$$TPNH + H^+ + FH_2 \xrightarrow[\text{reductase}]{\text{dihydrofolate}} FH_4 + TPN^+ \quad (1)$$

When reaction 1 was carried out in T_2O (0.59 curie of T_2O per ml.), the folate-H_4 isolated by column chromatography on DEAE-cellulose[9] was unlabeled. The tritium from the medium must have been transferred to an exchangeable position, *i.e.*, N-5 or N-8. This excludes the 5,8-dihydro structure.

If dithionite reduction of folate resulted in the formation of 5,8-folate-H_2, hydrogen would be incorporated into exchangeable positions only, *i.e.*, N-5 and N-8. When the dithionite reduction of folate was carried out in T_2O (0.67 curie of T_2O per ml.), the dihydro derivative isolated by column chromatography contained tritium (specific activity of folate-H_2/specific activity of one tritium atom of T_2O, 0.064). Since the tritium had been incorporated into a nonexchangeable position, the results of this experiment provide additional evidence against the 5,8-dihydro formulation. Although the marked dilution might be attributed to an indirect incorporation of label, it is a frequent finding with tritium because of isotope discrimination. To distinguish between the two alternatives dithionite reduction of folate was repeated in 99.8% D_2O.

Deuterium Incorporation Experiments.—Two preparations of folic acid were reduced to folate-H_2 with dithionite and ascorbate in D_2O. The folate-H_2 obtained was dissolved and reprecipitated several times in H_2O, lyophilized, and dried to constant weight over P_2O_5 *in vacuo* at 40°. One preparation was lyophilized as the ammonium salt and the other as the free acid. In order to exclude the possibility of isotope exchange, folate-H_2 was prepared by reduction in H_2O and then equilibrated in either D_2O and ascorbate or in a mixture of D_2O, ascorbate, and dithionite. The compound was dissolved and precipitated several times from H_2O and dried over P_2O_5 *in vacuo* at 40°.

The data in Table I clearly indicate that the dithionite reduction of folate to folate-H_2 results in the incorporation of approximately one atom of deuterium (80–90% of theory) per molecule of folate-H_2. This high level of deuterium incorporation rules out the unlikely possibility that the deuterated folate-H_2 could have arisen indirectly by oxidation of deuterated folate-H_4. It is therefore concluded that the deuterium was *directly* incorporated into a nonexchangeable position during the conversion of folate to folate-H_2. This eliminates the 5,8-dihydro assignment.

Nuclear Magnetic Resonance Studies.—An unambiguous demonstration of the fact that one of the hydrogens added in the reduction of folate to folate-H_2 is attached to carbon is possible by n.m.r. The n.m.r. spectrum of folate in D_2O consists of five groups of peaks (I) a single proton singlet at −1.82 p.p.m. (with respect to benzene as an external standard) which can be assigned to the H-7 of pteridine; (II) an A_2B_2 quartet from the *p*-aminobenzoic acid moiety centered at −0.47 p.p.m.; (III) a single proton triplet from the α-CH group of glutamic acid at +2.3 p.p.m.; (IV) a two-proton singlet at +2.6 p.p.m. attributable to the bridge CH_2 groups; and (V) a group of poorly resolved lines from the ABCD system of the two methylenes of glutamic acid at about +4.45 p.p.m. The assignments are based on the known spectra of glutamic acid[10] and *p*-aminobenzoic acid[11] and are unequivocal. In the spectrum of folate-H_2 the single proton singlet (I) is replaced by a *two-proton* singlet (VI) at +3.05 p.p.m., other peaks remaining the same. Thus, the two protons (the original H-7 and the added proton) are (1) equivalent since a single resonance line is observed and (2) attached to a fully saturated carbon since no resonance absorption from unsaturated systems is observed in this region of the spectrum. This rules out both a 5,8- and a 5,6-dihydro structure. The former would have given rise to a spectrum with the same number of protons as folate, the H-7 singlet appearing in the unsaturated region, −0.4 to +1.5 p.p.m. The spectrum of the latter would have shown a doublet for H-7 in the unsaturated region and a more complex pattern (ABX, AB_2, or ABC) for the C-6H, C-9H_2 system. The chemical shift of the new peak (VI) corresponds closely to the shift of the ring methylene in isosepiapterin, allowing for differences in substituents, solvent, and references.[12] The only reasonable interpretation is that (VI) represents a methylene group in position 7 on the pteridine ring.

Fu and Chinoperos have independently concluded that the 7,8-dihydro structure is the correct one based on ultraviolet spectral evidence.[13]

TABLE I
INCORPORATION OF DEUTERIUM INTO DIHYDROFOLATE PREPARED BY DITHIONITE REDUCTION OF FOLATE

Sample	Description	Atom % excess Found	Atom % excess Theory
1	FH_2[a] (diammonium salt)	3.00	2.86[e]
2	FH_2[b] (free acid)	2.97	3.84[f]
3	FH_2[c] (free acid)	0.30	None
4	FH_2[d] (free acid)	0.45	None

[a] Folate-H_2 was reduced according to the procedure of Blakley[8] in 99.8% D_2O, redissolved, and reprecipitated from H_2O–ascorbate and then washed three times with 0.001 *N* HCl. The compound was dissolved in a few milliliters of H_2O by cautious addition of solid NH_4HCO_3, lyophilized, and dried to constant weight *in vacuo* at 40° over P_2O_5. [b] Folate-H_2 was prepared as in *a*, dissolved, and reprecipitated once from ascorbate–D_2O and twice from ascorbate–H_2O. The sample was lyophilized as the free acid and dried to constant weight *in vacuo* at 40° over P_2O_5. [c] Folate-H_2 was prepared in H_2O,[8] dissolved, and precipitated twice from ascorbate–D_2O, twice from ascorbate–H_2O, washed three times with 0.001 *N* HCl, lyophilized, and dried to constant weight as in *b*. [d] Folate-H_2 was prepared in H_2O,[8] dissolved, and precipitated once from ascorbate–dithionite–D_2O, once from ascorbate–D_2O, and twice from ascorbate–H_2O. The sample was washed three times with 0.001 *N* HCl, lyophilized, and dried to constant weight as in *b*. [e] Based on $C_{19}H_{27}O_6N_9 \cdot 2.5\ H_2O$. [f] Based on $C_{19}H_{21}O_6N_7 \cdot 2.5\ H_2O$.

Acknowledgments.—This investigation was supported in part by grants from the National Science Foundation (G-6478, G-19296) and the National Institutes of Health (CA-05997).

(10) O. Jardetzky and C. D. Jardetzky, *J. Biol. Chem.*, **233**, 383 (1959).

(11) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co. Inc., New York, N. Y., 1959.

(12) H. S. Forrest and S. Nawa, *Nature*, **196**, 372 (1962).

(13) S.-C. J. Fu and E. Chinoperos, 145th National Meeting of the American Chemical Society, September, 1963.

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The Stereochemistry of Ion Pair Return Associated with Solvolysis of *p*-Chlorobenzhydryl *p*-Nitrobenzoate[1]

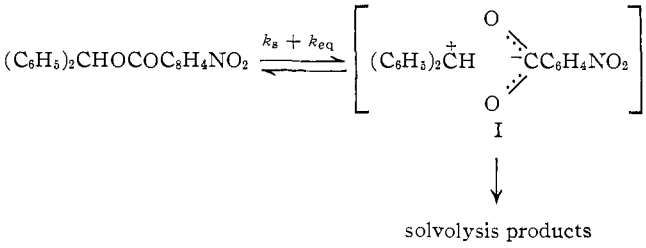
Sir:

We have shown recently that ion pair return associated with solvolysis (alkyl–oxygen cleavage) of O^{18}-labeled benzhydryl[2] and allylic[3] *p*-nitrobenzoates

(1) A preliminary report of this work was presented at the 141st National Meeting of the American Chemical Society, Washington, D. C., March, 1962.

(2) (a) H. L. Goering and J. F. Levy, *Tetrahedron Letters*, 644 (1961); (b) H. L. Goering and J. F. Levy, *J. Am. Chem. Soc.*, **84**, 3853 (1962).

results in first-order equilibration of the carboxyl oxygen atoms in the unsolvolyzed ester. In the case of benzhydryl *p*-nitrobenzoate-carbonyl-O^{18} in 90% aqueous acetone at 118.6°, both solvolysis (reaction 1) and intramolecular oxygen equilibration (reaction 2) are first order and k_{eq} is about three times larger than k_s. Evidently the carboxyl oxygen atoms in the ion pair intermediate(s) (I) are equivalent,[4] and thus, the carboxyl oxygen atoms are randomized in the ester reformed by ion pair return. According to this interpretation oxygen equilibration (k_{eq}) is a measure of total reformation of substrate by ion pair return and $k_s + k_{eq}$ is the rate constant for ionization.[5]



Winstein and co-workers[8] recently have reported the important discovery that ion pair return associated with ionization of optically active *p*-chlorobenzhydryl chloride results in racemization of the substrate. However, as was pointed out,[8] in such systems racemization does not necessarily correspond to total ion pair return because return with preservation of configuration is not detected. The distinguishing feature about the oxygen-equilibration method is that total ion pair return is measured regardless of stereochemistry. We now wish to report the results of an investigation in which we have used this method to study the stereochemistry of ion pair return associated with solvolysis of optically active *p*-chlorobenzhydryl *p*-nitrobenzoate in 80 and 90% aqueous acetone.

Solvolysis of optically active *p*-chlorobenzhydryl *p*-nitrobenzoate (reaction 1) in 80 and 90% aqueous acetone[9] is accompanied by randomization of the carboxyl oxygen atoms (reaction 2) and racemization of the unsolvolyzed ester (reaction 3). All of these transformations are first order and the last two are intramolecular—the carboxylate group remains associated with the same *p*-chlorobenzhydryl group. First-order rate constants for these three processes in the two solvents at 99.6° are given in Table I.[10]

$$\text{ROCOAr} \xrightarrow{k_s} \text{ROH} + \text{HOCOAr} \quad (1)$$

$$\text{ROCO}^{18}\text{Ar} \xrightarrow{k_{eq}} \text{RO}^{18}\text{CO}^{18}\text{Ar} \quad (2)$$

$$(+)\text{-ROCOAr} \xrightarrow{k_{rac}} dl\text{-ROCOAr} \quad (3)$$

The data presented in Table I show the relative rates of reactions 1–3 in 90 and 80% acetone. It is significant that the effects of varying structure[11] and solvent on the rates and relative rates[12] are consistent with the view that oxygen equilibration and racemization are the result of reformation of ester by ion pair return.

TABLE I

RATE CONSTANTS FOR SOLVOLYSIS (k_s), OXYGEN EQUILIBRATION (k_{eq}), AND RACEMIZATION (k_{rac}) ASSOCIATED WITH THE SOLVOLYSIS OF *p*-CHLOROBENZHYDRYL *p*-NITROBENZOATE IN AQUEOUS ACETONE AT 99.6°[a]

	90% acetone 10^3 hr.$^{-1}$	80% acetone 10^3 hr.$^{-1}$
k_s	0.50 ± 0.02	4.41 ± 0.06
k_{eq}	1.27 ± .02	6.5 ± .2
k_{rac}	0.48 ± .03	2.8 ± .1

[a] For all experiments the initial concentration of ester was about 0.03 *M*.

The stereochemistry of ion pair return can be determined from the relative rates of reactions 2 and 3. Reaction 2 measures total ion pair return and reaction 3 measures racemization associated with this return. From the values of k_{eq} and k_{rac} it is apparent that return proceeds with predominating retention of configuration but that there is substantial racemization. Quantitatively, the data show that in 90% acetone 72% of the ion pair intermediate returns to substrate. Of the portion that returns 81% gives the original optical isomer and 19% is converted to the enantiomer. Or to put it another way, return results in 62% retention of configuration and 38% racemization. In 80% acetone, 60% of the intermediate returns and return involves 57% preservation of configuration and 43% racemization. These results then show that less than half of the ion pair return results in racemization of the substrate.

That reactions 2 and 3 are intramolecular is shown by the first-order behavior—if exchange between ester and *p*-nitrobenzoic acid (produced by the solvolysis) contributed to racemization and oxygen equilibration the rates would drift up sharply. This was also confirmed by exchange experiments. The second-order rate constants for exchange between ester and C^{14}-labeled *p*-nitrobenzoic acid (determined as described earlier[2b]) are 0.116 ± 0.008 × 10^{-2} and 0.26 ± 0.01 × 10^{-2} l. mole^{-1} hr.$^{-1}$ for 90% and 80% acetone. With these constants, the fraction of unsolvolyzed ester that has undergone exchange can be determined for any point during the reaction.[2b] These calculations show that in 90% acetone, after 700 hr., <0.4% of the unsolvolyzed ester has been reformed by an intermolecular pathway. At this point, racemization of the unsolvolyzed ester is 29% complete and oxygen equilibration is 57% complete. Exchange is even slower, relative to ion pair return and racemization, in 80% acetone.

In another problem we are studying the effect of nucleophiles on the stereochemistry of ion pair return

(3) H. L. Goering and M. M. Pombo, *J. Am. Chem. Soc.*, **82**, 2515 (1960); H. L. Goering and J. T. Doi, *ibid.*, **82**, 5850 (1960); H. L. Goering, M. M. Pombo, and K. D. McMichael, *ibid.*, **85**, 965 (1963).

(4) As has been pointed out,[2b] this assumption provides a useful operational criterion for ionization because, in systems of this type, ionization that does not result in randomization of the carboxyl oxygen atoms cannot be detected by present methods.

(5) In connection with this interpretation it is significant that evidence has been presented that ion pair intermediates are involved in the intramolecular isomerization of (a) benzhydryl thiocyanates to the corresponding isothiocyanates[6] and (b) benzhydryl thionbenzoates to the corresponding thiolbenzoates.[7] These reactions differ from the present one in that ion pair return results in irreversible formation of rearrangement products rather than reversible formation of substrate.

(6) A. Iliceto, A. Fava, and U. Mazzucato, *Tetrahedron Letters*, **11**, 27 (1960); A. Iliceto, A. Fava, U. Mazzucato, and O. Rossetto, *J. Am. Chem. Soc.*, **83**, 2729 (1961).

(7) S. G. Smith, *Tetrahedron Letters*, 979 (1962).

(8) S. Winstein, J. S. Gall, M. Hojo, and S. Smith, *J. Am. Chem. Soc.*, **82**, 1010 (1960); S. Winstein and J. S. Gall, *Tetrahedron Letters*, **2**, 31 (1960); S. Winstein, M. Hojo, and S. Smith, *ibid.*, **22**, 12 (1960); S. Winstein, A. Ledwith, and M. Hojo, *ibid.*, 341 (1961). See also Y. Pocker, *Proc. Chem. Soc.*, 140 (1961).

(9) Solvent composition based on the volumes of the pure components (25°) prior to mixing.

(10) Rate constants (k_s, k_{eq}, and k_{rac}) were determined as described earlier.[2,3] The rate of racemization was also determined directly from the specific rotations of samples of isolated unsolvolyzed ester. Values of k_{rac} obtained by the two methods were in good agreement.

(11) Rates of solvolysis and oxygen equilibration are about half as large for the *p*-chlorobenzhydryl ester as for the unsubstituted ester[2] as would be expected [*cf.* M. S. Silver, *J. Am. Chem. Soc.*, **83**, 404 (1961)].

(12) The ratio of ion pair return to solvolysis (k_{eq}/k_s) is 40% smaller for 80% acetone than for 90% acetone. For solvolysis of optically active α,γ-dimethylallyl acid phthalate the ratio of return to solvolysis (k_{rac}/k_s) is also 40% smaller for 80% acetone than for 90% acetone (H. L. Goering and R. W. Greiner, *ibid.*, **79**, 3464 (1957)).

to obtain evidence as to whether a single intermediate is involved (which returns with partial loss of configuration) or if two intermediates are involved[13] (one which returns with preservation of configuration and the other with partial or complete loss of configuration).

We also have examined the solvolysis product, *p*-chlorobenzhydrol. For solvolysis in 80% acetone the alcohol is 8% as optically pure (retention of configuration) as the average optical purity[14] of the ester from which it was derived. This means that solvolysis proceeds with 92% loss of configuration and 8% retention of configuration. Solvolysis in 90% acetone proceeds with 10% retention of configuration. In this connection it is significant that control experiments demonstrated that (a) solvolysis involves at least 99% alkyl–oxygen cleavage and (b) the alcohol does not undergo any change (racemization or oxygen exchange) under the conditions of the solvolysis. The partial retention suggests that the present solvolysis is related to the microscopic reverse of the cleavage of α-phenylethyl aryl ethers with hydrogen chloride reported by Hart and co-workers.[15]

Acknowledgment.—This work was supported in part by a grant from the National Science Foundation, Grant No. G194244, and in part by the Research Committee of the Graduate School with funds given by the Wisconsin Alumni Research Foundation.

(13) See S. Winstein, P. E. Klinedinst, Jr., and E. Clippinger, *J. Am. Chem. Soc.*, **83**, 4986 (1961).

(14) Determined as described by S. Winstein and D. Trifan, *ibid.*, **74**, 1154 (1952).

(15) H. Hart and R. J. Elia, *ibid.*, **83**, 985 (1961); H. Hart and H. S. Eleuterio, *ibid.*, **76**, 1379 (1954). See also K. Okamoto, K. Takeuchi, and H. Shingu, *Bull. Chem. Soc. Japan*, **35**, 525 (1962).

(16) (a) National Science Foundation Fellow, 1961–1962; Shell Foundation Fellow, 1960–1961. (b) National Science Foundation Fellow, 1959–1962; Minnesota Mining and Manufacturing Company Fellow, 1962–1963.

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The Identification and Synthesis of the 4-Aminosugar from *Chromobacterium violaceum*

Sir:

We wish to report the complete identification and synthesis of viosamine, 4,6-dideoxy-4-amino-D-glucose, isolated from the lipo-polysaccharide of *C. violaceum*, NCTC 7917.[1] This 4-aminosugar represents the first of a new class of carbohydrates isolated and identified from the special polysaccharides of bacteria.

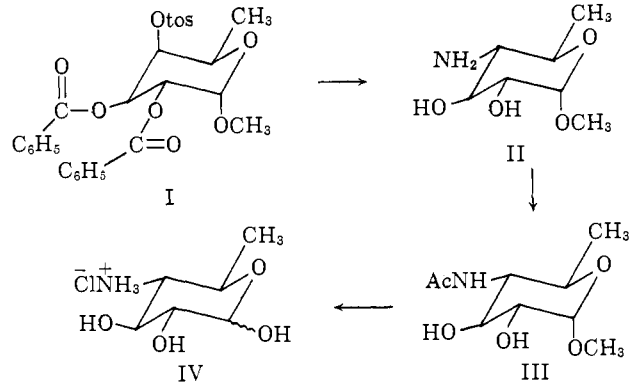
Further, we suggest that this class of carbohydrate may be of wide occurrence and importance, since, in addition to its presence in the lipopolysaccharide of *C. violaceum*, the N,N-dimethyl derivative has been shown to occur in the antibiotic amicetin, and since the properties of viosamine indicate that it is identical with one of the new aminosugars isolated by Strominger[2] as a thymidine diphosphate nucleotide conjugate (TDP-X_2) from *Escherichia coli B*.

(1) R. W. Wheat, E. L. Rollins, and J. M. Leatherwood, *Biochem. Biophys. Res. Commun.*, **9**, 120 (1962).

(2) T. Okazaki, R. Okazaki, J. L. Strominger, and S. Suzuki, *ibid.*, **7**, 300 (1962). A private communication from J. L. Strominger indicates N-acetyl viosamine appears to be identical with X_2 by paper chromatography in the three solvents reported in his article and by comparison of infrared spectra and that viosamine is identical with deacylated X_2 in the three solvent systems of ref. 1.

Natural crystalline viosamine hydrochloride was isolated as previously described,[1] m.p. 132–138° dec., $[\alpha]^{27}D$ −9° initial → +21° (24 hr., *c* 1, H_2O).

Synthetic viosamine hydrochloride was prepared starting with methyl-6-deoxy-4-tosyl-α-D-galactopyranoside dibenzoate[3] (I), m.p. 157–158°. Treatment with azide ion[4] followed by reduction with platinum catalyst and saponification with barium hydroxide gave methyl-4,6-dideoxy-4-amino-α-D-glucopyranoside (II), m.p. 117–118°; $[\alpha]^{23}D$ +144° (*c* 0.85, H_2O). The crystalline N-acetate III was prepared, m.p. 188–189.5°; $[\alpha]^{21}D$ +151° (*c* 0.43, H_2O). The free amine I and the N-acetate III were subjected to periodate studies to confirm their structure; II consumed 2 moles of periodate at a rate essentially identical with that of methyl-α-D-glucopyranoside. The N-acetate III consumed one mole of periodate at a much slower rate.



I II III IV

Many attempts to hydrolyze the α-methyl glycoside II failed to give a crystalline product, as did the many attempts to convert the natural viosamine into its α-methyl glycoside. Hydrolysis of the α-methyl glycoside N-acetate III was successful, using 2.5 *N* hydrochloric acid for 6.5 hr. at 100°. Crystalline synthetic viosamine hydrochloride (IV) was isolated in 58% yield after cation exchange chromatography, m.p. 130–138° dec., $[\alpha]^{21}D$ −12°, 8 min. → +20° (22 hr., *c* 0.76, H_2O). The decomposition point and optical rotation data correspond with the natural material. The infrared spectra of the natural and synthetic material were superimposable and the R_f values were identical in three systems: 1-butanol–acetic acid–water (5:1:2), R_f 0.14; phenol–water (3:1), R_f 0.27; and 1-butanol–ethanol–water–ammonium hydroxide (5:1.4:3:0.1), R_f 0.44.

The n.m.r. spectrum of the free aminosugar hydrochloride in deuterium oxide supported the gluco-configuration.

Acknowledgment.—Supported by Grant No. E1659-06 of the NIAID, United States Public Health Service, at Duke University, and by Grant No. A-769 and CA 3772 from the National Institutes of Health, at Wayne State University.

(3) C. L. Stevens, P. Blumbergs, and F. A. Daniher, *J. Am. Chem. Soc.* **85**, 1552 (1963).

(4) *Cf.* E. J. Reist, R. R. Spencer, B. R. Baker, and L. Goodman, *Chem. Ind.* (London), 1794 (1962), who show that a 4-mesyl group in the galacto series is displaced with inversion by azide ion in dimethylformamide solvent.

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