Properties of the Ultraviolet-Absorbing Lipids Produced by Rat Adrenals *in vitro*

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In their publication on the microestimation of steroids produced by rat adrenals *in vitro*, Elliott, Birmingham, Schally & Schonbaum (1954) pointed out that consistently higher values were obtained when the steroids were measured by ultraviolet absorption (corrected for the effect of interfering substances), than by reduction of a tetrazolium derivative. Addition of adrenocorticotrophic hormone to the medium increased the steroid values obtained by both methods as well as the absolute difference between the values. They concluded that, in addition to known corticoids, the isolated rat adrenal produces material with an α : β -unsaturated ketone group but without a reducing α -ketol side chain, and that the formation of this material was stimulated by adrenocorticotrophic hormone. More recently it was shown that a large proportion of the ultraviolet-absorbing lipids produced by the rat adrenal is located in the zone called X by Heard *et al.* (1954), after chromatography in toluene-propylene glycol, and that this material does not reduce tetrazolium but gives a typical Porter-Silber spectrum (Birmingham & Kurlents, 1959). It appears to be identical with the non-reducing Porter-Silber chromogen isolated from rat-adrenal-vein blood by Reif & Longwell (1958), with a mobility of 0.62 in the toluene-methanol-water (4:3:1, by vol.) system of Bush. A non-reducing ultraviolet-absorbing lipid with similar mobility had been found earlier in rat blood by Bush (1953) and by Vogt (1955).

Aside from zone X, three major ultraviolet-absorbing regions are obtained when lipids from incubated rat adrenals are chromatographed in toluene-propylene glycol. These have been designated by Heard *et al.* (1954) as zones F, E and B and have been associated with cortisol, cortisone, aldosterone, and with corticosterone and *epi*-corticosterone (Heard *et al.* 1954, 1956; Eisenstein, 1956). The production of appreciable amounts of material with mobility of 11-deoxycorticosterone has also been reported (Koritz, Péron & Dorfman, 1957; Roberts, 1957). In a recent study of the spectra obtained after exposure of eluates from zones F and E to the Porter-Silber reagent no evidence for the liberation of cortisol or cortisone by the rat adrenal could be obtained (Birmingham & Kurlents, 1959).

The present investigation deals with a closer characterization of the ultraviolet-absorbing lipids produced by isolated rat adrenals and with effects upon their elaboration of stimulation by adrenocorticotrophic hormone, of prolonged incubation and of variation in the substrates and ions of the medium. Parts of this work have been presented at the First Meeting of the Canadian Federation of Biological Sciences in June 1958 and at the 43rd Annual Meeting of the Federation of American Societies for Experimental Biology in April 1959.

METHODS

Incubation and chromatography

Adrenals from 8-30 male Sprague-Dawley rats, anaesthetized with sodium pentobarbital, were quartered and incubated at 38° in Ringer bicarbonate-glucose solution for 2-10 hr. with hourly changes of the medium. Adrenocorticotrophic hormone (ACTH; U.S.P. or Connaught lot no. 34) was used at concentrations of 100-200 milliunits/100 mg. of tissue. The methylene chloride extracts of the incubation media were chromatographed in the toluene-propylene glycol system of Burton, Zaffaroni & Keutmann (1951) on strips of Whatman no. 42 filter paper at 31° for 48-60 hr. For the estimation of lipids less polar than corticosterone the time of development was adjusted to prevent the solvent front from running off the paper, or the effluent was rechromatographed. Acetylated lipids were prepared and developed in the benzene-formamide system as outlined by Zaffaroni & Burton (1951).

Detection of steroids on paper

Ultraviolet-absorbing lipids were located by scanning the paper strips in the Beckman model DU spectrophotometer at 240 m μ . A diaphragm placed in the light path confined the width of the paper exposed to the ultraviolet light to 3 mm. Reducing lipids were made visible with an alkaline solution of the tetrazolium derivative M. and B. 1767 (May and Baker Ltd.) [2 mg. of 2:5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride + 2 ml. of 95% (v/v) ethanol + 5 ml. of 2.5N-NaOH], Porter-Silber chromogens by passing the paper through the Porter-Silber reagent (Birmingham, 1959). With the latter procedure a bright-yellow colour developed immediately at room temperature with the 21-aldehydes of steroids, or in 0.5-2 hr. with steroids containing the dihydroxyacetone side chain. The following substances could be detected with this reagent in amounts of at least 2 μ g./cm.²: cortisol, cortisone, 11-deoxy-17-hydroxycorticosterone and their acetates; Schiff-positive, non-reducing oxidation products of cortisol, cortisone

11-deoxy-17-hydroxycorticosterone, corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone and their acetates. With 50 μ g. of corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone and 17-hydroxyprogesterone no colour was obtained.

Other tests on paper included the Zimmermann test and the reaction for 17:20-diols by oxidation with lead tetraacetate, followed by the Zimmermann test (Bush, 1955), the vanillin test (McAleer & Kozlowski, 1956), exposure to concentrated sulphuric acid (Zaffaroni & Burton, 1951) and the Schiff test (Birmingham, 1959). In the last-named procedure the paper strips were passed through a 1% solution of *p*-rosaniline hydrochloride in sulphurous acid. Freshly prepared 21-aldehydes of cortisol, cortisone, 11-deoxy-17-hydroxycorticosterone, corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone (10 μ g./cm.²) caused the development of a purple spot which appeared as the rest of the strip turned pink. The corresponding ketols did not react in amounts of 50 μ g./cm.², nor did aldosterone. It was noted that freshly prepared aldehydes reacted at a lower concentration than material which had been stored in the cold in methanol, or which had been chromatographed. The aldehydes were prepared by a procedure adapted from that of Beyler & Hoffman (1957) for the preparation of 21-aldehydes of corticosterone and 11-dehydrocorticosterone. The steroid (10–20 mg.) was dissolved in 4 ml. of methanol. A 3% (w/v) solution of cupric acetate in 80% (v/v) methanol (1 ml.) and 1 drop of acetic acid were added and the mixture was heated in a water bath at 60°. Spot tests were performed at intervals on portions of 10 μ l. Solutions containing originally 11-dehydrocorticosterone and 11-deoxycorticosterone gave negative reactions with tetrazolium after 15 min. Solutions of corticosterone, 11-deoxy-17-hydroxycorticosterone, cortisone and cortisol ceased to reduce after 30 min. The formation of a 21-aldehyde was assumed when the steroids spotted on paper gave a negative reaction with the tetrazolium derivative, a rapidly developing bright-yellow colour with the Porter-Silber reagent and a positive Schiff test.

Tests on eluted lipids

Analytical procedures on eluted lipids included ultraviolet-absorption measurements to define a peak at

240 $m\mu$, reduction of the tetrazolium derivative M. and B. 1767, and the Porter-Silber reaction as outlined in previous publications (Elliott *et al.* 1954; Birmingham & Kurlents, 1958); the formation of dinitrophenylhydrazones (Weichselbaum & Margraf, 1955); formation of 17-oxo steroids before and after oxidation by sodium bismuthate (Bush & Willoughby, 1957) and by chromic acid (Wilson & Fairbanks, 1955); reaction with diphenylamine (Clark, 1955) and the determination of sulphuric acid spectra (Bernstein & Lenhard, 1953). For the last-named procedure a further purification of the eluates by application on a silica-gel column and gradient elution with benzene-ethyl acetate was sometimes necessary. Photometric readings were obtained in the beginning of this study with the Beckman model DU spectrophotometer and later with the DK-2 recording spectrophotometer. With microcuvettes, readings in 0.3 ml. of solution could be obtained.

RESULTS

Distribution of ultraviolet-absorbing lipids on the chromatogram

It is apparent from Table 1 that the relative amounts of lipid recovered from the various zones depend upon the state of the gland. Averages of between 2 and 4 μ g./100 mg. of adrenal/2 hr. were obtained in the absence of ACTH from each of the four main zones, and around 1 μ g. from a zone corresponding to the location of 11-dehydrocorticosterone (zone A). When adrenals were incubated with ACTH the amount of lipid recovered from zones X and B increased 3–11 times, whereas there was little effect on the contents of zones F, E and A. In addition, about 0.3–1 μ g./100 mg. of adrenal/2 hr. of an ultraviolet-absorbing, reducing material with the mobility of 11-deoxycorticosterone in the toluene-propylene glycol system was obtained occasionally from the medium of adrenals incubated with ACTH (see also Fig. 3).

Table 1. *Ultraviolet-absorbing lipids produced by rat adrenals incubated with and without adrenocorticotrophic hormone, and separated by paper chromatography in toluene-propylene glycol*

Amounts given under 'average recovery' are based on ultraviolet absorption; the values have not been corrected for a loss of about 10% in the extraction of the incubation medium with methylene chloride and for losses during chromatography. The recovery after elution of 10 samples of standard cortisol averaged 78 ± 12 (s.d.) %, and that of seven samples of corticosterone 87 ± 13 %. The combined amount of adrenal lipid eluted from zones F, E, X and B averaged 72 ± 13 % of the total ultraviolet-absorbing lipid applied, in 22 chromatograms. Figures given in parentheses represent numbers of experiments.

Zone	Average recovery (μ g./100 mg. fresh wt./2 hr. $\pm$ s.e.)		Ratios of stimulated to unstimulated production by the same glands				
	No ACTH	ACTH	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
F	3.2 ± 0.74 (5)	3.7 ± 0.31 (8)	1.3	1.3	1.7	—	1.7
						2.5 (F + E)	
E	2.8 ± 0.42 (6)	3.1 ± 0.34 (9)	1.4	1.1	1.7	—	1.1
X	2.6 ± 0.58 (7)	10.6 ± 0.89 (10)	3.5	3.3	7.9	5.2	11.1
B	4.0 ± 0.48 (8)	20.4 ± 1.69 (11)	3.4	4.6	9.1	6.4	11.4
A	0.9 ± 0.20 (3)	1.6 ± 0.21 (4)	1.2	—	—	1.7	2.0
DOC	0.0 (2)	0.6 ± 0.33 (3)					
Total	13.5	40.0					

Identity of ultraviolet-absorbing lipids

Ultraviolet-absorption spectra. The eluates from zones F, X, B and DOC had absorption maxima near 240 mμ, those of zones E and A near 238 mμ. The eluate of the solvent front did not give a curve characteristic for a Δ⁴-3-ketone and absorbed ultraviolet light maximally in the region below 225 mμ.

Reducing power and phenylhydrazone formation. The ratios of reducing lipid, and of Porter-Silber chromogen to ultraviolet-absorbing lipid, obtained with eluates from the different zones are given in Table 2. Expected ratios near unity of reducing lipid to ultraviolet-absorbing lipid, and of Porter-Silber chromogen to ultraviolet-absorbing lipid, were obtained with all chromatographed steroid standards containing respectively an α-ketol and an α-dihydroxyacetone side chain. Adrenal lipids

eluted from zones E and B gave average ratios of reducing to ultraviolet-absorbing lipid near 0.8, those of zones F and X less than 0.2. The ratios of Porter-Silber chromogen to ultraviolet-absorbing lipid obtained from zones F, E and B were less than 0.3, those of zone X near 0.9. These data indicate that cortisol and cortisone account for little, if any, of the ultraviolet-absorbing lipid found in the two more polar zones and that the Porter-Silber chromogens are represented by the non-reducing lipid in zone X.

The dinitrophenylhydrazones of steroids in alkaline solution yield absorption spectra that

Fig. 1. Absorption spectra of the dinitrophenylhydrazones of adrenal lipids from zones F, E, X and B (solid lines); of the dinitrophenylhydrazones of corticosterone, 11-deoxycorticosterone, progesterone and 11-hydroxyprogesterone (○); of cortisol, 17-hydroxy-11-deoxycorticosterone and cortisone (broken lines, curves 1, 2 and 3); of 17-hydroxy-11-deoxycorticosterone-21-aldehyde, and of 11-dehydrocorticosterone (●, curves 4 and 5). Curves 6 and 7, spectra obtained with dehydroisoandrosterone and cortolone. The dinitrophenylhydrazones were prepared by a modification of the procedure of Gornall & MacDonald (Weichselbaum & Margraf, 1955). Concentration of lipid, 10 μg./ml. (adrenal lipid based on ultraviolet absorption).

Fig. 2. Sulphuric acid spectra of rat-adrenal lipids eluted from zones B, A and DOC of the toluene-propylene glycol chromatogram and of chromatographed and eluted corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone. —, Adrenal lipids; ---, reference steroids.

Table 2. Ratios of reducing lipid and of Porter-Silber chromogen to ultraviolet-absorbing lipid obtained with extracts of incubation media of rat-adrenal glands and with reference steroids chromatographed in toluene-propylene glycol

The reference steroids in zones F, E and B were cortisol, cortisone and corticosterone respectively. Results are given ± S.D. Figures in parentheses represent numbers of experiments.

Zone	Reducing lipid/ultraviolet-absorbing lipid		Porter-Silber chromogen/ultraviolet-absorbing lipid	
	Adrenal lipid	Reference steroid	Adrenal lipid	Reference steroid
F	0.19 ± 0.11 (3)	0.92 ± 0.02 (2)	0.04 ± 0.04 (2)	1.00 ± 0.00 (2)
E	0.87 ± 0.15 (3)	1.00 ± 0.17 (2)	0.18 ± 0.08 (2)	1.11 (1)
X	0.14 ± 0.06 (2)	—	0.86 ± 0.22 (6)	—
B	0.75 ± 0.14 (7)	0.86 (1)	0.28 (1)	—
A	0.86 (1)	—	—	—

depend to some extent upon the structure of the steroid (Gornall & MacDonald, 1953). The spectra obtained after reaction of dinitrophenylhydrazine for 90 min. at 59° with chromatographed eluted steroid standards and with adrenal lipids are shown in Fig. 1. Adrenal lipids from zones F, E, X and B showed absorption maxima near 460 m μ , as did standard corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone, progesterone and 17 α -hydroxyprogesterone. The maximum of cortisone, 11-deoxy-17-hydroxycorticosterone and cortisol on the other hand was at 480 m μ and the maximum absorption was higher than that of the steroids lacking a dihydroxyacetone side chain. Higher absorption values were also obtained with C-21 aldehydes of steroids containing a ketol or a dihydroxyacetone side chain, but with a maximum located near 490 m μ .

Sulphuric acid spectra. The sulphuric acid spectra of the lipids eluted from zones F and E show an absorption maximum near 280 m μ , but lack the pronounced maxima in the wavelengths above 400 obtained with cortisol and cortisone. The lipids in zones B, A and DOC gave similar

spectra to those of corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone respectively (Fig. 2). The spectrum of the eluate from zone X had only one maximum, located at 290 m μ .

Reaction with diphenylamine. An absorption maximum at 530 m μ was not obtained with adrenal lipids from zones F, E, X and B exposed to diphenylamine, suggesting the absence of a ketone group at C-11 from the major lipid components of these zones.

17-Oxo steroid formation. No 17-oxo steroid could be detected in any region of the toluene-propylene glycol chromatogram including the solvent front (Fig. 3).

17-Oxogenic steroids. Ultraviolet-absorbing lipids eluted from zones F, E, X and B, and respotted in amounts of 20 μ g. over an area of less than 1 cm.², failed to give the Zimmermann test after oxidation with lead tetra-acetate, whereas a strong positive reaction was obtained with 10 μ g. of cortolone. Oxidation of the eluates from these zones with sodium bismuthate or chromic acid did not liberate a 17-oxo steroid.

Fig. 3. Ultraviolet-absorbing areas and colour reactions obtained with paper chromatograms of adrenal lipids (—) developed in toluene-propylene glycol, of reference steroids (---) and of the paper blank prepared from extracted medium (---). (b) and (c) Respotted effluents containing all the material less polar than corticosterone; (a) effluents containing corticosterone as well. Open bars, reducing areas; stippled bar, area giving a positive Zimmermann test. Strips from each chromatogram were passed through the tetrazolium, Porter-Silber and Zimmermann reagents. B, Corticosterone; DHA, dehydroisandrosterone; DOC, 11-deoxycorticosterone; A, 11-dehydrocorticosterone; SF, solvent front.

Acetylation. The number and position of ultraviolet-absorbing regions obtained after acetylation at room temperature of lipids in zones F, E, X and B, and their colour reactions, are compared with those of acetylated reference steroids in Table 3. Zone F yielded two ultraviolet-absorbing, non-reducing regions considerably less polar in the benzene-formamide system than cortisol acetate. A major, ultraviolet-absorbing, reducing substance with R_f near 0.8 and a small non-reducing region close to the solvent front were obtained with lipid eluted from zone E. A mixture of 20 μ g. of the acetylated lipid with R_f near 0.8 and 20 μ g. of aldosterone diacetate did not separate on chromatography in benzene-formamide. Zone B yielded an acetate in the exact location of corticosterone acetate. When the Porter-Silber chromogen eluted from zone X was acetylated and chromatographed in benzene-formamide, two ultraviolet-absorbing substances giving a positive Porter-Silber reaction were obtained, one with R_f near 0.5, the other with R_f near 0.9. Mixed chromatography indicated that the slow-moving material was slightly more polar than the acetate of 11-deoxy-17-hydroxycorticosterone, from which it could be distinguished by its lack of reducing power. It moved with the same speed as corticosterone acetate but differed from it by giving a positive Porter-Silber spot test and a negative reaction with tetrazolium. In agreement with the results of Reineke (1956), corticosterone acetate was found, upon mixed chromatography, to be more polar than 11-deoxy-17-hydroxycorticosterone acetate, reversing the order of mobility of the parent compounds.

Comparison with 21-aldehydes. The 21-aldehydes of corticosterone and 11-dehydrocorticosterone form phenylhydrazones with an absorption maximum at 410 $m\mu$ (Silber & Porter, 1957) and do not reduce tetrazolium (Simpson & Tait, 1953; Beyler & Hoffman, 1957). Several steroids containing an α -ketol side chain were therefore oxidized with cupric acetate in methanol after the procedure adapted from Beyler & Hoffman (1957). The mobilities in toluene-propylene glycol of the substances contained in the methanol solution after oxidation of the different steroids with cupric acetate, and their reaction with tetrazolium and with the Porter-Silber reagent, are indicated in Table 3. The main non-reducing products obtained after oxidation of cortisol and cortisone and giving the Porter-Silber reaction had mobilities similar to the parent compounds. The chromogen derived from corticosterone moved with one-third of the speed of corticosterone. The oxidation of 11-deoxy-17-hydroxycorticosterone resulted in a non-reducing Schiff-positive Porter-Silber chromogen with the exact mobility of the non-reducing Porter-Silber-positive lipid secreted by the rat adrenal. With all reference

steroids, additional non-reducing substances were obtained that did not, however, except cortisol, give the Porter-Silber reaction. Their mobilities are indicated in Table 3.

When the oxidation product of 11-deoxy-17-hydroxycorticosterone was acetylated with excess of acetic anhydride at room temperature and chromatographed in benzene-formamide, a phenylhydrazone was found with the same mobility as the rapidly moving component obtained after acetylation of the adrenal chromogen. The Porter-Silber-positive acetylation products of the 21-aldehydes of 11-dehydro- and of 11-deoxy-corticosterone were also located near the solvent front. None of the oxidized steroids examined gave a Porter-Silber-positive acetylation product with R_f near 0.5.

Although the oxidation product of 11-deoxy-17-hydroxycorticosterone had the same mobility as the adrenal Porter-Silber chromogen in the toluene-propylene glycol system and gave a positive spot test with phenylhydrazine, examination of the rate of formation of the phenylhydrazone from eluted material and various other tests revealed distinct differences between the two compounds. The adrenal lipid as well as the two derivatives of different mobilities obtained after acetylation formed the phenylhydrazone slowly and at the same rate as cortisol and cortisone. The oxidation product of 11-deoxy-17-hydroxycorticosterone, on the other hand, developed an absorption maximum well within 1 hr., located at 425 rather than 410 $m\mu$. The absorption was only about one-third of that obtained with the phenylhydrazone prepared from comparable amounts of the adrenal lipid, of cortisol and of the rapidly reacting 21-aldehyde of corticosterone. In several experiments the derivative of 11-deoxy-17-hydroxycorticosterone ceased to absorb after 24-48 hr. The dinitrophenylhydrazone of the adrenal lipid in alkaline solution had an absorption maximum near 460 $m\mu$, that of 11-deoxy-17-hydroxycorticosterone-21-aldehyde at 490 $m\mu$ (Fig. 1).

After exposure to concentrated sulphuric acid 20 μ g. of the derivative of 11-deoxy-17-hydroxycorticosterone became orange-brown, whereas a bright-yellow colour was obtained with the adrenal lipid as well as with 20 μ g. of corticosterone and of cortisol. The characteristic red spot was given by 11-deoxy-17-hydroxycorticosterone. Cortisol, 11-dehydrocorticosterone and 11-dehydrocorticosterone-21-aldehyde remained colourless, corticosterone-21-aldehyde became yellow-brown. The oxidation product of 11-deoxy-17-hydroxycorticosterone gave a sulphuric acid spectrum with a maximum near 290 $m\mu$ and minor peaks at 345 and 460 $m\mu$. The spectrum of the adrenal lipid and of both acetylated derivatives had only one maximum, located near 290 $m\mu$.

In the vanillin spot test the adrenal lipid from zone X ($20 \mu\text{g./cm.}^2$) gave a bright-yellow colour, whereas both 11-deoxy-17-hydroxycorticosterone and its aldehyde became wine-red. A pale yellow was obtained with cortisol, cortolone and 11-deoxycorticosterone, a dark brown with corticosterone-21-aldehyde. Corticosterone, 11-dehydrocorticosterone, cortisone, progesterone and 11β -hydroxyprogesterone remained colourless, whereas 17α -hydroxyprogesterone gave an orange colour, reported by McAleer & Kozlowski (1956) to be typical for 17-hydroxy-20-oxo-21-methylsteroids.

A positive Schiff reaction was never obtained with adrenal lipids.

Bioassays. Lipids eluted from zones X and B were examined for their ability to depress the number of eosinophils in adrenalectomized mice,

as an index of their effect on carbohydrate metabolism. In the Speirs-Meyer (1951) assay $25 \mu\text{g.}$ of eluate from zone B caused a fall of 81 % in eosinophils, whereas the same dose of lipid from zone X was completely inactive. A fall of 77 % was obtained with $8 \mu\text{g.}$ of cortisone.

The effect of lipids eluted from zones E, X and B on the ratio of urinary sodium to potassium was measured by the isotope method of Simpson & Tait (1952), eight rats at each of three doses of standard 11-deoxycorticosterone acetate and eight rats at one dose of adrenal lipid being used. The material from zone E, assayed at a dose of $0.1 \mu\text{g./rat.}$ had an effect comparable with that of $3 \mu\text{g.}$ of 11-deoxycorticosterone acetate. The lipid from zone B was inactive at a dose of $5 \mu\text{g./rat.}$ Equivocal results were obtained with the eluate from zone X. In

Table 3. *Mobilities, reducing power and phenylhydrazone formation by rat-adrenal lipids and their derivatives and by reference steroids*

r, Reducing; p, positive Porter-Silber test. Mobilities relative to the solvent front, or to corticosterone (B), are given with the standard error and the number of observations where it exceeds one. Values given in bold type indicate location of the major ultraviolet-absorbing area. Acetylated products were developed in the benzene-formamide system, all others in toluene-propylene glycol. Reference steroids in zones: F, cortisol; E, cortisone (except those marked with an asterisk); S, 11-deoxy-17-hydroxycorticosterone; B, corticosterone; A, 11-dehydrocorticosterone; DOC, 11-deoxycorticosterone. The preparation of oxidation products and acetylated derivatives is described in the text.

	Zone F	Zone E	Zone X	Zone S
Adrenal lipid	B0-09 $\pm$ 0.009(18)	B0-28 $\pm$ 0.018(18)r	B0-59 $\pm$ 0.014(21)p	—
Reference steroid	B0-09 $\pm$ 0.006(19)rp	B0-24 $\pm$ 0.006(19)rp B0-30r*	—	B0-77 $\pm$ 0.011(6)rp
Reference steroid oxidized with Cu^{2+} ions	B0-11 $\pm$ 0.01(2)p B0-39 $\pm$ 0.00(2)p B1-25 $\pm$ 0.05(2)	B0-30 $\pm$ 0.00(2)p B1-15 $\pm$ 0.05(2)	—	B0-61 $\pm$ 0.016(10)p B1-40 $\pm$ 0.00(2)
Acetylated adrenal lipid	0.64 0.90	0.76r 0.90	0.53 $\pm$ 0.023(9)p 0.87 $\pm$ 0.019(8)p	—
Acetylated reference steroid	0.07 $\pm$ 0.03(2)rp 0.23rp	0.28 $\pm$ 0.018(4)rp 0.70 $\pm$ 0.076(4)r*	—	0.62 $\pm$ 0.053(7)rp
Reference steroid oxidized with Cu^{2+} ions and acetylated	—	0.19r 0.31p	—	0.49 0.89 $\pm$ 0.021(4)p
	Zone B	Zone A	Zone DOC	
Adrenal lipid	B0-98 $\pm$ 0.024(17)r	0.32 $\pm$ 0.019(5)r	0.63 $\pm$ 0.026(3)r	
Reference steroid	(B1-0)r	0.31 $\pm$ 0.009(4)r	0.67 $\pm$ 0.013(5)r	
Reference steroid oxidized with Cu^{2+} ions	B0-05 $\pm$ 0.00(3) B0-18 $\pm$ 0.012(10) B0-33 $\pm$ 0.021(17)p B0-95 $\pm$ 0.029(4) B1-20	B0-35 $\pm$ 0.00(2) B0-74 $\pm$ 0.012(4)p	B0-80 0.30p	
Acetylated adrenal lipid	0.62r	—	—	
Acetylated reference steroid	0.57 $\pm$ 0.029(5)r	0.60r	0.84 $\pm$ 0.033(5)r	
Reference steroid oxidized with Cu^{2+} ions and acetylated	0.26p	0.55 0.89p	0.78p	

* Aldosterone and derivative.

three assays, at doses of 2.5, 5 and 5 $\mu\text{g./rat}$, effects corresponding to 1.9, 2.2 and 0.5 $\mu\text{g.}$ respectively of 11-deoxycorticosterone acetate were obtained.

Time course

The adrenal produces ultraviolet-absorbing lipids at a constant rate for at least 4–5 hr. endogenously or in response to added ACTH, provided that the medium is renewed frequently to prevent accumulation of steroids and inactivation of ACTH (Saffran & Bayliss, 1953; Birmingham & Kurlents, 1958). The rate of production over a 10 hr. period of each major lipid fraction in the presence of ACTH is shown in Fig. 4. After 4–6 hr. the rate declines but the percentages of the different fractions remain constant and are characteristic for the stimulated gland.

Effects of calcium and of carbohydrate substrates

Calcium and glucose are required for the response of the rat adrenal to added ACTH (Birmingham, Elliott & Valère, 1953; Péron & Koritz, 1958; Schonbaum, Birmingham & Saffran, 1956). Neither calcium nor glucose is effective alone (Table 4).

Fig. 4. Two experiments showing the time course of the production of ultraviolet-absorbing lipids of different mobilities by rat adrenals incubated with ACTH (200 milliunits/100 mg.). The medium was changed at hourly intervals and the production during 2 successive hours was pooled. Percentages of total eluate recovered in zones F, E, X and B during the first 2-hr. period: 9, 5, 33, 53 and 7, 6, 24, 62; during the fifth period: 12, 4, 30, 54 and 7, 4, 21, 68.

Both substances increase principally the production of corticosterone and the Porter-Silber chromogen, as does inosine (Tables 4 and 5). Ribose had no effect on the response to ACTH, in contrast with a previously published result (Schonbaum *et al.* 1956). Similar to the findings with calcium and glucose, inosine did not increase the production of steroids significantly in the absence of added ACTH.

DISCUSSION

The data indicate that the rat adrenal produces corticosterone, 11-dehydrocorticosterone and aldosterone *in vitro*. That the two first-mentioned substances are the main components of the eluates obtained from zones B and A of the toluene-propylene glycol chromatogram is apparent from the typical spectra obtained with sulphuric acid and from the ratios of ultraviolet-absorbing to reducing lipid. The material eluted from zone E had the following properties in common with aldosterone: mobility in the toluene-propylene glycol system, mobility of the acetylated derivative in benzene-formamide, ultraviolet- and sulphuric acid-absorption spectra, the ratio of ultraviolet absorption to reducing power, a negative Porter-Silber reaction and biological activity in the bioassay of Simpson & Tait at a dose of 0.1 $\mu\text{g./rat}$. Although a definite conclusion from only one assay at one dose is not justified, the material appeared to have one-third of the activity of aldosterone. The region may therefore also contain reducing, ultraviolet-absorbing and Porter-Silber-negative material without biological activity. Haynes, Koritz & Péron (1959) have recently renamed this zone aldosterone and mentioned that no cortisone was found to contaminate the aldosterone isolated from this region.

The identity of the material in zone F is not known. The lipid presumably contains an $\alpha:\beta$ -unsaturated ketone group as it absorbs maximally at 240 $m\mu$ in the ultraviolet range and its sulphuric acid spectrum has a peak near 290 $m\mu$. It does not seem to contain an α -ketol or α -dihydroxyacetone side chain as it does not reduce tetrazolium and does not give a Porter-Silber reaction. Mialhe-Voloss & Baulieu (1958) have reported the production of a Δ^4 -3-ketone-20:21-diol with the mobility of cortisol by incubated rat adrenals and Southcott, Bandy, Newsom & Darrach (1956) obtained 20- α -dihydrocorticosterone (pregn-4-ene-11 β :20 α :21-triol-3-one) in this region from extracts of mouse blood. Neither 20- α - nor 20- β -dihydrocorticosterone appeared to be among the lipids elaborated by rat adrenals *in vitro*. The sulphuric acid spectra of both isomers, prepared by reduction of corticosterone and separated by chromatography for 7 days as described by Southcott *et al.*,

had two pronounced maxima, near 290 and 390 $m\mu$ as opposed to the spectrum obtained from the adrenal lipid. However, the failure of the material to form a 17-oxo steroid after treatment with oxidizing agents, and the relatively low polarity of its acetylated derivatives, are compatible with a 20:21-diol structure. The absorption spectrum obtained with the diphenylamine reagent suggests the absence of a ketone function at C-11.

The lipid in zone X also remains to be identified. Ultraviolet-absorption spectra in methanol and in sulphuric acid, lack of reducing power and the

spectrum obtained with diphenylamine are compatible with a steroid containing a Δ^4 -3-ketone and lacking a ketone at C-11 as well as a readily reacting α -ketol side chain. The lipid is not 17-oxogenic and therefore does not appear to contain adjacent oxygen functions at C-17 and C-20. Acetylation of the material yielded two derivatives and it is therefore possible that more than one component is located in this zone. However, as both derivatives appeared to have the properties of the parent lipid, one component may be represented with more than one site available for acetylation. Differences in

Table 4. *Effects of calcium and of glucose on the output, by rat adrenals, of lipids of different mobilities in the toluene-propylene glycol system*

Adrenals were pre-incubated for 30 min. in glucose- and calcium-free medium. For the experimental period all media contained ACTH (200 milliunits/100 mg. fresh wt.) and, where indicated, 2.5 mM-calcium and 10 mM-glucose. The tissue was incubated for four successive 1 hr. periods with hourly changes of medium. Two separate experiments are shown. In experiment (a) all values are based on ultraviolet absorption. In experiment (b) lipids in zone X were determined by the Porter-Silber method, in all other zones by reduction of the tetrazolium derivative M. and B. 1767. All figures are expressed as $\mu\text{g./100 mg. fresh wt./2 hr.}$

Column no....	1	2	3	4	Calcium effect		Glucose effect		Effect of calcium + glucose (4-1)
					No glucose (2-1)	With glucose (4-3)	No calcium (3-1)	With calcium (4-2)	
Calcium ...	-	+	-	+					
Glucose ...	-	-	+	+					
Zone									
F (a)	1.7	1.0	2.3	3.5	-0.7	1.2	0.6	2.5	1.8
E (a)	0.7	0.9	1.5	2.2	0.2	0.7	0.8	1.3	1.5
(b)	0.8	1.1	2.6	1.7	0.3	-0.9	1.8	0.6	0.9
X (a)	1.3	1.9	1.1	6.7	0.6	5.6	-0.2	4.8	5.4
(b)	4.5	5.7	1.7	12.3	1.2	10.6	-2.8	6.6	7.8
B (a)	4.3	6.0	5.3	20.4	1.7	15.1	1.0	14.4	16.1
(b)	—	9.9	5.8	25.2	—	19.4	—	15.3	—
A (b)	1.5	1.1	0.8	2.2	-0.4	1.4	-0.7	1.1	0.7
Total (a)	8.0	9.8	10.2	32.8	1.8	22.6	2.2	23.0	24.8

Table 5. *Effects of different substrates on the output of rat-adrenal lipids in vitro*

All values are expressed as $\mu\text{g./100 mg. fresh wt./2 hr.}$; figures in parentheses are values obtained with the Porter-Silber reaction; all other values are based on ultraviolet absorption. ACTH, 200 milliunits/100 mg. fresh wt. Glucose, ribose, inosine, 10 mM; calcium, when present, 2.5 mM. In Expt. 3 adrenals were quartered and pre-incubated in glucose- and calcium-free Ringer bicarbonate solution; in all other experiments adrenals were halved and pre-incubated in glucose-free Ringer bicarbonate solution.

As the variation in production by samples from the same tissue in the absence of ACTH was always less than 30% and usually less than 15% in medium of the same composition, the values obtained by ultraviolet absorption or by the Porter-Silber reaction in the presence of ACTH indicate a significant increase.

No ACTH	Expt. 1	Expt. 3	Expt. 4	Expt. 5	Expt. 6
No addition	21, 22	—	—	—	—
Inosine	22, 27	16 (6)	—	—	—
Inosine, no calcium	—	11 (3)	—	—	—
Glucose	—	16 (8)	—	—	—
Glucose, no calcium	—	9 (4)	—	—	—
With ACTH	Expt. 2				
No addition	38, 38	—	66	43	54
Inosine	50, 54	36 (19)	77	74	66
Inosine, no calcium	—	18 (9)	—	—	—
Glucose	—	31 (15)	87	83	78
Glucose, no calcium	—	19 (8)	—	—	—
Ribose	—	—	55	55	49

rate of phenylhydrazone formation, in the reaction with sulphuric acid and in other spot tests, make it unlikely that the 21-aldehyde of 11-deoxy-17-hydroxycorticosterone forms a part of the lipid in zone X even though other properties are similar. The negative vanillin test suggests the absence of a 17-hydroxy-20-oxo-21-methyl function. There is thus no evidence so far that the rat adrenal produces a steroid with a hydroxyl group on C-17.

The presence of 6 β -hydroxy-11-deoxycorticosterone in zone X (Koritz & Péron, 1958) is unlikely as this compound is reducing, and it has a maximum near 345 m μ in sulphuric acid exceeding that near 290 m μ (Bernstein & Lenhard, 1954).

The Porter-Silber chromogen is present in adrenal-vein blood (Reif & Longwell, 1958). It may be excreted in an altered form as no Porter-Silber reaction has been obtained with extracts of rat urine (Silber & Porter, 1957). It is not extracted with alkali (unpublished observations; see also Reif & Longwell).

Factors affecting the production of corticosterone, such as ACTH, glucose, calcium, inosine and pantothenic acid (Longwell, Reif & Hansbury, 1958), affect the production of the Porter-Silber chromogen to the same extent. Whether the last-named substance is a precursor or derivative of corticosterone or whether it is elaborated independently remains to be determined. No evidence for an increased production of the chromogen by the addition of corticosterone, and vice versa, has been obtained so far (Birmingham & Kurlents, 1958, and unpublished observations).

A possible effect of this ACTH-dependent Porter-Silber chromogen on electrolyte metabolism is of interest. Liberation of this material at a higher than normal rate into the vein blood from enucleated regenerated adrenals *in situ* and its production in higher than normal amounts by regenerated adrenals incubated with [4-¹⁴C]progesterone have been reported (Laplante, Chappel, Stachenko & Giroud, 1959).

The lipid eluted from zone DOC had the same mobility, ultraviolet-absorption spectrum in methanol and reducing power as 11-deoxycorticosterone, and a similar, if not very distinctive, sulphuric acid spectrum. The poor yield of the substance has precluded further attempts at identification. The lack of a pronounced absorption in sulphuric acid at 380 m μ rules out the presence of 11 β -hydroxy-androst-4-ene-3:17-dione as does also the negative Zimmermann reaction. These results are similar to those obtained by Reif & Longwell (1958) with the lipids of rat-adrenal-vein blood that have R_f 0.86 in the toluene-methanol-water system of Bush. The relative amounts recovered by Longwell *et al.* (1958) from the various zones are also strikingly similar to those obtained in the present work with

glands stimulated by addition of ACTH to the medium. This is in line with the conclusion that the secretions *in situ* are those of an already stressed gland (Bush, 1953; Vogt, 1957). Useful information on factors affecting the secretion of the resting gland and on the requirements for the response to ACTH may thus be obtained by the method *in vitro* even though the rate of production is only one-fifth to one-tenth of that of the adrenal gland *in situ*. One reason for this difference is the accumulation during incubation of steroids and possibly other interfering substances, and the loss of activity of added ACTH.

The failure to detect cortisol, cortisone or 11-deoxy-17-hydroxycorticosterone in these studies and the poor recovery of material with the properties of 11-deoxycorticosterone, differ from the finding by Roberts (1957) of substances with chromatographic, spectrophotometric and staining characteristics of cortisol, cortisone and 11-deoxy-17-hydroxycorticosterone, and of the secretion by unstimulated rat adrenals *in vitro* of nearly equal amounts of material with mobilities of corticosterone and 11-deoxycorticosterone.

Haynes *et al.* (1959) have shown that adenosine 3':5'-monophosphate is more effective than ACTH in stimulating the steroid production by incubated rat adrenals and conclude that the main function of ACTH is the activation of phosphorylase, which, by accelerating breakdown of glycogen and subsequent C-1 oxidation, generates the reduced triphosphopyridine nucleotide required for steroid synthesis. The role of glucose remains, however, to be clarified. In the presence of hexokinase it should become accessible to C-1 oxidation, obviating the need for the activation of phosphorylase. Yet it cannot stimulate steroid production in the absence of ACTH, not even when oxidized triphosphopyridine nucleotide is added (Koritz & Péron, 1958). This might be explained by an effect of ACTH at the hexokinase stage. The inability of adrenals to respond to ACTH in the absence of glucose agrees with this interpretation, but the successful substitution of inosine or adenosine (Schonbaum, Davidson, Large & Casselman, 1959) for glucose does not. It would be of interest to know whether adenosine 3':5'-monophosphate acts in the absence of glucose or of inosine and calcium. Calcium, but not glucose, has recently been found to be required for the extra-adrenal lipolytic action of ACTH (Lopez, White & Engel, 1959).

SUMMARY

1. Ultraviolet-absorbing lipids produced by incubated rat adrenals were separated by paper chromatography in toluene-propylene glycol, and some physical, chemical and biological properties were examined.

2. Lipids with chromatographic mobility, absorption spectra in methanol and in sulphuric acid, and with the reducing power of corticosterone, aldosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone were recovered.

3. No evidence was found for the production of lipids with a dihydroxyacetone side chain, of 17-oxo steroids and of 17-oxogenic steroids.

4. A non-reducing Porter-Silber chromogen was recovered with a polarity slightly greater than that of 11-deoxy-17-hydroxycorticosterone and with maximum absorption in methanol at 240 m μ and in sulphuric acid at 290 m μ . Acetylation of this material yielded two components, with R_F values 0.5 and 0.9 in benzene-formamide; other properties were the same as those of the parent compound.

5. The lipid with the mobility of cortisol in toluene-propylene glycol had properties expected of a Δ^4 -3-ketone, did not reduce tetrazolium and its spectrum in sulphuric acid differed from those of both isomers of pregn-4-ene-11 β :(20):21-triol-3-one. Upon acetylation, products of low polarity in benzene-formamide were obtained.

6. A dose of 0.1 μ g. of lipid with the mobility of aldosterone depressed the urinary ratio of sodium to potassium in the rat by the same amount as 3 μ g. of deoxycorticosterone acetate. The Porter-Silber chromogen gave variable results and the lipid with the mobility of corticosterone was inactive. In the eosinopenia assay the Porter-Silber chromogen was inactive at a dose of 25 μ g./rat. A similar dose of the lipid with the mobility of corticosterone was one-third as active as cortisone.

7. The production of the Porter-Silber chromogen and of the lipid with the properties of corticosterone was greatly enhanced by adrenocorticotrophic hormone and by factors increasing the response to adrenocorticotrophic hormone, such as calcium, inosine and glucose. The effect on the other lipids was small or absent.

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Biological Synthesis of L-Ascorbic Acid in Animal Tissues: Conversion of D-Glucuronolactone and L-Gulonolactone into L-Ascorbic acid

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The synthesis of L-ascorbic acid in animal tissues is believed (Horowitz & King, 1953*a, b*; Isherwood, Chen & Mapson, 1954; Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1957, 1958*b*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960*a*) to follow the sequence:

The enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid has previously been reported to be located in the microsomal fractions of the liver homogenates of the rat and goat, and of kidney homogenates of the chick (Burns *et al.* 1956; Chatterjee *et al.* 1958*b, c*; Roy & Guha, 1958). The synthesis from D-glucuronolactone, irrespective of the species studied, is greatly stimulated in the presence of sodium or potassium cyanide but the conversion of L-gulonolactone into L-ascorbic acid does not require the presence of cyanide and is, in fact, inhibited by it (Chatterjee *et al.* 1958*c*). The function of cyanide therefore lies apparently in the reduction of D-glucuronolactone to L-gulonolactone and it probably acts by activating the thiol enzyme involved in this reduction (Chatterjee *et al.* 1958*c*).

The rate of conversion of D-glucuronolactone into L-ascorbic acid by the microsomes from any of the species mentioned above is maximum with 50 mM-cyanide, the rate of synthesis decreasing with decreasing cyanide concentration. With rat-liver and chick-kidney microsomes no appreciable synthesis is observed with 5 mM-potassium cyanide even when the time of incubation is prolonged from 1.5 to 3.0 hr. It has, however, been observed that the synthesis by rat-liver microsomes takes place also in the presence of 5 mM or even a lower concentration of cyanide if reduced diphosphopyridine

nucleotide, reduced triphosphopyridine nucleotide, glutathione or a chelating agent such as sodium pyrophosphate, $\alpha\alpha'$ -dipyridyl, 8-hydroxyquinoline or the boiled rat-liver supernatant is added to the system (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1958*a*). Substances such as reduced di- and

tri-phosphopyridine nucleotide and glutathione probably function by stimulating the action of cyanide in reducing the thiol-enzyme for the conversion of D-glucuronolactone into L-gulonolactone, which constitutes the first phase of the reaction. The metal-binding agents such as sodium pyrophosphate, $\alpha\alpha'$ -dipyridyl and 8-hydroxyquinoline, and the boiled supernatant have been reported previously (Chatterjee *et al.* 1958*a*; Chatterjee *et al.* 1960*a*) to enhance the conversion of L-gulonolactone into L-ascorbic acid. It is likely therefore that these agents promote the formation of L-ascorbic acid from D-glucuronolactone by stimulating the second phase of the reaction, namely conversion of L-gulonolactone into L-ascorbic acid.

Goat-liver microsomes behave somewhat differently from rat-liver and chick-kidney microsomes. Although the maximum conversion of D-glucuronolactone into L-ascorbic acid by the goat-liver microsomes takes place with 50 mM-KCN, appreciable synthesis is also observed with 5 mM or an even lower concentration of cyanide if the time of incubation is prolonged from 1.5 hr. to 3.0 hr. This synthesis by the goat-liver microsomes is not further augmented by any of the aforesaid agents.

The lactone forms of the precursors, D-glucuronolactone and L-gulonolactone, have been found to be specifically active in the microsomal synthesis of