the aid of carrier and counted. The amount of radioactivity has been expressed as μmoles of added serine.

The results (Table II) show that homogenates also effect a rapid conversion of serine to phosphorylserine and that this activity resides almost exclusively in the supernatant fraction. The extent of the reaction, about 4–6 μmoles per g. liver per hour, is not significantly altered by dialysis or subsequent re-addition of ATP. When carrier phosphorylserine was added at the end of the incubation (Homogenate I) less than 0.02 μmole of radioserine was recovered as phosphorylserine.[3] Hence there is an apparent need for phosphorylserine in the incubation medium to serve either as a trap or to participate in an exchange of phosphate groups.

TABLE II

FORMATION OF PHOSPHORYLSERINE FROM L-SERINE-3-C^{14} IN LIVER HOMOGENATES AND CELLULAR FRACTIONS

Liver fraction	Added serine phosphorylated, μM./hr./g. liver
Homogenates	
I[a]	0.016
II[b]	4.2
Cell fractions[c]	
Homogenate	4.4
Mitochondria	0.03
Microsomes	0.2
Supernatant	6.8
Supernatant fraction[d]	
Undialyzed	4.0
Dialyzed—ATP added	3.0
Dialyzed—no ATP added	3.6

[a] 1 g. of liver homogenate incubated 1 hour with 10–13 μM. L-serine-3-C^{14}, containing 4.67×10^4 c.p.m. per μM., in phosphate buffer, pH 7.0. Final volume 9.3 ml. 50 μM. DL-phosphorylserine carrier added at end of incubation. Average of 3 experiments. [b] Conditions same as above, except that carrier phosphorylserine added at start of incubation. Average of 2 experiments. [c] Cell fractions prepared according to G. Hogeboom in N. Kaplan and S. Colowick, "Methods in Enzymology," Vol. I, Academic Press, New York, N. Y., 1955, p. 16. Each fraction incubated 1 hour in final volume of 10 ml. with 25 μM. L-serine-3-C^{14} containing 1.4×10^3 c.p.m. per μM. 2 μM. ATP and 50 μM. DL-phosphorylserine added at start of incubation. A similar experiment gave nearly identical results. [d] Dialyzed 6 hr., with stirring, against 20 l. of 0.05 M phosphate buffer. Conditions as above except that final incubation volume was 16 ml. and 5 μM ATP was added except where absence is indicated.

Phosphorylserine has been shown to be an intermediate in the synthesis of serine from carbohydrate precursors.[4] The present study shows that phosphorylserine is normally present in liver and is, in part, derived from serine. From the results of the *in vitro* experiments the question arises as to what extent this conversion of serine to phosphorylserine represents a net phosphorylation or an exchange reaction.

Phosphorylserine may be involved in the conversion of serine to carbohydrates and in the synthesis of phosphatidylserine, in analogy to the synthesis of lecithin from phosphorylcholine.[5]

These and other possible pathways of phosphorylserine metabolism are currently under study.

(3) This value is high owing to isotopic contamination in the isolation procedure.

(4) A. Ichihara and D. M. Greenberg, *J. Biol. Chem.*, **224**, 331 (1957).

(5) E. P. Kennedy and S. B. Weiss, *ibid.*, **222**, 193 (1956).

(6) Predoctoral Fellow of the National Paraplegia Foundation for 1957–1958.

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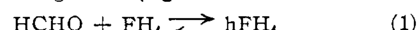
THE ENZYMATIC SYNTHESIS OF HYDROXYMETHYLTETRAHYDROFOLIC ACID (ACTIVE HYDROXYMETHYL)[1,2]

Sir:

The chemical synthesis of "active hydroxymethyl" (hFH_4)[3] according to equation 1 has been reported by several investigators,[4,5,6] but the multiplicity of binding sites (*e.g.*, N^3, N^5, N^8 and N^{10})

$$HCHO + FH_4 \rightleftharpoons hFH_4 \qquad (1)$$



on FH_4 makes it difficult to prepare hFH_4 uniquely by the simple admixing of HCHO and FH_4. An unambiguous synthesis of hFH_4 may be achieved, however, with the *formaldehyde-activating* enzyme, which has been purified from extracts of pigeon liver acetone powder by adsorption (at pH 6.2) and elution (pH 7.5) from calcium phosphate gel, and by ammonium sulfate fractionation at pH 8 (55–75% fraction).

When FH_4 at a concentration of 7×10^{-5} M (λ_{max} at 298 mμ, $\epsilon = 22 \times 10^6$ cm.^{2} mole^{-1})[7] in phosphate buffer, pH 7.5, containing 10^{-2} M mercaptoethanol[8] is mixed with a 4- to 5-fold excess of HCHO in the presence of purified enzyme (50 γ), the spectrum of FH_4 is shifted rapidly to that of hFH_4 (λ_{max} at 290 mμ, $\epsilon = 26 \times 10^6$ cm.^{2} mole^{-1}). At this level of HCHO, no appreciable chemical reaction can be detected spectrophotometrically during the same time period (5–10 minutes) in the absence of enzyme.

hFH_4 can be separated from FH_4 by chromatography on Solka-Floc columns, or on Whatman No. 1 paper using 0.1 M phosphate buffer, pH 8, as the solvent system[5] (hFH_4, R_f = 0.25; FH_4, R_f = 0.13).

The authenticity of hFH_4 after isolation has been established by enzymatic assays. In the presence

(1) Paper V in the series "Folic Acid Coenzymes and Active One-Carbon Units"; for Paper IV see M. J. Osborn and F. M. Huennekens, *Biochim. Biophys. Acta*, in press.

(2) Supported by research grants from the Life Insurance Medical Research Fund and the United States Public Health Service (CY-3310).

(3) FH_4 represents 5,6,7,8-tetrahydrofolic acid; hFH_4 and fFH_4, hydroxymethyl and formyl FH_4 (position of the C_1 group not specified); h^5FH_4, $h^{10}FH_4$, N^5- and N^{10}-hydroxymethyl FH_4; $h^{5-10}FH_4$, N^5,N^{10}-hydroxymethyl FH_4; f^5FH_4, $f^{10}FH_4$, and $f^{5-10}FH_4$, the corresponding formyl derivatives of FH_4.

(4) R. L. Kisliuk, *J. Biol. Chem.*, **227**, 805 (1957).

(5) G. R. Greenberg and L. Jaenicke, "The Chemistry and Biology of Purines," ed. by G. E. W. Wolstenholme and C. M. O'Connor, Little, Brown and Company, Boston, 1957, pp. 204–232.

(6) R. L. Blakley, *Biochim. Biophys. Acta*, **23**, 654 (1957).

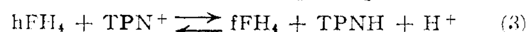
(7) Y. Hatefi, P. T. Talbert, M. J. Osborn and F. M. Huennekens, submitted to *Biochemical Preparations*.

(8) In the presence of mercaptoethanol FH_4 is stabilized considerably against oxidation to dihydrofolic acid (λ_{max} at 282 mμ at pH 7.5).

of *serine hydroxymethylase*[9] and glycine, hFH_4 is converted to FH_4 (equation 2)

$$\text{Glycine} + hFH_4 \rightleftharpoons \text{Serine} + FH_4 \qquad (2)$$

Treatment of hFH_4 with *hFH_4 dehydrogenase*[9] and TPN produces equivalent amounts of "active formyl" and TPNH according to equation 3



$$hFH_4 + TPN^+ \rightleftharpoons fFH_4 + TPNH + H^+ \qquad (3)$$

The results of two typical experiments may be summarized as follows: (a) 0.070 μmole of hFH_4 produced 0.066 μmole of TPNH; and (b) 0.024 μmole of hFH_4 was converted to 0.026 μmole of fFH_4 (measured as f^{5-10} FH_4).

hFH_4 is decomposed by treatment with hydroxylamine or acetylacetone[10]; the latter reagent can be used to estimate the bound HCHO in hFH_4. The stability constant ($K_{diss} \cong 10^{-3}$ for reaction 1 in reverse) of hFH_4, and the resistance of the compound to air-oxidation, suggests that hFH_4 is the N^5,N^{10}-hydroxymethyl[11] bridge compound ($h^{5-10}FH_4$) rather than h^5FH_4 or $h^{10}FH_4$. This conclusion has been reached also by other investigators,[4,5] and is consistent with our previous finding,[12] that with purified preparations of hFH_4 dehydrogenase, $f^{5-10}FH_4$, rather than f^5FH_4 or $f^{10}FH_4$, is the reaction product in equation 3.

(9) Y. Hatefi, M. J. Osborn, L. D. Kay and F. M. Huennekens, *J. Biol. Chem.*, **227**, 637 (1957).

(10) T. Nash, *Biochem. J.*, **55**, 416 (1953).

(11) Because the term "active hydroxymethyl" is already established in the literature, it is retained here even though the structure $h^{5-10}FH_4$ represents a methylene, rather than a hydroxymethyl, derivative of FH_4.

(12) M. J. Osborn and F. M. Huennekens, *Biochim. Biophys. Acta*, in press.

(13) Research Fellow of the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

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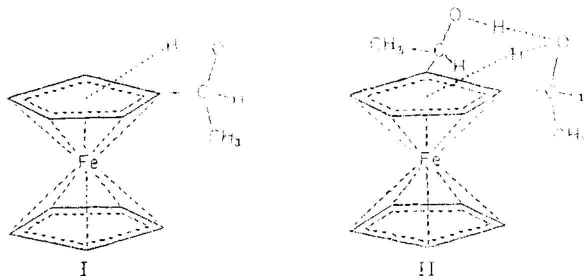
INTRAMOLECULAR AROMATIC RING–HYDROGEN BONDING[1]

Sir:

Evidence has been available indicating the occurrence of intermolecular hydrogen bonding between alcohol solutes and benzene aromatic solvents through observation in the overtone[2] and fundamental[3] spectral region of the greater shift of the hydroxyl stretching frequency in these solvents with respect to the vapor state than in CCl_4 solvent. However, there has not yet been reported any instance of the important general case of an intramolecular structure involving hydrogen bonding to an aromatic ring. Such an instance would additionally provide a clarifying distinction between the previously combined roles of the aromatic system as both solvent and reactant.

Interesting examples of this type of intramolecular hydrogen bonding to the π-electron systems of both ferrocene and benzene aromatic rings have now been observed in the case of several ferrocenyl and phenyl alcohols through infrared absorption studies using a Perkin–Elmer Model 21 spectrometer with LiF optics. For example, the spectrum of α-hydroxyethylferrocene in CCl_4 exhibits both the free hydroxyl absorption band at 3617 cm.$^{-1}$ and another concentration-independent, but temperature-dependent, hydroxyl absorption band of greater intensity at 3574 cm.$^{-1}$ corresponding to that fraction of the molecules at equilibrium having a ring–hydrogen bonded structure of the probable representation I. Similarly, β-phenylethanol exhibits the same corresponding absorptions at 3630 and 3601 cm.$^{-1}$,[4] respectively, but with reversed intensities.



I II

The homoannular-diacetylferrocene first prepared by Rosenblum and Woodward[5] and presumed to have the 1,3-structure was shown here to have the 1,2-structure[6] on the basis of an intramolecular hydrogen bond between the two hydroxyl functions of the corresponding diol, 1,2-di-α-hydroxyethylferrocene. In the present connection, however, the point of interest is that in addition to the intra-hydroxyl band at 3457 cm.$^{-1}$, the only stretching frequency of the second hydroxyl group observed is at 3588 cm.$^{-1}$, *i.e.*, in the ring–hydrogen bonded state (II). Similar free hydroxyl approach for interaction with the aromatic ring is permitted here as in I, while in the contrasting parallel case of the intramolecularly hydrogen bonded 1,1'-di-α-hydroxyethylferrocene, the structure does not permit close approach of the free hydroxyl to either of the two aromatic rings resulting in a characteristic free band at 3621 cm.$^{-1}$.[7]

The hydrogen bonding role of the ferrocene ring also was demonstrated intermolecularly in CCl_4 with a solution 0.1 *M* in both ferrocene and *n*-butanol through the appearance of a new, additional weak hydroxyl absorption at 3597 cm.$^{-1}$.

With the magnitude of $\Delta\nu$ as a measure of the strength of a hydrogen bond,[8,9] it is clear from the $\Delta\nu$ value for α-hydroxyethylferrocene (43 cm.$^{-1}$) compared to the value for β-phenylethanol (29 cm.$^{-1}$) that the π-electron system of the ferrocene

(1) This work was supported in part by the Army and Navy under Signal Corps Contract No. DA-036-39SC-70154.

(2) L. H. Jones and R. M. Badger, THIS JOURNAL, **73**, 3132 (1951).

(3) M. Tamres, *ibid.*, **74**, 3375 (1952).

(4) H. Kwart, private communication, also has informed us of his observation of these two bands.

(5) M. Rosenblum, Ph.D. Thesis, Harvard University, 1953.

(6) J. H. Richards and T. J. Curphey, *Chemistry and Industry*, 1456 (1956), have independently made this same 1,2-structure assignment *via* anhydride formation from the corresponding diacid.

(7) Other additionally interesting aspects of the spectra associated with the hydroxyl groups of these diols together with a more detailed and quantitative account of these over-all results will be presented subsequently.

(8) R. M. Badger, *J. Chem. Phys.*, **8**, 288 (1940).

(9) L. P. Kuhn, THIS JOURNAL, **74**, 2492 (1952).