Biosynthesis of Corticoids in Guinea Pig Adrenal Slices. [1-^{14}C]Acetate Incorporation into Adrenal Steroids and Sterols*

R. B. Billiar,† A. Oriol-Bosch,‡ and K. B. Eik-Nes

ABSTRACT: Guinea pig adrenal slices were incubated in Krebs-Ringer phosphate buffer containing 200 mg % glucose and [1-^{14}C]acetate. Exposure of the slices to high concentrations of [1-^{14}C]acetate resulted in the incorporation of ^{14}C into free and esterified adrenal tissue sterols. Radioactive cortisol, corticosterone, cortisone, and 11-deoxycorticosterone could be isolated and identified in the incubation medium from such experiments.

The addition of adrenocorticotropic hormone to the incubation medium increased the incorporation of [1-^{14}C]acetate into ^{14}C adrenal steroids. When adrenal slices were incubated with [1-^{14}C]acetate, the concentration of carbon 14 was higher in isolated 17-deoxycorticosteroids than in the isolated 17-hydroxycorticosteroids, although the total mass of isolated cortisol in such experiments was greater than that of isolated corticosterone. Factor(s) related to the incubation times and conditions used in this investigation may be responsible. Finally, adrenal slices incubated for 6 hours exhibited biosynthetic capacity when incubated for an additional 2 hours.

Conversion *in vitro* of [^{14}C]acetate to [^{14}C]sterol by beef adrenal cortical slices was demonstrated as early as 1948 (Srere *et al.*, 1948). Zaffaroni *et al.* (1951) and Hechter *et al.* (1953) perfused bovine adrenals with [^{14}C]acetate and [^{14}C]cholesterol and isolated [^{14}C]-adrenocorticosteroids in the perfusate. Bovine (Haynes *et al.*, 1954) and porcine (Haines, 1952) adrenal cortical slices metabolized [^{14}C]acetate to [^{14}C]corticosteroids, and human fetal adrenal slices converted [^{14}C]acetate to C-19 steroids (Bloch and Benirschke, 1959). Finally, [^{14}C]acetate incorporation into adrenocorticosteroids has also been suggested to occur in porcine and bovine adrenal cell-free preparations (Bligh *et al.*, 1955; Bryson and Sweat, 1962).

In a preliminary note, Burstein and Nadel (1956) reported the conversion *in vivo* of [1-^{14}C]acetate to [^{14}C]cortisol in scorbutic guinea pigs. Hamster adrenal slices apparently could not incorporate [^{14}C]acetate into adrenocorticoids, but [1-^{14}C]acetate was converted to [^{14}C]cortisol *in vivo* (Schindler and Knigge, 1959). Thus no successful experiments have been reported on [1-^{14}C]acetate conversion to adrenal steroids by the adrenal glands of laboratory animals *in vitro*. Since the physiological status of such animals can be controlled and is known to the investigator, adrenals from a laboratory animal should be better for experimental purposes than adrenals obtained from slaughterhouse sources. We wish to report our experience with [1-^{14}C]-acetate incorporation into sterols and steroids by guinea pig adrenal slices.

Experimental Procedure

The tissue preparation used and incubation conditions have been described elsewhere (Billiar *et al.*, 1965). In short-term incubations the incubation flasks were oxygenated for 3 minutes with 95% O_2–5% CO_2, tightly stoppered, and incubated for 1 hour at 37° with agitation (80 cpm). The medium was removed, the slices were washed with 2–3 ml of fresh medium, the rinses were discarded, and 10 ml of fresh medium containing sodium [1-^{14}C]acetate, with or without ACTH,[1] was added. After oxygenation the flasks were incubated for 2 hours. The medium was then quantitatively transferred to extraction tubes and the slices were rinsed several times. The medium and rinses were combined, extracted, and analyzed for steroids. The washed tissue was homogenized in about 10 ml of an ethanol-acetone-ether (4:4:1, v/v/v) mixture, and this homogenate was utilized for sterol determinations.

In long-term incubations, after a sufficient amount of

* From the Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City. *Received December 7, 1964; revised May 3, 1965.* This work was submitted to the faculty of the University of Utah in partial fulfillment of the requirements of Doctor of Philosophy (R.B.B.). Financial support for this work was provided by a U.S. Public Health Service predoctoral training grant (TL GM 152-05) and a postdoctoral training grant (T4 CA 5000).

† Present address: Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts.

‡ Postdoctoral trainee of a USPHS-sponsored training program in steroid biochemistry. Present address: Department of Physiology, University of Madrid, Spain.

[1] Abbreviations used in this work: ACTH, adrenocorticotropic hormone; P-S, Porter-Silber color reaction for 17-hydroxycorticosteroids; 11β-hydroxyandrostenedione, 11β-hydroxyandrost-4-ene-3,17-dione.

adrenal tissue was present in each flask, the tissues were oxygenated and then incubated for 2 hours at 37° in the presence or absence of 50 μc (*ca.* 0.5–1.0 μmole) [1-^{14}C]acetate per flask. The medium was then removed and either discarded or analyzed for steroids. The tissues were washed with 2 ml of medium, the rinses discarded, and the slices incubated for an additional 2 hours in the presence or absence of 50 μc (*ca.* 0.5–1.0 μmole) [1-^{14}C]acetate per flask. This latter procedure was repeated, giving a total incubation period of 6 hours. At the initiation of a final 2-hour incubation (6–8 hour period) ACTH and [7-^{3}H]-progesterone were added to some flasks, and [1-^{14}C]-acetate was added in all the flasks. ACTH was dissolved in the incubation medium while [^{3}H]progesterone was added to the flasks in 0.1 ml ethanol. At the end of the final 2-hour incubation (total incubation time of 8 hours), the medium and rinses of the adrenal slices were extracted and analyzed for steroids and the washed tissue homogenized and used for sterol estimation.

The methods employed for the isolation and quantification of steroids in the incubation medium have been outlined in a previous paper (Billiar *et al.*, 1965). In Figures 1 and 2 are depicted the paper chromatographic systems used.

In order to purify cortisol acetate (Figure 1) and corticosterone acetate (Figure 2) for colorimetric analysis, these compounds were partitioned between ethyl acetate and 0.1 volume of 0.1 N sodium hydroxide. Cortisol acetate was then estimated by a micromodification of the Porter-Silber reaction (Porter and Silber, 1950). Corticosterone acetate was determined by the procedure of Lewbart and Mattox (1960). The lower limit of detection for both methods was 0.1 μg.

The tomatine method of Kabara *et al.* (1961) was used to isolate free and esterified sterols in the adrenal slices (Billiar *et al.*, 1965). Glacial acetic acid solutions of the sterol-tomatine complex were taken to dryness, redissolved in *N*-dimethylformamide, and analyzed on a Barber-Colman gas chromatograph. An SE-30 (0.75%) coated gas chromatography column was used to analyze the sterol complex. The column was operated at 214° and at a gas pressure of 25 psi (argon). When the tomatinide obtained from about 40 mg of adrenal slices was applied to this column, only 1 peak, having a retention time of 13 minutes, was recorded. This corresponded to the retention time of a purified cholesterol standard. The tomatine moiety of the complex apparently did not interfere with the analysis. This column, however, will not separate cholesterol from all other sterols (e.g., dihydrocholesterol).

An acetone-ethanol-ether (4:4:1) extract of the adrenal slices was concentrated to 4 ml, 0.25 ml methanol saturated with potassium hydroxide was added, and each sample was saponified for 1 hour at 50°. Five ml of water was added to each sample, and the organic solvent mixture was removed under a stream of nitrogen at 45°. The resulting water phase was extracted two times with 10 ml of chloroform-ether (1:3, v/v), and the organic extract was backwashed two times with 2 ml of water and then taken to dryness.

A modification[2] of the aluminum oxide column described by Schneider *et al.* (1957) was used to isolate a squalene fraction. Aluminum oxide was stirred with water (7% by weight) and then dried. Two g of this deactivated aluminum oxide was poured into a 1-cm wide column, and the aluminum oxide was washed with 15–25 ml dried Skellysolve B. The residue from the chloroform-ether extract was applied to the column in 2 ml of Skellysolve B. Two 1-ml Skellysolve B rinses of the sample tube were added to the column, and the column was developed with Skellysolve B. Sterols were retained by the aluminum oxide, and squalene was eluted with Skellysolve B.

The squalene fraction eluted from the column was then chromatographed on a 1% XE-60 coated gas chromatography column operated at 180° and an argon pressure maintained at 18 psi (Nishizawa and Eik-Nes, 1964). Authentic squalene had a retention time of 20.4 minutes. A single compound with retention time of 20.4 minutes was present in the biological samples. The peak area on the gas chromatographic tracing was calculated as described (Nishizawa and Eik-Nes, 1964). The concentration of squalene in the samples was estimated by comparing the peak area of the unknown squalene with those of known concentrations of authentic squalene chromatographed on the same column on the same day. The sensitivity setting of the recorder of the gas chromatograph was such that 0.1 μg authentic squalene gave a peak area of 1.1 cm^2. In the aliquots of the biological samples chromatographed from 0.3 to 1.0 μg squalene was present.

The effluent gas corresponding to the squalene-like compound in the biological samples was collected in toluene as described by Nishizawa and Eik-Nes (1964), and carbon-14 counting was done on the condensate in a Packard liquid scintillation spectrophotometer.

The techniques used for paper chromatography, derivative formation of steroids, and crystallization to constant specific activity have been reported by our laboratory (Billiar *et al.*, 1965; Oriol-Bosch and Eik-Nes, 1964; Hagen and Eik-Nes, 1964). Estimation of carbon 14 and tritium was done by counting known portions of the purified sample (*ca.* 50–100% of the total sample) in a Packard liquid scintillation spectrometer. The samples were dissolved in 10 ml of a solution containing 4 g 2,5-diphenyloxazole and 40 mg 2,2-*p*-phenylenebis(5-phenyloxazole) in 1 liter of toluene. Tritium counts in channel II were 0.4% of those in channel I, and the carbon-14 counts in channel I were 38% of those in channel II. It was not necessary to correct for quenching. In samples containing both tritium and carbon 14, the concentration of isotopes was estimated as described by Okita *et al.* (1957). The average counting efficiency for tritium and carbon 14 was 13 and 42%, respectively, in such experiments. All samples were counted for a sufficient period of time to

[2] A. Salokangas, H. Rilling, and L. T. Samuels, private communication, 1964.

give a standard error of the mean of the counts recorded of less than 5%. Each sample was counted four times and the background counts varied from 16 to 45 dpm. All counts recorded in this report have been corrected for background counts.

Sodium [1-^{14}C]acetate (24–55 mc/mmole) and [7-^{3}H]-progesterone (1 mc/0.033 mg) were obtained from New England Nuclear Corp. After incubation of [7-^{3}H]-progesterone in 200 mg % glucose in Krebs-Ringer phosphate buffer (but in the absence of tissue) for 2 hours at 37°, extraction, base wash, and chromatography in hexane-formamide, there was only one radioactive area corresponding to progesterone when the chromatogram was scanned with a windowless Geiger counter.

Approximately 30,000 dpm of sodium [1-^{14}C]acetate was examined in a paper ionophoresis system of pyridine–acetic acid–water (10:0.4:90, v/v/v) at a voltage of 5 v/cm, *p*H 6.5, and for a time of 3 hours. Only one radioactive area (23 cm from the application line toward the anode) could be detected either on the fine or course counting scales of a window Geiger tube scanner.

The ACTH used in these studies was kindly provided by Drs. M. Glenn of the Upjohn Company and J. Fisher of Armour Laboratories.

Reagents were all analytical grade, and organic solvents were redistilled before use. The tomatine was purchased from Chemical Concentrates, Fort Washington, Pa., and Nutritional Biochemicals Corp., Cleveland, Ohio. The tomatine reagent solution was filtered through Whatman No. 1 filter paper and stored in a brown bottle at 4°. Aluminum oxide, Grade I (Woelm), was supplied by Alupharm Chemicals, New Orleans, La.

Results

Incorporation of [1-^{14}C]Acetate into Adrenocorticoids Using Short-Term Incubations and Low Acetate Concentrations

Incorporation of [1-^{14}C]acetate into adrenal steroids was only considered demonstrated when a significant radioactive area which had a chromatographic mobility of an authentic corticoid could be measured on the paper chromatogram by a recording strip counter with thin window Geiger tube. The lower limit of sensitivity of the strip counter was approximately 500 dpm/cm^2. Using these criteria, no significant acetate incorporation into the commonly encountered adrenocorticoids was observed when either a small amount (100–250 mg) or a large amount (400–600 mg) of adrenal slices was incubated with relatively low concentrations of [1-^{14}C]acetate (0.25–1.0 μmole) for 2 hours. [1-^{14}C]Acetate incorporation into the tomatine-precipitable sterol fraction of the adrenal tissue was, however, repeatedly observed in such incubations (Table I). [1-^{14}C]Acetate was also incorporated into a compound which behaved chromatographically like squalene on aluminum oxide and gas chromatography columns (Table II). The total squalene concentration

TABLE I: Influence of ACTH on [1-^{14}C]Acetate Incorporation into Adrenal Gland Sterols.[a]

	[1-^{14}C]Acetate Incorporation	
	Free Sterol (dpm/μmole)[b]	Esterified Sterol (dpm/μmole)[b]
Control	73,000	8,000
ACTH	57,000	6,000
P value[c]	<0.001	<0.005

[a] Adrenal slices (100–250 mg) were incubated for 1 hour in 200 mg % glucose Krebs-Ringer phosphate buffer (*p*H 7.4) solution. The medium was removed and fresh medium containing 110 × 10^6 dpm [1-^{14}C]-acetate (*ca.* 1 μmole) was added. When used, 2 USP ACTH units/50 mg adrenal fresh weight were added. The incubation was then performed for 2 hours at 37°. The figures are means from 13 experiments. The incubation media from these experiments were analyzed for Porter-Silber chromogens. Mean concentrations of 0.2 and 1.1 μg/100 mg adrenal tissue were found for the control and the ACTH experiments, respectively. These values show a <0.001 probability of being the same. The adrenal tissue contained approximately 0.3 mg free sterol/100 mg wet weight and 2.3 mg esterified sterol/100 mg wet weight. [b] A molecular weight of 387 was used to calculate the sterol specific activity. [c] Probability that control and ACTH values are the same as determined by the Student t test.

TABLE II: Effect of ACTH on [1-^{14}C]Acetate Incorporation into Squalene in Guinea Pig Adrenal Slices.[a]

Experiment	P-S Chromogens in Incubation Medium (μg/100 mg adrenal)		Squalene in Adrenal Slices (dpm/μg squalene)[b]	
	Control	ACTH	Control	ACTH
1	0.9	3.6	1100	900
2	0.4	2.8	1200	500
3	1.1	1.9	1200	1100
4	0.7	2.2	1500	1100
5	0.9	1.9	700	800
Mean	0.8	2.5	1130	880

[a] The experimental details are described in Table I except that the slices were utilized for squalene determinations and 55 × 10^6 dpm [1-^{14}C]acetate was used per flask. [b] The method for isolation of squalene and determination of its specific activity are described in the text.

TABLE III: [1-^{14}C]Acetate Incorporation into Adrenal Sterols *in Vitro*.[a]

Flask	Addition of 110 × 10^6 dpm [1-^{14}C]Acetate	Total [1-^{14}C]Acetate Added		Sterols	
		dpm	μmole	Free dpm/μmole[b]	Ester dpm/μmole[b]
1	Each of four 2-hour periods	440 × 10^6	4	118,000	45,000
2	First three 2-hour periods Absent in the last 2-hour period	330 × 10^6	3	74,000	30,000
3	Present during the two first 2-hour periods only	220 × 10^6	2	47,000	16,000
4	Present during the last 2-hour period only	110 × 10^6	1	37,000	9,000

[a] Adrenal slices (*ca.* 450 mg) were incubated in 200 mg % glucose Krebs-Ringer phosphate buffer for four successive 2-hour periods. Fresh medium was added each 2 hours and [1-^{14}C]acetate was added at the beginning of the 2-hour incubations as indicated in the table. At the initiation of the final incubation, ACTH (4 USP units/100 mg) was added to each flask. The medium from each 2-hour incubation of each flask was analyzed for [^{14}C]steroids. After completion of the fourth 2-hour incubation the slices were used for [^{14}C]sterol specific activity determinations. [b] A molecular weight of 387 was used to calculate the sterol specific activity. The average concentration of free and esterified cholesterol of guinea pig adrenals has been indicated in the text of Table I.

in the adrenal gland was, however, not estimated. The data indicate that addition of ACTH to the incubation medium lowered the specific activity of both free and esterified sterol, and probably of the squalene-like compound.

Incorporation of [1-^{14}C]Acetate into Adrenal Steroids by Increasing the Time of Adrenal Incubation or Concentration of [1-^{14}C]Acetate

In long-term incubations (Experimental Procedure) [1-^{14}C]acetate was incorporated into adrenal tissue sterols (Table III), and adrenal slices incubated for 6 hours without [1-^{14}C]acetate could still incorporate this compound into adrenal sterols during a subsequent 2-hour incubation (flask 4, Table III). Analysis of the incubation medium (data not shown) of each flask in 2-hour periods revealed that significant ^{14}C incorporation into adrenal steroids occurred only when the slices were exposed to at least 150 μc (3 μmoles) [1-^{14}C]acetate for 6–8 hours (flasks 1 and 2, Table III); ACTH was added only at the initiation of the final 2-hour incubation period (6–8 hours).

The effect of repeated exposure of adrenal slices to [1-^{14}C]acetate was examined with respect to [^{14}C]-cortisol specific activity (Table IV). This was possible by combining the incubation media from 4 flasks. Although the adrenal release of cortisol decreased with increasing incubation time, the tissue was still able to utilize [1-^{14}C]acetate for cortisol formation during the last 2 hours of incubation.

TABLE IV: Incorporation of [1-^{14}C]Acetate into Cortisol as a Function of Incubation Time.[a]

Incubation Period	Cortisol		
	μg/g Adrenal[a] (a)	dpm/g Adrenal (b)	Specific Activity b/a
0–2 hour	3.0	1300	430
2–4 hour	0.8	2100	2600
4–6 hour	0.5	3100	6200

[a] About 1 g adrenal slices in each of 4 flasks was incubated for three 2-hour periods as described in Table III, but no ACTH was added to any of the flasks. [1-^{14}C]Acetate (*ca.* 1 μmole, 110 × 10^6 dpm) was added per flask at the initiation of each 2-hour incubation. At the end of a 2-hour incubation period the media of the 4 flasks were combined and analyzed for cortisol (Billiar *et al.*, 1965). [b] Determined by the Porter-Silber reaction.

Isolation and Identification of Radioactive Adrenal Steroids from Incubations of Adrenal Slices with [1-^{14}C]-Acetate and [7α-^{3}H]Progesterone

From experiments as outlined in the legend of Table V, the following radioactive adrenal steroids could be distinguished after first being purified by paper chromatography in different solvent systems (Figures 1 and 2).

[^{14}C]*Cortisol.* The material behaving chromatographically like [^{14}C]cortisol acetate gave a positive Porter-Silber chromogen curve. Oxidation of the acetate with chromium trioxide in glacial acetic acid produced a compound with the chromatographic mobility of authentic cortisone acetate. A portion of the [^{14}C]-cortisol acetate was also crystallized to constant specific activity from different solvent combinations (first

TABLE V: [1-^{14}C]Acetate Incorporation into Adrenal Steroids and Sterols.[a]

Compound	Incubation Conditions		
	One 2-Hour Period with 200 μc [1-^{14}C]-Acetate and ACTH	Four 2-Hour Incubation Periods with 50 μc [1-^{14}C]Acetate Added Every Second Hour ACTH	Control
"X"	1,900	1,400	900
Cortisol	600	1,500	500
"Z"	3,400	3,200	3,200
Corticosterone	1,300	5,400	800
"R"	3,200	1,300	400
11β-Hydroxyandrostenedione	700	Lost	500
11-Deoxycorticosterone	1,500	2,100	600
Free sterol tissue	65,000	190,000	231,000
Esterified sterol tissue	56,000	114,000	169,000

[a] Data on the formation of sterols and steroids are given in dpm of ^{14}C/100 mg adrenal fresh weight. In the short-term incubation experiment 600 mg adrenal slices was incubated with 440 × 10^6 dpm [1-^{14}C]acetate (*ca.* 4 μmoles) and 2 USP units ACTH/100 mg adrenal weight for 2 hours. In the long-term incubation experiment *ca.* 600 mg adrenal slices was incubated for four 2-hour periods with 110 × 10^6 dpm [1-^{14}C]acetate (*ca.* 1 μmole) added at the initiation of each 2 hours of incubation. The ACTH stimulation in the long-term incubation experiment was achieved by adding 2 USP units ACTH/100 mg adrenal weight during the last 2-hour incubation. Also at this point of the long-term incubation 0.1 μg [7-^{3}H]progesterone/100 mg adrenal weight was added to all the flasks containing ACTH. Only the medium from the last incubation period was analyzed for steroids and the tissue slices were used for sterol estimation. Each figure is the average of duplicates. The procedures used for steroid and sterol isolation and quantification are given in Billiar *et al.* (1965), and 85% of the isolated radioactivity was counted by liquid scintillation spectrophotometry.

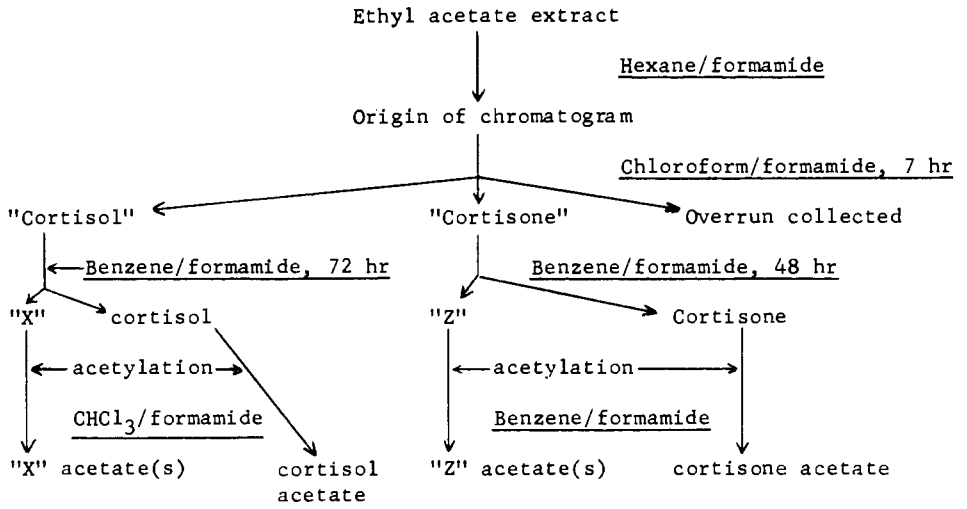

FIGURE 1: Flowsheet depicting procedures used for the isolation of cortisol, cortisone, and unknown compounds "X" and "Z."

crystallization 1280 dpm/mg, second crystallization 1240 dpm/mg, third crystallization 1210 dpm/mg, fourth crystallization 1260 dpm/mg).

[^{14}C,^{3}H]*Cortisone.* Although cortisone was not routinely isolated and was generally present in negligible amounts, sufficient material was present in a 6–8 hour ACTH experiment (*cf.* Table V) for its identification. Authentic cortisone acetate was mixed with the biological sample derived from adrenal incubations with [1-^{14}C]-acetate and [7-^{3}H]progesterone, and the mixture was crystallized from different solvent combinations (ratio ^{3}H/^{14}C: first crystallization 9.8, second crystallization

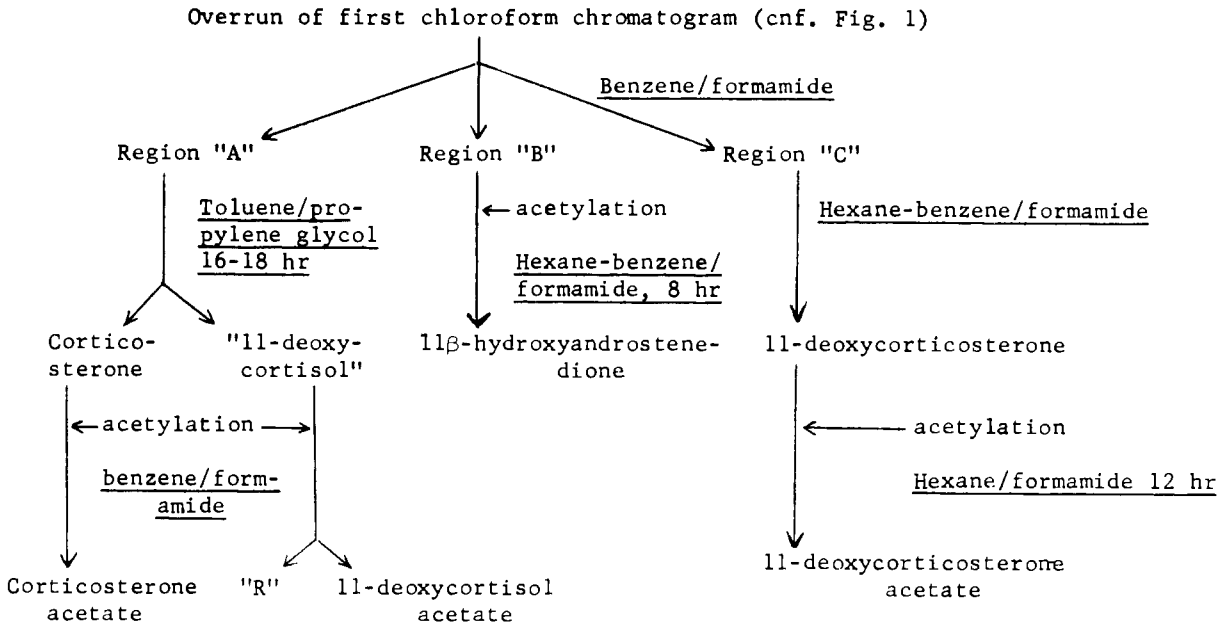

FIGURE 2: Flowsheet depicting procedures used for the isolation of corticosterone, 11-deoxycortisol, 11β-hydroxyandrostenedione, 11-deoxycorticosterone, and an unknown compound "R."

9.1, third crystallization 9.0; cpm of ^{3}H/mg: 980, 1000, 990; cpm of ^{14}C/mg: 100, 110, 110, for the first, second, and third crystallizations, respectively).

[^{14}C,^{3}H]*Corticosterone.* Isolated [^{14}C,^{3}H]corticosterone was mixed with authentic unlabeled corticosterone and the mixture was chromatographed for a total of 24 hours in hexane-benzene (1:1)-formamide. The radioactivity and the single ultraviolet absorbing area ran with the same mobility. The mixed sample was acetylated and chromatographed in hexane-benzene-formamide for a total of 8 hours; the single ultraviolet area and the radioactivity again did not separate. The acetate was saponified with sodium carbonate, and the saponified product was chromatographed in benzene-formamide. Again the single ultraviolet absorbing substance did not separate from the radioactivity, both having the same chromatographic mobility as authentic corticosterone.

When [^{14}C,^{3}H]corticosterone acetate was oxidized with chromium trioxide, a compound chromatographing similar to authentic 11-dehydrocorticosterone acetate in benzene-formamide was formed.

[^{14}C,^{3}H]Corticosterone acetate (Figure 2) was mixed with authentic unlabeled corticosterone acetate and crystallized to constant specific activity (ratio $^{3}H/^{14}C$: first crystallization 24, second crystallization 24, third crystallization 25; cpm of ^{3}H/mg: 26,800, 24,500, 25,900; cpm of ^{14}C/mg: 1100, 1050, 1030, for the first, second, and third crystallizations, respectively).

[^{14}C,^{3}H]*11-Deoxycortisol.* Since relatively small quantities of ^{14}C from [^{14}C]acetate were incorporated into a compound with chromatographic properties similar to those of authentic 11-deoxycortisol, the identity and radiochemical purity of this compound cannot be considered established.

[^{14}C]*11β-Hydroxyandrostenedione.* Chromic oxide oxidation of the isolated metabolite gave quantitative conversion to a compound with the chromatographic mobility of authentic 11-ketoandrostenedione. However, the specific activity of the isolated C-19 metabolite was still decreasing, albeit slightly, after four successive crystallizations (first crystallization 260 dpm/mg, second crystallization 225 dpm/mg, third crystallization 220 dpm/mg, fourth crystallization 205 dpm/mg).

[^{14}C,^{3}H]*11-Deoxycorticosterone.* [^{14}C,^{3}H]11-Deoxycorticosterone isolated as its acetate was mixed with authentic 11-deoxycorticosterone acetate and chromatographed in hexane-benzene-formamide; the single ultraviolet absorbing area and the single radioactive area corresponded. When the [^{14}C,^{3}H]11-deoxycorticosterone acetate isolated from an adrenal incubation with [1-^{14}C]acetate and [7-^{3}H]progesterone as substrates was mixed with authentic unlabeled 11-deoxycorticosterone acetate and crystallized from different organic solvents, it retained a constant specific activity (ratio $^{3}H/^{14}C$: first crystallization 69, second crystallization 69, third crystallization 70, fourth crystallization 70; cpm of ^{3}H/mg: 51,800, 52,700, 50,400, 50,700; cpm of ^{14}C/mg: 750, 760, 720, 720, for the first, second, third, and fourth crystallizations, respectively).

The following unknown compounds were also observed:

Compound "X"-^{14}C was separated from cortisol as shown in Figure 1. Upon acetylation "X" formed a low polar derivative. Partial acetylation (Dominguez *et al.*, 1963) yielded three radioactive areas of approximately equal quantity with R_F values of 0.17, 0.35, and 0.55 when chromatographed in chloroform-formamide. The R_F of compound "X" in this system of paper chromatography was 0.17. Sodium carbonate

saponification of "X" acetate(s) resulted in the formation of three radioactive areas, R_F 0.8, 0.2, and 0.0, in benzene-formamide. It is suggested that compound "X" is a reduced derivative(s) of the corticoids.

Compound "Z" had acetylation and saponification properties similar to those described for compound "X" and showed the same relative polarity relationship to cortisone as "X" did to cortisol.

Compound "R"-^{14}C could be separated from 11-deoxycortisol by acetylation (Figure 2) since it apparently did not form an acetate. An interesting property of compound "R" was its high rate of formation in short-term incubations (Table V). Also the addition of ACTH in 2-hour incubations enhanced the formation of [^{14}C]"R" from [1-^{14}C]acetate (data not presented).

Additionally, ^{14}C activity was occasionally observed on the chromatograms in compound(s) more polar than cortisol and also in a compound(s) with chromatographic mobility similar to authentic 11-dehydrocorticosterone and adrenosterone. However, the 11β-hydroxy compounds always contained more ^{14}C activity than the 11-keto analogs, and further identification of these compounds has not been pursued.

A fraction of the total carbon-14 activity moved with the solvent front in the first hexane-formamide chromatogram (Figure 1). Also, radioactivity remained at the application site of the first toluene–propylene glycol chromatogram in Figure 2. These two regions accounted for approximately 10–20% of the total ^{14}C activity isolated in the corticoid fraction. Rechromatography of these regions in high and low polar paper chromatographic systems did not alter their mobilities. No further effort was devoted to their identification.

Addition of ACTH to the incubation medium stimulated the incorporation of ^{14}C precursors from [1-^{14}C]acetate into both the 17-deoxy, and 17-hydroxylated corticosteroids (Table V). The ^{14}C contained in compounds isolated from the 6–8-hour incubation medium with ACTH added represented approximately 10% of the amount of ^{14}C present in the free sterol fraction of the adrenal slices at the end of the incubation. Furthermore, more ^{14}C activity was present in both the steroid and sterol fractions of the 6–8-hour incubation with ACTH than in a 2-hour ACTH incubation (Table V).

Slices preincubated with [1-^{14}C]acetate were also incubated with [7-^{3}H]progesterone and ACTH in the 6–8-hour period. The ratio of ^{3}H/^{14}C (Table VI) was lower in "end products" (viz., cortisol and corticosterone) than in their respective precursors (viz., 11-deoxycortisol and 11-deoxycorticosterone). In an incubation study similar to that reported in Table V, 0.01 μg [7-^{3}H]progesterone instead of 0.1 μg [7-^{3}H]-progesterone was added per 100 mg adrenal weight during the last 2 hours of incubation, and essentially the same precursor-product relationship as recorded in Table VI was observed.

Tissue Activity. It can be argued that slices incubated for 6 hours would lose biosynthetic capacity. It is evident from Table VII that slices incubated for this period of time could convert [^{3}H]progesterone to [^{3}H]steroids during an additional hour of incubation.

TABLE VI: Incorporation of [7-^{3}H]Progesterone and [1-^{14}C]Acetate into Steroids by Adrenal Slices Stimulated with ACTH.[a]

Compound	dpm/100 mg Adrenal Weight ^{14}C	dpm/100 mg Adrenal Weight ^{3}H	^{3}H/^{14}C in Isolated Compound
Cortisol	1,500	148,000	98
11-Deoxycortisol	300	144,000	480
Corticosterone	5,200	806,000	154
11-Deoxycorticosterone	2,100	837,000	397

[a] Adrenal slices (600 mg) were incubated for four 2-hour periods with 110 × 10^{6} dpm [1-^{14}C]acetate (*ca.* 1 μmole) added at the initiation of each 2 hours of incubation. During the last 2 hours of incubation 2 USP units ACTH and 0.1 μg [7-^{3}H]progesterone/100 mg slices were also added. The data of this table are averages from duplicate experiments, and 95% of the isolated radioactivity was counted by liquid scintillation spectrophotometry.

In the present studies more ^{14}C was present in the 17-deoxycorticoids than in the 17-hydroxylated corticoids. Although we could repeatedly estimate the specific activity of the isolated cortisol, we could not obtain sufficient corticosterone for measurement of its specific activity even though the lower limit for detection of the color reactions for both compounds was the same. The specific radioactivity of [^{14}C]corticosterone must, therefore, in this incubation system be higher than that of the [^{14}C]cortisol.

At the beginning of this investigation the assumption was made that analysis of the incubation medium for steroids would be an adequate index of adrenal steroid production. The major part of labeled corticosterone can be found in the incubation medium (Table VIII), and thus the assumption seems justified.

Discussion

Guinea pig adrenal slices survived and released steroids in a synthetic medium for 8 hours, and sterol biosynthesis from [1-^{14}C]acetate occurred at a brisk rate even when incubated for 6–8 hours. The specific activity of the tissue sterols isolated in long-term experiments (four 2-hour repeated incubations), exposing the adrenals to [1-^{14}C]acetate during the last 2-hour period only, compared well with the specific activity of sterol isolated from a 2-hour experiment with approximately the same concentration of [1-^{14}C]acetate. Additionally, when 200 μc [1-^{14}C]acetate was incubated with adrenal slices for 2 hours, the amount of ^{14}C incorporated into tissue sterol and medium steroids was lower than that obtained in incubations lasting 8

TABLE VII: Time Course of [1-^{14}C]Acetate and [7-^{3}H]Progesterone Incorporation into Adrenal Steroids.[a]

Compound	Time of Incubation					
	With [^{14}C]Acetate			With [^{3}H]Progesterone		
	6.25 hours	6.5 hours	7 hours	15 min	30 min	60 min
Cortisol	<50	100	100	500	1,000	1,100
11-Deoxycortisol	<50	<50	<50	4,800	5,000	8,400
Corticosterone	250	250	550	11,800	14,500	20,500
11-Deoxycorticosterone	60	60	160	16,500	20,600	27,700
	^{14}C: dpm/100 mg adrenal			^{3}H: dpm/100 mg adrenal		

[a] About 600 mg adrenal slices per flask was incubated for three 2-hour periods with 110 × 10^6 dpm [1-^{14}C]acetate (*ca.* 1 μmole) added at the initiation of each 2-hour incubation. The medium was discarded after each 2-hour incubation. [1-^{14}C]Acetate (*ca.* 1 μmole, 110 × 10^6 dpm), 0.1 μg [7-^{3}H]progesterone/100 mg adrenal weight, and 3 USP ACTH units/100 mg adrenal weight were then added to each flask. Duplicate flasks were incubated for 15, 30, and 60 minutes, and the media were then analyzed for adrenal steroids as outlined in Figures 1 and 2. The data in the table are averages of duplicates. The slices of these incubations were used for sterol estimation, the supernatant of the esterified sterol fraction (Kabara *et al.*, 1961) was partitioned between hexane and 70% methanol–30% water, and the hexane was discarded. The methanol was evaporated to its aqueous phase, which was extracted with ethyl acetate and analyzed for adrenal steroids (Billiar *et al.*, 1965). About 95% of the radioactivity isolated was counted by liquid scintillation spectrophotometry. The ^{14}C activity was counted 8 times and for 10 minutes each time.

TABLE VIII: Distribution of Radioactive Corticosterone in Adrenal Slices and Incubation Medium.[a]

Time	Distribution (dpm)			
	Tissue		Medium	
	^{14}C	^{3}H	^{14}C	^{3}H
15 minutes	<50	7,100	2700	133,000
30 minutes	<50	7,500	2600	167,000
60 minutes	<50	13,700	6400	250,000

[a] The conditions of this experiment are stated in the text of Table VII.

hours in which 50 μc [1-^{14}C]acetate was added each second hour of incubation. The adrenal pool sizes of the sterol precursors in long- and short-term incubations are not known. Nevertheless, adrenal steroids containing significant ^{14}C activity could be isolated both in long- and short-term experiments, provided that the concentration of ^{14}C from [1-^{14}C]acetate was high in the adrenal tissue sterols.

We have furthermore shown the conversion of [1-^{14}C]acetate to a ^{14}C-containing compound with the chromatographic properties of authentic squalene. The addition of ACTH to the incubation medium decreased the specific activity of adrenal tissue squalene and also the specific activity of free and esterified tissue sterols (Tables I and II). Since only one time period of incubation was used in this part of the study, no conclusions are justified as to the mechanism of ACTH influence on the specific activity of these compounds. However, not all of the tomatine-precipitated sterol may participate in adrenal steroid biosynthesis.

Bloch and Benirschke (1959) mentioned in their publication on [1-^{14}C]acetate incorporation into steroids by human fetal adrenal slices that the total acetate incorporation was enhanced if the duration of incubation was increased from 4 to 12 hours. However no quantitative data were presented. Since it has been claimed that the adrenocorticoids may have a direct inhibitory effect on the adrenal cortex (Birmingham and Kurlents, 1958), we preferred dividing the long preincubation time with [1-^{14}C]acetate into 2-hour incubation periods. That adrenal slices incubated for 6 hours are still biosynthetically active is clearly shown in Table VII.

Most of the radioactivity in steroids from long-term experiments was in the form of adrenal "secretory" compounds, i.e., cortisol and corticosterone. Furthermore, as routinely encountered in adrenal experiments *in vitro* or *in vivo*, production of 11β-hydroxyl compounds was higher than that of the 11-keto analogs. The evidence presented for the radiochemical purity of cortisol, cortisone, corticosterone, and 11-deoxycorticosterone suggests that the routine isolation procedures used in this investigation were adequate.

Hofmann (1962) has claimed that corticosterone biosynthesis does not readily occur in guinea pig adrenal slices or whole homogenates although corticosterone biosynthesis from progesterone occurred when the guinea pig adrenal mitochondria were incubated with progesterone in the absence of the microsomal fraction (Hofmann, 1962). Bush (1951) indicated the presence of corticosterone in guinea pig blood. Dyrenfurth and McLeod (1961) observed a high rate of corticosterone secretion of guinea pig adrenals *in vitro*, and Chart

et al. (1962) reported the conversion of progesterone to corticosterone and 11-deoxycorticosterone in this species. Oriol-Bosch and Eik-Nes (1964) and Fajer *et al.* (1963) also isolated [^{14}C]corticosterone after incubating guinea pig adrenal slices with [4-^{14}C]-progesterone.

Indeed, more [^{14}C]corticosterone was present than [^{14}C]cortisol in the 6–8 hour incubations, and the specific activity of [^{14}C]corticosterone (although not measurable by the techniques employed) appeared to be higher than that of [^{14}C]cortisol. Berliner *et al.* (1958) also found a greater synthesis of [^{14}C]corticosterone than [^{14}C]cortisol in human adrenal homogenates with [^{14}C]progesterone as substrate. It has also been reported that the salt concentration in the incubation medium may alter the metabolism of progesterone to cortisol and corticosterone by adrenal homogenates (Eichhorn and Hechter, 1959), although the metabolism of cholesterol to these steroids is not influenced by such changes (Eichhorn and Hechter, 1959). Berliner *et al.* (1961) observed that bovine adrenal reticuloendothelial cells produced 17-hydroxylation of progesterone whereas relatively little hydroxylation occurred on other sites of the molecule. Finally, preliminary investigations indicate that the pyridine nucleotide concentration may affect steroid biosynthetic pathways (Villee, 1963; Berliner *et al.*, 1962). However, it would be premature to attribute the [^{14}C]corticosterone:[^{14}C]cortisol ratios observed by us to any one or a combination of the above factors, and independent pathways may be utilized by the guinea pig adrenal *in vitro* for the synthesis of [^{14}C]17-deoxycorticoids and [^{14}C]17-hydroxycorticoids.

In experiments in which both [7-^{3}H]progesterone and [1-^{14}C]acetate were used as corticoid precursors (Table VI), 11-deoxycortisol and 11-deoxycorticosterone had higher ^{3}H/^{14}C ratios than cortisol and corticosterone, respectively. Since all the ^{3}H/^{14}C ratios in this experiment were greater than 50, the data of Table VI should be interpreted with care. A time study (Table VII) indicated that [^{3}H]progesterone was metabolized at least as rapidly as the adrenal ^{14}C precursors synthesized from [1-^{14}C]acetate. The tissue supply of precursor was not exhausted in these experiments since [^{3}H]progesterone could be isolated from the tissue at the end of each time period studied, and [^{14}C]sterols were also present in the adrenal gland at the end of 8-hour incubations. It is difficult to interpret data where one precursor (i.e., ^{14}C precursors) may be continuously available but further removed in the biosynthetic scheme from the products than the single pulse addition of another precursor (i.e., [^{3}H]progesterone) closer in the metabolic pathway to product. The results could, however, suggest that the ^{14}C precursors and the [^{3}H]progesterone are converted to the corticoids, in part, by different routes. The ^{14}C precursors might utilize a Δ^5,3-hydroxyl pathway which could have different kinetic characteristics from those of an adrenal Δ^4,3-keto pathway used for [7-^{3}H]progesterone metabolism. Also, the metabolism of the ^{14}C precursor and of [7-^{3}H]progesterone in these experiments could occur by the same metabolic path, but the biosynthetic enzymes of this common pathway may be located, in part, at different cellular and/or subcellular sites in the adrenal with regard to the biotransformation of ^{14}C precursors and of [7-^{3}H]progesterone to the corticosteroids.

Studies *in vivo* by Lossow *et al.* (1962) indicated that adrenal tissue can esterify cholesterol. Recently Dailey *et al.* (1962) and Longcope and Williams (1963) have demonstrated that adrenal homogenates could also esterify [^{14}C]cholesterol. In our studies we have confirmed and extended these observations since significant ^{14}C activity from [1-^{14}C]acetate was isolated in the esterified sterol fraction of guinea pig adrenal slices. In short-term experiments (Table V) about the same amount of ^{14}C was isolated in the esterified fraction as in the free sterol fraction. However, since there was approximately eight times more sterol ester present than free sterol in the adrenals, the specific activity of the free fraction was higher than that of the ester fraction (Table I) when acetate was used as substrate for the sterol. The metabolic relationship between free or conjugated adrenal sterol and steroidogenesis is presently not known.

Acknowledgments

The assistance of Dr. E. Nishizawa with the gas chromatographic analysis and many aspects of the methodology employed is gratefully acknowledged. The excellent technical assistance of E. Kristensen and M. Woodward is appreciated.

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The Lipids of *Ruvettus pretiosus* Muscle and Liver*

Judd C. Nevenzel, Waldtraut Rodegker, and James F. Mead†

ABSTRACT: The muscle of the gempylid fish, *Ruvettus pretiosus*, contains 14.7% (wet wt) of lipid, which is predominantly wax esters of 34 and 36 carbon atoms with one and two double bonds. The liver lipids contain only about 2% wax esters.

Contrary to a previous report, the muscle lipid does not contain hydroxy fatty acids. Gas-liquid chromatographic analyses are reported for the fatty acids of several lipid fractions, including the muscle wax esters, for the long-chain alcohols of the muscle wax esters, and for the unhydrolyzed wax esters.

The flesh of *Ruvettus pretiosus*, a fish of the family Gempylidae, was reported (see Gudger, 1925) to have strong purgative properties, hence the trivial name "castor oil fish." Cox and Reid (1932) analyzed samples of oil from specimens caught in the Ellice Islands of the western Pacific Ocean and reported (a) that the oil consisted mostly of the wax esters cetyl oleate and oleyl oleate, and (b) that hydroxyoleic acid constituted approximately 13% of the total fatty acids. They also found the oil to be a mild laxative for rats, although ingestion of an amount of the crude *Ruvettus* oil which was within the range of dosage levels usually prescribed for castor oil produced no effect in the junior author. Our purpose in reexamining the lipids of *R. pretiosus* was to confirm the presence and establish the structures of the hydroxy fatty acids present, and to investigate the wax esters for comparison with unpublished data previously obtained for a second gempylid fish, *Lepidocybium flavo-brunneum*.

Experimental Methods and Results

Chromatographic Techniques. Thin-layer chromatography on silica gel G (E. Merck A. G., Darmstadt, Germany; available in the U.S.A. from Brinkmann Instruments, Inc., Great Neck, N.Y.) or Anasil S (Analytical Engineering Laboratories Inc., Hamden, Conn.) was used for the qualitative assay of the fractions of

* From the Laboratory of Nuclear Medicine and Radiation Biology, Department of Biophysics and Nuclear Medicine, School of Medicine, Center for the Health Sciences, University of California, Los Angeles. *Received November 20, 1964; revised May 10, 1965.* These studies were supported by a contract (AT(04-1)GEN-12) between the U.S. Atomic Energy Commission and the University of California.

† Supported in part by a U.S. Public Health Service research career award (GM-K6-19,177) from the Division of General Medical Sciences, National Institutes of Health.