a mixture of 60–70° ligroin and chloroform to give 67% of colorless needles, m.p. 121.0–121.5°.

Anal. Calcd. for $C_7H_7O_3N$ (153): C, 54.9; H, 4.6. Found: C, 54.9; H, 4.6.

Isonicotinyl Hydrazide-1-Oxide.—Reaction of the above ester with a twofold excess of hydrazine hydrate in warm methanol gave a 90% yield of the desired hydrazide, m.p. 229° dec., lit.[25,26] 227° dec., 233–234°.

Cyanomethyl Nicotinate-1-oxide.—Nicotinic acid-1-oxide was prepared by the method of Ochiai[16] to give 75% of the acid, m.p. 250–251°, lit.[27] 249°. To a suspension of 10 g. (0.072 mole) of this acid in 100 ml. of dry ethyl acetate was added 15 ml. (0.108 mole) of triethylamine and 8.16 g. (0.108 mole) of chloroacetonitrile. The gummy mixture formed on the addition of triethylamine dissolved upon the addition of the chloroacetonitrile. The resultant clear yellow solution was held at reflux for 90 minutes with stirring in order to break up the precipitate which formed. The stirred suspension was cooled in an ice-bath, filtered and the residue washed with 10 ml. of ethyl acetate. The residue was air dried, then suspended in 85 ml. of cold absolute methanol to remove the triethylamine hydrochloride, filtered, washed with 25 ml. of cold methanol and dried *in vacuo*. The product was recrystallized from ethyl acetate to give 10.0 g. (78%) of ester, m.p. 160–161°.

Anal. Calcd. for $C_8H_6O_3N_2$ (178): C, 53.9; H, 3.4; N, 15.7. Found: C, 53.7, 53.9; H, 3.6, 3.4; N, 15.8, 15.7.

Enzyme Experiments.—The general method has been described previously.[21] Other pertinent details are summarized in Table I. In addition to the experiments described in Table I the value of K_I for α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alaninate was evaluated against α-N-benzoyl-L-valine methyl ester with $[S]_0 = 2.04 \times 10^{-3}$ *M*, $[I] = 0.877 \times 10^{-3}$ *M*, [E] = 0.15 mg. protein-nitrogen per ml. and [NaCl] = 0.02 *M*. The value of v_0 obtained was 2.86×10^{-5} *M*/min. and the value of $[S]_0[E]/v_0$, 10.7 min./mg. protein–nitrogen per ml. Since for the same values of $[S]_0$, [E] and [NaCl], but with [I] = 0, $v_0 = 2.96 \times 10^{-5}$ *M*/min. and $[S]_0[E]/v_0$ = 10.3 min./mg. protein–nitrogen per ml., it may be inferred that K_I for α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alaninate is 20×10^{-3} *M* at *p*H 7.9 and 25°.

PASADENA, CALIFORNIA

(25) M. Shimizu, T. Naito, G. Ohta, T. Yoshikawa and R. Dohmori, *J. Pharm. Soc., Japan*, **72**, 1474 (1952); *C. A.*, **47**, 8077h (1953).

(26) M. Colonna and C. Runti, *Ann. chim. (Rome)*, **43**, 87 (1953); *C. A.*, **48**, 3359i (1954).

(27) G. R. Clemo and H. Koenig, *J. Chem. Soc.*, S231 (1949).

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

Citrovorum Factor Cyclodehydrase[1]

BY JAMES M. PETERS AND DAVID M. GREENBERG

RECEIVED NOVEMBER 26, 1957

An enzyme has been separated from sheep liver acetone powders which converts citrovorum factor in the presence of adenosine triphosphate to a compound with an absorption spectrum similar to, but not identical with, that of $N^{5,10}$-methenyltetrahydrofolic acid. The spectrum of the enzymatically-formed product has its absorption maximum at 343 mμ, instead of at 354 mμ, which is the absorption maximum of $N^{5,10}$-methenyltetrahydrofolic acid. The enzymatically-formed material also differs from $N^{5,10}$-methenyltetrahydrofolic acid by being stable at *p*H 6.8. It is not readily decomposed by oxygen. The enzyme has its optimum activity at about *p*H 4 and requires a free sulfhydryl group for activity. The Michaelis constant of the enzyme for citrovorum factor was determined to be 1.7×10^{-6} *M*. The residual protein fraction, after extraction of the citrovorum factor cyclodehydrase, was found to act synergistically with citrovorum factor cyclodehydrase in converting citrovorum factor to a compound which could not be converted to $N^{5,10}$-methenyltetrahydrofolic acid by acidification.

Introduction

Recently we reported the partial purification and certain properties of a multi-enzyme system of sheep liver concerned with the conversion of citrovorum factor (N^5-formyltetrahydrofolic acid) to a serine aldolase cofactor.[2] Subsequent investigations have enabled us to separate an enzyme from sheep liver acetone powders which converts citrovorum factor to a compound with an absorption spectrum similar to that of anhydrocitrovorum factor (anhydroleucovorin, $N^{5,10}$-methyltetrahydrofolic acid). The enzyme was prepared by passing an aqueous extract of the acetone powder through an hydroxylapatite column (the aqueous eluate contained the enzyme) or by adsorption of the aqueous acetone powder extract on calcium phosphate gel (gel:protein ratio 1.2 or more; protein concentration 1% or less) followed by centrifugation of the gel (the supernatant fluid contained the

TABLE I

SUMMARY OF PURIFICATION OF SHEEP LIVER CITROVORUM FACTOR CYCLODEHYDRASE

Fraction	Mg. protein per ml.	Apparent enzyme units[a] per ml.	Apparent specific activity
Aqueous extract of whole liver	22	0	0
Aqueous extract of acetone powder	24	30	1
Calcium phosphate gel fraction[b]	2	32	16
40–60% satd. $(NH_4)_2SO_4$ fraction	105	2015	19
Calcium phosphate gel fraction of 45–55% $(NH_4)_2SO_4$ fraction	2	47	24
Calcium phosphate gel fraction after treatment with CM-cellulose[c]	1	72	72

[a] One unit of citrovorum factor cyclodehydrase is defined as that amount of protein which will bring about a change in the optical density at 343 mμ of 0.001 unit in 30 minutes when incubated with 0.2 μmole leucovorin, 2 μmoles ATP, 2 μmoles $MgSO_4$ and 100 μmoles of sodium citrate buffer, *p*H 6.0, in 3.0 ml. at 25°. [b] Calcium phosphate gel fraction = eluate from aqueous extract of acetone powder and calcium phosphate gel (gel:protein ratio = 1.2 or more; protein concentration 1% or less). [c] The calcium phosphate gel fraction was negatively adsorbed at *p*H 5.4 on carboxymethyl-cellulose.[3]

(1) Taken in part from a dissertation submitted by James M. Peters in partial fulfillment of the requirements for the Ph.D. degree in Physiological Chemistry, University of California, June, 1957. Presented in part: 10th Meeting of the Pacific Slope Biochemical Conference, August 31, 1957, Berkeley, California, and 132nd Meeting of the American Chemical Society, September 9, 1957, New York, New York. Aided by research grants from the National Cancer Institute (CY-3175), United States Public Health Service and the American Cancer Society, California Division (151).

(2) J. M. Peters and D. M. Greenberg, *J. Biol. Chem.*, **226**, 329 (1957).

enzyme). The citrovorum factor cyclodehydrase activity also is associated with the protein fraction which precipitates between 45 and 55% saturation with ammonium sulfate at *p*H 7. Further purification of the enzyme was achieved by negative adsorption on a cation exchanger, carboxymethylcellulose[3] at *p*H 5.4 (Table I).

The enzyme was adsorbed on carboxymethylcellulose at *p*H values below 4.5, indicating that its isoelectric point probably lies in the *p*H region 4.5 to 5.4.

The enzyme assay was based on the accumulation of a product with an absorption maximum at 343 mμ. The optical density of citrovorum factor cyclodehydrase preparations increased rapidly upon the addition of leucovorin (*dl*-citrovorum factor) and ATP[4] together, while no such increase was observed when either leucovorin or ATP alone was added (Fig. 1). The rate of accumulation of the product of citrovorum factor as a function of protein concentration is shown in Fig. 2.

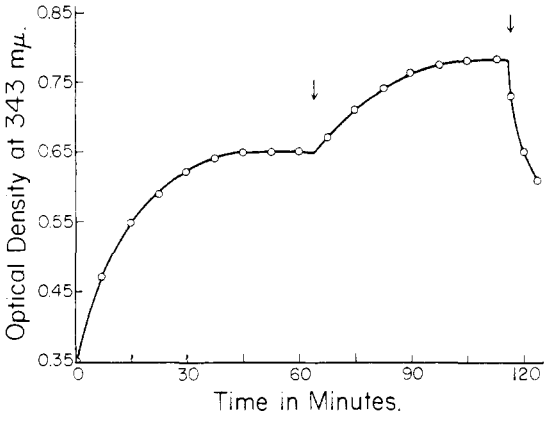

Fig. 1.—Formation of a product from citrovorum factor by citrovorum factor cyclodehydrase and utilization by another enzyme of the citrovorum factor-converting system. Initial cuvette contents: 3 mg. of citrovorum factor cyclodehydrase, 2 μmoles of ATP, 2 μmoles of $MgSO_4$ and 250 μg. of calcium leucovorin. Additions: first arrow, 250 μg. of calcium leucovorin; second arrow, 0.1 ml. of calcium phosphate gel extract (refer to text). Readings corrected for optical density changes due to the additions.

A protein fraction was prepared by extraction of the calcium phosphate gel, which remained after the separation of citrovorum factor cyclodehydrase, with 0.01 *M* sodium citrate buffer at *p*H 6.5. This fraction and the citrovorum factor cyclodehydrase fraction were assayed individually and together for their ability to convert citrovorum factor to a compound which could not be converted to anhydrocitrovorum factor by acidification.[5] The results are shown in Table II and indicate a synergism between the citrovorum factor-converting system and citrovorum factor cyclodehydrase.

(3) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956); M. B. Rhodes, P. R. Azari and R. A. Feeney, *J. Biol. Chem.*, **230**, 399 (1958).

(4) Abbreviations used are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TPN, triphosphopyridine nucleotide; THF, tetrahydrofolic acid.

(5) M. May, T. J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland and W. Shive, THIS JOURNAL, **73**, 3067 (1951).

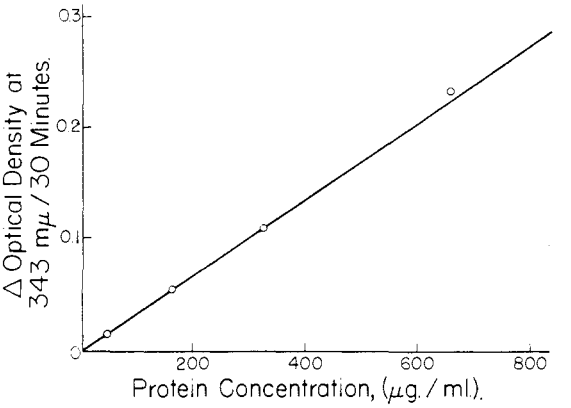

Fig. 2.—The rate of intermediate formation as a function of protein concentration. Cuvette contents: 1.5 ml. of 0.1 *M* sodium citrate buffer, *p*H 4.0, 125 μg. of calcium leucovorin, 2 μmoles of $MgSO_4$, 2 μmoles of ATP, and citrovorum factor cyclodehydrase in a total volume of 3.0 ml.

Evidence that the product formed from citrovorum factor is not anhydrocitrovorum factor is the finding that the enzymatically formed product is stable at *p*H 6.8. Anhydroleucovorin formed from leucovorin by acidification[5] rapidly disappears upon adjustment of the *p*H to values above 5. The enzymatically formed product is also stable to oxygenation. The similarity of this compound to anhydroleucovorin suggests that it also may be an imidazolinium compound, intermediate in the transfer of the formyl group of citrovorum factor from the N^5- to the N^{10}-position.

TABLE II

SYNERGISM BETWEEN THE CITROVORUM FACTOR CONVERTING SYSTEM AND CITROVORUM FACTOR CYCLODEHYDRASE

The vessels contained 0.47 μmole of calcium leucovorin, 0.25 mg. of TPN, 5 μmoles of ATP, 5 μmoles of $MnCl_2$, 0.2 ml. of 0.05 *M* sodium citrate buffer, *p*H 6.6, and protein fractions as indicated in a total volume of 1.0 ml. Aerobic incubation 20 min. at 37°. Reactions terminated by addition of 1.0 ml. of 4% $HClO_4$. Each value represents the average of duplicate determinations.

Protein fraction	Leucovorin disappearing[a] μmoles	mμmoles/mg. protein
Citrovorum factor cyclodehydrase (0.4 mg.)	0	0
Sodium citrate extract of gel (2.1 mg.)	0.03	14.3
Combined fractions (2.5 mg.)	0.06	24.0

[a] Leucovorin was determined by conversion to anhydroleucovorin, by the addition of the deproteinizing acid. A 30-minute period was allowed for complete conversion to the anhydro compound.

The intermediate was separated from ATP and partially separated from unreacted leucovorin by chromatography on a Dowex-1 chloride column[6] (Fig. 3) and the spectra of the two fractions which absorb light at 354 mμ are recorded in Fig. 4. The 0.01 *N* HCl which was used for the elution of the column converted leucovorin to the anhydro compound and the spectrum of this derivative is in close agreement with that reported by May, *et al.*[5] As shown in Fig. 3, conversion of leucovorin to the

(6) (a) W. E. Cohn, in "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Volume III, Academic Press, New York, N. Y., 1957, pp. 867-869; (b) pp. 731-738.

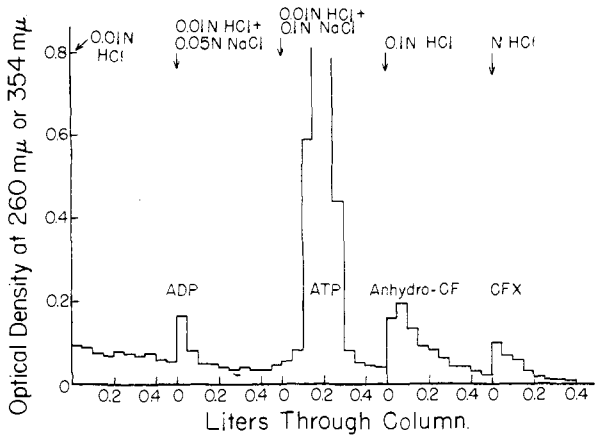

Fig. 3.—Separation of ATP (and ADP) from leucovorin and the enzymatically-formed intermediate on a Dowex-1 chloride column.[6]

anhydro derivative is not in itself sufficient to cause its elution from the column. The second fraction had a spectrum similar to that of the anhydro derivative but differed in that it showed a maximum at 240 mμ and that the maximum in the 360 mμ region was shifted to a shorter wave length than that observed for anhydroleucovorin. The two fractions were readily distinguishable from one another by the ratio of the optical density readings at 354 and 343 mμ. The 354/343 ratio was greater than unity in the anhydroleucovorin fractions and less than unity in the fractions obtained after elution of the anhydro compound. The fraction which is designated as CFX in Fig. 3 was also stable at neutral *p*H (as judged by its absorption at 343 mμ), whereas the absorption peak at 354 mμ of the anhydro-CF fraction decreased rapidly upon adjustment of the *p*H to values above 5. Further attempts at identification of the intermediate have not yet been successful. The occurrence of ADP in the reaction mixture and the absence of an absorption peak at 260 mμ in the spectrum of the intermediate suggest that it is not an adenyl derivative of citrovorum factor but possibly a phosphorylated compound.

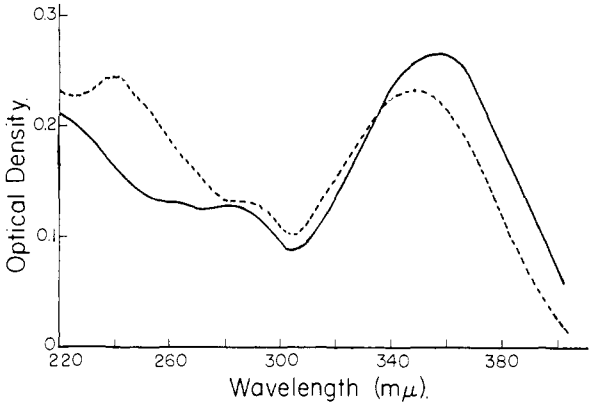

Fig. 4.—Spectra of anhydroleucovorin, ———, and the enzymatically-formed intermediate, - - - -. The optical density readings were taken immediately following elution.

The *p*H–activity curve of citrovorum factor cyclodehydrase preparations (Fig. 5) was determined from the change in optical density at 343 mμ in a 30-minute period over the significant *p*H range. In preparing the curve a correction was applied for the increase in optical density at 343 mμ due to the spontaneous formation of anhydroleucovorin from leucovorin in 30 minutes between *p*H 3.0 and 4.5 (shown in Fig. 5). The rate of the spontaneous reaction as a function of *p*H was determined for *p*H values down to 0.7, employing HCl–KCl and glycine–HCl buffers,[7] and found to be exponentially proportional to the hydrogen ion concentration.

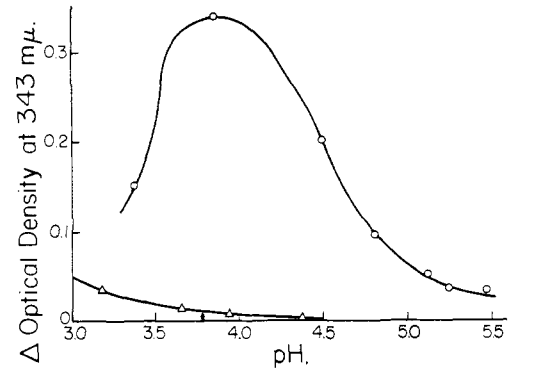

Fig. 5.—*p*H–activity curve of citrovorum factor cyclodehydrase. Cuvette contents: 1.0 ml. of 0.1 *M* sodium citrate buffer, 1 mg. of citrovorum factor cyclodehydrase, 125 μg. of calcium leucovorin, 2 μmoles of $MgSO_4$ and 2 μmoles of ATP in a total volume of 3.0 ml. (Δ, spontaneous formation of anhydroleucovorin from leucovorin).

The rate of formation of the intermediate as a function of the citrovorum factor concentration (50% of the leucovorin concentration) was determined and the values were used to calculate a

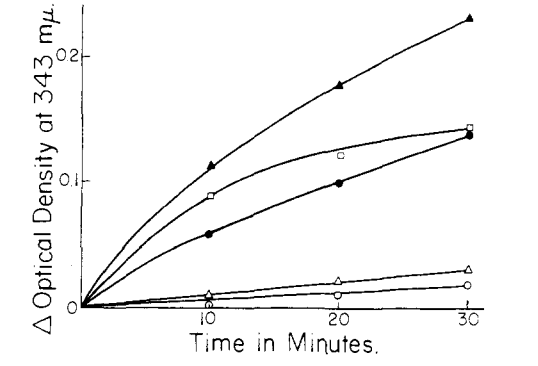

Fig. 6.—Inhibition of citrovorum factor cyclodehydrase by sulfhydryl reagents and reversal of inhibition by glutathione. Cuvette contents: 2 mg. of citrovorum factor cyclodehydrase (preincubated with inhibitor), 2 μmoles of ATP, 2 μmoles of $MgSO_4$, and 177 μg. of calcium leucovorin. Additions: O, *p*-chloromercuribenzoate (3×10^{-4} *M*); Δ, *o*-iodosobenzoate (3×10^{-4} *M*); ●, *o*-iodosobenzoate (3×10^{-4} *M*) plus glutathione (3×10^{-3} *M*); □, *p*-chloromercuribenzoate (3×10^{-4} *M*) plus glutathione (3×10^{-3} *M*); ▲, glutathione (3×10^{-3} *M*).

(7) G. Gomori, ref. 6, Vol. I, Academic Press, New York, N. Y., 1955, pp. 138–139.

Michaelis–Menten constant having the value of $1.7 \times 10^{-6} M$.

The requirement of sulfhydryl groups for citrovorum factor cyclodehydrase activity is indicated by the inhibitory effect of *p*-chloromercuribenzoate and *o*-iodosobenzoate on the rate of formation of the intermediate compound from citrovorum factor and the reversal of inhibition in both cases by reduced glutathione (Fig. 6).

Rabinowitz and Pricer[8] have reported the occurrence in *Clostridium cylindrosporum* of an enzyme, formimino-THF cyclodeaminase, which catalyzes a reaction which is analogous to that proposed for citrovorum factor cyclodehydrase. ATP is not required in the reaction they describe.

Experimental

Apparatus and Materials.—A Beckman model DU spectrophotometer and 1 cm. Corex cuvettes were used for the enzyme assays. 1 cm. quartz cuvettes were used for the determinations of protein concentration from the ultraviolet absorption by the method of Warburg and Christian[9] and a Cary recording spectrophotometer was used for the comparison of the spectra of anhydroleucovorin and the enzymatically-formed intermediate in the region 320–360 mμ. A Beckman model G *p*H meter was used for *p*H determinations.

Calcium leucovorin was generously supplied by the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y., and Solka-Floc BW 200 used for the preparation of carboxymethyl-cellulose[3] (sodium form), was a gift of the Brown Company, Boston, Mass. ATP was obtained from the Sigma Chemical Company, St. Louis, Mo.

Sheep liver acetone powder was prepared as previously described.[2] Hydroxylapatite was prepared by the method of Tiselius, *et al.*,[10] and calcium phosphate gel was prepared by the method of Keilin and Hartree.[11] Dowex-1 (200–400 mesh) (Microchemical Specialties Company, Berkeley, Calif.) was converted to the chloride form and columns were prepared by the method of Cohn.[6b]

(8) J. C. Rabinowitz and W. E. Pricer, Jr., THIS JOURNAL, **78**, 5702 (1956).

(9) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941–1942).

(10) A. Tiselius, S. Hjertén and Ö. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

(11) D. Keilin and E. F. Hartree, *Proc. Roy. Soc. London, Series B*, **124**, 397 (1938).

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[CONTRIBUTION FROM THE CHEMICAL RESEARCH DEPARTMENT, SCHERING CORP.]

Introduction of Oxygen into Ring B of Corticoids

BY A. L. NUSSBAUM, G. BRABAZON, T. L. POPPER AND E. P. OLIVETO

RECEIVED JANUARY 2, 1958

Epoxidation of 6-dehydroprednisone acetate gave the 6α,7α-epoxide as well as some 17-ketone V. The former was converted to the 7α-hydroxy derivative either directly with chromous acetate or *via* a bromohydrin. Corresponding reactions with 6-dehydrocortisone acetate led to 7α-hydroxycortisone acetate. The latter was converted microbiologically to the new 7α-hydroxyprednisone. The Δ^4-compound dehydrates normally, but the $\Delta^{1,4}$-diene is resistant to dehydration. Arguments in support of the indicated structures are presented.

The introduction of an additional double bond into the prednisone molecule by Gould, *et al.*,[1] and by Agnello and Laubach[2] furnished a means for further modification of the steroidal B ring in structures possessing physiological activity. Preferential attack of the 6,7-double bond by peracids was observed when 17α,21-dihydroxy-$\Delta^{1,4,6}$-pregnatrien-3,11,20-trione 21-acetate[1,2] (I) was exposed to such reagents. Two products were isolated: the 6α,7α-epoxide IIa and its corresponding 17-ketone V.

The assignment of structure of substance IIa follows from the following considerations: Elementary analysis of both 21-acetate and the corresponding 21-ol IIb showed the presence of an additional oxygen atom in the molecule. The ultraviolet spectrum of the cross-conjugated trienone starting material had disappeared, and the new substance had instead a single absorption maximum at 240 mμ. This excluded attack at either of the two ring A double bonds, a consideration that was confirmed by the infrared spectrum. The latter showed absorption peaks at 6.18 and 6.24 μ, characteristic of cross-conjugated dienones,[3] and at 11.88 μ, assigned to the epoxide function.[4] The nature of the newly-introduced oxygen was further indicated by reversion of IIa to starting material upon treatment with hydriodic acid.[5] Stereochemical assignment of the epoxide function to the α-side of the molecule rests on steric considerations (rule of the rear[6]) as well as the nature of certain transformation products now to be described.

Halohydrins III were prepared by standard procedures. Thus, a bromohydrin IIIa, a chlorohydrin IIIc and a fluorohydrin IIIe, as well as the corresponding diacetates IIIb, d and f were obtained. Normal oxide opening would lead to the halohydrins formulated as in III, Chart I (6β-halo, 7α-hydroxy) and a consideration of their ultraviolet absorption spectra lends support to this formulation. There is a progressive drop in the α,β-unsaturated ketone absorption; thus, bromohydrin IIIa has its maximum at 244 mμ, chlorohydrin IIIc at 240 mμ and fluorohydrin IIIe at 236 mμ. A similar picture has been observed by Bird and co-

(1) D. Gould, E. L. Shapiro, H. L. Herzog, M. J. Gentles, E. B. Hershberg, W. Charney, M. Gilmore, S. Tolksdorf, M. Eisler, P. L. Perlman and M. Pechet, THIS JOURNAL, **79**, 502 (1957).

(2) E. J. Agnello and G. D. Laubach, *ibid.*, **79**, 1257 (1957).

(3) J. Fried, R. W. Thoma and A. Klingsberg, *ibid.*, **75**, 5764 (1953).

(4) W. A. Patterson, *Anal. Chem.*, **26**, 823 (1954), finds an absorption band at 11.6 μ for a series of simple aliphatic epoxides.

(5) E. P. Oliveto, C. Gerold and E. B. Hershberg, THIS JOURNAL, **79**, 3596 (1957).

(6) L. Fieser, *Experientia*, **6**, 312 (1950).

(7) Oxides generally open to the *trans*-diaxial halohydrins; see, for instance, A. Fürst and P. A. Plattner, Abstracts, 12th International Congress of Pure and Applied Chemistry, New York, 1951, p. 409. Abnormal ring openings, however, have been reported: *cf. inter al.*, W. S. Knowles and Q. E. Thompson, THIS JOURNAL, **79**, 3212 (1957).