The Reaction of Tetrahydropteroylglutamic Acid and Related Hydropteridines with Formaldehyde

By R. L. BLAKLEY

Department of Biochemistry, John Curtin School of Medical Research,
Australian National University, Canberra, A.C.T.

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Since the discovery that tetrahydropteroylglutamic acid (I) stimulates the synthesis of serine from glycine and formaldehyde or formate in liver-enzyme preparations (Blakley, 1954*a, b*; Kisliuk & Sakami, 1954), there has been considerable speculation on the chemical mechanism by which this acid activates formaldehyde in this reaction. On the basis of known reactions of formaldehyde with 1:2-diamines it seemed likely that formaldehyde would condense with two amine groups to form an imidazolidine structure, *N*⁵*N*¹⁰-methylenetetrahydropteroylglutamate (II) (Blakley, 1954*b*), although an alternative is the formation of *N*⁵-hydroxymethyltetrahydropteroylglutamate (III), as suggested by Kisliuk & Sakami (1954).

Tetrahydropteroylglutamic acid is extremely sensitive to oxidation and in air rapidly breaks

down to xanthopterin, pteroylglutamic acid and other products (Blakley, 1957*a*). On the other hand, leucovorin, the *N*⁵-formyl derivative of the acid, is relatively stable to oxidation, the formyl group protecting the hydrogenated pyrazine ring. It might be expected therefore that *N*⁵*N*¹⁰-methylenetetrahydropteroylglutamate would also be resistant to oxidation. But formaldehyde in low concentrations did not protect tetrahydropteroylglutamate from oxidation to any significant extent (Blakley, 1957*a*); it was therefore concluded that under these conditions the *N*⁵*N*¹⁰-methylene derivative is not formed in appreciable amounts.

On the other hand, Kisliuk (1957), in a study of the non-enzymic binding of formaldehyde by tetrahydropteroylglutamic acid and other hydropteridines, concluded that a single molecule of formaldehyde must combine with both *N*-5 and *N*-10 of the tetrahydropteroylglutamic acid molecule forming the bridged structure (II). According to his results *N*¹⁰-methyl- and *N*⁵-formyl-tetrahydropteroylglutamic acid and 2-amino-4-hydroxy-6-methyltetrahydropteridine were unable to bind significant amounts of formaldehyde; it was concluded that when combination of a single molecule of formaldehyde with two amine groups of tetrahydropteroylglutamate occurred a stable product was formed. As preliminary results from this Laboratory on the combination of H¹⁴CHO with hydropteridines (Blakley, 1957*c*) had indicated a combination of formaldehyde both with *N*¹⁰-formyltetrahydropteroylglutamic acid and with 2-amino-4-hydroxy-6-methyltetrahydropteridine, further study of the combination of formaldehyde with these and other hydropteridines seemed desirable. Moreover, since all the tetrahydropteridines previously examined for reaction with formaldehyde are attacked extremely rapidly by dissolved oxygen, it was of interest to investigate at least one stable tetrahydropteridine and for this purpose 4-hydroxy-6-methyltetrahydropteridine has been synthesized. The results of these studies, here presented, indicate that formaldehyde combines non-enzymically with all hydropteridines, but that the extent of combination with tetrahydropteroylglutamic acid is greater than with any other hydropteridine studied.

Osborne, Vercamer, Talbert & Huennekens (1957) have reported the isolation from acetone-dried pigeon liver of an enzyme which catalyses the combination of formaldehyde and tetrahydropteroylglutamic acid as shown by spectrophotometric studies. Partially purified serine trans-hydroxymethylase preparations used in this Laboratory have been examined for such an enzyme and attention has been directed to the question whether non-enzymic combination of formaldehyde and tetrahydropteroylglutamic acid is sufficiently rapid to account for observed rates of enzymic serine synthesis. A brief summary of these results has been published elsewhere (Blakley, 1958).

EXPERIMENTAL

Materials

Pteridines. Commercial samples of folic acid were purified as previously described (Blakley, 1957a). A specimen gave the following analysis (Found: C, 48.3; H, 4.81; N, 20.6. $C_{19}H_{19}O_6N_7 \cdot 2H_2O$ requires C, 47.8; H, 4.86; N, 20.6%). Samples of pteric acid, 2-amino-4-hydroxy-6-methylpteridine, leucovorin and N^{10} -methylpteroylglutamic acid were generously donated by Dr H. P. Broquist of Lederle Laboratories, Pearl River, New York. The following compounds were synthesized by Dr D. J. Brown of the Department of Medical Chemistry of this University: 4:5-diaminopyrimidine (Brown, 1952); 2-deaminopteroylglutamic acid (Brown, 1953); 4-hydroxy-6-methylpteridine (Albert, Brown & Cheeseman, 1952); N^8 -benzyl-2-chloro-4-methyltetrahydropteridine (Brook & Ramage, 1955); tetrahydroquinoxaline (Merz & Ris, 1887).

N^{10} -Formylpteroylglutamic acid. Since published methods for the synthesis of N^{10} -formylpteroylglutamic acid gave products which were often found by paper electrophoresis to be grossly contaminated, the following procedure was adopted. Pteroylglutamic acid (approximately 0.5 g. of purified material) was dissolved in 98% formic acid (20 ml.) without heating and allowed to stand for 2 days at 20°. The formic acid was removed by freeze-drying and the product dissolved in hot water (200 ml.) and filtered. The filtrate, together with the material which precipitated from it on cooling, was immediately freeze-dried, to yield a fluffy cream residue. When the product was subjected to chromatography and electrophoresis on paper, examination of the papers in ultraviolet light revealed the presence of one major component with a blue fluorescence, two other fluorescing components which were present only in small amounts and a small amount of folic acid. The impurities were removed by chromatography on Whatman no. 3 sheets (20 mg. of pteridine/sheet) with 0.1M-ammonium formate as solvent. The major fluorescing band was eluted with 0.1M-ammonium formate buffer (pH 9) and the eluate freeze-dried. The residue was dissolved in a small volume of hot water and N^{10} -formylpteroylglutamic acid precipitated by adjusting to pH 4 with formic acid and allowing the flask containing the solution to stand in a large beaker of hot water in the dark until the whole system had cooled to room temperature. The precipitate was recovered by centrifuging, washed with cold water and finally dissolved in hot water (20 ml.) and freeze-dried. Residual am-

monium formate was sublimed off at 100° under vacuum (5×10^{-2} mm. Hg). The product, dissolved in 0.01N-NaOH to give a concentration of 10.8 μ g./ml., had absorption maxima at 256 and 365 m μ and minima at 225 and 305 m μ . The molecular extinction at 256 m μ was 46 000, which is considerably higher than that for material prepared by other methods (cf. Pohland, Flynn, Jones & Shive, 1951). After standing overnight in N-NaOH at room temperature, the compound was quantitatively converted into pteroylglutamic acid as shown by spectra and paper chromatography.

Tetrahydropteroylglutamic acid (tetrahydroPGA), dihydropteroylglutamic acid (dihydroPGA) and tetrahydro- N^{10} -formylpteroylglutamic acid (N^{10} -formyltetrahydroPGA) were prepared by catalytic hydrogenation as previously described (Blakley, 1957a). DihydroPGA was also prepared by the method of Futterman (1957). If an aqueous solution of tetrahydroPGA is stored under H_2 in the presence of platinum catalyst (20 mg. for 4 ml. of 0.01M-tetrahydroPGA), further uptake of H_2 occurs until 6 molecular proportions of H_2 have been consumed. The rate at which this occurs varies with the state of aggregation of the catalyst but is reduced to a very low level by storing solutions under a low pressure of H_2 (20 mm. Hg) at 0°; this procedure was therefore adopted for keeping tetrahydroPGA solutions for periods up to a week. Little liberation of aromatic amine occurred (0.08 mole/mole of pteridine) during hydrogenation beyond the tetrahydro stage. In the enzymic synthesis of serine this material had low coenzyme activity, the Michaelis constant being 1×10^{-3} M (cf. tetrahydroPGA below).

Dihydroptericoic acid and 2-amino-4-hydroxy-6-methyltetrahydropteridine were prepared by catalytic hydrogenation in aqueous 0.01N-NaOH.

4-Hydroxy-6-methyltetrahydropteridine. To a suspension of 4-hydroxy-6-methylpteridine (1.6 g.) in water (50 ml.) at room temperature solid sodium borohydride (0.57 g.) was added slowly, with stirring, over a period of 15 min. The solution was allowed to stand for a further 15 min. at room temperature before being freeze-dried. The residue was extracted with several portions of hot propan-1-ol (total 70 ml.), insoluble material being removed by centrifuging after each extraction. The combined extracts were concentrated by heating under a stream of N_2 until the volume was about 10 ml. Sufficient water was added to the hot mixture to give a clear solution, which was then allowed to stand overnight at 0°. The crystals which formed were filtered off in the cold and washed with cold propan-1-ol. After drying *in vacuo* over P_2O_5 the yield of the cream-coloured crystals was 0.65 g. Further crops of crystals were obtained by concentrating the mother liquor. A portion of this material was twice recrystallized from propan-1-ol-water (1:1). After drying for 30 min. at 140° over P_2O_5 (5×10^{-2} mm. Hg) the following analysis was obtained (Found: C, 50.2; H, 6.01; N, 33.3. $C_7H_{10}ON_4$ requires C, 50.6; H, 6.07; N, 33.7%).

When the recrystallized material was subjected to chromatography in several solvent systems and the chromatograms were inspected in ultraviolet light, only one spot could be detected. This had a faint-blue fluorescence. Titration of a 5 mM-solution with 0.1N-KOH revealed an acidic group with pK 10.20 ± 0.03 and indicated the presence of only one compound. The material melted at 112–116° (uncorr.).

*N*⁵-Formyl-4-hydroxy-6-methyltetrahydropteridine. 0.65 g. of 4-hydroxy-6-methyltetrahydropteridine, once-crystallized, was dissolved in 98% formic acid (8 ml.); acetic anhydride (2 ml.) was added and the solution kept overnight at room temperature. The solution was evaporated to dryness under a low pressure of N_2 , 5 ml. of ethanol added and evaporation to dryness repeated. The residue was extracted with boiling ethanol (15 ml.) and then dissolved in the minimum volume of hot water. The ethanolic and aqueous solutions were mixed and allowed to stand overnight at 0°. The white crystals which formed were filtered off and dried *in vacuo* over P_2O_5 at room temperature. Yield was 0.435 g. The material was recrystallized from ethanol-water and dried for 1 hr. at 140° over P_2O_5 (5×10^{-2} mm. Hg) and gave the following analysis (Found: C, 49.8; H, 5.11; N, 28.4. $C_8H_{10}O_2N_4$ requires C, 49.5; H, 5.19; N, 28.8%). In a melting-point tube the crystals softened at 251° and melted with decomposition at 257–259° (uncorr.).

Serine transhydroxymethylase. This was prepared by the method previously described (Blakley, 1955), except that advantage was taken of the stabilizing effect of ethylenediaminetetra-acetic acid (EDTA) on the enzyme to achieve a useful fractionation with acetone. By this means a preparation was obtained with activity four to five times that obtained previously.

The rabbit-liver powder (20 g.), prepared as before, was extracted for 1 min. in a Waring Blendor with 300 ml. of cold 0.01 M-potassium phosphate buffer (pH 7.5) containing mM-EDTA. The mixture was centrifuged and the supernatant heated at 53° as previously described. The supernatant from this treatment was brought to pH 6.4 by the addition of 0.03 vol. of M-potassium phosphate, pH 6.3, followed by 0.0075 vol. of M- KH_2PO_4 , and then fractionated with acetone (purified by boiling with potassium permanganate and distilling over dry potassium carbonate), the temperature of the solution being progressively lowered from 0° to -4° as the acetone concentration was increased to 29% (v/v). The material which precipitated was removed by centrifuging at -5° and discarded. Acetone was added to the supernatant to bring the acetone concentration to 40% (v/v), the temperature being simultaneously lowered from -4° to -7°. The precipitate was removed by centrifuging and redissolved in 10 ml. of ice-cold 0.01 M-potassium phosphate buffer (pH 7.3) containing mM-EDTA. To this solution was added 1.0 vol. of an ice-cold solution of saturated ammonium sulphate containing mM-EDTA and adjusted with aqueous NH_3 solution (sp.gr. 0.880) so that, on dilution with an equal volume of water, it was pH 8. The precipitate was removed by centrifuging at 0° and discarded. More saturated ammonium sulphate solution was added to the supernatant to bring the final concentration of ammonium sulphate to 66% saturated. The precipitate was removed by centrifuging and redissolved in 10 ml. of cold 0.01 M-potassium phosphate buffer (pH 7.3) containing mM-EDTA. The solution was then fractionated with cold, saturated ammonium sulphate containing mM-EDTA and 50 ml. of M-potassium phosphate buffer (pH 7.3)/l. On dilution with an equal volume of water it was pH 6.4. Sufficient of the ammonium sulphate solution was added to bring the concentration of ammonium sulphate to 45% saturated. The precipitate was removed by centrifuging at 0° and discarded. More of the ammonium sulphate solution was added to the supernatant to bring the concentration of ammonium sulphate in the solution to

60% saturated. The precipitate was collected by centrifuging at 0°, and dissolved in 0.01 M-potassium phosphate buffer (pH 7.3, containing mM-EDTA) and the solution dialysed overnight against the same buffer.

Methods

Determination of enzyme activity. This was carried out manometrically as previously described (Blakley, 1955).

Determination of the binding of formaldehyde by hydropteridines. The reaction mixture contained 0.1 M-potassium phosphate buffer (pH 7.2), pteridine and formaldehyde in a total volume of 2.0 ml. After reaction at 20° (for 5 min. unless otherwise stated) the mixture was pipetted on to a column (3 cm. $\times$ 1 cm.) of Nuchar C (granular grade, West Virginia Pulp and Paper Co., New York). Before use the charcoal was boiled with 10% HCl, washed free of acid, dried at 95° and sieved to remove material finer than 40 mesh.

The reaction tube was washed with 2 ml. of water and the washings were pipetted on to the charcoal column after the reaction mixture had all entered the column. This procedure was repeated and the column was then washed with water until 20 ml. of effluent had been collected. The unbound formaldehyde which appeared in the effluent was determined in a sample by the method of Nash (1953). Bound formaldehyde was calculated by difference. Blank determinations were carried out in the same way except that no formaldehyde was added to the reaction mixture. Recovery of known amounts of formaldehyde in the absence of hydropteridine was also checked regularly.

Measurement of reaction rates. The reaction mixture containing formaldehyde (4 μ moles) and buffer was added to each of a series of conical flasks. At zero time the hydropteridine solution was added. After reaction at 20° for the stated periods 400 mg. of acid-washed Nuchar C was added to each and thoroughly mixed with the solution. Each mixture was poured into a chromatography column containing a cotton-wool plug, the effluent collected and again passed through the column. The charcoal was then washed with water until a total of 20 ml. of effluent had been collected. The formaldehyde contained in the filtered effluent was then determined by the method of Nash (1953).

Buffers. Potassium phosphate and sodium acetate buffers of the stated (anion) concentrations and pH were prepared from the data of Green (1933). Borate was used as the sodium salt.

RESULTS

Determinations of the amount of formaldehyde bound by reaction with various hydropteridines yielded the results presented in Table 1. Tetrahydroquinoxaline was also tested because of its close structural relationship to tetrahydropteridine, which is difficult to prepare.

Measurement of formaldehyde-binding by tetrahydroPGA and its derivatives was complicated by the fact that solutions of these compounds produce a colour in the formaldehyde estimation. Thus with a freshly prepared solution of tetrahydroPGA the colour produced was equivalent to almost 2 moles of formaldehyde for each mole of tetrahydroPGA, making it impossible to determine formaldehyde

directly in the presence of tetrahydroPGA. No explanation can be offered for the discrepancy between this result and the claim made by others that formaldehyde bound by tetrahydroPGA may be estimated directly by this colorimetric method (Osborne *et al.* 1957). If the solution of tetrahydroPGA was first run through a 3 cm. column of charcoal, as in the actual procedure adopted, 0.1–0.4 mole of 'formaldehyde' appeared in the effluent for each mole of tetrahydroPGA in the original solution. *N*¹⁰-MethyltetrahydroPGA gave a particularly high blank value and only approximate results could be obtained with this substance. The 'formaldehyde blank' arising from hydropteridines has been subtracted from observed values in calculating free formaldehyde in solution. Although this relatively high blank value has limited the accuracy of the results for these compounds, it has not affected the validity of the conclusions. The blank value with 6-methylhydropteridines and tetrahydroquinoxaline was negligible.

Since reasonably reproducible results were obtained for the dissociation constants of the products formed by the reaction of the various hydropteridines with formaldehyde in widely varying proportions, it may be concluded that only minor disturbances of the base-formaldehyde equilibrium

could have occurred during the washing of the column. Moreover, further prolonged washing of the column with water after 20 ml. of effluent had been collected removed a negligible amount of additional formaldehyde, even though a considerable proportion of the original formaldehyde was still bound on the column. This indicates that once the base-formaldehyde complexes were adsorbed on the charcoal they no longer dissociated to release formaldehyde.

The extent of the reaction of tetrahydroPGA with formaldehyde was unequalled by any other of the compounds tested, each mole of tetrahydroPGA binding 0.7–0.95 mole of formaldehyde. This is in agreement with the results obtained with *H*¹⁴CHO (Blakley, 1957c). Since in solution tetrahydroPGA reacts rapidly with dissolved oxygen, it seemed possible that stoichiometric reaction between formaldehyde and tetrahydroPGA might be obtained if the latter were protected from oxygen. Accordingly the reaction was performed in the presence of 0.01M-ascorbic acid, but no increase in the amount of bound formaldehyde was obtained. To check whether products of the oxidation of tetrahydroPGA had an appreciable capacity to bind formaldehyde, the reaction of formaldehyde with L(+)-*p*-aminobenzoylglutamic acid and with

Table 1. *Binding of formaldehyde by hydropteridines and tetrahydroquinoxaline*

For method and conditions of reaction see text. Figures in parentheses are approximations.

Base	Base concn. (mm)	HCHO concn. (mm)	HCHO bound (% of total)	Dissociation constant
4-Hydroxy-6-methyltetrahydropteridine	50 5 50 25 25*	5.0 5.0 2.5 2.5 2.5	61 17 60 32 24	0.031 0.020 0.032 0.051 0.077
<i>N</i> ⁵ -Formyl-4-hydroxy-6-methyltetrahydropteridine	50	5.0	10	(0.4)
2-Amino-4-hydroxy-6-methyltetrahydropteridine	10 50 20†	2.5 5.0 5.0	20 66 25	0.038 0.024 0.056
8-Benzyl-2-chloro-4-methyltetrahydropteridine	33†	5.0	10	0.29
Tetrahydroquinoxaline	5* 50	2.5 2.5	32 82	0.009 0.011
'Dihydropteroic acid'	10	5	92	0.00047
Dihydropteroylglutamic acid	5 5‡	5.0 5.0	27 25	0.0099 0.011
Tetrahydropteroylglutamic acid	5 5‡	5.0 5.0	85 70	0.00013 0.00064
<i>N</i> ¹⁰ -Formyltetrahydropteroylglutamic acid	5‡	5.0	24	0.012
<i>N</i> ¹⁰ -Methyltetrahydropteroylglutamic acid	22	2.2	(38)	(0.035)
<i>N</i> ⁵ -Formyltetrahydropteroylglutamic acid (leucovorin)	17	2.5	4	(0.4)
2-Deaminotetrahydropteroylglutamic acid	5 10	5.0 5.0	18 36	0.019 0.015

* Reaction was performed in ethanol-water (3:1) instead of in water.

† Reaction was performed in ethanol-water (19:1) instead of in water.

‡ Reaction mixture contained 0.01M-ascorbic acid.

4:5-diaminopyrimidine was investigated. Both compounds reacted with formaldehyde, giving products with dissociation constants of 0.0036 and 0.064M respectively. Consequently it is unlikely that the binding of formaldehyde by products of the degradation of tetrahydroPGA has caused a serious error in the figure for the amount of formaldehyde bound by the latter compound. The incompleteness of the reaction between formaldehyde and tetrahydroPGA is consistent with a reversible reaction, and the data give the approximate value of the dissociation constant, defined by

$$K = [\text{HCHO}] [\text{tetrahydroPGA}] / [\text{product}],$$

as 0.0004 (Table 1).

A group of compounds related to tetrahydroPGA were found to bind 0.04–0.46 mole of formaldehyde/mole of base, corresponding to dissociation constants of 0.017–0.0005 (Table 1). It may be noted that dihydroPGA, N^{10} -formyltetrahydroPGA and (as far as can be determined) N^{10} -methyltetrahydroPGA bind significant amounts of formaldehyde, whereas N^8 -formyltetrahydroPGA (leucovorin) binds only very small amounts.

The very slight ability of leucovorin to bind formaldehyde indicates that N-10, N-8, N-3 and the 2-amino group of the tetrahydroPGA molecule have little reactivity towards formaldehyde. In N^{10} -formyltetrahydroPGA and N^{10} -methyltetrahydroPGA a methylene-bridge product similar to (II) is not possible, so that formaldehyde bound is presumably combined as an N-5-hydroxymethyl group. Because of difficulty in measuring the small hydrogen uptake by the sample of pteric acid, the 'dihydroptericoic acid' may have contained considerable amounts of tetrahydroptericoic acid. This would be consistent with the large amount of formaldehyde bound by the hydroptericoic acid.

Tetrahydroquinoxaline and 6-methyltetrahydropteridines combined 0.02–0.17 mole of formaldehyde/mole of base, corresponding to dissociation constants of 0.01–0.04, indicating that while the *p*-methylaminobenzoyl-L-glutamic acid side-chain considerably increases the affinity of hydropteridines for formaldehyde, appreciable reaction still occurs between formaldehyde and compounds lacking the long side-chain. In such cases also, N^8 -hydroxymethyltetrahydropteridines are the most likely products.

N^8 -Formyl-4-hydroxy-6-methyltetrahydropteridine, like N^8 -formyltetrahydroPGA, bound negligible quantities of formaldehyde, again indicating that a free N-5-amine group is necessary for reaction with formaldehyde.

The fact that N^8 -benzyl-2-chloro-4-methyltetrahydropteridine did not combine with formaldehyde might be due to the influence of the N-8-benzyl group on the reactivity of N-5. Further studies with

related compounds are necessary, however, for an adequate understanding of the lack of reactivity of this compound.

Effect of pH and formaldehyde concentration on formaldehyde-binding by tetrahydropteroylglutamic acid

The amount of formaldehyde bound by tetrahydroPGA in the presence of various concentrations of formaldehyde is shown in Table 2. As the excess of formaldehyde present was increased the errors in the estimate of the amount bound became proportionately greater. It may be seen, however, that a fourfold increase in formaldehyde concentration did not significantly increase the amount of bound formaldehyde beyond 1 mole/mole of tetrahydroPGA.

When tetrahydroPGA (5 mM) was allowed to react with formaldehyde (10 mM) for 1 min. the amount of formaldehyde bound was close to one molecular proportion at all pH values tested between pH 3.8 and 13.0 (Table 2). Somewhat less formaldehyde was bound at pH 1. These results suggest that the N-5-amine group of tetrahydroPGA has a pK of about 1.

Rate of reaction between tetrahydropteroylglutamic acid and formaldehyde

Measurements at the lowest concentrations (0.2 mM) of tetrahydroPGA and formaldehyde at which analyses could be performed indicated that combination of these compounds was complete in about 10 min. (Fig. 1), and that 50% of the formaldehyde had reacted in the first minute. Reaction rates were too rapid to permit investigation of the effect of increased formaldehyde concentration. Since the reaction velocity will be

Table 2. *Effect of pH and formaldehyde concentration on the amount of formaldehyde bound by tetrahydropteroylglutamic acid*

TetrahydroPGA at a concentration of 5 mM was caused to react with formaldehyde at the concentrations indicated in a total volume of 2 ml. under standard conditions as described in the Methods section.

Formaldehyde concn. (mM)	Buffer	pH	Formaldehyde bound (μmoles)
5	0.1M-Phosphate	7.4	9.1
10		7.4	10.8
20		7.4	11.8
10		7.4	9.8
10		6.3	8.3
10	0.1M-Acetate	5.0	9.3
10	0.01M-Acetate	3.8	8.8
10	0.005M-Borate	9.2	8.9
10	0.1N-NaOH	13	8.7
5	0.1M-Phosphate	7.3	7.9
5	0.01M-Acetate	3.8	6.1
5	0.1N-HCl	1.0	5.3

proportional to formaldehyde concentration, it may be calculated that, with a formaldehyde concentration of 0.01M as used in the enzyme system for serine synthesis, 5 μ moles of formaldehyde/ml. will combine with tetrahydroPGA/min. at 20°, or about 16 μ moles/ml./min. at 37°. As this is far in excess of any rates of enzymic serine synthesis attained *in vitro*, the data are consistent with the hypothesis that non-enzymic combination of tetrahydroPGA and formaldehyde precedes enzymic reaction of the product with glycine during the enzymic synthesis of serine.

When serine transhydroxymethylase was added to the reaction mixture the rate of combination of formaldehyde and tetrahydroPGA was diminished rather than increased (Table 3).

Fig. 1. Rate of combination of formaldehyde with tetrahydroPGA. At zero time tetrahydroPGA (final concentration 0.4 mM) was added to a solution of formaldehyde (0.4 mM) in potassium phosphate buffer, pH 7.2 (25 mM), containing ascorbic acid (2.5 mM). Total volume, 10 ml. The reaction was terminated by addition of charcoal and the mixture immediately treated as described in the Methods section.

Table 3. *Effect of serine transhydroxymethylase on binding of formaldehyde by tetrahydropteroylglutamic acid*

The reaction was performed in conical flasks; each contained formaldehyde (0.4 mM), ascorbic acid (5 mM) and potassium phosphate buffer (0.1 M, pH 7.2) in a final volume of 10 ml. Where enzyme was present 0.30 ml. (1.08 mg. of protein) of a preparation of specific activity 12 (μ moles of serine/mg. of protein/hr.) was employed. The reaction was started by addition of tetrahydroPGA (final concentration 0.4 mM) and terminated by shaking with 400 mg. of charcoal. Other conditions were as described in the Methods section.

Time (min.)	Formaldehyde disappearance (μ moles)	
	No enzyme	With enzyme
0.5	2.4	1.3
1.0	2.7	2.0

Rate of reaction of other hydropteridines with formaldehyde

Because of the smaller amounts of formaldehyde bound by other hydropteridines, measurement of their reaction rates was more difficult, but from Table 4 it may be seen that at 20° the reaction was substantially complete in 0.5 min. when the reactants were present at concentrations between 0.1 and 2.5 mM.

The rate of reaction of 4-hydroxy-6-methyltetrahydropteridine with formaldehyde is shown in more detail in Fig. 2. This figure shows that 4 mM-pteridine reached equilibrium with 0.4 mM-formaldehyde in 2–5 min. and that the reaction velocity is therefore of the same order as for the reaction between 0.4 mM-formaldehyde and 0.4 mM-tetrahydroPGA.

Table 4. *Rate of reaction of dihydropteroylglutamic acid and N¹⁰-formyltetrahydropteroylglutamic acid with formaldehyde*

The pteridine was caused to react with 0.4 mM-HCHO in a total volume of 10 ml. under standard conditions as described in the Methods section.

Hydropteridine	Concn. (mM)	Formaldehyde disappearance (μ moles)	
		0.5 min.	15 min.
DihydroPGA	1.0	1.6	1.6
DihydroPGA	2.5	2.2	2.6
N ¹⁰ -FormyltetrahydroPGA	1.0	1.4	1.7

Fig. 2. Rate of combination of formaldehyde with 4-hydroxy-6-methyltetrahydropteridine. At zero time the pteridine (final concentration 4 mM) was added to a solution of formaldehyde (0.4 mM) in potassium phosphate buffer, pH 7.2 (0.1 M). Total volume, 10 ml. The reaction was terminated by addition of charcoal and the mixture immediately treated as described in the Methods section.

Enzyme activation by tetrahydro-, dihydro- and N^{10} -formyltetrahydro-pteroylglutamic acid

The very great difference between the dissociation constant of the compound formed by tetrahydroPGA and those of the compounds formed by dihydroPGA and N^{10} -formyltetrahydroPGA is surprising in view of previous results indicating comparable enzyme activation by tetrahydroPGA and dihydroPGA and appreciable activation even by N^{10} -formyltetrahydroPGA (Blakley, 1957*b*). It was therefore decided to reinvestigate the Michaelis constants for these compounds with N^{10} -formyltetrahydroPGA prepared from the highly purified N^{10} -formylpteroylglutamic acid and the more highly purified preparations of enzyme now available.

It was found that N^{10} -formyltetrahydroPGA prepared by hydrogenation of highly purified N^{10} -formylpteroylglutamic acid caused no measurable activation of the enzyme, so that activation previously obtained with crude N^{10} -formyltetrahydroPGA must be attributed to its contamination by tetrahydroPGA. DihydroPGA prepared by catalytic hydrogenation becomes contaminated with tetrahydroPGA if the reaction is allowed to proceed too long (Blakley, 1957*a*). When rigid precautions were taken to reduce such contamination of the dihydroPGA to a minimum, the activation of the enzymic synthesis of serine caused by the dihydroPGA was slight and variable, and frequently diminished as the enzymic reaction proceeded. Such results suggest that the activity of these dihydroPGA preparations was also due to traces of tetrahydroPGA which could easily become oxidized before or during the course of the enzymic reaction. As a check, dihydroPGA was synthesized by the reduction of pteroylglutamic acid with dithionite, as described by Futterman (1957). These preparations, which gave a spectrum identical with that of the material produced by catalytic hydrogenation, caused no detectable activation of the enzymic synthesis of serine.

It may be concluded therefore that tetrahydroPGA and its conjugates are the only compounds capable of activating the enzymic synthesis of serine. Re-determination of the Michaelis constant for tetrahydroPGA gave the value of 1.1×10^{-4} M, in fairly good agreement with previous results (Blakley, 1957*b*).

DISCUSSION

It has been established that formaldehyde in dilute solution reacts with 4-hydroxy-6-methyltetrahydropteridine, 2-amino-4-hydroxy-6-methyltetrahydropteridine, tetrahydroquinoxaline and N^{10} -formyltetrahydropteroylglutamic acid. It

seems highly probable that the products of the combination of formaldehyde with these compounds are the corresponding N^5 -hydroxymethyl derivatives. Each of these bases binds only a relatively small proportion of formaldehyde even when equimolecular proportions of formaldehyde and base are allowed to react, suggesting equilibria of the type:

The corresponding dissociation constants defined by

$$K = [\text{HCHO}] [\text{base}] / [\text{product}]$$

are of the order of 0.01–0.04. Attainment of equilibrium is relatively rapid, being complete in a few minutes when the reactants are present at concentrations of 0.1–1.0 mM. As expected on this formulation, N^5 -formyl derivatives of hydropteridines bound little formaldehyde.

The reaction of 5 mM-formaldehyde with an equimolar concentration of tetrahydroPGA approaches much nearer to completion than does the reaction of formaldehyde with 6-methylhydropteridines or N^{10} -substituted tetrahydroPGA at similar concentrations. The reason for this cannot be ascertained with certainty from the data presented above, although they exclude the possibility that it is due to the binding of more than one molecule of formaldehyde by each molecule of tetrahydroPGA. Even when a fourfold excess of formaldehyde was added to solutions of tetrahydroPGA only approximately one molecular proportion of formaldehyde was removed from the solution. Furthermore, when N^5 -formyltetrahydroPGA (leucovorin) was treated with formaldehyde, very little binding of formaldehyde occurred, indicating that no other grouping reacts as readily with formaldehyde as does the N^5 -amine group.

The suggestion made some years ago (Blakley, 1954*b*) that tetrahydroPGA might react with formaldehyde to form N^5N^{10} -methylenetetrahydroPGA (II) has been currently revived by several authors to explain the unusual reactivity of tetrahydroPGA towards formaldehyde. The great difference between the binding of formaldehyde by tetrahydroPGA and the binding by 4-hydroxy-6-methyltetrahydropteridine and N^5 - or N^{10} -formyltetrahydroPGA is emphasized by the results presented above. The dissociation constant for the product of the interaction of tetrahydroPGA and formaldehyde is approx. 4×10^{-4} , in contrast with

It was found that serine transhydroxymethylase preparations did not catalyse the combination of formaldehyde and tetrahydroPGA, and that the latter non-enzymic reaction proceeded much faster than the enzymic synthesis of serine under the

same conditions of temperature, pH and concentration of reactants. Although it cannot be decided from the available data whether it is *N*⁵-hydroxymethyltetrahydroPGA or *N*⁵*N*¹⁰-methylenetetrahydroPGA which participates in the enzymic synthesis of serine, a simple mechanism would be involved if *N*⁵-hydroxymethyltetrahydroPGA were the active form.

SUMMARY

1. 4-Hydroxy-6-methyltetrahydropteridine has been synthesized and shown to bind formaldehyde in dilute solution. The evidence indicates a reversible reaction, the dissociation constant for the product being about 0.03.

2. 2-Amino-4-hydroxy-6-methyltetrahydropteridine and tetrahydroquinoxaline react similarly with formaldehyde, the corresponding dissociation constants being 0.04 and 0.01 respectively.

3. It is concluded that such hydropteridines undergo a reversible reaction with formaldehyde to form *N*⁵-hydroxymethyl derivatives. This conclusion is strengthened by the fact that there is little binding of formaldehyde by *N*⁵-formyl-4-hydroxy-6-methyltetrahydropteridine.

4. With 4-hydroxy-6-methyltetrahydropteridine, equilibrium is reached in a few minutes at room temperature when the initial concentration of pteridine is 4 mM and the initial concentration of formaldehyde is 0.5 mM.

5. Tetrahydropteroylglutamic acid binds 0.7–0.95 molecular proportion of formaldehyde, corresponding to the formation of a product with a dissociation constant of 4×10^{-4} or lower, the reaction being completed in 5–7 min. when the reactants have an initial concentration of 0.4 mM.

6. There is no detectable binding of formaldehyde by *N*⁵-formyltetrahydropteroylglutamic acid, but the dissociation constants for the compounds formed by *N*¹⁰-formyltetrahydropteroylglutamic acid and dihydropteroylglutamic acid were found to be 0.01.

7. A new procedure has been developed for preparation of serine transhydroxymethylase. The more highly purified enzyme obtained by this method has been used to re-determine the Michaelis constant for tetrahydropteroylglutamic acid. Dihydropteroylglutamic acid and *N*¹⁰-formyltetra-

hydropteroylglutamic acid did not activate the enzyme.

8. The non-enzymic combination of formaldehyde and tetrahydropteroylglutamic acid proceeded so rapidly that its rate greatly exceeded the maximum rate of enzymic serine synthesis obtained under the same conditions *in vitro*. It may therefore be assumed that non-enzymic combination of formaldehyde and tetrahydropteroylglutamic acid is the first step in serine synthesis. Serine transhydroxymethylase preparations did not accelerate combination of formaldehyde and tetrahydropteroylglutamic acid.

9. It is concluded that tetrahydropteroylglutamic acid and formaldehyde react reversibly, possibly forming *N*⁵*N*¹⁰-methylenetetrahydropteroylglutamic acid. In view of the weakness with which N-10 of tetrahydropteroylglutamic acid binds formaldehyde, *N*⁵-hydroxymethyltetrahydropteroylglutamic acid may be an intermediate in the formation and dissociation of *N*⁵*N*¹⁰-methylenetetrahydropteroylglutamic acid.

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