Metabolism of Adrenal Cholesterol in Man

II. IN VITRO STUDIES INCLUDING A COMPARISON OF ADRENAL CHOLESTEROL SYNTHESIS WITH THE SYNTHESIS OF THE GLUCOCORTICOSTEROID HORMONES

ABRAHAM BORKOWSKI, CLAUDE DELCROIX, and SAM LEVIN

From the Service of Medicine and Clinical Investigation, Institut Jules Bordet, Centre Anti-Cancéreux de l'Université Libre de Bruxelles, and Central Laboratory of Nuclear Medicine, Brussels, Belgium

ABSTRACT The synthesis of adrenal cholesterol, its esterification and the synthesis of the glucocorticosteroid hormones were studied in vitro on human adrenal tissue.

It was found that the synthesis of adrenal cholesterol may normally be small in the zona "fasciculata," particularly when compared with the synthesis of the glucocorticosteroid hormones, that it is several times higher in the zona "reticularis" where esterified cholesterol is less abundant, and that under ACTH stimulation it increases strikingly and proportionally to the degree of esterified adrenal cholesterol depletion.

On the other hand, the relative rate of esterification as well as the concentration of free adrenal cholesterol are remarkably stable: they do not differ according to the adrenal zonation and are unaffected by ACTH. Furthermore, from a qualitative point of view, the relative proportions of Δ_1 and Δ_2 cholesteryl esters formed *in situ* are similar to those anticipated from their relative concentrations, suggesting that the characteristic fatty acid distribution of the adrenal cholesteryl esters results from an in situ esterification rather than from a selective uptake of the plasma cholesteryl esters. Besides, the in vitro esterification reveals a propensity to the formation of the most unsaturated cholesteryl esters.

Regarding hydrocortisone and corticosterone, their synthesis tends to be more elevated in the zona "fasciculata." Despite its higher cholesterol concentration the zona "fasciculata" should not therefore be viewed as a quiescent functional complement to the zona "reticularis" and the cortical distribution of glucocorticosteroid hormone synthesis is quite distinct from that of adrenal cholesterol synthesis.

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INTRODUCTION

The concentration of adrenal cholesterol is quite variable from species to species (1); the synthesis of adrenal cholesterol might vary accordingly. In vitro studies in the squirrel monkey (2) indicate that in the primate the rate of cholesterol synthesis might normally be much lower in the adrenal cortex than in the liver but an immediate conversion of newly synthesized adrenal cholesterol into steroid hormones was not excluded. As far as man is concerned, the in vivo kinetic study of plasma and adrenal cholesterol equilibration under control conditions also suggests that adrenal cholesterol comes mainly from plasma and that the synthesis of cholesterol by the adrenal cortex is relatively small; it raises furthermore the problem of possible differences between the zona fasciculata and the zona reticularis (3).

On the other hand, according to the same kinetic study in man, esterified adrenal cholesterol is produced within the adrenal cortex by the esterification of free adrenal cholesterol and does not result from a direct and selective accumulation of the plasma cholesteryl esters. Apparently, it is this local esterification, together with the hydrolysis of the large pool of esterified adrenal cholesterol which precisely regulates the amounts of free adrenal cholesterol available for the synthesis of the steroid hormones and for the stability of the cellular membranes.

Briefly stated, esterified adrenal cholesterol normally serves as a buffer to free adrenal cholesterol and constitutes a rapid source of supply according to the immediate functional demands whereas the inflow of plasma cholesterol serves as a purveyor to both free and esterified adrenal cholesterol but functions in a more continuous and less flexible fashion; as for the local synthesis it seems, under control conditions, to play a secondary role.

The purpose of the work reported here is to test the validity of this scheme and to bring some further light on the functional significance, mode of regulation and zonal distribution of adrenal cholesterol synthesis. It constitutes an in vitro study, in human adrenal glands removed for the treatment of generalized mammary carcinoma, of cholesterol synthesis, esterification, intracellular equilibration, and conversion into steroid hormones.

METHODS

A. In vitro synthesis of adrenal cholesterol from acetate-2-^{14}C. The adrenal glands obtained in the operating room were immediately placed in a solution of Krebs-Ringer-bicarbonate and processed in the cold room as previously described (3, 4). The slices of adrenal tissue were then incubated, during 3 hr, at 37°C, in a metabolic incubator, under constant agitation and in an atmosphere of 95% oxygen and 5% carbon dioxide. Each incubation flask contained 200–300 mg of adrenal cortex in 3 ml of a Krebs-Ringer-bicarbonate solution at pH 7.4, 60 μCi of acetate-2-^{14}C (SA 55 mCi/mmole; Amersham, England), and 19.096 μmole of sodium acetate. The concentration of glucose in the medium was 2 g/liter.

The incubation was stopped on ice and after homogenisation in the medium, the tissues were extracted with 25 vol of acetone-ethanol 1:1 (5). The precipitated proteins were separated by centrifugation and their nitrogen content was measured by standard Kjeldahl procedures (6) whereas the corresponding concentrations of free and total cholesterol were measured in a precise fraction of the acetone-ethanol extract; in the rest of the extract, free and esterified adrenal cholesterol were separated from each other by thin-layer chromatography (7), the various adrenal cholesteryl esters were fractionated further on silica gel impregnated with 5% Ag NO_3, and the specific activities (SA) were determined on the digitonides after purification by the method of Schwenk and Werthessen (8). The adequacy of this purification was confirmed by the demonstration of the constancy of adrenal cholesterol digitonides specific activities after thin-layer chromatography and two successive dibrominations: these specific activities were not significantly modified by two supplementary crystallisations of cholesterol in methanol. The synthesis of cholesterol was calculated from the number of dpm of labeled cholesterol obtained per milligram of protein nitrogen, i.e. from the product of cholesterol concentrations with cholesterol specific activities. This synthesis was expressed in pmoles of acetate-2-^{14}C incorporation per milligram of protein nitrogen during the duration of the incubation. The absolute value of cholesterol synthesis could in turn be calculated from the absolute amount of methyl carbon incorporated and from the contribution of this carbon to cholesterol molecular weight (9). The adrenal glands were obtained from eight control patients, from six patients who had been treated by ACTH (Cortrophine Z; Organon Inc., Holland) at a dose of 40–120 U i.m. per day for the 2–3 days preceeding surgery, and from six patients who had been given 4 mg of dexamethasone per day during the 2 days preceeding surgery.

B. In vitro synthesis of hydrocortisone, corticosterone, and aldosterone. The adrenal tissue (100–300 mg per flask) was incubated as described in *A*, but the incubation lasted 2 hr and followed a 1 hr preincubation, and no labeled acetate was added to the milieu.

The secretion and the tissue concentration of hydrocortisone, corticosterone, and aldosterone were measured by acetylation with tritiated acetic anhydride (Amersham-SA 100 μCi/μmole) according to the method of Kliman and Peterson (10) as modified by Davis, Burwell, Casper, and Bartter (11); besides, after the chromic acid oxidation, a sixth chromatography was carried out using the E_1 or the E_4 system of Eberlein and Bongiovanni (12). The correction for losses was obtained by the addition to the medium, or to the tissue at the time of homogenisation, of tracer amounts of the corresponding ^{14}C-labeled steroids previously purified by paper chromatography in a Bush 5 system (13) (Amersham, England; hydrocortisone-4-^{14}C: SA 56.8 mCi/mmole, corticosterone-4-^{14}C: SA 56.7 mCi/mmole, and aldosterone-4-^{14}C: SA 56.7 mCi/mmole).

C. Incorporation of acetate-2-^{14}C in newly synthesized hormones. The adrenal slices were cut into small pieces and divided into two equivalent homogeneous fractions:

(*a*) The first fraction was preincubated and incubated as described in *B* for the measurement of hydrocortisone and corticosterone secretion per milligram of protein nitrogen; the concentration of acetate added to the Krebs-Ringer-bicarbonate solution was of 1.115 μmole/ml.

(*b*) The second fraction was preincubated and incubated identically except for the addition of 60 μCi of acetate-2-^{14}C to the incubation medium. The synthesis of cholesterol-^{14}C and the incorporation of acetate-2-^{14}C into hydrocortisone were measured separately. Hydrocortisone secreted in the medium was isolated by chromatography in a Bush 5 (13) system, was acetylated thereafter with cold acetic anhydride and chromatographed again in a system of cyclohexane-benzene-methanol-water (4-3-4-1); tracer amounts of tritiated hydrocortisone were added to the medium for correction of losses resulting from extraction and purification and the tracer itself was purified before use by paper chromatography in a Bush 5 system.

The incorporation of acetate-2-^{14}C into hydrocortisone was obtained from the secretion of hydrocortisone per milligram of protein nitrogen in the first fraction of adrenal tissue and from the number of ^{14}C dpm secreted as hydrocortisone in the second fraction of the same adrenal tissue.

D. Intracellular equilibration of newly synthesized cholesterol. After incubation with acetate-2-^{14}C the adrenal tissue was fractionated as previously described (4) in order to follow the intracellular equilibration of newly synthesized cholesterol. The isolated subcellular fractions were further characterized by their content of DNA, RNA, and cytochrome oxidase per milligram of protein nitrogen. RNA, DNA, and proteins were extracted by the method of Schmidt and Thannhauser (14). DNA was measured as described by Ceriotti (15). The absorption spectrum of the extracted RNA was verified in a Cary 15 spectrophotometer and the concentration determined thereafter from the absorption at 260 nm. In a known fraction of the subcellular organelles, the cytochrome oxidase was measured by the

method of Smith (16), cytochrome C being first reduced on a column of Duolite S_{10} as described by Chantrenne (17).

RESULTS

A. In vitro synthesis of adrenal cholesterol from acetate-2-^{14}C

Incorporation of acetate-2-^{14}C into cholesterol according to acetate concentration. As shown by Fig. 1, for a same number of microcuries of labeled acetate per milligram of incubation medium (20 μCi/ml) and in a same adrenal tissue, the incorporation of acetaate-2-^{14}C into free and esterified adrenal cholesterol measured after a 3 hr incubation remains fairly constant when the concentration of acetate in the medium is made to vary from 6.365 μmoles/ml to 1.115 μmole/ml; in other words, since the in vitro synthesis of cholesterol does not fall at lower acetate concentrations, it appears to be a reaction of zero order, in the concentration range studied.

Comparison of cholesterol synthesis in the zonas "reticularis" and "fasciculata" control conditions. Our results, obtained from eight different adrenal glands (Table I), indicate that everything else being equal the synthesis of cholesterol is several times more important in the zona reticularis than in the zona fasciculata ($P < 0.001$). This synthesis is also quite variable from gland to gland, particularly in the so-called zona "reticularis," perhaps because of variable degrees of contamination of one zone by the other. The zonal distribution of adrenal cholesterol synthesis is the opposite of that of adrenal cholesterol concentration and more precisely of that of esterified adrenal cholesterol concentration which is significantly higher ($P < 0.01$) in the zona fasciculata than in the zona reticularis.

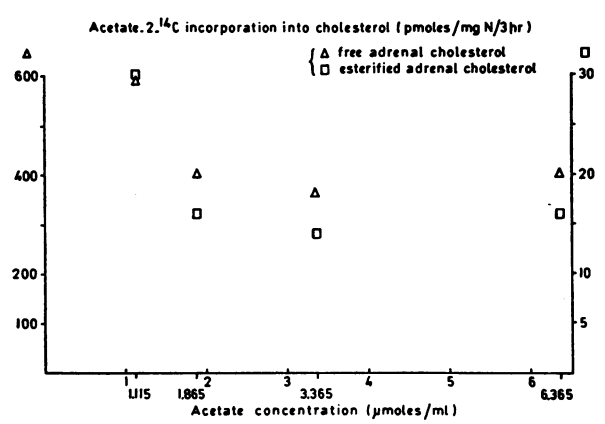

FIGURE 1 Acetate concentration and cholesterol synthesis. The incorporation of acetate-2-^{14}C into free (Δ) and esterified (□) adrenal cholesterol remains fairly constant when the concentration of acetate in the incubation medium is made to vary from 6.365 to 1.115 μmoles/ml (Krebs-Ringer-bicarbonate; 20 μCi acetate-2-^{14}C/ml.

TABLE I
Normal Adrenal Glands

Study	Zone‡	Acetate-2-^{14}C incorporation into cholesterol* Free	Esterified	Total	Ratio of esterified/free	Cholesterol concentrations Free	Esterified
		pmoles/mg nitrogen per 3 hr				*mg/mg nitrogen*	
1	R	179	13	192	0.07	0.07	0.91
	F	102	15	117	0.15	0.13	2.09
2	R	453	70	523	0.15	0.07	0.54
	F	191	6	197	0.03	0.13	0.72
3	R	486	92	578	0.19	0.24	1.57
	F	64	25	89	0.40	0.27	2.57
4	R	459	151	610	0.33	0.22	1.54
	F	79	25	104	0.29	0.20	1.74
5	R	799	80	879	0.10	0.19	1.38
	F	339	40	379	0.12	0.24	2.50
6	R	998	122	1,120	0.12	0.21	1.42
	F	309	44	353	0.14	0.22	2.56
7	R	3,222	234	3,456	0.07	0.26	1.60
	F	485	42	527	0.09	0.42	2.44
8	R	7,290	934	8,224	0.13	0.18	1.10
	F	916	90	1,006	0.10	0.19	1.72
Means	R	1,736	212	1,948	0.14	0.18	1.25
	F	311	36	347	0.16	0.22	2.04

* 3 hr incubation in a Krebs-Ringer-bicarbonate medium; sodium acetate, 6.365 μmoles/ml; acetate-2-^{14}C, 20 μCi/ml.
‡ R, reticularis; F, fasciculata.

Cholesterol synthesis in Table I is expressed in terms of pmoles of acetate-2-^{14}C incorporated into cholesterol per milligram of protein nitrogen during a 3 hr incubation; when converted in terms of weight it actually appears to be very low with a mean value of 0.05 μg of cholesterol per milligram of protein nitrogen in the zona reticularis and of 0.009 μg in the zona fasciculata.

Interestingly enough, as also shown in Table I, the concentration of free adrenal cholesterol and its rate of esterification are quite similar in the two adrenal zones.

Influence of ACTH stimulation and of dexamethasone suppression. Under the influence of ACTH stimulation the adrenal cortex becomes delipidated and the morphological differences between the adrenal zones tend to disappear (18). Correspondingly (Table II) we find that the concentration of esterified adrenal cholesterol falls significantly in our six patients ($P < 0.01$) and becomes similar in the inner and outer cortex. The modifications of total cholesterol synthesis (Table II) are symmetrical to those of esterified cholesterol concentration: the total adrenal cholesterol synthesis increases strikingly ($P < 0.005$ in the zona "reticularis" and $P < 0.001$ in the zona "fasciculata") and in proportion to the degree of esterified adrenal cholesterol depletion (Fig. 2); it also becomes similar in the inner and outer cortex.

TABLE II

Adrenal Glands under ACTH or Dexamethasone

Zone‡		Acetate-2-^{14}C incorporation into cholesterol*				Cholesterol concentrations	
		Free	Esterified	Total	Ratio of esterified/free	Free	Esterified
		pmoles/mg nitrogen/3 hr				*mg/mg nitrogen*	
Adrenal glands under ACTH			(n = 6)			(n = 6)	
R	Means	10,714	2,204	12,916	0.21	0.17	0.33
	Ranges	(2,480–22,814)	(344–3,770)	(2,824–26,230)	(0.14–0.33)	(0.12–0.20)	(0.14–1.20)
F	Means	8,153	1,312	9,464	0.17	0.18	0.41
	Ranges	(1,256–19,763)	(188–2,238)	(1,444–21,714)	(0.10–0.27)	(0.14–0.23)	(0.17–1.43)
Adrenal glands under dexamethasone			(n = 6)			(n = 8)	
R	Means		undetectable			0.18	1.94
	Ranges					(0.13–0.28)	(0.93–3.19)
F	Means		undetectable			0.23	2.81
	Ranges					(0.14–0.34)	(1.42–4.66)

* 3 hr incubation in a Krebs-Ringer-bicarbonate medium; sodium acetate; 6.365 μmoles/ml; acetate-2-^{14}C, 20 μCi/ml.
‡ R, reticularis; F, fasciculata.

Despite this striking stimulation which suggests that acetate is a good precursor of adrenal cholesterol in our experimental system, when expressed in absolute terms, the in vitro cholesterol synthesis remains moderate with a mean figure of 0.360 μg in the zona reticularis," of 0.260 μg in the zona "fasciculata," per milligram of protein nitrogen and during a 3 hr incubation; however, in the most delipidated adrenal glands, this in vitro cholesterol synthesis can reach 0.700 μg/mg N/3 hr, a figure which is still low but practically reaches the range of the glucocorticosteroid hormone secretion obtained in vitro in the zona reticularis of control adrenal glands (see below-section B of Results).

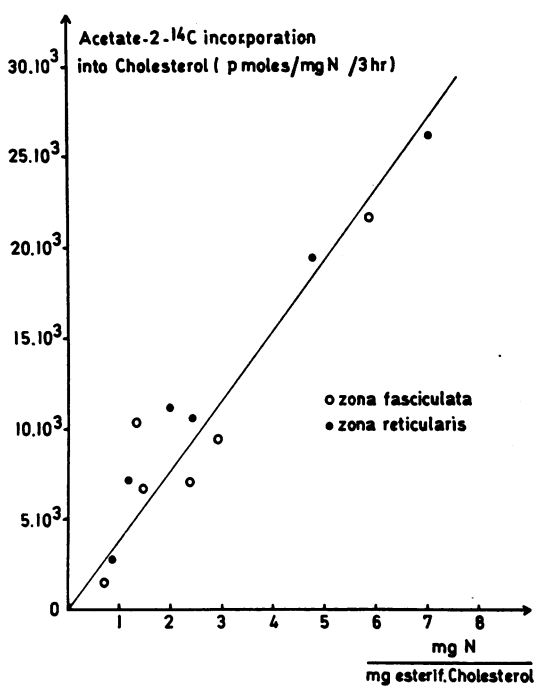

FIGURE 2 Cholesterol synthesis and cholesterol concentration under ACTH. In delipidated adrenal glands the incorporation of acetate-2-^{14}C into cholesterol is proportional to the degree of esterified cholesterol depletion. The correlation coefficient is 0.92.

Finally, it is remarkable that under ACTH stimulation the concentration of free adrenal cholesterol and the rate of free adrenal cholesterol esterification remain very constant and are practically identical to those found under control conditions (Tables I and II).

Under dexamethasone (Table II) the synthesis of adrenal cholesterol becomes undetectable. With regard to esterified adrenal cholesterol concentration in the eight patients investigated, it is slightly elevated in the zona "reticularis" ($P < 0.05$) but not significantly elevated in the zona "fasciculata" when compared with the control adrenal glands.

Qualitative aspects of the esterification of cholesterol synthesized in vitro. In four control adrenal glands and in three which had been stimulated by ACTH the various cholesteryl esters were separated from each other after incubation with acetate-2-^{14}C in order to compare their specific activities.

For the purpose of presentation, these specific activities were normalized to that of the saturated or Δ_0 form: it can be seen in Table III that they are fairly similar to each other except, as in vivo, for a tendency to increase progressively with the unsaturation of the fatty

acid moiety and except for a considerable increase in the most unsaturated cholesteryl esters. It is remarkable in this regard that the specific activities of cholesterol in the Δ_1 and Δ_2 esters are practically identical to each other, i.e., that the relative amounts of free adrenal cholesterol esterified *in situ* with Δ_1 and Δ_2 fatty acids are nearly equivalent to the relative concentrations of the corresponding adrenal cholesteryl esters which accumulate in vivo (19). It is also remarkable (Fig. 3) that the relative proportions of the various adrenal cholesteryl esters to be formed *in situ* do not vary with time during the incubation: thus there is no evidence of any interconversion. Finally, it is noteworthy (Table III) that the relative concentrations of the various adrenal cholesterol esters are not modified by ACTH despite variable and sometimes considerable degrees of adrenal cholesterol depletion.

Intracellular equilibration of cholesterol synthesized in vitro. As shown in Table IV, except for the fraction which is trapped in the lipid droplets and which is less readily available for exchange, free cholesterol equilibrates rapidly within the adrenal cell; the esterification must take place on the subcellular organelles although most of the esterified adrenal cholesterol is found in the supernatant and in the lipid droplets. With regard to the later two subcellular fractions it should be noted that their distinction is particularly arbitrary since the supernatant and the lipid droplets were thoroughly mixed with each other during the homogenisation of the adrenal tissue. Table IV also gives some indications as to the purity of the other subcellular fractions investigated: in terms of DNA, RNA, and cytochrome oxidase

TABLE III

*Fractionation of the Adrenal Cholesteryl Esters after Incubation**

	Control adrenal glands (n = 4)		Adrenal glands under ACTH (n = 3)	
	Specific activities‡	Concentrations	Specific activities‡	Concentrations
		%		%
Δ_4§ Means	4.92	18.5	4.73	13.6
Ranges	(3.78–6.39)	(11.7–29)	(4.10–5.46)	(11.2–17)
Δ_3 Means	1.52	12.0	1.83	12.3
Ranges	(1.15–1.92)	(8.6–17)	(1.69–2.01)	(11.7–14.3)
Δ_2 Means	1.38	11.8	1.62	11.3
Ranges	(1.14–1.63)	(9–15)	(1.60–1.64)	(10–13.1)
Δ_1 Means	1.10	40.1	1.52	41.3
Ranges	(0.89–1.40)	(33–47.5)	(1.40–1.61)	(39.1–43.8)
Δ_0 Means	1.00	17.0	1.00	20.3
Ranges		(11–19.3)		(16–22.9)
Concentrations of the esters, *mg cholesterol/mg N*				
Means	1.53		0.63	
Ranges	(1.41–2.62)		(0.46–0.89)	

* Conditions of incubation as in Table I.
‡ Normalized to Δ_0 and expressed in dpm/mg cholesterol.
§ Number of double bonds of the fatty acid moiety.

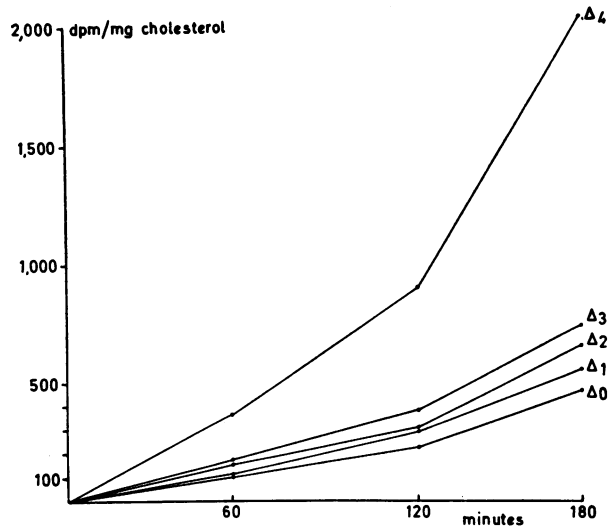

FIGURE 3 Influence of time on cholesteryl esters specific activities. During a 3 hr incubation the relative specific activities of the various adrenal cholesteryl esters remain constant. (The whole cortical tissue of two adrenal glands was combined for this analysis).

per milligram of protein nitrogen these fractions are not pure but may be viewed predominantly as nuclei, microsomes, or mitochondria.

B. In vitro synthesis of glucocorticosteroid and mineralocorticosteroid hormones under control conditions

As shown in Table V the absolute values of hydrocortisone and corticosterone secretion are much higher than those of the corresponding cholesterol synthesis although this comparison might not be perfectly valid since the steroid hormones are produced from cholesterol which is already abundantly present within the adrenal cell whereas cholesterol is synthesized de novo from acetate: such a comparison therefore does not take into account the relative duration of the two syntheses and the problem of the efficiency of acetate as a precursor to cholesterol. In any case, the cortical distribution of hydrocortisone and corticosterone secretion is certainly different from that of cholesterol synthesis, since, everything else being equal, this secretion tends to be more important in the zona fasciculata than in the zona reticularis ($P < 0.01$). On the other hand the in vitro secretion of the adrenal steroid hormones undoubtedly reflects new synthesis since the concentrations of these hormones in the adrenal tissue are relatively low and do not vary significantly during the incubation. Indeed the mean hydrocortisone, corticosterone, and aldosterone concentrations measured before incubation in the zona "reticularis" of the first four pairs of adrenal glands shown in Table V amount respectively to: 0.30–

TABLE IV

*Intracellular Equilibration of Cholesterol Synthesized in Situ**

Fraction	Specific activities of adrenal cholesterol						Characteristics of the subcellular fractions‡				
	Loo§		Deh§		Lul§						
	Free	Esterified	Free	Esterified	Free	Esterified	DNA	RNA	Cytochrome oxidase	Free cholesterol	Esterified cholesterol
	dpm/mg		*dpm/mg*		*dpm/mg*		*mg/mg N*	*mg/mg N*	*k/mg N*	*mg/mg N and % of total*	*mg/mg N and % of total*
Nuclei	51,237	286	69,259	2,987	659,464	29,250	0.405	0.079	0.010	0.10 20	1.69 21
Mitochondria	45,199	666	47,250	2,543	630,724	89,873	0.056	0.097	0.113	0.09 6	0.39 1
Microsomes	66,676	1,459	70,023	3,103	928,808	89,400	0.017	0.348	0.016	0.28 31	0.33 2
Supernatant	29,336	—	38,409	2,743	669,769	—	0.015	0.060			
										{0.71 {42	{20.85 {76
Lipid droplets	3,879	56	6,523	193	—	—					

* 3 hr incubation—same conditions as Table I.

‡ DNA, RNA, cytochrome oxidase: means from three different adrenal glands where the concentrations of unfractionated DNA and RNA were, respectively, 0.104 and 0.161/mg N; cholesterol: measured in one adrenal gland where the concentrations of unfractionated free and esterified cholesterol were, respectively, 0.19 mg/mg N and 2.95 mg/mg N.

§ Loo and Deh: control studies; Lul: under ACTH.

$\| k = \frac{1}{t} \cdot \log \frac{(Cyt^{++})_0}{(Cyt^{++})_t}$.

0.20 and 0.01 μg/mg of protein nitrogen; in the zona "fasciculata" we find correspondently: 0.66–0.51 and 0.04 μg/mg of protein nitrogen. After the incubation in the same tissues the above values become: 0.24–0.11 and 0.00 μg in the zona "reticularis," 0.47–0.21 and 0.00 μg in the zona "fasciculata."

C. Direct comparison of glucocorticosteroid secretion with cholesterol synthesis under control conditions

Four pairs of adrenal glands in which an appropriate separation of the adrenal zones could not be obtained were subdivided into two identical parts: the first of these two parts was incubated with cold acetate for the measurement of the hormone secretion, the second part was incubated with labeled acetate for the measurement of cholesterol synthesis and of acetate-2-^{14}C incorporation into the steroid hormones.

As confirmed in Table VI, the absolute synthesis of cholesterol is negligible when compared with the corresponding hormone secretion. Besides Table VI indicates that there is no important elective incorporation of acetate-2-^{14}C into the steroid hormones: in other words and consequently, the low magnitude of the in vitro cholesterol synthesis and its characteristic zonal distribution are not attributable to an elective incorporation of labeled acetate into the steroid hormones.

DISCUSSION

The extrapolation of in vitro biosynthetic data to the whole living organism is dangerous, particularly when acetate is utilized as a metabolic precursor since its activation to acetyl CoA might vary from tissue to tissue. However, our in vitro observations suggest very strongly that adrenal cholesterol synthesis must be low under normal conditions, much lower than the needs for steroid hormone production, particularly in the zona fasciculata, whereas under appropriate ACTH stimulation it can become as efficient as the normal hepatic cholesterol synthesis. Indeed, if one milligram of protein nitrogen corresponds to 75–100 mg of adrenal tissue (reference 18 and personal observations), our data indicate that the mean in vitro conversion of acetate-2-^{14}C to cholesterol within the zona fasciculata is normally of the order of 5,000–3,500 pmoles/g per 3 hr. These figures are practically identical to those found by Bhattathiry and Siperstein (20) in normal hepatic tissue obtained by liver biopsy in patients submitted to a high-cholesterol diet, i.e., when the human hepatic cholesterol synthesis is almost completely suppressed. On the other

hand, under ACTH stimulation we find that the conversion of acetate-2-^{14}C to adrenal cholesterol increases considerably and can even exceed 300,000 pmoles/g per 3 hr, a figure which is now practically identical to those obtained by Bhattathiry and Siperstein (20) in the liver from patients submitted to a normal cholesterol diet: indeed, under these circumstances the hepatic incorporation of acetate-1-^{14}C into cholesterol was found to average 282,000 pmoles/g per 2 hr. Furthermore, the normal rates of acetate incorporation into adrenal and hepatic cholesterol of man are both of the same order of magnitude as those obtained by Dietschy and Wilson (2) in the adrenal glands and in the liver of the squirrel monkey, respectively.

Our experimental observations indicate that however small the usual rate of adrenal cholesterol synthesis, it is several times higher in the zona reticularis than in the zona fasciculata. They indicate also that this low rate of adrenal cholesterol synthesis is not an artifact due to a preferential and immediate conversion of newly synthesized cholesterol into steroid hormones; neither does the zonal difference in adrenal cholesterol synthesis result from an opposite zonal difference in this preferential conversion: the latter is too small to interfere with the calculation of the former.

What then regulates adrenal cholesterol synthesis, what might be its functional significance and why such a cortical gradient? With regard to the regulation and to the cortical gradient our results indicate that the rate of adrenal cholesterol synthesis is not a direct function of the needs for cholesterol as precursor to the glucocorticosteroid hormones. In fact, cholesterol synthesis is much more important in the zona reticularis although the latter, everything else being equal, tends to produce less hydrocortisone and less corticosterone than the zona fasciculata. On the other hand, one is impressed by the relationship which is repeatedly found between adrenal cholesterol synthesis and adrenal cholesterol concentration: under normal conditions the synthesis is higher in the zona reticularis which contains less cholesterol, and under ACTH stimulation the synthesis increases in direct proportion to the degree of adrenal cholesterol depletion. These observations might indicate instead that the synthesis of cholesterol in the adrenal cortex is regulated as in the liver by its intracellular concentration through a mechanism of feedback inhibition (21, 22).

TABLE V

*In Vitro Secretion of the Adrenal Steroid Hormones**

Study	Zone‡	Aldosterone	Corticosterone	Hydrocortisone
			μg/mg protein N/2 hr	
Volv	R	0.02	0.29	1.64
	F	0.02	0.56	4.20
DeN	R	0.02	0.22	1.77
	F	0.06	0.67	3.76
DeV	R	0.01	0.18	0.98
	F	0.00	0.39	1.73
Nic	R	0.00	0.11	0.58
	F	—	0.22	1.13
Jon	R	—	0.10	0.74
	F	—	—	1.02
Spart	R	0.00	0.12	0.83
	F	0.01	0.18	2.06
Raez	R	0.00	0.04	0.55
	F	0.01	0.19	1.16
Behn	R	—	0.42	3.70
	F	—	0.93	5.00
Sur	R	—	0.51	3.70
	F	0.07	0.69	3.50
			ACTH§	ACTH§
Fau	R	0.00	0.13 → 0.24	1.03 → 1.28
	F	0.05	0.89 → 1.69	2.54 → 4.28
Moe	R	0.03	0.14 → 0.37	1.39 → 3.61
	F	0.04	0.25 → 0.95	1.73 → 5.17
Duch	R	0.01	0.15 → 0.18	0.73 → 1.09
	F	0.02	0.47 → 0.51	1.13 → 1.11
Means	R	0.01	0.21	1.47
	F	0.03	0.49	2.41

* 2 hr incubation after a 1 hr preincubation in a Krebs-Ringer-bicarbonate medium.

‡ R: "reticularis"; F: "fasciculata".

§ 3 U of ACTH per ml of incubation medium (Cortrosyn: 24 first aminoacids of ACTH).

TABLE VI

*Comparison of Cholesterol Synthesis with the Synthesis of the Glucocorticosteroid Hormones**

Study	Acetate-2-^{14}C incorporation into cholesterol			Acetate-2-^{14}C incorporation into hydrocortisone‡	Cholesterol synthesis	Hormone synthesis	
	Free	Esterified	Total			Hydrocortisone	Corticosterone
	p moles/mg N/2 hr			*p moles/mg N/2 hr*	*ng/mg N/2 hr*	*μg/mg N/2 hr*	
	186	11	197	4	4	2.03	0.48
Leu	256	4	260	2	7	0.68	0.22
Dam	104	10	114	5	3	4.76	1.00
Jan	371	9	380	3	10	0.54	0.16
Means	229	9	238	4	6	2.00	0.46

* 2 hr incubation in Krebs-Ringer-bicarbonate medium; acetate, 1.115 μmole/ml; acetate-2-^{14}C, 20 μCi/ml; the incubation followed a 1 hr preincubation without labeled acetate; no labeled acetate either for the measurement of hormone synthesis.

‡ Purified by paper chromatography on Bush 5 (after acetylation plus an additional chromatography the remaining counts are insufficient for an accurate measurement).

With regard to functional significance the synthesis of free adrenal cholesterol seems complementary to the hydrolysis of esterified adrenal cholesterol when, under the influence of ACTH stimulation, the stores of the cholesteryl esters become progressively depleted. However, if the in vitro equivalence of cholesterol synthesis in the most delipidated adrenal glands with that of normal hepatic tissue is extrapolated to the living man on a whole organ basis (23) it can be calculated that the over-all rate of cholesterol synthesis by the adrenal cortex, under optimal conditions, should not exceed 20 or at most 30 mg/day, which is still moderate when compared with the steroid hormone production under intense ACTH stimulation (24); our in vitro finding that the highest syntheses of adrenal cholesterol under intense ACTH stimulation hardly reach the lowest control values for hydrocortisone secretion support the same inference. While, as already mentioned, such extrapolations have definite limitations, they suggest that even in delipidated glands the passive inflow of plasma cholesterol remains the most important source of adrenal cholesterol; the advantage of a moderate local cholesterol synthesis might then reside in a greater flexibility and sensibility of adjustment to the instantaneous demands at a time when the depleted stores of esterified adrenal cholesterol can no longer play this role of buffer.

Our data also provide some information with regard to intracellular equilibration and esterification of adrenal cholesterol. Like free adrenal cholesterol coming from plasma (4), free adrenal cholesterol synthesized *in situ* is found to equilibrate rapidly within the adrenal cell, except for the pool which is trapped in the lipid droplets and seems less readily available for intracellular exchange; in addition, it is rapidly esterified and, interestingly enough, the percentage of esterification is the same in the two adrenal zones and remains constant despite ACTH stimulation; the importance of the esterification therefore appears to be a constant function of free adrenal cholesterol concentration and seems independent of the levels of free cholesterol synthesis, esterified cholesterol concentration, and steroid hormone production. This lack of regulation of cholesterol esterification by esterified cholesterol concentration suggests that esterified cholesterol could accumulate within the adrenal cells when for some metabolic or mechanical reason increased amounts of free plasma cholesterol are made available to the intracellular esterifying enzymes without correspondently increased hydrolysis; such might be the case under ACTH plus aminogluthetimide and explain the observations of Dexter, Fishman, Ney, and Liddle (25); such might also be the case in adrenal lipoid hyperplasia where a congenital defect in cholesterol side-chain cleavage induces presumably ACTH hypersecretion.

From a qualitative point of view and with the exception of the most unsaturated cholesteryl esters (Δ_4) the pattern of the in vitro esterification is comparable to that previously found in vivo (4). Indeed, the specific activities of cholesterol in the various esters are fairly similar to each other, indicating that the distribution of the newly formed cholesteryl esters is similar to that already present before incubation; this is particularly striking for the Δ_1 and Δ_2 cholesteryl esters (essentially oleate and linoleate) which are the most abundant and are found in reversed proportions in plasma (26) and in the adrenal cortex (19). Furthermore, despite their similarity, the specific activities of cholesterol in the various esters tend to increase progressively with the unsaturation of the fatty acid moiety: this was also found in vivo. Finally, it is remarkable that the relative values of the cholesterol specific activities in the different esters do not vary with time. The correspondance between the in vivo and in vitro observations suggests, as previously discussed that the characteristic distribution of the adrenal cholesteryl esters, results from an *in situ* esterification rather than from a selective and direct uptake of the plasma cholesteryl esters, the differences of specific activity according to the unsaturation of the fatty acid moiety perhaps being due to differences of affinity according to the fatty acid involved in this esterification. On the other hand, the constancy in time of the relative specific activities argues against the hypothesis of a non selective uptake of the plasma cholesteryl esters followed by intra-adrenal interconversions (27).

The in vitro system reveals a striking propensity for the accumulation of the Δ_4-unsaturated cholesteryl esters of which arachidonate is certainly the most representative (19). We have no explanation for the difference between the in vitro and the in vivo observations with regard to the latter point. Some experimental evidence suggests that the fatty acid moiety of the adrenal cholesteryl esters formed *in situ* is provided by an intracellular pool of free fatty acids (28): it is then possible that in vitro, because of the absence of a free fatty acid uptake from plasma, the composition of this intracellular pool is modified. In any case, such a propensity to form the most unsaturated cholesteryl esters must explain their relative abundance in the adrenal cortex. Furthermore, the accumulation of these esters might be a means of storing arachidonic acid which is a precursor of the prostaglandins (29); the latter in turn might regulate the adrenal vasculature and blood perfusion; by their ACTH-like effects the prostaglandins might also exert a more direct control on steroid hormone production (30).

Finally, with regard to the functional significance of the characteristic fatty acid distribution of the adrenal cholesteryl esters it is remarkable that this distribution is

not modified by ACTH. In other words, under ACTH stimulation there is no preferential utilization of any of the many adrenal cholesteryl esters for steroid hormone synthesis.

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