excess reagent). The insoluble urea was removed, the solvent was replaced by ethyl acetate, and the solution was washed with dilute acid and aqueous potassium bicarbonate. The addition of petroleum ether afforded 87% of crystalline carbobenzoxy-glycyl-L-phenylalanylglycine ethyl ester; m.p. 118–119°, $[\alpha]^{27}D$ −13.5° [ethanol] (reported[2]: m.p. 116–118°, $[\alpha]^{25}D$ −12°). In a similar fashion we have prepared a variety of dipeptide derivatives, including the following examples.

In methylene chloride, phthaloyl-L-phenylalanyl-glycine ethyl ester was produced in 92% yield; m.p. 161–162°, $[\alpha]^{26.6}D$ −146°, (reported[3]: m.p. 161–162°, $[\alpha]^{29.5}D$ −146°). In aqueous tetra-hydrofuran, a product of the same quality was obtained in 72% yield. Phthaloyl-L-alanyl-L-proline benzyl ester (74%) was isolated with m.p. 101–102°, $[\alpha]^{26.5}D$ −135° [ethanol]. Anal. Calcd. for $C_{23}H_{22}N_2O_5$: C, 67.98; H, 5.42; N, 6.90. Found: C, 68.07; H, 5.52; N, 6.77. Carbobenz-oxy-L-serine and ethyl glycinate coupled to give carbobenzoxy-L-serylglycine ethyl ester (59%) in tetrahydrofuran: m.p. 106–107°, [ethanol], reported,[4] m.p. 105–107°. Phthaloyl-L-phenylalanyl-L-leucine ethyl ester (91% yield) had a m.p. of 109–110°, $[\alpha]^{25.4}D$ −115° [ethanol]. Anal. Calcd. for $C_{25}H_{28}N_2O_5$: C, 68.78; H, 6.47; N, 6.42. Found: C, 68.50; H, 6.59; N, 6.48.

DEPARTMENT OF CHEMISTRY   JOHN C. SHEEHAN
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
CAMBRIDGE 39, MASSACHUSETTS   GEORGE P. HESS[5]

(2) G. Anderson and R. Young. THIS JOURNAL, **74**, 5307 (1952).

(3) J. Sheehan. D. Chapman and R. Roth. ibid., **74**, 3822 (1952).

(4) J. S. Fruton. J. Biol. Chem., **146**, 463 (1942).

(5) Aided by a fellowship from the National Foundation for Infantile Paralysis.

9α-HALO-11β-HYDROXY AND 11-KETO DERIVATIVES OF PROGESTERONE, DESOXYCORTICOSTERONE AND 17α-HYDROXYPROGESTERONE

Sir:

In previous communications[1,2] there have been described the synthesis of 9α-halogenated deriva-tives of cortisone and hydrocortisone and shown that the glucocorticoid activity of these substances increased with decreasing atomic weight of the halogen atom. The most active member of that series, 9α-fluorohydrocortisone acetate possessed about 11 times the activity of cortisone acetate in the rat liver glycogen assay. Soon thereafter it was found that in addition to being potent gluco-corticoids these compounds were highly effective in controlling electrolyte balance and in maintaining life in the rat[3], dog[4,5] and in man.[4,6]

It appeared of great interest to ascertain what influence variations in the side-chain might have upon the adrenocorticoid activity of such halogen-

(1) J. Fried and E. F. Sabo, THIS JOURNAL, **75**, 2273 (1953).

(2) J. Fried and E. F. Sabo, ibid., **76**, 1455 (1954).

(3) A. Borman. F. M. Singer and P. Numerof. Proc. Soc. Exp. Biol. Med., **86**, 570 (1954).

(4) G. W. Liddle. M. M. Pechet and F. C. Bartter. Science, **120**, 496 (1954).

(5) W. W. Swingle, C. Baker. M. Eisler. S. J. Le Brie and L. J. Brannick. Proc. Soc. Exp. Biol. Med., In press.

(6) A. Goldfien. G. W. Thorn, P. M. Beigleman and J. C. Laidlaw. J. Clin. Endocrinology, **14**, 782 (1954).

ated derivatives. For this purpose we have prepared the 9α-halo derivatives (halogen = Br, Cl, F) of 11β-hydroxyprogesterone, 11β,17α-di-hydroxyprogesterone and corticosterone acetate and of the corresponding 11-ketones by a synthetic route paralleling that described in our earlier publi-cations.[1,2] This synthesis proceeds from the 11-mesylates of the requisite 11α-hydroxy derivatives[7] (11α-hydroxyprogesterone mesylate, m.p. 165–167°; $[\alpha]^{23}D$ +135° (c, 0.77 in CHCl$_3$); λ_{max}^{alc} 238 mμ (ϵ = 17,200); Anal. C, 64.81; H, 7.63; S, 7.48. Epicorticosterone 11α-mesylate 21-acetate, m.p. 156–157°; $[\alpha]^{23}D$ +144° (c, 0.92 in CHCl$_3$); λ_{max}^{alc} 238 mμ (ϵ = 16,600); Anal. C, 61.52; H, 7.07. 11α,17α-Dihydroxyprogesterone 11α-mesyl-ate, m.p. 150–152°; $[\alpha]^{23}D$ +64° (c, 0.49 in CHCl$_3$); λ_{max}^{alc} 238 mμ (ϵ = 18,200); Anal. C, 62.11; H, 7.71; S, 7.11), via the 9,11-unsaturated steroids (9(11)-dehydro-17α-hydroxyprogesterone, m.p. 214–216°; $[\alpha]^{23}D$ +67° (c, 0.82 in CHCl$_3$); λ_{max}^{alc} 239 mμ (ϵ = 18,450); Anal. C, 76.52; H, 8.46), to the 9α,11β-bromohydrins (see table). The latter on treatment with base yielded the 9β,11β-epoxides (9β,11β-oxidoprogesterone, amorphous, $[\alpha]^{23}D$ +61° (c, 1.55 in CHCl$_3$); λ_{max}^{alc} 243 mμ (ϵ 13,600). 9β,11β-Oxidodesoxycorticosterone acetate, m.p. 137–138°; $[\alpha]^{23}D$ +61° (c, 0.66 in CHCl$_3$); λ_{max}^{alc} 243 mμ (ϵ = 15,100); Anal. C, 71.81; H, 8.10. 9β,11β-Oxido-17α-hydroxyprogesterone, m.p. 183–184°; $[\alpha]^{23}D$ −32° (c, 1.02 in CHCl$_3$); λ_{max}^{alc} 243 mμ (ϵ = 16,600); Anal. C, 72.99; H, 8.11), which upon reaction with the requisite hydrogen halides formed the 9α-chloro- and 9α-fluoro-11β-hydroxy derivatives. Oxidation with chromic acid furnished the corre-sponding 11-ketones. Alternatively, the 9α-chloro-derivatives could be prepared by allowing the 9(11)-unsaturated steroids to react with N,N'-dichloro-dimethylhydantoin in the presence of perchloric acid.[8]
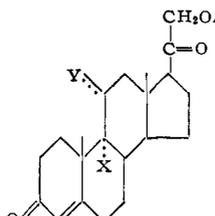
The physical properties of the halogenated steroids and the activities of representative com-pounds in the liver glycogen and sodium retention assays in the adrenalectomized rat are listed in the accompanying table. As had been observed pre-viously in the 9α-halohydrocortisone series both gluco- and mineralocorticoid activities were found to increase with decreasing atomic weight of the halogen atom. No significant differences were noted between the activities of the 11β-hydroxy and 11-keto derivatives. Outstanding among the compounds tested were 9α-fluoro-11β-hydroxy and 11-ketoprogesterone, which although lacking both the 17- and 21-hydroxyl groups approximately equalled cortisone acetate in glucocorticoid activ-ity. The most potent mineralocorticoids of this series were 9α-fluorocorticosterone acetate and 9α-

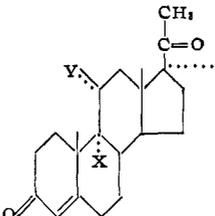(7) J. Fried. R. W. Thoma. J. R. Gerke. J. E. Herz. M. N. Donin and D. Perlman. THIS JOURNAL, **74**, 3962 (1952).

(8) The course of this reaction was dependent on the nature of the side chain. Thus, 9(11)-dehydro-17α-hydroxyprogesterone afforded the desired chlorohydrin in about 50% yield. On the other hand, treatment of 9(11)-dehydroprogesterone with N,N'-dichlorohydantoin resulted in a mixture containing more than one atom equivalent of chlorine from which 9α-chloro-11β-hydroxyprogesterone could be isolated only after reduction with chromous chloride. It appears likely that the extra chlorine atom reducible by chromous chloride is located in the 17-position.

PHYSICAL PROPERTIES AND ADRENOCORTICOID ACTIVITY OF 9α-HALO STEROIDS
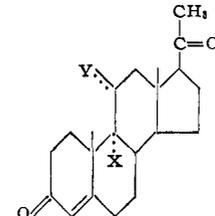
Steroid	M.p., °C.	$[\alpha]_D^{CHCl_3}$	Analysis, % C H	Liver glycogen[a]	Sodium retention[b]
X = Br, Y = OH	148–150°	+193°	61.91; 6.92	...	<0.02
X = Br, Y = O	165–167°	+318°	61.80; 6.46	...	...
X = Cl, Y = OH	204°	+205°	69.00; 7.73	0.35 (0.27–0.44)	<0.02
X = Cl, Y = O	211–212°	+314°	70.03; 7.21	...	...
X = F, Y = OH	216–217°	+197°	72.49; 8.13[d]	0.85 (0.69–1.04)	1.14 (0.53–2.48)
X = F, Y = O	174–175°	+209°	72.37; 7.66	1.10 (0.87–1.38)	2.84 (1.26–6.36)

Steroid	M.p., °C.	$[\alpha]_D^{CHCl_3}$	Analysis, % C H	Liver glycogen[a]	Sodium retention[b]
X = Br, Y = OH	152–153°	+183°	59.15; 6.70	...	<0.1
X = Br, Y = O	171–172°	+285°	59.26; 6.03	0.07 (0.03–0.15)	...
X = Cl, Y = OH	192°	+197°	65.52; 7.24	1.15 (0.67–1.96)	0.87 (0.48–1.57)
X = Cl, Y = O	233–234°	+279°	65.52; 6.82	...	1.1 (0.5–2.5)
X = F, Y = OH	212–213°	+192°	68.31; 7.60[e]	4.58 (3.33–6.30)	26.2 (10.9–66.0)
X = F, Y = O	208–210°	+189°	68.53; 6.96	5.90 (4.83–7.21)	20.8 (9.6–44.7)

Steroid	M.p., °C.	$[\alpha]_D^{CHCl_3}$	Analysis, % C H	Liver glycogen[a]	Sodium retention[b]
X = Br, Y = OH	189–191°	+128°	59.59; 6.81	...	...
X = Br, Y = O	...	...	...	...	...
X = Cl, Y = OH	242–243°	+138°[e]	66.18; 7.38	0.06 (0.04–0.08)	<0.02
X = Cl, Y = O	260–261°	+223°[e]	66.12; 7.45	...	...
X = F, Y = OH	275–277°	+136°[e]	69.50; 7.97[f]	1.88 (1.47–2.40)	<0.1
X = F, Y = O	252–255°	+145°[e]	69.91; 7.66	0.81 (0.67–0.98)	<0.1

[a] Rat liver glycogen assay (Pabst, Sheppard and Kuizenga, *Endocrinology*, **41**, 55 (1947)); cortisone acetate = 1. The figures in parentheses represent the 95% confidence intervals calculated by the method of C. I. Bliss (The Statistics of Bioassay, Academic Press, New York, N. Y., 1952). [b] Sodium retention assay in the adrenalectomized rat (Borman, Singer and Numerof, *Proc. Soc. Exp. Biol. Med.*, **86**, 570 (1954)); desoxycorticosterone acetate = 1. [e] Solvent, dioxane. [d] F, 5.48. [e] F, 4.81. [f] F, 5.21.

fluorodehydrocorticosterone acetate, which possessed activities of the order of that observed with aldosterone.[9]

Certain regularities in the physical properties of this series of compounds are worthy of comment. Thus, the ultraviolet absorption maximum associated with the αβ-unsaturated ketone system in ring A suffers progressive hypsochromic shifts

(9) Aldosterone has been reported to be 20–30 times as active as desoxycorticosterone in sodium retention tests in the adrenalectomized rat. (P. Desaulles, J. Tripod and W. Schuler. *Schweiz. Med. Wschr.*, **83**, 1088 (1953); J. Axelrod, J. E. Cates, B. B. Johnson and J. A. Luetscher. Jr., *Endocrinology*, **55**, 568 (1954)).

when the bromine atom in the 9α-bromo-11β-hydroxy derivatives (λ_{max}^{alc} 243 mμ) is replaced by chlorine (240 mμ) and fluorine (238 mμ). Oxidation of the 11β-hydroxy- to the 11-ketoderivatives produces additional hypsochromic shifts of 4 to 6 mμ similar to those noted previously for the halogen-free compounds.[10] The differences in molecular rotation between the 11-keto and 11β-hydroxy derivatives are considerably greater for the bromo and chloro derivatives ($\Delta[M]_D^{CHCl_3}$ +340°

(10) R. Antonucci, S. Bernstein. M. Heller, R. Lenhard, R. Littell and J. H. Williams, *J. Org. Chem.*, **18**, 70 (1953).

to $+500°$) than for the corresponding halogen-free steroids ($+190°$ to $+230°$), while the reverse is true for the fluoro-compounds ($-10°$ to $40°$).

THE SQUIBB INSTITUTE
FOR MEDICAL RESEARCH
NEW BRUNSWICK, NEW JERSEY

JOSEF FRIED
JOSEF E. HERZ
EMILY F. SABO
ALECK BORMAN
FRANK M. SINGER
PAUL NUMEROF

ENZYMATIC FORMATION OF CORTICOSTEROID GLUCURONIDES[1]

Sir:

The metabolites of steroid[2a] and thyroid[2b,c] hormones are known to be excreted in significant amounts as conjugates of glucuronic acid. In previous studies, glucuronide synthesis of *o*-aminophenol and menthol has been observed in cell free preparations of liver and shown to require the presence of uridine diphosphate glucuronic acid (UDPGA).[3]

We should like to report the enzymatic formation of glucuronides of corticosteroids, such as tetrahydrocortisone.[4] This reaction is catalyzed by an enzyme system in the microsomes of mammalian liver of several species and also requires UDPGA as the glucuronide donor. In addition to tetrahydrocortisone glucuronide, uridine diphosphate (UDP) is a product of the conjugation (Table I). The reaction would appear to proceed as shown

UDPGA + Tetrahydrocortisone ⟶

Tetrahydrocortisone glucuronide + UDP
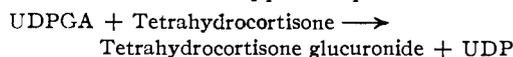
Attempts to demonstrate reversibility of this reaction have thus far been unsuccessful.

Incubation of tetrahydrocortisone with microsomes of guinea pig liver and UDPGA resulted in disappearance of free tetrahydrocortisone (extracted with methylene chloride and measured by the method of Porter and Silber[5] which was not observed in the absence of UDPGA, or when UDPGA was replaced by uridine diphosphate glucose (UDPG) or glucuronolactone (Table I). After extraction with methylene chloride, the aqueous residue (adjusted to pH 2-3) was treated with butanol[6] to extract any steroid glucuronide present. This butanol fraction, after distillation *in vacuo*, yielded Porter–Silber reacting material and gave a positive carbazole reaction for glu-

(1) We wish to express our appreciation to Dr. Jack L. Strominger and Dr. Herman M. Kalckar for the many helpful suggestions throughout this study, and for their generous supply of UDPG and UDPG dehydrogenase; and to Dr. Gordon Tomkins for his constant and valuable advice.

(2) (a) For references see L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," 3rd ed., Reinhold Publ. Corp., New York, N. Y., 1949; (b) A. Taurog, F. N. Briggs, and I. L. Chaikoff, *J. Biol. Chem.*, **194**, 655 (1952); (c) J. Roche, O. Michel, R. Michel, and J. Tata, *Biochem. Biophys. Acta*, **13**, 471 (1954).

(3) (a) G. J. Dutton and I. D. E. Storey, *Biochem. J.*, **53**, xxxvii (1953); (b) **57**, 275 (1954); (c) E. E. B. Smith and G. T. Mills, *Biochem. Biophys. Acta*, **13**, 386 (1954); (d) J. L. Strominger, H. M. Kalckar, J. Axelrod and E. S. Maxwell, THIS JOURNAL, **76**, 6411 (1954).

(4) Tetrahydrocortisone = pregnane-3α,17α,21-triol-11,20-dione; tetrahydro-hydrocortisone = pregnane-3α,11β,17α,21-tetrol-20-one; cortisone = Δ⁴-pregnene-17,21-diol-3,11,20-trione.

(5) C. C. Porter and R. H. Silber, *J. Biol. Chem.*, **185**, 201 (1950); R. H. Silber and C. C. Porter, *ibid.*, **210**, 923 (1954).

(6) S. L. Cohen, *ibid.*, **192**, 147 (1952).

curonic acid.[7] Alternatively, when the aqueous residue was incubated with purified bacterial β-glucuronidase,[8] subsequent re-extraction with methylene chloride resulted in almost quantitative recovery[5] of a steroid with an R_f corresponding to tetrahydrocortisone on paper chromatography (modified benzene–aqueous methanol system of Bush[9]). These observations were taken as evidence that the free tetrahydrocortisone, which disappeared upon incubation in the complete system, was converted to tetrahydrocortisone glucuronide.

Similar results have been obtained with tetrahydrohydrocortisone as the substrate. However, cortisone is not metabolized in this system. This would suggest that reduction of the 3-ketone to the 3-hydroxyl group is necessary for conjugation to occur and that the probable site of coupling on the steroid molecule is at the 3-hydroxyl position, as in the case of pregnanediol 3-β-d-glucuronide.[10]

The appearance of UDP in the reaction mixture was detected by means of paper chromatography.[11] UDP was also measured spectrophotometrically by following the disappearance of reduced diphosphopyridine nucleotide (DPNH) in the phosphopyruvate–pyruvate phosphokinase system coupled to the DPNH-lactic dehydrogenase system.[12]

TABLE I

ENZYMATIC SYNTHESIS OF TETRAHYDROCORTISONE GLUCURONIDE

Reaction mixture consisted of 0.2 ml. 0.5M phosphate buffer pH 7.4; 0.2 μM. tetrahydrocortisone; 1 ml. guinea pig liver microsomal preparation (30 mg. protein per ml.); total volume 3 ml.; incubated 30 min., at 38° in air; additions as indicated below.

Additions, μM	μM Tetrahydrocortisone		μM UDP formed[e]
	Disappearing on incubation[a]	Recovered with β-gluc-uronidase[b]	
UDPGA[d] (0.065)	0.046	0.042	0.040
UDPG (0.1)	0.000	0.000	0.000
Glucuronolactone (0.1)	0.000	0.000	0.000

[a] Steroid determined in CH_3Cl extracts before and after incubation by the method of Porter and Silber.[5] [b] Reaction mixture extracted after incubation with CH_3Cl to remove free steroid; aqueous residue then incubated with β-glucuronidase (40 units per ml.) at 37° for 36 hours; steroid extracted again with CH_3Cl and measured as in footnote.[a] [e] Determined spectrophotometrically with pyruvate phosphokinase and lactic dehydrogenase.[12] [d] Generated enzymatically[3d] from UDPG with UDPG dehydrogenase and DPN⁺.

In addition to the formation of corticosteroid glucuronides, we have obtained preliminary evidence for the synthesis of glucuronides of phenolphthalein and thyroxine by the enzyme system described. Disappearance of phenolphthalein occurred upon incubation and was detected by the reduction in the optical density at 540 mμ (in alkali). Subsequent hydrolysis with β-glucuronidase resulted in a return of the optical density to its original value. When I^{131}-labeled L-thyroxine was

(7) Z. Dische, *ibid.*, **183**, 489 (1950).

(8) H. J. Buehler, P. A. Katzman and E. A. Doisy, *Fed. Proc.*, **8**, 189 (1949).

(9) I. E. Bush, *Biochem. J.*, **50**, 370 (1952).

(10) R. D. H. Heard, M. M. Hoffman and G. E. Mack, *J. Biol. Chem.*, **155**, 607 (1944).

(11) A. C. Palidini and L. F. Leloir, *Biochem. J.*, **51**, 426 (1952).

(12) A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **193**, 481 (1951); A. Kornberg, "Phosphorus Metabolism," The Johns Hopkins Press, Baltimore, Md., 1951, Vol. I, p. 392.