hyde with a solution containing $[HFe(CO)_4]^-$ ion. Benzyl alcohol was obtained in 33% yield.

DIV. OF SOLID FUELS TECHNOLOGY
BRANCH OF COAL-TO-OIL RESEARCH HEINZ W. STERNBERG
CENTRAL EXPERIMENT STA. RAYMOND MARKBY
U. S. BUREAU OF MINES IRVING WENDER
BRUCETON, PA.

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INTERMEDIATE STEPS IN THE FORMYLATION OF TETRAHYDROFOLIC ACID BY FORMIMINOGLUTAMIC ACID IN RABBIT LIVER

Sir:

The involvement of folic acid in the metabolism of formimino compounds was first indicated by the accumulation of the histidine metabolite,[1] formiminoglutamic acid (FIGLU), in the urine of folic-deficient rats.[2] Subsequently, FIGLU was shown to be a suitable substrate for the formylation of folic acid[3a,b] and of THF in liver preparations, with the formation of 10-formyl-THF.[3c,4]

THF has also been shown to be a cofactor in the metabolism of formiminoglycine (FIG) in extracts of *Clostridium cylindrosporum* and *Clostridium acidiurici*[5a,b] with the formation of 10-formyl-THF.[5c] Rabinowitz and Pricer[6] have now demonstrated that three enzymatic steps are involved, and that 5-formimino-THF and 5,10-methenyl-THF are intermediates. In the present communication it is shown that these three enzymatic steps occur in the metabolism of formiminoglutamic acid in extracts of rabbit liver acetone powder:

$$\text{FIGLU} + \text{THF} \xrightarrow{\text{I}} [\text{5-Formimino-THF}] \xrightarrow{\text{II}}$$

$$\text{5,10-methenyl-THF} \xrightarrow{\text{III}} \text{10-formyl-THF}$$

Enzyme I converts FIGLU and THF to 5-formimino-THF with essentially no change in the optical density at 355 mμ. Upon treatment with acid or enzyme II, 5,10-methenyl-THF is produced, with an increase in the optical density at this wave length (Table I, Fig. 1). By the action of enzyme III (Fig. 1) 5,10-methenyl-THF is converted to 10-formyl-THF, with the simultaneous decrease in the optical density at 355 mμ. Treatment of 10-formyl-THF with acid results in the non-enzymatic formation of 5,10-methenyl-THF.[7]

Enzyme I is readily separated from enzyme III by ammonium sulfate fractionation (enzyme I:0-

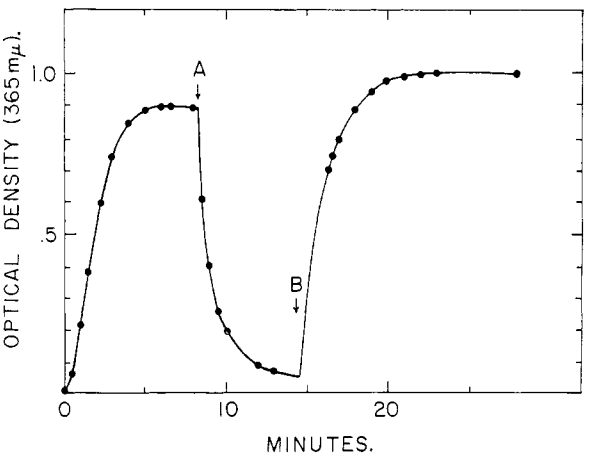

Fig. 1.—The incubation mixture contained 5 μmoles of Na FIGLU, 0.16 μmole of *dl*-THF, 10 μmoles of phosphate buffer (*p*H 7.2), 60 μmoles of mercaptoethanol, 300 γ of liver enzyme (I + II), and water in a total volume of 1 ml. The optical density was measured directly against a blank cell without FIGLU (light path, 1 cm.). At A, 300 γ of enzyme III were added; at B perchloric acid was added to a final concentration of 2.3%. Control experiments without FIGLU or without enzyme showed essentially no changes in O.D. 365 mμ. The optical density obtained represents approximately a 70% yield, assuming that only one optical isomer is active; the low yield is probably due to impurities in the THF preparation used. The rate of formation of 5,10-methenyl-THF when acid was added at B is similar to the rate found with synthetic 10-formyl-THF.

0.8M; enzyme III:1.2-2M); it still contains enzyme II activity, even after 150-fold purification. Under the experimental conditions of Table I, however, the activity of enzyme II is sufficiently less than that of enzyme I to permit accumulation of the formimino-THF intermediate. Although this intermediate compound has not been characterized completely, it behaves like the 5-formimino-THF obtained from FIG + THF[6] in its conversion to 5,10-methenyl-THF when treated with enzyme II or with acid.[8]

Enzyme I from liver will react with formiminoglutamic acid, but not with formiminoglycine, while the transferase from *C. cylindrosporum* will react with formiminoglycine, but not with formiminoglutamic acid. 5-Formimino-THF, formed by either enzyme, is converted to 5,10-methenyl-THF by enzyme II from liver or from *C. cylindrosporum*. The evidence that this compound is 5,10-methenyl-THF is based on the similarity of the enzymatic product and synthetic[9] 5,10-methenyl-THF in spectral characteristics in neutral and acid *p*H (absorption maxima: 355 mμ and 350 mμ,[7] respectively), in their conversion to 10-formyl-THF by en-

(1) For references on the structure of FIGLU, and on its role in histidine metabolism, see B. Borek and H. Waelsch, THIS JOURNAL, **75**, 1772 (1953); *J. Biol. Chem.*, **205**, 459 (1953); H. Tabor and A. H. Mehler, *ibid.*, **210**, 559 (1954).

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(3) (a) K. Slavik and V. Matoulkova, *Coll. Czechoslov. Chem. Commun.*, **17**, 1032 (1954); (b) A. Miller and H. Waelsch, *Biochim. Biophys. Acta*, **17**, 278 (1955); (c) A. Miller and H. Waelsch, *Arch. Biochem. Biophys.*, **63**, 263 (1956).

(4) Abbreviations are the same as used in the accompanying paper.[6]

(5) (a) R. D. Sagers, J. V. Beck, W. Gruber and I. C. Gunsalus, THIS JOURNAL, **78**, 694 (1956); (b) J. C. Rabinowitz and W. E. Pricer, Jr., *ibid.*, **78**, 1513 (1956); (c) J. C. Rabinowitz and W. E. Pricer, Jr., *ibid.*, **78**, 4176 (1956).

(6) J. C. Rabinowitz and W. E. Pricer, Jr., *ibid.*, **78**, 5702 (1956).

(7) M. May, *et al.*, *ibid.*, **73**, 3067 (1951); D. B. Cosulich, *et al.*, *ibid.*, **74**, 3252 (1952).

(8) The rate of conversion of the enzymatically-formed 5-formimino-THF to 5,10-methenyl-THF in acid (2.3% perchloric acid, 25°) is approximately 25% in 4 minutes and 50% in 10 minutes. This is similar to the rate of cyclization observed with 5-formyl-THF; the cyclization of 10-formyl-THF in acid occurs more rapidly, and is essentially complete in 8 minutes under these conditions.

(9) Synthetic 5,10-methenyl-THF was prepared[7] by incubating 5-formyl-THF in a 0.1 *N* HCl-0.05 *M* mercaptoethanol mixture. We wish to thank Dr. Harry Broquist of the Lederle Laboratories for a generous gift of 5-formyl-THF (calcium leucovorin).

TABLE I

EVIDENCE FOR THE ENZYMATIC FORMATION OF 5-FORMIMINO-THF AND ITS CONVERSION TO 5,10-METHENYL-THF

The incubation mixture, containing 25 μmoles of Na FIGLU, 4.8 μmoles of *dl*-THF, 60 μmoles of mercaptoethanol, 10 μmoles of K phosphate buffer (*p*H 7.2) and 100 γ of enzyme I (liver) in a final volume of 1 ml., was incubated at room temperature in an atmosphere of helium. At various times 0.05-ml. aliquots were diluted to 1 ml. with a 0.01 *M* phosphate buffer (*p*H 7.2)–0.05 *M* mercaptoethanol mixture, and the optical density determined immediately (line A). Simultaneously a similar dilution was made in another cuvette containing an excess of enzyme II (*C. cylindrosporum*), and the optical density determined after 3 minutes (line B). Line C presents the result obtained when the dilution was made in 2.3% perchloric acid, and read after 60 minutes at 365 mμ. The ΔO.D.365 mμ reported in these experiments has been corrected for an initial value of 0.103. This value did not show any change during the incubation period in simultaneous control experiments without FIGLU.

	Δ Optical density (365 mμ)[c]						
Incubation time min.	2.5	6	9	15	20	25	30
A. Enzyme I[a]	.010	.030	.030	.100	.130	.150	
B. Enzyme I[a] + Enzyme II[b]	.115	.280	.360	.490	.595	.595	
C. EnzymeI[a] + Acid							.600

[a] Source liver: the liver preparations still contain some enzyme II activity, which accounts for the slow increase in optical density on line A. The presence of enzyme II in various preparations was conveniently assayed by measuring the formation of 5,10-methenyl-THF in incubation mixtures containing an excess of formiminoglycine and enzyme I from *C. cylindrosporum*. The rate of increase at 365 mμ was proportional to the amount of enzyme II added; no ΔO.D.$_{365}$ mμ was observed in the absence of enzyme II. [b] Source: *C. cylindrosporum:* no Δ O.D.$_{365}$ mμ was observed in a comparable experiment when FIGLU and THF were incubated with this enzyme II, in the absence of enzyme I. [c] The absorption maximum was 355 mμ in line B and 350 mμ in line C, 365 mμ was arbitrarily used for following the changes in optical density reported in Table I and Fig. 1.

zyme III (from liver or *C. cylindrosporum*)[10], and in their slow non-enzymatic conversion to 10-formyl-THF at neutral *p*H, which is markedly accelerated by phosphate.

These observations on the formation of 10-formyl THF by a system involving formiminoglutamic acid explain the accumulation of formiminoglutamic acid in folic-deficient rats, and indicate a pathway for the previously-described incorporation of the isotope from (2-C^{14}-imidazole) histidine into various "one-carbon" compounds.[11]

Further work is necessary to determine the relationship of the enzymatic steps described here to the enzymatic conversion of FIGLU to formamide (in *Aerobacter aerogenes*[12a] and in *C. tetanomorphum*[12b]) and to formylglutamic acid (in *Pseudomonas*[1]).

(10) Although the enzymatic product is completely converted to 10-formyl-THF by enzyme III, only 50% of the synthetic 5,10-methenyl-THF is converted, presumably due to the racemic nature of the latter.

(11) J. C. Reid and M. O. Landefeld, *Arch. Biochem.*, **34**, 219 (1951); D. B. Sprinson and D. Rittenberg, *J. Biol. Chem.*, **198**, 655 (1952); M. Toporek, L. L. Miller and W. F. Bale, *ibid.*, **198**, 839 (1952).

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NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES
NATIONAL INSTITUTES OF HEALTH — HERBERT TABOR
U. S. DEPARTMENT OF HEALTH — JESSE C. RABINOWITZ
EDUCATION AND WELFARE
BETHESDA 14, MARYLAND

RECEIVED SEPTEMBER 6, 1956

THE DEHYDRATION OF 9-HYDROXYTETRAHYDRO-*exo*-DICYCLOPENTADIENE

Sir:

In 1948 Bruson and Riener[1] reported the dehydration of 9-hydroxytetrahydro-*exo*-dicyclopentadiene[2] (I) with phosphoric acid and suggested that the product of the reaction was 1,2-dihydro-*exo*-dicyclopentadiene (II). We have recently prepared an authentic sample of II by the action of alcoholic potash or sodium amide in liquid ammonia upon either 9-chloro-(III) or 9-bromotetrahydro-*exo*-

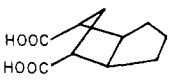
I

II

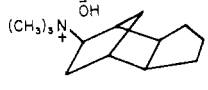
III, X = Cl
IV, X = Br

dicyclopentadiene[3] (IV) and have found this olefin to be different from that obtained by Bruson and Riener. The structure of II was established (1) by the ready formation of a phenyl azide adduct, m.p. 140–140.5° (uncor.) (reported[4] 142°); (2) by catalytic hydrogenation to the known tetrahydro-*exo*-dicyclopentadiene; (3) by permanganate oxidation in acetone solution to the dicarboxylic acid[5] V, the *trans*-acid of which was shown to be identical with the *trans*-acid from the oxidation of 9-ketotetrahydro-*endo*-dicyclopentadiene[6]; and (4) by its identity with the olefin produced upon pyrolysis of the quaternary ammonium hydroxide VI.

We also wish to report that the olefin which Bruson and Riener prepared by the dehydration reaction has been shown to be 9,10-dihydro-*exo*-dicyclopentadiene (VII). The infrared spectrum of this



V



VI

VII

olefin is identical in every respect with an authentic sample of VII which was first described by Bruson and Riener[5] and later investigated by Bartlett and Schneider.[7] Like olefin II, that of Bruson and Riener was converted readily into tetrahydro-*exo*-dicyclopentadiene, but, unlike II, gave no adduct with phenyl azide even after four days.

Another observation of interest here is the smooth conversion of 1,2-dihydro-*exo*-dicyclopentadiene (II) into the 9,10-dihydroisomer (VII) in the presence of phosphoric acid at elevated temperature.

DEPARTMENT OF CHEMISTRY — PELHAM WILDER, JR.
DUKE UNIVERSITY — GEORGE T. YOUNGBLOOD
DURHAM, NORTH CAROLINA

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(2) Previously referred to as hydroxytetrahydro-*nor*-dicyclopentadiene (*ibid.*, **79**, 727 (1945)).

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