

While all our attempts have met with failure, we prefer, however, to put a question mark in the step acetate ——→cholesterol as shown in Figure 16, rather than to dismiss altogether its possible occurrence under other experimental conditions that we have not been able to explore thus far.

If cholesterol, however, is the first station in the pathway of corticosteroidogenesis in frog and turtle interrenals, as our studies so strongly indicate, plasma free cholesterol could be the source of this precursor from which the interrenal cells of these vertebrates elaborate their adrenocortical hormones.

This speculation is based on the postulation by Morris and Chaikoff (99) and Krum et al (100) that plasma cholesterol might be directly utilized to synthesize corticosteroids by the

in vitro experimental conditions investigated. It should be recalled, that, with the exception of the temperature of incubation, these conditions were almost identical to those of in vitro mammalian or avian adrenal systems achieving the incorporation or the 2-carbon fragment into C_{27} sterol and/or cortical hormones. We have even carried out studies in absence of glucose in the medium after the successful studies by Savard's group (225) with the idea of not diluting our radioacetate with radioinert 2-carbon fragments that might result from the in vitro catabolism of exogenous glucose (234).

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If cholesterol, however, is the first station in the pathway of corticosteroidogenesis in frog and turtle interrenals, as our studies so strongly indicate, plasma free cholesterol could be the source of this precursor from which the interrenal cells of these vertebrates elaborate their adrenocortical hormones.

This speculation is based on the postulation by Morris and Chaikoff (99) and Krum et al (100) that plasma cholesterol might be directly utilized to synthesize corticosteroids by the

adrenal gland. This situation has been postulated for the mammalian adrenal glands which utilize acetate for the elaboration of adrenal cholesterol and corticosteroids. This idea seems even more attractive for the frog and turtle interrenals which appear to lack the enzymatic systems concerned with the acetate——→cholesterol step. However, the radiocholesterol feeding studies that led the above mentioned authors to suggest this alternative in corticosteroidogenesis in mammals have not been carried out with amphibians or reptilians to our knowledge.

It is pertinent to recall in these regards that Sandor et al (21) have made similar speculation in their <u>in vitro</u> studies with cardinal vein strippings (containing interrenal tissue) from the <u>Anguilla anguilla</u> (eel) conducted in presence of radioacetate.

Compounds II and III have been included in Figure 16 as theoretical intermediates between cholesterol and Δ^5-pregnenolone extrapolating the data contributed by Shimizu et al (108-110) to the elucidation of the mechanism of the side-chain scission of cholesterol by mammalian adrenal preparations. In our studies with frog and turtle interrenals, we have not been able to demonstrate their presence in the studies with radiocholesterol. It is possible that these hydroxylated derivatives of cholesterol are rapidly metabolized into Δ^5-pregnenolone,

thus accounting for the difficulty in their detection. It should be pointed out that compounds II and II have also failed to appear in incubation studies carried out in this Department with cholesterol-4-^{14}C using different bovine (78) and rat (217) adrenal preparations or rat (235) testicular homogenates.

Compound VI in Figure 16 is 11β-OH-progesterone. This compound is a logical intermediate in the metabolism of progesterone into corticosterone, aldosterone and 18-OH-corticosterone. However, most <u>in vitro</u> studies with adrenal from various classes of vertebrates fail to prove its presence in the incubation medium (21, 22, 46, 47, 57, 78, 79). Our studies with surviving sections and homogenates yielded extremely small amounts of this metabolite. However, in incubations with mitochondria from frog and turtle interrenals, this compound appeared to be a major metabolite of progesterone-4-^{14}C. This seems to indicate that 11β-OH-progesterone is a possible station in the elaboration of corticosterone (and perhaps other corticosteroids) and that the failure to detect it in preparations with a full array of steroid enzyme (surviving sections and homogenates) is due to its rapid conversion into corticosterone or other metabolites (49).

Regarding our mitochondrial preparations, there is an issue that we would like to discuss at this point. The issue

is the presence of 21-hydroxylase activity in this mitochondrial preparation as evinced by the isolation of metabolites like corticosterone, 18-OH-corticosterone and aldosterone. 21-Hydroxylase has been proven to be associated with the microsomal and supernatant fractions of mammalian adrenals (134). Our studies indicate that this is also the case for frog and turtle interrenals. The isolation of 21-hydroxylated steroids from mitochondrial preparations indicates their contamination with microsomes in spite of our efforts to produce as pure a mitochondrial pellet as possible. This contamination seems almost inevitable with our present-day techniques for the ultracentrifugal separation of adrenal organelles. An example of this situation is offered by the studies of Halkerston et al (106) in which the incubation of cholesterol-4-^{14}C with carefully isolated mitochondrial pellets resulted in the detection of metabolites like cortisol in whose elaboration microsomal-bound 17α- and 21-hydroxylases are involved.

The metabolic activity displayed by fraction P1 (nuclei and debris) is also probably due to contamination, in this case with both mitochondria and microsomes.

Recapitulating, the Rana catesbeiana (American bullfrog) and the Chrysemys picta picta (painted turtle) interrenals possess 20-22 'desmolase activity', Δ^5-3β-hydroxysteroid-de-

hydrogenase-isomerase and 11β-, 18- and 21-hydroxylases.

These interrenals appear to lack the ability to utilize radioacetate. This is a major distinction from the process of steroidogenesis observed in aves and non-cortisol producing mammals.

They do not possess demonstrable 17α-hydroxylase activity like most amphibians, reptilians and aves investigated and even some mammals (rat, mouse).

This lack of 17α-hydroxylase distinguishes the frog and turtle interrenals from fishes whose adrenal homologues do not incorporate acetate into corticosteroids either, but possess 17α-hydroxylase activity as demonstrated by the isolation and characterization of cortisol (21).

VI SUMMARY AND CONCLUSIONS

The results obtained in the in vitro studies carried out with frog and turtle interrenal preparations will be summarized together. These investigations have revealed that:

A. These two interrenal tissues possess 11β-18- and 21 steroid hydroxylases.

B. No evidence for 17α-hydroxylase activity has been found in either type of interrenal.

C. The localization of steroid 11β- and 18-hydroxylases is in the frog adrenal mitochondria. In the case of turtle interrenal 11β-hydroxylase, activity is also associated with the mitochondria. The mitochondrial localization of 18-hydroxylase activity could not be clearly established in the case of turtle interrenals.

D. 21-hydroxylation is mainly associated with microsomal fraction of these two interrenals. Supernatants and mitochondrial fractions also displayed the same type of enzymatic activity.

E. These hydroxylations have been stimulated and/or mediated in both tissues by preformed or 'in situ' generated NADPH.

F. The generation of NADPH from exogenous NADP plus G-6-P suggested the presence of hexosemonophosphate shunt dehydrogenases in both interrenals. This was corraborated by histochemical studies which revealed the presence of G-6-P dehydro-

genase and 6-phosphogluconate dehydrogenase in frog and turtle interrenal preparations.

G. 3', 5' AMP (substituted or unsubstituted) failed to promote the degree of hydroxylations in frog interrenals.

H. The rate of steroid hydroxylations is almost proportional to the time of incubation up to two hours, thereafter leveling off.

I. Δ^5-3β-hydroxysteroid-dehydrogenase-isomerase is present in both types of interrenals. This enzymatic complex appears to be localized in all subcellular fractions.

J. Either NAD or NADP plus G-6-P support the Δ^5-3β-ol ——→ Δ^4-3keto transformation in both types of adrenals. NAD promotes this transformation into progesterone mainly, while NADPH generating system induces the appearance of poly-hydroxylated Δ^4-3keto metabolites.

K. The side chain scission of radiocholesterol is not demonstrable in surviving sections under a variety of experimental conditions.

L. Homogenates and mitochondrial fraction of both tissues possess cleavage activity. The cleavage mechanism is supported by NADPH either preformed or generated 'in situ'.

M. Fumarate alone supports steroid hydroxylations, Δ^5-3β-hydroxysteroid-dehydrogenase-isomerase in both interrenals.

N. No incorporation of radioacetate into cholesterol or corti-

costeroids could be demonstrated in either tissue under various experimental conditions explored.

O. The following metabolites have been isolated and fully characterized in studies with both tissues: 18-OH-corticosterone, aldosterone, corticosterone, 11β-OH-progesterone, 11-deoxycorticosterone and progesterone. In addition, 11-dehydrocorticosterone (frog studies) and Δ^5-pregnenolone (turtle incubations) have been isolated. The criteria for characterization of most metabolites have been: a) microcrystallization to constant specific activity and constant ^3H:^{14}C ratios, b) doubly labelled derivatives showing a constant ^3H:^{14}C ratio in serial chromatography.

P. Under most experimental conditions, corticosterone and aldosterone are the major metabolites isolated from both types of tissues.

Q. With the exception of their inability to incorporate radioacetate into corticosteroids, the process of steroidogenesis in frog and turtle interrenals seems to be very similar to that encountered in avian and non-cortisol producing mammalian adrenal gland.

CLAIMS OF ORIGINALITY

The *in vitro* studies contained in this thesis have revealed for the first time:

1. The apparent inability of frog and turtle interrenals to utilize radioacetate for the elaboration of cholesterol and corticosteroids.
2. The capacity of homogenized preparation of these two interrenals to metabolize radiocholesterol into C_{21} steroids. The cleavage mechanism is associated with the mitochondria and is an NADPH-dependent reaction.
3. The distribution of Δ^5-3β-hydroxysteroid-dehydrogenase-isomerase in all subcellular compartments of frog and turtle interrenal tissue.
4. The association of 11β-hydroxylase with the mitochondrial fraction of both types of interrenals.
5. The association of 21-hydroxylase in microsomal and supernatant fractions from these interrenals.
6. The presence of G-6-P dehydrogenase and 6-phosphogluconate dehydrogenase in these tissues as shown by histochemical studies. These findings explain the capacity of these interrenals to generate NADPH from exogenous NADP plus G-6-P.
7. The isolation of 11β-OH-progesterone as a conversion product from frog and turtle incubation studies. This metabolite has

been considered a theoretical intermediate in the scheme of corticosteroidogenesis in amphibian and reptilian interrenals. 8. The isolation of Δ^5-pregnenolone as a cleavage product of radiocholesterol in turtle interrenal incubations.

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