guinea pigs. 1. The active material resided in the alcohol soluble fraction and was absent from the protein and acetone soluble fractions. 2. The anti-inflammatory effect did not appear to be mediated by the adrenals. 3. Feeding egg yolk and yolk alcohol solubles resulted in an elevation of serum total lipids with no increase in cholesterol to phospholipid ratio. Inactive yolk fractions caused elevations of serum total lipids, phospholipids, cholesterol, and cholesterol to phospholipid ratio. 4. Serum levels of total N and non-protein N were unaffected by yolk alcohol solubles.

The authors wish to express their thanks to Drs. John R. Allen and Joseph C. Street for their assistance in preparing egg yolk fractions and conducting the anti-inflammatory assays.

1. Assn. of Official Agricultural Chemists, *Official and Tentative Method of Analysis*, 7th ed., Washington, D. C., 1950,

2. Birkhang, K., *Ann. Rev. Tuberc.*, 1954, v69, 511.

3. Coburn, A. F., Graham, C. E., and Haninger, J., *J. Exp. Med.*, 1954, v100, 425.

4. Dury, A., *Am. J. Med. Sci.*, 1955, v203, 427.

5. Dury, A., and DiLuzio, N. R., *Am. J. Physiol.*, 1955, v182, 45.

6. Fisher, N., Harrington, C., and Long, D. A., *Lancet*, 1951, vVII, 522.

7. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, v38, 81.

8. French, C. E., Allen, J. R., and Davis, K. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 41.

9. Germuth, F. G., Jr., and Ottinger, B., *ibid.*, 1950, v74, 815.

10. Hansted, C., and Anderson, H., *Acta Paediat.*, 1953, v42, 386.

11. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, 12th Ed., Philadelphia, The Blakiston Co., 1947, p540.

12. Kerr, W. R., and Robertson, M., *J. Hygiene*, 1952, v50, 354.

13. Keys, A., *J. Biol. Chem.*, 1940, v132, 181.

14. Ma, T. S., and Zuazaga, G., *J. Ind. and Eng. Chem.*, (Anal. Ed.) 1942, v14, 280.

15. McIntire, F. C., Roth, L. W., and Sproull, M., *Am. J. Physiol.*, 1951, v167, 233.

16. Menkin, V., *Sci.*, 1954, v120, 1026.

17. Pula, D., and Marmorston, J., 1941, *Natural Resistance and Clinical Medicine*, Chap. 18, Boston, Little Brown & Co.

18. Selye, H., *The Physiology and Pathology of Exposure to Stress*, Acta, Montreal, Canada, 1950.

19. Street, J. C., Allen, J. R., Chang, I., and French, C. E., Am. Chem. Soc. 128th Meeting, 1955, 32C.

20. Stumpf, H. H., and Wilena, S. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 219.

21. Ungar, G., Damgaard, E., and Weinstein, H. G., *Am. J. Physiol.*, 1951, v166, 340.

22. Vollmer, E. P., *J. Infect. Dis.*, 1951, v88, 27.

23. Zlatkis, A., Zak, B., and Boyle, A. J., *J. Lab. Clin. Med.*, 1953, v41, 486.

Received August 8, 1956. P.S.E.B.M., 1956, v93.

## $\Delta^1$-Hydrocortisone and Hydrocortisone: Plasma 17-Hydroxycorticosteroid Concentrations in the Dog Following Oral and Intravenous Administration. (22759)

ELLIOTT J. COLLINS, ARLINGTON A. FORIST, AND ESTHER B. NADOLSKI
*Research Laboratories, The Upjohn Company, Kalamazoo, Mich.*

$\Delta^1$-Hydrocortisone ($\Delta^{1,4}$-pregnadiene-11$\beta$, 17$\alpha$, 21-triol-3,20-dione) has received considerable attention as a potent synthetic analogue of hydrocortisone ($\Delta^4$-pregnene-11$\beta$, 17$\alpha$, 21-triol-3, 20-dione). Clinical studies have indicated $\Delta^1$-hydrocortisone to be 3 to 5 times as potent as the parent steroid(1-3). Evaluation of glucocorticoid activity (liver glycogen deposition, eosinopenic response, thymus involution) has shown a similar ratio (4). Introduction of unsaturation at the $C_1$ position of the steroid A-ring could conceivably alter the rates of absorption and/or metabolism of the compound. In the present study, this possibility has been examined experimentally in the dog by determining the changes in plasma 17-hydroxycorticosteroid (17-OHCS) concentrations as a function of time following oral and intravenous administration of $\Delta^1$-hydrocortisone and hydrocortisone. Subsequent to this study, a similar evaluation of $\Delta^1$-hydrocortisone in the human has been reported(5).

*Materials and methods.* Male mongrel

dogs weighing 15-20 kg were used in these studies. The animals were fasted for 16-18 hours prior to the steroid administration and food but not water was withheld during the blood sampling period. In experiments involving oral administration, the preparations tested were as follows: (a) $\Delta^1$-hydrocortisone: 5 mg (5 expts), 20 mg (4 expts), and 40 mg (4 expts); (b) hydrocortisone: 10 mg (4 expts), 20 mg (23 expts), and 40 mg (4 expts). Following administration of the compressed tablets, 3-6 blood samples (25-45 ml) were collected over periods of 2-5 hours from the external jugular vein without anesthesia. For the intravenous studies, the steroids were administered as the free alcohols (1 mg/ml in 10% ethanol-isotonic saline) and as the 21-hemisuccinate sodium salts (equivalent to 1 mg of free steroid ml in isotonic saline) by a rapid infusion into the antecubital vein at a dose of 2 mg kg. During a 3-4 hour period, 7-8 blood samples were collected. Plasma 17-OHCS concentrations were determined by a modification of the Nelson-Samuels procedure(6,7,8). In order to avoid frequent occurrence of cloudy solutions in the spectrophotometric step, it was necessary to partition the residue from the chloroform extract of dog plasma between 70% ethanol and Skellysolve B. The 70% ethanol extractables were then carried through Florisil chromatography as described by Nelson and Samuels(6). The spectrophotometric procedure was modified to include a direct correction for non-specific, acid-produced chromogens(8) similar to that originally recommended by Porter and Silber (9).

*Results.* Before applying routine 17-OHCS procedures in studies involving $\Delta^1$-hydrocortisone, it was necessary to know the reactivity of this steroid with the phenylhydrazine-sulfuric acid reagent as well as its behavior on Florisil columns. The absorption spectra of the phenylhydrazine-sulfuric acid chromogens obtained from $\Delta^1$-hydrocortisone and hydrocortisone are shown in Fig. 1. Absorptivity at the maximum is less for $\Delta^1$-hydrocortisone than for hydrocortisone and there is a slight shift of the maximum toward longer wavelengths due to the effect of the unsaturation

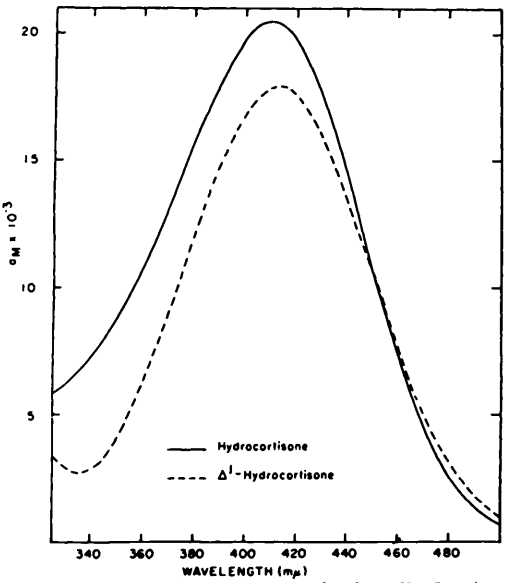


FIG. 1. Absorption spectra of phenylhydrazine-sulfuric acid chromogens from $\Delta^1$-hydrocortisone and hydrocortisone ($a_M$ calculated on basis of steroid conc. in reaction mixture).

at $C_1$. Routine measurements for both steroids were made at 410 mμ. By the procedure employed(8), the calibration curves at this wave length obey Beer's law over a wide range, and $\Delta^1$-hydrocortisone is 87% as chromogenic as hydrocortisone. Recovery of $\Delta^1$-hydrocortisone from Florisil columns was slightly but consistently less than hydrocortisone. With a typical batch of Florisil, recoveries (mean ± s.e.m.) were as follows: $\Delta^1$-hydrocortisone, 88.1 ± 1.1% (35 samples); hydrocortisone, 90.4 ± 1.4% (14 samples). For these reasons, the steroid administered was used as reference standard for chromatography and spectrophotometry.

To interpret data following intravenous administration of the steroid hemisuccinates, the behavior of hydrocortisone-21-hemisuccinate during Florisil chromatography was examined. None of the ester was eluted by either 2% or 25% methanol-in-chloroform. Further elution with 40% methanol-in-chloroform recovered about 30% of the ester. Therefore, even though some of the ester was extracted from plasma, it was removed during chromatography and only free 17-OHCS were measured. Previous studies have shown that neutral esters such as the acetate and cyclopentylpro-

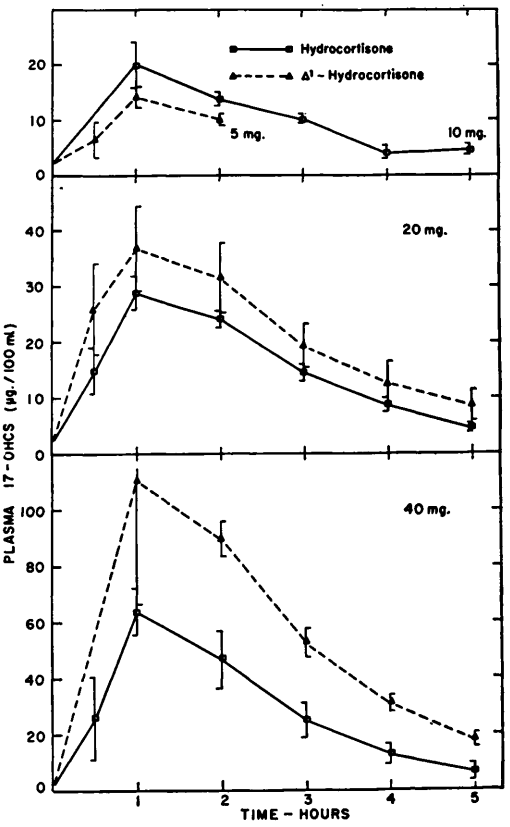


FIG. 2. Plasma 17-OHCS conc. (mean ± S.E.) vs. time following oral admin. of $\Delta^1$-hydrocortisone and hydrocortisone.

pionate also are not eluted from Florisil in the 17-OHCS-containing 25% methanol-in-chloroform fraction(8).

Mean plasma 17-OHCS concentrations as a function of time following the oral administration of $\Delta^1$-hydrocortisone and hydrocortisone are shown in Fig. 2. At a dose of 5 mg, $\Delta^1$-hydrocortisone produced elevations in plasma 17-OHCS concentrations of about 70% of those obtained with 10 mg of hydrocortisone. At the 20 mg level, $\Delta^1$-hydrocortisone gave plasma 17-OHCS levels which were higher at the maximum and which remained higher throughout the experimental period. At the 40 mg dose, the higher maximum values and prolonged elevations were even more striking. In addition, although 20 mg of $\Delta^1$-hydrocortisone produced maximum plasma 17-OHCS concentrations of only about 60% of those observed after 40 mg of hydrocortisone, the response curves crossed after about 4 hours. This indicated a difference in the rates of removal of the 2 steroids from the circulation.

Plasma 17-OHCS half-life values obtained following the intravenous administration of $\Delta^1$-hydrocortisone and hydrocortisone as the free steroids and as the sodium hemisuccinates are shown in Table I. Typical first order regression curves (method of least squares) are shown in Fig. 3. The mean half-lives of $\Delta^1$-hydrocortisone and hydrocortisone were 71.3 and 52.1 minutes respectively. When administered as the sodium hemisuccinates, the mean plasma 17-OHCS half-lives were 79.7 and 67.0 minutes respectively. Therefore, $\Delta^1$-hydrocortisone is removed from the circulation more slowly than hydrocortisone in the dog.

*Discussion.* The data presented indicate that orally administered $\Delta^1$-hydrocortisone produced greater and more prolonged elevations of plasma 17-OHCS in the dog than did equivalent doses of hydrocortisone. The half-life of intravenously administered $\Delta^1$-hydrocortisone was also longer than that of hydrocortisone (ratio 1.37). Further, a comparison of the ratios of plasma 17-OHCS concentrations one hour after oral administration shows the following: 10 mg (assuming linearity of

TABLE I. Half-Lives of $\Delta^1$-Hydrocortisone and Hydrocortisone in the Dog.

| | Half-life (min. ± stand. dev.) | | | |
|---|---|---|---|---|
| | $\Delta^1$-Hydrocortisone | | Hydrocortisone | |
| Dog | Alcohol | Hemisuccinate | Alcohol | Hemisuccinate |
| 500 | 69.1 ± 2.1 | 89.8 ± 6.4 | 51.0 ± 2.2 | 59.7 ± 5.4 |
| 503 | 70.4 ± 2.0 | — | 45.1 ± .9 | — |
| 505 | 76.2 ± 3.9 | 72.8 ± 3.0 | 59.2 ± 1.4 | 87.3 ± 2.8 |
| 508 | 69.3 ± 1.6 | — | 53.1 ± 2.0 | 57.7 ± 2.2 |
| 502 | — | 76.4 ± 3.1 | — | 63.1 ± 2.2 |
| Mean ± S.E. | 71.3 ± 1.7 | 79.7 ± 5.2 | 52.1 ± 2.9 | 67.0 ± 6.9 |

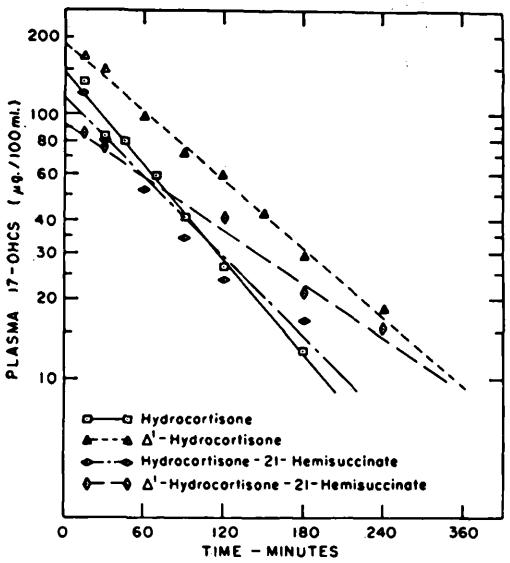


FIG. 3. Typical first order regression curves following intrav. admin. of $\Delta^1$-hydrocortisone, hydrocortisone and the respective hemisuccinates.

response between 5 and 10 mg), 1.42; 20 mg, 1.28; and 40 mg, 1.73. Similarity of these ratios to the ratio of half-lives suggests that there is no appreciable difference in the rates of absorption of $\Delta^1$-hydrocortisone and hydrocortisone from the gastrointestinal tract of the dog. It therefore appears that the higher and more prolonged elevations of plasma 17-OHCS following oral administration are a reflection of the reduced rate of metabolism of the $\Delta^1$-steroid.

With the intravenously administered steroid hemisuccinates, the plasma 17-OHCS half-lives were greater than observed with the corresponding free alcohols (Table I). These values are not half-lives of the administered esters since only free 17-OHCS are measured by the analytical procedure. Apparently, hydrolysis of the hemisuccinates proceeds at a rapid but finite rate and the procedure determines the intermediate in a series of consecutive reactions such as: steroid hemisuccinate → 17-OHCS→ metabolite(s). Jakoby and Tomkins(10) have studied the metabolism of pregnan-*21*-ol-*3,20*-dione-*21*-hemisuccinate by rat liver preparations and have reported that hydrolysis of the ester, followed by reduction of the $C_3$ carbonyl, analogous to the scheme above, represents the main metabolic pathway. However, they also observed considerable reduction of the $C_3$ carbonyl without prior hydrolysis. The $\Delta^1$-hydrocortisone and hydrocortisone hemisuccinates probably behaved similarly. The somewhat larger standard deviations obtained (Table I) when the data are fitted as a first order regression (Fig. 3) indicate that the plasma 17-OHCS half-lives so obtained represent the simplification of a complex series of consecutive reactions. It may only be concluded that intravenous administration of the steroid esters results in somewhat lower but, due to continuing hydrolysis of the esters, more prolonged elevations of plasma 17-OHCS.

Metabolism of corticosteroids containing the $\Delta^4$-3-ketone structure proceeds via reduction of first the $C_4$ unsaturation and then the $C_3$ carbonyl to produce tetrahydro derivatives which are subsequently conjugated(11). Alternatively, some reduction of the $C_{20}$ carbonyl may also occur(12,13,14). The longer half-life of $\Delta^1$-hydrocortisone may reflect the rate of an additional reduction at $C_1$ prior to the normal metabolism or may reflect a change in the relative proportions of the alternative processes. The recent studies by Liddle and Richard(15) and by Silber and Morgan(16) have shown that alteration of ring A or substitution of fluorine at $C_9$ of the hydrocortisone molecule results in a reduced rate of metabolism. These investigators have suggested, on the basis of urinary excretion studies, that changes in the steroid molecule alter its susceptibility to the usual enzymatic attack on ring A. Glenn* has observed that the 2-methyl-, 9$\alpha$-fluoro-, and $\Delta^1$-derivatives of hydrocortisone are metabolized at a markedly reduced rate, when compared with hydrocortisone, in a microsome-supernatant fraction of rat liver. Hence, it appears that enhanced potencies of these steroids are related to a reduced rate of metabolism in the animal. Final evaluation of the processes involved awaits further experimentation.

*Summary.* The effects of orally and intravenously administered $\Delta^1$-hydrocortisone and hydrocortisone on plasma 17-OHCS concentrations in the dog have been studied. $\Delta^1$-

* Unpublished data.

Hydrocortisone produced higher and more prolonged plasma 17-OHCS elevations than did equivalent doses of hydrocortisone administered orally. The half-life of $\Delta^1$-hydrocortisone was found to be 1.37 times that of hydrocortisone. Intravenous administration of these steroids as the hemisuccinates gave lower but somewhat more prolonged elevations in plasma 17-OHCS concentrations than the corresponding free alcohols. It was concluded that $\Delta^1$-hydrocortisone was metabolized more slowly than hydrocortisone and suggested that this may explain, at least in part, the enhanced potency of the $\Delta^1$-steroid.

1. Bunim, J. J., Pechet, M. M., and Bollett, A. J., *J. Am. Med. Assn.*, 1955, v157, 311.

2. Spies, T. D., Stone, R. E., Lopez, G. G., Tellechea, C. M. D., Toca, R. L., Reboredo, A., and Suarez, R. M., *ibid.*, 1955, v159, 645.

3. Spies, T. D., Stone, R. E., and Spies, H. A., Jr., *G. P.*, 1955, v12, 73.

4. Thorn, G. W., Renold, A. E., Morse, W. I., Goldfien, A., and Reddy, W. J., *Ann. Int. Med.*, 1955, v43, 979.

5. Ely, R. S., Done, A. K., and Kelley, V. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 503.

6. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 519.

7. Eik-Nes, K., Nelson, D. H., and Samuels, L. T., *ibid.*, 1953, v13, 1280.

8. Kelley, V. C., Done, A. K., Ainger, L. E., Ely, R. S., Forist, A. A., and Collins, E. J., *J. Pediatrics*, 1956, v49, 26.

9. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, 1950, v185, 201.

10. Jakoby, W. B., and Tomkins, G., *Science*, 1956, v123, 940.

11. Tomkins, G., and Isselbacher, K. J., *J. Am. Chem. Soc.*, 1954, v76, 3100.

12. Glenn, E. M., and Recknagel, R. O., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 153.

13. Recknagel, R. O., and Glenn, E. M., *ibid.*, 1955, v89, 156.

14. Glenn, E. M., Siu, P. M. L., and Recknagel, R. O., *ibid.*, 1956, v91, 332.

15. Liddle, G. W., and Richard, J. E., *Science*, 1956, v123, 324.

16. Silber, R. H., and Morgan, E. R., *Clin. Chem.*, 1956, v2, 170.

Received August 27, 1956. P.S.E.B.M., 1956, v93.

## Titration of Influenza Virus and Viral Antibodies by Preserved Human Erythrocytes.* (22760)

CHARLES D. COX AND E. C. PIRTLE

*Department of Microbiology, University of S. Dakota School of Medicine, Vermillion*

Procedures for titration of influenza viruses and their antibodies by use of chicken and human erythrocytes in tube hemagglutination (HA) and hemagglutination inhibition (HAI) tests have been well established(1-8). Most of the presently used tube HA and HAI titrations are read by the pattern method(5) and employ freshly washed chicken or human erythrocyte suspensions. Flick(9) reported the use of formalin treated human erythrocytes in studying the hemagglutinating capacity of influenza A virus; however, these cells exhibited spontaneous agglutination and could not be read by the pattern technic. Previous work in this laboratory(10) has demonstrated effective preservation of sheep erythrocytes for use in various serological systems. This report describes the preparation and use of preserved human erythrocytes in the titration of influenza viruses and their antibodies.

*Materials and methods.* Diluent used in all serological tests consisted of 0.137 M NaCl, 0.01 M $Na_2HPO_4$, and 0.003 M $KH_2PO_4$ in distilled water, the pH being 7.2 $\pm$ 0.05. Blood group O, Rh+ human erythrocytes were used in all tests. Those used as fresh cell suspensions were obtained by bleeding into an equal volume of Alsever's solution, and before use they were washed 3 times in 40 volumes of diluent and resuspended to a final concentration of 0.5%. Egg-adapted departmental strains of the type A (PR8 and IA43),

* This investigation was supported in part by research grant from National Microbiological Institute.