relative prophylactic and therapeutic effectiveness of M-8450 in monkeys also requires additional investigation although it appears that its antiviral activity is greater when administered before virus, in agreement with the findings in mice(2) and in tissue culture(3). In its present state of development M-8450 does not appear to have direct therapeutic application. However, its effectiveness in both preventing infection with and prolonging the incubation period of poliomyelitis in monkeys seems to justify further investigation of its properties and mechanism of action.

Summary. The crude filtrate of a penicillium mold, M-8450, was shown to have antiviral action in cynomolgus monkeys inoculated subcutaneously with the Mahoney strain of Type 1 poliomyelitis. Treatment with at least 100 ml of this crude filtrate reduced the morbidity and increased the incubation period of infected animals. Less intensive treatment appeared to be less effective. The antiviral action of this substance appears to merit further study.

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Presence of 17-Ketosteroids in Adrenal Perfusates.* (20800)

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Studies on the nature and effects of adrenocortical steroids have indicated that the adrenal cortex synthesizes C_{19} as well as C_{21} steroids. Reichstein and coworkers isolated Δ^4-androstene-3,17-dione(1), Δ^4-androstene-3,11,17-trione (adrenosterone)(2), and androstane-3β,11β-diol-17-one(3) from adrenal extracts. Cow adrenal venous blood was shown by Gassner *et al.* to contain an androgenic steroid characterized as an α,β unsaturated, hydroxylated, 17-ketosteroid(4). Bush reported the possible presence of Δ^4-androstene-11β-ol-3,17-dione in the adrenal venous blood of rats, sheep and cats(5), but more recently considers his compound to be 17-hydroxyprogesterone(6). In the systematic investigation of steroidal compounds obtained during adrenal perfusions Hechter and his group found several Zimmermann reactive zones on chromatograms run on the perfusates(7). Recently Δ^4-androstene-11β-ol-3,17-dione has been identified in human adrenal venous blood(8) and urine(9) after ACTH administration. The data presented here represents a preliminary report on the presence of Δ^4-androstene-3,17-dione, Δ^4-androstene-3,11,17-trione, and Δ^4-androstene-11β-ol-3,17-dione in blood perfused through beef adrenals.

Experimental. Six liters of citrated beef blood, to which 120,000 units penicillin and 0.6 g streptomycin had been added, were perfused through 6 beef adrenals for 5 cycles as described by Hechter and coworkers(10). After the first cycle, 25 mg ACTH (Armour) was dissolved in propylene glycol-saline and infused into blood over 3 hours. Acetic acid-1-C^{14} was added to the blood prior to perfusion in one experiment (perfusion no. 5). A total of 5 perfusions was conducted. The perfusates were extracted with 3 portions of twice the volume of redistilled ethyl acetate by gently shaking the blood and solvent together(11). The extracts were evaporated to dryness under

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reduced pressure; the residues partitioned between 90% methanol and pentane, and the residues from the methanolic fractions transferred to diethyl ether.

In perfusions 1 and 5, the ether fractions were washed with water, dried over anhydrous Na_2SO_4, and brought to dryness. Separation of the neutral fractions into ketonic and non-ketonic fractions was carried out with the Girard T reagent. The ketonic fractions were further fractionated to characterize individual steroids. The material in the ether fractions from perfusions 2, 3, and 4 was transferred into benzene and fractionated by silica-gel chromatography(12). Appropriate fractions were analyzed qualitatively by paper chromatography and quantitatively by the Zimmermann reaction for the 17-ketosteroids.

Aliquots were taken from all samples for total 17-ketosteroid determinations. The remainder was placed on propylene-glycol impregnated Whatman No. 1 filter paper strips and chromatographed twice for 12-48 hours in the ligroin-propylene glycol solvent system (13). The location of individual compounds was determined by examination of the paper under ultraviolet light, and reacting the paper strips with a) the Zimmermann reagent and b) 2,4-dinitrophenylhydrazine in 1 N HCl solution(14). The Zimmerman reactive zones of the paper chromatograms from perfusate No. 5 were subjected to further analysis. The individual steroids were eluted from paper with redistilled methanol and dried. Employing paper chromatographic mobility as the criterion for identification, the eluates were chromatographed a) as the free compounds on propylene glycol impregnated paper in the solvent system ligroin-benzene (1:1), b) as the hydrazone derivatives, formed after reacting with Girard reagent T, in the system butanol-water (84:16)(14), c) as the acetate derivatives, formed after reacting the eluates with acetic anhydride in pyridine(15), in the solvent system ligroin-ethylene glycol phenyl ether(16), and d) as the free compounds after oxidation with chromic acid(17) in the ligroin-propylene glycol solvent system. The absorption curve between 220 and 500 $m\mu$ of the chromogens produced by treatment of the eluates with concentrated H_2SO_4(18) was also obtained.

Three blood control perfusion experiments were done. In control experiment 1, 10 l of blood with added C^{14}-labeled acetate circulated through the perfusion apparatus for 3 hours. The blood was then extracted with ethyl acetate, partitioned between pentane and 90% methanol, and tested for Zimmermann reactive material as described above. In control Experiment 2, 10 mg each of corticosterone (Kendall's Cpd. B) and 11-desoxy-17-hydroxy-corticosterone (Reichstein's Cpd. S), were mixed with 1.3 l of citrated blood. Similarly, 10 mg each of 17-hydroxycorticosterone (Kendall's Cpd. F) and 11-dehydro-17-hydroxycorticosterone (Kendall's Cpd. E) were mixed with 1.5 l of blood in control No. 3. The 2 blood samples were extracted immediately with ethyl acetate, partitioned between 90% methanol and pentane, separated into ketonic and non-ketonic fractions, and the ketonic residue chromatographed on paper. Individual Zimmermann reactive zones were eluted from paper and their Zimmermann reactive material determined quantitatively.

Results. The perfusates contained between 0.35 and 1.5 mg total 17-ketosteroids. The data suggest that silica-gel chromatography results in greater purification of steroid extracts than the procedure involving Girard separation. Recovery experiments, in which 1 mg of Δ^4-androstene-3,17-dione was added to 2 l of blood, in duplicate, resulted in 63% and 68% recovery. The ratio of Zimmermann reactive substances to formaldehydrogenic substances in blood varied between 1:35 and 1:50.

Paper chromatography in ligroin-propylene glycol of the five perfusates gave 9 distinct Zimmermann reactive zones. In Table I, the relative mobilities (R_T) (mobility relative to androsterone = 1.00 cm/hr), the colors produced upon reaction with the Zimmermann reagent and 2,4-dinitrophenylhydrazine, and the presence or absence of ultraviolet light absorption by the reactive areas are listed. Only those zones with identical R_T values found in two or more perfusates are included in Table I. Except in the case of the rapidly moving compound I, the R_T values among

TABLE I. Relative Mobilities (R_T) of Compounds I-V and Standards in Ligroin-Propylene Glycol.

Compounds in perfusates	No. of perfusates containing compounds	R_T	Ultra-violet absorption	Color reaction	
				Zimm.	DNPH
I	2	2.3, 2.8*	—	B-P	Y
II	3	1.70	+	P	Or
III	4	.42	+	P	Or
IV	4	.10	+	P	Or
V	2	.083	—	B	—
Standards					
androstane-3,17-dione		2.5*	—	P	Y
Δ^4-androstene-3,17-dione		1.70	+	P	Or
Δ^4-androstene-3,11,17-trione		.45	+	P	Or
Δ^4-androstene-11β-ol-3,17-dione		.10	+	P	Or

* Mobility of androstane-3,17-dione in ligroin-propylene glycol varies by 25% from the mean.
The following abbreviations are used: Zimm. = Zimmermann reagent, DNPH = 2,4-dinitrophenylhydrazine in 1 N HCl, B = blue, P = purple, Y = yellow, Or = orange.

TABLE II. Mobilities of Compounds I-IV from Perfusion 5 and Their Derivatives in Various Solvent Systems.

Compound	Free compound	Derivative	
		Girard hydrazone	CrO_3 oxidation product
	Solvent system		
	Ligroin-Bz (1:1)-Prop. G.	Butanol-water (84:16)	Ligroin-Prop. G.
	Mobility (cm/hr)		
I	—	.83	—
II	—	.14	—
III	.43	.08	.47
IV	.17	.06	.42
Δ^4-androstene-11β-ol-3,17-dione	.15	—	.10
Δ^4-androstene-3,11,17-trione	.53	.07	.41
Δ^4-androstene-3,17-dione	—	.15	1.65
testosterone	.58	.85	—
Δ^4-androstene-3-one	—	.98	—

The following abbreviations are used: Bz = benzene, Prop. G. = propylene glycol.

replicate samples differed by 10% or less. These paper tests indicated that all compounds contained a ketone group at carbon atoms 3 and 17 and α,β unsaturation in the structure of compounds II, III and IV. Interfering material eluted from paper with the individual compounds prevented their spectrophotometric determination at 240 mμ.

Five Zimmermann reactive compounds were found in perfusate No. 5 (perfusion with C^{14}-labeled acetate). Compounds I to IV, when subjected to different conditions and treatment, exhibited characteristic mobilities which are presented in Table II. The mobilities of the trimethylammoniumacethydrazone derivatives of compounds I and II were identical with those of testosterone and Δ^4-androstene-3,17-dione, respectively. The reaction product of compounds I and II with acetic anhydride in pyridine did not move appreciably on filter paper in the system ligroin-ethylene glycol phenyl ether during a 21-hour period. Testosterone acetate and androsterone acetate moved 0.24 and 0.50 cm/hr, respectively, in the same system, indicating that no monoacetates had been formed. The rates of movement of compounds III and IV in the ligroin-benzene (1:1)-propylene glycol system corresponded to the mobilities of the standards.

Δ^4-androstene-3,11,17-trione and Δ^4-androstene-11β-ol-3,17-dione, respectively. The hydrazones of both compounds III and IV exhibited a mobility identical with Δ^4-androstene-11-one-3,17-dihydrazone. Oxidation of compounds III and IV with chromic acid and chromatography of the oxidation products gave a single Zimmermann reactive area with an R_T value of 0.45 $\pm$ 0.03 (ligroin-propylene glycol system) corresponding to the mobility of Δ^4-androstene-3,11,17-trione. In all instances, the Zimmermann reactive and K_2PtI_6 reactive zones on paper were found to be radioactive. Determinations of specific activities were not possible because the variable weight of the non-steroidal portion of the methanol eluate frequently exceeded that of the steroidal portion. Nevertheless, when consecutive tests were carried out on one aliquot of an eluate, the activity did not change significantly.

The absorption curve of the chromogen of compound I in concentrated H_2SO_4 could not be identified; that of compound II exhibited a minimum at 240 mμ and a maximum at 310 mμ. The spectra of both the crystalline Δ^4-androstene-3,17-dione and androstane-3,17-dione chromogens contained one minimum at 240 mμ and one maximum at 300 mμ.

An aliquot of labeled compound III (contained in 6.6 mg of eluted material) was diluted with 2.0 mg of crystalline, non-labeled Δ^4-androstene-3,11,17-trione. This sample was chromatographed 4 times in the ligroin-propylene glycol system, once in the ligroin-benzene (1:1)-propylene glycol system, and once as the dihydrazone derivative in butanol-water. The C^{14} activity remained associated with the Zimmermann or K_2PtI_6 reactive zone in every chromatogram. The infra-red absorption spectrum of the diluted compound III was identical with Δ^4-androstene-3,11,17-trione.

The purified perfusate of control experiment 1 contained less than 50 μg of total 17-ketosteroids. No Zimmermann reactive zones were noted on the paper chromatograms. The chromatograms of the residues from control Experiments 2 (Compounds B and S added to blood) and 3 (added Compounds E and F) contained one Zimmermann reactive zone each with respective R_T values of 1.00 and 0.41. These two 17-ketosteroids were found quantitatively as contaminants of the crystalline Compounds S and E added to blood in the control experiments.

Discussion. Characterization of Zimmermann reactive substances found in the adrenal perfusates required use of highly sensitive analytical methods because of the small quantities of material present (30-250 μg per Zimmermann reactive zone). Paper chromatography meets this requirement. Identification of compounds I to IV was based primarily on the chromatographic behavior of the free compounds and their derivatives in different solvent systems, and by the use of several color tests. Although identification by these methods is not as definitive as by classical procedures (melting point of free steroids and derivatives, infrared absorption curve, optical rotation), the correctness of the identifications made on the basis of the sum of the results obtained from the 4-5 tests used in this investigation carries a significant degree of certainty.

The color reactions and mobility of the free compound I indicated a saturated 3,17-diketone, possibly androstane-3,17-dione or etiocholane-3,17-dione. Formation of a monohydrazone and the presence of a non-reactive polar group, which also did not form an acetate, added to the evidence for the probable nature of compound I. Similarly, employing the same criteria and tests, compound II and Δ^4-androstene-3,17-dione gave identical reactions. The excellent reproducibility of the mobility of Δ^4-androstene-3,17-dione in ligroin-propylene glycol as compared to androstane-3,17-dione and the added parameter of H_2SO_4 chromogen absorption curves, which agreed within experimental error, allow compound II to be identified as Δ^4-androstene-3,17-dione with a high degree of certainty.

The behavior of compounds III and IV indicated both to be Δ^4-3,17-diketones containing a third oxygen function which did not form an acetate or hydrazone. Compound III was not affected by chromic acid oxidation while compound IV was converted to compound III. These results indicate that compound III has a carbonyl group and compound

IV a β-hydroxyl group attached to C-11, neither of which would react with acetic acid in pyridine or Girard reagent T. The mobilities of the free compounds III and IV in the two solvent systems employed were identical with Δ^4-androstene-3,11,17-trione and Δ^4-androstene-11β-ol-3,17-dione, respectively. The results with the mixture of non-labeled Δ^4-androstene-3,11,17-trione and labeled compound III further strengthen the conclusion that these two substances are identical.

Compound V was present in insufficient amounts to attempt its characterization. Its mobility and ultraviolet light absorption would suggest a C_{19} steroid with three oxygen functions including a Δ^4-3-ketone.

The large amounts of formaldehydrogenic material found in the perfusates compared to the amounts of 17-ketosteroids suggested the possibility that compounds II, III and IV were chemical degradation products of the C_{21} steroids formed by the adrenal gland during perfusions. The absence of Δ^4-androstene-11β-ol-3,17-dione, Δ^4-androstene-3,17-dione and Δ^4-androstene-3,11,17-trione in the control samples containing 10 mg each of Compounds B, S, E and F which agrees with the absence of Δ^4-androstene-3,11,17-trione in blood from cortisone perfused adrenal glands (11) strongly suggests that these three 17-ketosteroids were not formed during the course of purifying and analyzing the perfusates. Rather, they are of adrenal origin.

Summary. Bovine adrenal glands were perfused with blood containing added ACTH and the perfusates purified for study of their C_{19} steroid contents. Individual steroids present in the purified extracts were detected and characterized by studying the behavior of the free compounds and their derivatives by paper chromatographic techniques involving several solvent systems and color tests. By these methods, five 17-ketosteroids were found in adrenal perfusates, three of which exhibited properties identical to standard samples of Δ^4-androstene-3,17-dione, Δ^4-androstene-3,11,17-trione and Δ^4-androstene-11β-ol-3,17-dione. The conclusion is reached that the methods used here allow the identification of these three steroids in adrenal perfusates with a high degree of certainty.

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