While $N^{10}$-CHOFAH$_4$ has been shown to occur in these reactions, it is conceivable that it is interconvertible with a more labile derivative. $N^{10}$-CHOFA is converted to $N^{10}$-CHOFAH$_4$ by a reducing system. In Table II $^{14}$C-formyl-labeled $N^{10}$-CHOFA is shown to transformylate to glycine to form serine. A system forming reduced pyridine nucleotide is required. Transformylation for synthesis of histidine and purine derivatives also is shown.

These experiments have been repeated with labeled $N^{10}$-CHOFAH$_4$ and gave essentially similar results, but addition of DPNH was still necessary for serine synthesis. Beginning either with serine and FAH$_4$ or with $N^{10}$-CHOFAH$_4$ and DPNH a hydroxymethylfolic acid compound was formed*. The conversion to the hydroxymethyl level by reaction with reduced pyridine nucleotide can be coupled in this system with reduction of DPN to DPNH by a tetrahydrofolate compound. Thus in the presence of substrate amounts of formate, glycine, ATP and DPNH, serine synthesis occurred with catalytic quantities of FAH$_4$ (0.01 moles/mole serine). However when substrate amounts of FAH$_4$ were used, only catalytic concentrations of DPNH (0.05 moles/mole serine) were required. While it is clear that a stable cofactor acts in one-carbon transfer reactions[3, 7, 10-12], these studies provide evidence that $N^{10}$-CHOFAH$_4$ can function as a general transformylating agent, that its formyl group is in equilibrium by an oxidation-reduction system with the $\beta$-carbon of serine through a hydroxymethyl level derivative and that CF is not directly involved in this interconversion.

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* On isolation this compound was found to be oxidized to $N^{10}$-hydroxymethylfolic acid which was identified by comparison with the authentic compound synthesized by two different methods.

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## On the occurrence of $N^{10}$-formyltetrahydrofolic acid by enzymic formylation of tetrahydrofolic acid and on the mechanism of this reaction

Pigeon[1,2] and pig[3] liver extracts catalyze an ATP*-dependent reaction between tetrahydrofolic acid and formate to yield a product which transformylates directly to 5-amino-4-imidazolecarboxamide-5'-phosphoriboside[1,4] to form inosine-5'-phosphate in the absence of ATP. During purification this compound was converted to a compound having the properties of $N^{10}$-formyldihydrofolic acid[2] and then to $N^{10}$-formylfolic acid[1,2,3,5,6,7]. This communication provides evidence that the overall reaction, catalyzed by an enzyme system which we propose to call tetrahydrofolate formylase, may be formulated as follows:

$$HCOOH + FAH_4 + ATP \longrightarrow CHOFAH_4 + ADP + H_3PO_4$$

The evidence that CHOFAH$_4$ is $N^{10}$-CHOFAH$_4$ or a closely related compound is: 1. synthetic $N^{10}$-CHOFAH$_4$, transformylates to imidazolecarboxamide ribotide in the absence of ATP, 2. CHOFAH$_4$ can be converted quantitatively to the $N^5$–$N^{10}$-*imidazolinium* derivative of formyltetrahydrofolic

* Abbreviations: FAH$_4$, tetrahydrofolic acid; CHOFAH$_4$, formyltetrahydrofolic acid; $N^{10}$-CHOFAH$_4$, $N^{10}$-formyltetrahydrofolic acid; $N^{10}$-CHOFAH$_2$, $N^{10}$-formyldihydrofolic acid; $N^{10}$-CHOFA, $N^{10}$-formylfolic acid; CF, citrovorum factor; ATP, adenosinetriphosphate; ACF, the $N^5$–$N^{10}$-imidazolinium derivative of formyltetrahydrofolic acid (anhydrocitrovorum factor, anhydroleucovorin); EDTA, ethylenediaminetetraacetate.

acid, 3. $CHOFAH_4$ is oxidized to a compound having the properties of $N^{10}$-$CHOFAH_2$, and 4. $CHOFAH_4$ is converted chemically to CF. In addition it is shown that CF ($N^5$-formyltetrahydrofolic acid) is not the first product of the formylase reaction but appears to be derived from $N^{10}$-$CHOFAH_4$.

Table I presents a balance for the overall tetrahydrofolate formylase reaction.

TABLE I

FORMATION OF $N^{10}$-$CHOFAH_4$ AND ORTHOPHOSPHATE BY THE FORMYLASE REACTION

Conditions: 0.05 ml enzyme, 0.05 $\mu$moles $Na_4$ ATP, 0.7 $\mu$moles Na-PGA, 0.8 $\mu$moles Na-formate-$^{14}$C, 16,000 counts/$\mu$mole, 3 $\mu$moles $MgCl_2$, 0.4 mg EDTA, 1.25 $\mu$moles $FAH_4$, 10 $\mu$moles $KHCO_3$. Total volume 0.5 ml. The reaction was carried out under petroleum ether. Incubation 15 minutes, 37°. The reaction was stopped with 1.2 ml 0.66% $HClO_4$ and was kept under petroleum ether 30 min before measurement of ACF.

| *Additions* | *$^{14}$C formate Incorporated $\mu$moles* | *$N^{10}$-$CHOFAH_4$* Formed $\mu$moles* | *Orthophosphate** Total $\mu$moles* | *(a–b) $\mu$moles* |
|---|---|---|---|---|
| (a) Complete | 0.20 | 0.21 | 0.46 | 0.21 |
| (b) —$FAH_4$ | 0 | 0 | 0.25 | |
| (c) —ATP | 0.01 | 0.07 | 0.16 | |
| (d) Complete (zero time) | 0 | 0.05 | 0.18 | |

* Determined as ACF (after anaerobic acidification[8,9]) by the difference in optical density at 360 m$\mu$ between aliquots taken at zero time and after incubation and based on a change in the molecular extinction value of 22,000 between $FAH_4$ and ACF.

** By the method of GOMORI[10] after removal of interfering folic acid compounds with Dowex-50 ($H^+$ form).

*** Pigeon liver was homogenized with 5 volumes of 0.25 *M* sucrose containing 0.1% EDTA and centrifuged at 80,000 × *g* for 30 min. The extract was frozen overnight, allowed to stand 2 h at room temperature and any precipitate removed. The fraction precipitating between 0.25 to 0.60 of saturation with ammonium sulfate (pH 7.0) was dialyzed against 0.01 *M* $KHCO_3$ overnight at 4°. The enzyme system is stable to freezing and thawing.

TABLE II

CHEMICAL CONVERSION OF THE PRODUCT OF THE TETRAHYDROFOLATE FORMYLASE REACTION TO CITROVORUM FACTOR

Conditions: 1.0 ml pigeon liver extract (homogenized liver with 3 parts of 0.25 *M* sucrose, remove particulate fractions in Spinco centrifuge, pass through a Dowex-1-Cl column and dialyze overnight against 0.05 *M* $KHCO_3$), 6 $\mu$moles $FAH_4$, 9 $\mu$moles $MgCl_2$, 50 $\mu$moles $KHCO_3$, 2.5 $\mu$moles ATP, 35 $\mu$moles Na phosphoglycerate, 2.5 mg muscle extract fraction[2], 7 $\mu$moles $^{14}$C-formate 29,000 counts/$\mu$mole. Vol. 2.6 ml, temp. 38° C, time 42 min, gas phase, He. Reaction is stopped in boiling bath 1 min under He, filtered under He, $O_2$-free NaOH added to 0.1 *N* and mixture heated under He for 60 min at 100°*. Analyzed with *Leuconostic citrovorum* 8081. Each figure represents the average of two separate determinations.

| | *$\mu$g/ml filtrate* |
|---|---|
| CF* activity before alkali treatment | 50 |
| CF activity after alkali treatment | 285 |
| $^{14}$C formate incorporated, calculated as CF | 208 |

* CF has been isolated from such reaction mixtures by paper chromatography[3,5].

When such anaerobically prepared acid filtrates were chromatographed with 1 *M* formic acid as a solvent, the incorporated radioactivity was found as a pale, blue-fluorescing compound at $R_F$ 0.37, corresponding both in migration and, after elution with acid, in its absorption spectrum[8,9] to ACF. Since it is known that CF accounts for only a small percentage of the formate incorporated in these experiments, the ACF must be derived from $N^{10}$-$CHOFAH_4$ or a closely related compound. $CHOFAH_4$ is oxidized to the more stable $N^{10}$-$CHOFAH_2$ if oxygen is not excluded. When filtrates of the formylase reaction system are deproteinized by heat without exclusion of air and are subjected to ascending paper chromatography with 0.5 *M* formic acid and 0.1 *M* $K_2HPO_4$, in each solvent more than 90% of the incorporated $^{14}$C activity is found as a blue-fluorescing spot corresponding exactly with the migration of synthetic $N^{10}$-$CHOFAH_2$* ($R_F$ 0.37 and 0.58 in the two solvents

* Synthesized by reduction of $N^{10}$-CHOFA[11].

respectively). Only small quantities of $N^{10}$-CHOFA are present ($R_F$ 0.69 and 0.76 respectively)*. Eluates of the $^{14}C$ compound, and $N^{10}$-CHOFAH$_2$ show the same ultraviolet absorption[9] spectrum. If the product of the formylase reaction were $N^{10}$-CHOFAH$_4$, it should be converted to $N^5$-CHOFAH$_4$ (CF) through an internal rearrangement on heating anaerobically in dilute alkali[8,9]. Table II shows the results of such an experiment.

Within the limits of this type of experiment CHOFAH$_4$ appears to be completely converted to CF**. Since the microbiological analyses were carried out with *l* (L)-citrovorum factor[12] as a standard, the enzymically synthesized $N^{10}$-CHOFAH$_4$ must show the *l*-configuration at carbon atom 6. Some of the CF activity present before alkali treatment is due to the conversion of CHOFAH$_4$ to CF during heat-deproteinization since $N^{10}$-CHOFAH$_4$ undergoes such a transformation under these conditions at the pH of the mixture[9]. Under the conditions of this experiment (compare with Table I) there was a non-$^{14}C$ source of the one-carbon unit presumably because of the longer reaction time and the diminished activity of the formylase.

Synthetic $N^{10}$-CHOFAH$_4$[11] transformylates directly to 5-amino-4-imidazolecarboxamide-5'-phosphoriboside just as anhydroleucovorin[2] does. The latter hydrolyzes rapidly at pH 7 to $N^{10}$-CHOFAH$_4$[9]. Under the conditions of the formylase reaction the rearrangement of CF to $N^{10}$-CHOFAH$_4$[1,2,3] is much slower than the formation of $N^{10}$-CHOFAH$_4$. In acetone powder extracts as little as 0.25% of the formate incorporated is accounted for as CF***. Since it has been shown that CF is converted to $N^{10}$-CHOFAH$_4$[2,3,5] by an ATP-dependent reaction[1,2] it follows that at least 2 moles of inorganic phosphate should be formed if CF were the first product of the formylation reaction: one for the energy-requiring acylation and one for the $N^5$–$N^{10}$ conversion.

The experiments outlined here do not preclude the presence of a labile precursor of $N^{10}$-CHOFAH$_4$. It is not suggested that tetrahydrofolic acid as such is the natural carrier, but that it represents a convenient model system. The results strengthen the concept first proposed by GORDON *et al.*[14] that $N^{10}$-derivatives are the active cofactors[5,6]. Several laboratories have presented evidence for relatively stable one-carbon cofactors[2,3,15,16,17].

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* By careful anaerobic chromatography $N^{10}$-CHOFAH$_4$ was demonstrated as the product of this reaction in pig liver extracts[3,5].

** CF has been isolated from such reaction mixtures by paper chromatography[3,5].

*** Very recently it has been reported[13] that in an A-methopterin-resistant strain of *S. faecalis* folic acid is converted to a labile compound which non-enzymically forms CF and which appears to have properties similar to $N^{10}$-CHOFAH$_4$.

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