of a slight negative deviation from Beer's law it is advisable to draw straight lines between each pair of standard values rather than a single line through the whole set.

RESULTS

Fig. 1 shows the variation of extinction with the temperature of the first bath with the modified technique. The values obtained for an average run by the original method are included for comparison. At first bath temperatures below 35° the colour yields are not reproducible and below 25° the normal reddish pink is sometimes replaced by an orange oxidation product, resulting from the presence of undestroyed hydrogen peroxide. The

Fig. 1. Temperature of oxidation versus colour yield: A, 15 μ g. of hydroxyproline/ml.; B, 10 μ g. of hydroxyproline/ml.; C, 5 μ g. of hydroxyproline/ml. Broken lines refer to average values obtained by the original Neuman & Logan method at the concentrations given above. A 4 cm. cell was used.

points below 25° are the average of a number of determinations where the colour did appear and are included to give an idea of the order of the decreased colour yield.

The scatter amongst replicates is less with the modified method; for example, carrying out both methods with the same solutions gave standard deviations, amongst replicates for the three standards and unknown, of 0.74 and 0.49 % by the original and modified methods respectively. The modified method has often yielded standard deviations amongst replicates of the order 0.1–0.2 %, an order of accuracy which was not achieved with the original method over a considerable period. The reproducibility of the colour yield from one series to another has been very high over a prolonged period. The colour yields are represented by the plateaus in Fig. 1, and the reproducibility of the method is such that it serves as a guide to reagent quality. A decrease in colour yield by 4 % or more from the values shown indicates inferior or decomposing reagents; or, if acid is omitted from the hydroxyproline solutions, that microbiological decomposition of the amino acid is taking place.

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Spectrophotometric Studies on the Combination of Formaldehyde with Tetrahydropteroylglutamic Acid and Other Hydropteridines

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A previous paper (Blakley, 1959) described studies on the reaction of formaldehyde with tetrahydropteroylglutamic acid and other hydropteridines, which were undertaken to elucidate the coenzyme function of tetrahydropteroylglutamate in the synthesis of serine from glycine and formaldehyde.

It was found that formaldehyde reacted with all the hydropteridines studied, but the complexes

formed differed greatly in the extent to which they were dissociated. Formaldehyde was very firmly bound by tetrahydropteroylglutamate, the dissociation constant for the complex being at least 100 times lower than those for the complexes formed by simple hydropteridines. Since *N*⁸-formyltetrahydropteroylglutamate (leucovorin) and *N*¹⁰-formyltetrahydropteroylglutamate also formed

complexes with relatively high dissociation constants, it was concluded that tetrahydropteroylglutamate reacts reversibly with formaldehyde to form N^5N^{10} -methylenetetrahydropteroylglutamate, whereas all other hydropteridines studied (except possibly tetrahydroptericoic acid) formed hydroxymethyl derivatives. The point of attachment of the hydroxymethyl group in these derivatives was indicated by the fact that the N^5 -formyl derivative of a simple hydropteridine formed a complex with a dissociation constant considerably higher than that of the complex from the parent hydropteridine. It was concluded therefore that simple hydropteridines form highly dissociated N^5 -hydroxymethyl derivatives.

In this previous investigation the values of the dissociation constants were obtained from determinations of the free formaldehyde in equilibrium with known concentrations of hydropteridines. A limitation was placed on the accuracy of the results for tetrahydropteroylglutamate and related compounds by the fact that large blanks were obtained in the colorimetric determination of formaldehyde when these hydropteridines were present. Since only very low concentrations of formaldehyde existed in equilibrium with tetrahydropteroylglutamate, the relative error was highest in this case. As this was the reaction of primary interest, another approach seemed necessary. Spectrophotometric studies were therefore undertaken to obtain further information about the combination of formaldehyde with tetrahydropteroylglutamate and for comparative purposes the reaction of formaldehyde with other hydropteridines was also studied. Clear evidence has been obtained for the reversibility of the reaction between tetrahydropteroylglutamate and formaldehyde and a more accurate estimate obtained for the dissociation constant of the product. In addition, more data have been obtained on the reaction of formaldehyde with other pteridines and hydropteridines which shed light on the compounds formed.

EXPERIMENTAL

Materials

All pteridine derivatives were prepared as previously described (Blakley, 1959). The following additional compounds were prepared as indicated: 2-hydroxypyrimidine, Brown (1950); 2-methoxyypyrimidine, Brown & Short (1953).

Methods

Spectrophotometric readings. Manually operated instruments (Shimadzu and Unicam) were used in the original experiments and the results were checked with a Beckman DK2 ratio-recording spectrophotometer. The reference cell contained all the reagents present in the sample cell, except the pteridine or pyrimidine under study. During kinetic studies exactly the same additions of reagents were made to the reference cell as to the sample cell.

Measurement of pH. A glass electrode, with 5 mm-sodium borate to provide the reference pH, was used. Potassium phosphate buffer was prepared according to the data of Green (1933). The concentrations of phosphate and *N*-ethylmorpholine buffers given refer to anion and cation concentrations respectively.

Calculation of dissociation constants. In calculating the dissociation constants of the complexes formed by formaldehyde with various bases, the assumption was made that each complex was formed by reaction of equimolecular proportions of base and formaldehyde thus:

If the initial concentrations of base and formaldehyde, $[\text{B}]_i$ and $[\text{H} \cdot \text{CHO}]_i$ respectively, were known, then the equilibrium concentrations of these compounds, $[\text{B}]_{\text{equ}}$ and $[\text{H} \cdot \text{CHO}]_{\text{equ}}$ respectively, could be calculated from the equilibrium concentration of the complex, $[\text{complex}]_{\text{equ}}$. This follows from the fact that the decrease in concentration of base and of formaldehyde is equal to the equilibrium concentration of complex,

$$[\text{Complex}]_{\text{equ}} = [\text{B}]_i - [\text{B}]_{\text{equ}} = [\text{H} \cdot \text{CHO}]_i - [\text{H} \cdot \text{CHO}]_{\text{equ}}. \quad (2)$$

The dissociation constant, K , could then be calculated from the relationship

$$K = \frac{[\text{B}]_{\text{equ}} [\text{H} \cdot \text{CHO}]_{\text{equ}}}{[\text{Complex}]_{\text{equ}}}. \quad (3)$$

The equilibrium concentration of the complex was determined spectrophotometrically as follows. The extinction of the pure base at a concentration $[\text{B}]_i$ was determined at a wavelength where complex formation caused a large change in extinction. Let E_B be the determined extinction of the pure base at concentration $[\text{B}]_i$ at this wavelength. Base at a concentration $[\text{B}]_i$ was then equilibrated with a high concentration of formaldehyde. Assuming complete conversion of the base into complex, the extinction of the solution equalled E_C , the extinction of the complex at a concentration equal to $[\text{B}]_i$. If now base at a concentration $[\text{B}]_i$ was allowed to equilibrate with a low concentration of formaldehyde $[\text{H} \cdot \text{CHO}]_i$, the measured extinction E , due to the equilibrium mixture of base and complex, was intermediate between E_B and E_C . The proportion of complex in the mixture was given by the ratio $(E - E_B)/(E_C - E_B)$ so that the equilibrium concentration of complex is given by

$$[\text{Complex}]_{\text{equ}} = \frac{E - E_B}{E_C - E_B} [\text{B}]_i.$$

Substitution of this value in equations (2) and (3) enabled calculation of the dissociation constant. If the concentration of formaldehyde employed in the determination of E_C was not high enough, then some free base remained in the solution, leading to an error in E_C which resulted in a low value for $E_C - E_B$. On the other hand, care was necessary that the level of formaldehyde used was not so high that secondary reactions occurred leading to extraneous spectrum changes. With tetrahydropteroylglutamate the concentrations of formaldehyde used in determining E_C was 10 mm.

An alternative procedure was used in determining pK spectrophotometrically: values of the extinction at the selected wavelength were plotted against pH. From the graph the pH value was obtained at which the extinction was midway between those of the two ionic species. This pH was taken as equal to the pK .

RESULTS

4-Hydroxy-6-methyltetrahydropteridine. The spectrum of 4-hydroxy-6-methyltetrahydropteridine at pH 7.2 is shown in Fig. 1. Absorption maxima occurred at 220 and 288 $m\mu$ and a minimum of 245 $m\mu$. Although this compound was relatively stable in neutral solution the spectrum changed significantly over a period of hours. The shape of the absorption curve did not change but the extinction slowly decreased, indicating the formation of non-absorbing products.

On addition of formaldehyde to give a concentration of 25 mM the spectrum was shifted towards longer wavelengths with a slight reduction in the extinction maximum (Fig. 1). Rate studies showed that this reaction was 50% complete in about 2.5 min. so that its rate was about the same as that for the combination of tetrahydropteroylglutamic acid (tetrahydroPGA) and formaldehyde (cf. Blakley, 1959). More detailed studies showed that at high concentrations of formaldehyde the 220 $m\mu$ maximum is shifted to longer wavelengths with a simultaneous increase in the extinction maximum (Fig. 1). Moreover, the 288 $m\mu$ peak, which is

shifted to 292 $m\mu$ by 25 mM-formaldehyde, shifts back to shorter wavelength at higher formaldehyde concentrations. In addition, the absence of an isobestic point in this region of the spectrum (Fig. 1) indicates the formation of more than one product. An approximate calculation of the dissociation constant of the final product from the extinctions obtained at 235 $m\mu$ (see Methods section) indicated that the constant was about 0.2M. This is much higher than the value of 0.03M obtained for the N^5 -hydroxymethyl derivative by chemical analysis (Blakley, 1959), so that the changes in spectrum at the higher concentrations of formaldehyde corresponded to the formation of another compound. The spectrum change corresponding to the secondary reaction made it impossible to obtain a value for the dissociation constant of the N^5 -hydroxymethyl derivative by the spectrophotometric method.

 N^5 -Formyl-4-hydroxy-6-methyltetrahydropteridine

Since the spectrum changes obtained when 4-hydroxy-6-methyltetrahydropteridine was treated with high concentrations of formaldehyde appeared due to the combination of the latter at some site

Fig. 1. Absorption spectra of 4-hydroxy-6-methyltetrahydropteridine (0.04 mM) in 0.1M-potassium phosphate buffer, pH 7.2. —, No formaldehyde; ..., in the presence of 25 mM-formaldehyde; ---, in the presence of 0.15M-formaldehyde; -.-, in the presence of 5.0M-formaldehyde.

Fig. 2. Absorption spectra of N^5 -formyl-4-hydroxy-6-methyltetrahydropteridine (0.03 mM) in 0.1M-potassium phosphate buffer, pH 7.2. —, No formaldehyde; ---, in the presence of 0.15M-formaldehyde; ..., in the presence of 0.75M-formaldehyde; -.-, in the presence of 5.0M-formaldehyde.

other than N^5 of the hydropteridine, it seemed probable that N^5 -formyl-4-hydroxy-6-methyltetrahydropteridine would show spectrum changes in the presence of formaldehyde similar to the secondary change shown by the parent compound, and this was in fact found to be the case (Fig. 2). When the spectra obtained at various formaldehyde concentrations were superimposed isosbestic points were obtained at 221, 253 and 292 $m\mu$, indicating the formation of only one product. The close agreement between the dissociation constant (0.3M) calculated from the spectrum change for this compound, and that given above for the product formed by the parent 4-hydroxy-6-methyltetrahydropteridine at high formaldehyde concentrations, suggests that formaldehyde was reacting at the same site in the two molecules.

In the formyl derivative there are three possible sites for the reaction with formaldehyde; i.e. N^8 in the hydrogenated ring and N^1 and N^3 in the pyrimidine ring. The spectrum of 4-hydroxy-6-methylpteridine was investigated in an attempt to resolve this problem and was found also to undergo a shift in the presence of formaldehyde (Fig. 3), corresponding to formation of a product with dissociation constant approximately 0.3M. Pteroylglutamic acid, 2-deaminopteroylglutamic acid and N^8 -formyltetrahydropteroylglutamic acid (leucovorin)

displayed similar changes in spectra in the presence of formaldehyde at high concentrations, forming complexes with approximate dissociation constants of 0.3, 0.4 and 0.4M respectively. These results suggest that formaldehyde was reacting at a site in the pyrimidine ring in all the above-mentioned compounds.

Reaction of formaldehyde with pyrimidines. Since 4-hydroxypyrimidine exists mainly in the lactam (ketonic) form (scheme 1; I) at neutral pH (Brown, Hoerger & Mason, 1955), it seemed possible that similar tautomerism in 4-hydroxy-6-methylpteridine might allow reaction of formaldehyde at N^3 to give an N^3 -hydroxymethyl derivative (scheme 1; II). To test this hypothesis the effect of formaldehyde on 2-hydroxypyrimidine and on 2-methoxypyrimidine was investigated. At high concentrations of formaldehyde the extinction maximum of 2-hydroxypyrimidine was shifted to longer wavelengths and slightly increased (Fig. 4) in a manner exactly analogous to that described above for various 4-hydroxypteridines, but the spectrum of

Fig. 3. Absorption spectra of 4-hydroxy-6-methylpteridine (0.075 mM) in 0.1M-potassium phosphate buffer, pH 7.2. —, No formaldehyde; ---, in the presence of 0.15M-formaldehyde; - · - ·, in the presence of 5.0M-formaldehyde.

Fig. 4. Absorption spectra of 2-hydroxypyrimidine (0.1 mM) in 0.1M-potassium phosphate, pH 7.2. —, No formaldehyde; - · - ·, in the presence of 5.0M-formaldehyde.

2-methoxypyrimidine at pH 7.2 ($\lambda_{\max}$, 264 m μ , $\epsilon_{\max}$, 4700) was unchanged by 5M-formaldehyde. The spectrum change for 2-hydroxypyrimidine corresponded to a product with dissociation constant of 0.4M. These results strongly suggest formation of N^3 -hydroxymethyl-2-oxypyrimidine from 2-hydroxypyrimidine by the action of formaldehyde in high concentrations.

Spectra of tetrahydroquinoxaline and 2-chloro-4-methyl- N^8 -benzyltetrahydropteridine. In these two compounds there is no possibility of a reaction of formaldehyde with pyrimidine ring nitrogen. Tetrahydroquinoxaline has a benzene ring instead of a pyrimidine ring and in 2-chloro-4-methyl- N^8 -benzyltetrahydropteridine no hydrogen is able to migrate to N^3 . With the former compound the addition of 25 mM-formaldehyde to the solution at pH 7.2 caused the spectrum to be displaced towards longer wavelength, together with a decrease in the maximum extinction. At high formaldehyde concentrations, however, the maximum extinction was greater than for the untreated compound as well as being displaced towards longer wavelengths (Fig. 5). This suggests the formation of two derivatives, presumably N^5 -hydroxymethyl and N^5N^8 -bis-(hydroxymethyl). The dissociation constant calculated from the extinction at 230 m μ at various formaldehyde concentrations was about 0.08M, which is about ten times higher than that previously

assigned to the N^5 -hydroxymethyl compound (Blakley, 1959) and probably refers to the dissociation of the N^8 -hydroxymethyl group.

Formaldehyde decreased the extinction maximum of 2-chloro-4-methyl- N^8 -benzyltetrahydropteridine as shown in Fig. 6. From the extinction figures at 310 m μ a value of 0.5M was obtained for the dissociation constant, which is rather high compared with the value (0.29M) previously obtained (Blakley, 1959).

Spectrophotometric determination of pK of 4-hydroxy-6-methyltetrahydropteridine. If the N^5 -amine grouping of 4-hydroxy-6-methyltetrahydropteridine has a basic pK near the pH at which measurements of formaldehyde-binding have been made (pH 7.2), the measured binding of formaldehyde would be unduly low because of the existence of some of the hydropteridine in the protonated form which may not combine with formaldehyde. To investigate this possibility a spectroscopic investigation of the pK of this hydropteridine was made. Titration had already shown that the pK of the 4-hydroxy group was 10.2 (Blakley, 1959). The spectrum did not change significantly between this pH and pH 5.0; but below pH 5.0 the absorption maximum shifted from 288 to 260 m μ . By plotting

Fig. 5. Absorption spectra of tetrahydroquinoxaline (0.025 mm) in 0.1M-potassium phosphate, pH 7.2. —, No formaldehyde; ---, in the presence of 25 mM-formaldehyde; —·—, in the presence of 5.0M-formaldehyde.

Fig. 6. Absorption spectra of N^8 -benzyl-2-chloro-4-methyl-tetrahydropteridine (0.075 mm) in 0.1M-potassium phosphate, pH 7.2. —, No formaldehyde; ---, in the presence of 0.75M-formaldehyde; —·—, in the presence of 5.0M-formaldehyde.

the extinction at 260 $m\mu$ and at 290 $m\mu$ against pH (see Methods section), a value of 4.1 was obtained for the pK . Even if this pK were attributed to the N^5 -amine group (rather than to the N^8 -amine group to which it more probably belongs; see Discussion), protonation at pH 7.0 would be negligible.

Reaction of formaldehyde with tetrahydropteroyl-glutamic acid

In order to study the effect of formaldehyde on tetrahydroPGA, the instability of the latter had to be overcome. The spectrum of a solution of tetrahydroPGA at neutral pH changed rapidly with a shift of the extinction maximum from 298 to about 274 $m\mu$. This change, which indicated oxidative degradation of tetrahydroPGA, could be retarded by the addition of various stabilizing substances to the solution. Chelating agents such as ethylenediaminetetra-acetic acid (EDTA) and 8-hydroxyquinoline-5-sulphonic acid caused some stabilization, as seen in Fig. 7, but reducing agents such as thiols were rather more effective. By far the most efficient stabilizing compound was 2:3-dimercaptopropan-1-ol (BAL) which, in mM concentration, prevented oxidative degradation of tetrahydroPGA at neutral pH for several hours. In the presence of 0.5 mM-BAL only slight oxidation occurred over a period of 25 min. (Fig. 7).

The spectrum change shown by neutral solutions of tetrahydroPGA was also retarded by the presence of formaldehyde. Addition of formaldehyde to give a final concentration of 0.1 mM caused a significant decrease in the rate at which 0.03 mM-tetrahydro-

PGA was degraded, and in the presence of mM-formaldehyde degradation was almost completely arrested (Fig. 8).

This stabilizing effect of formaldehyde on tetrahydroPGA is interpreted as due to combination of formaldehyde with tetrahydroPGA to form a product which is not, or is very slowly, attacked by oxygen. Since oxidative degradation eventually proceeds to completion even when a large excess of formaldehyde is present, the formation of the oxygen-stable complex must be a reversible reaction. To obtain further data on tetrahydroPGA-formaldehyde combination the reaction has been studied in the presence of mM-BAL. At pH 7.2 addition of formaldehyde to such a stabilized solution of tetrahydroPGA causes an increase in the maximum extinction and a slight shift to shorter wavelength (Fig. 9). The extent of the spectrum shift varies with formaldehyde concentration, indicating a reversible reaction, but is complete in 10 mM-formaldehyde. In the absence of formaldehyde λ_{max} is 298 $m\mu$ and ϵ_{max} is 28 400, whereas in the presence of 10 mM-formaldehyde λ_{max} is 294 $m\mu$ and ϵ_{max} is 32 000. Isosbestic points may be seen to occur at 249 and 270 $m\mu$, an indication that only a single product is formed in significant amounts. In the presence of 10 mM-formaldehyde the same spectrum is obtained whether BAL is present or absent.

Fig. 7. Rate of change of the spectrum of tetrahydroPGA (0.03 mM) in 0.1M-potassium phosphate buffer, pH 7.2. A, In the presence of mM-BAL; B, in the presence of 0.5 mM-BAL; C, in the presence of 2 mM-2-mercaptoacetic acid; D, in the presence of 5 mM-2-mercaptoethanol; E, in the presence of mM-2-mercaptoethanol; F, no addition; G, in the presence of 0.2 mM-8-hydroxyquinoline-5-sulphonic acid.

Fig. 8. Rate of change of the spectrum of tetrahydroPGA (0.03 mM) in 0.1M-potassium phosphate buffer, pH 7.2. A, in the presence of 10 mM-formaldehyde; B, in the presence of mM-BAL and 10 mM-formaldehyde; C, in the presence of 0.1 mM-formaldehyde; D, no formaldehyde.

The rate of the spectrum change accompanying reaction of formaldehyde with tetrahydroPGA is shown in Fig. 10. In the experiment in which both reactants were at a concentration of 0.4 mM the reaction was 40 % complete in the first minute. This reaction rate is similar to that observed by measuring formaldehyde disappearance from the reaction mixture (Blakley, 1959).

At alkaline pH it was more difficult to observe the formaldehyde-tetrahydroPGA reaction spectrophotometrically, although previous evidence had indicated that the reaction proceeded even at pH 13.0 (Blakley, 1959). TetrahydroPGA, in the presence of mM-BAL, showed a considerable spectrum change over a period of 30 min. due to degradation, $\lambda_{\max}$ changing from 292 to 285 $m\mu$, which made observations on the formaldehyde reaction difficult. Owing to the absorption by the BAL, readings could not be made below 270 $m\mu$. The addition of formaldehyde (final concentration 10 mM) to a freshly prepared solution of 0.03 mM-tetrahydro-

PGA in mM-BAL and 0.01N-sodium hydroxide resulted in only a very slight increase in the maximum extinction, together with a broadening of the absorption peak (Fig. 11). Observations on the formaldehyde-tetrahydroPGA reaction at low pH were extremely difficult because sulphhydryl reagents no longer prevented oxidative degradation of tetrahydroPGA under these conditions, and other reducing agents which were tried were unsatisfactory.

Further evidence for the reversibility of the tetrahydroPGA-formaldehyde reaction. Data have been presented above which indicate that the reaction of tetrahydroPGA and formaldehyde is reversible. As a further test of reversibility the reaction between tetrahydroPGA (0.05 mM) and formaldehyde (0.15 mM) in the presence of mM-BAL was allowed to come to equilibrium, as shown by the recording of the extinction at 300 $m\mu$ (Fig. 12). A small volume of bisulphite solution was then added to both the reference and sample cells and the mixture rapidly stirred. The extinction at 300 $m\mu$ rapidly fell until a new equilibrium level had been reached. A final concentration of 0.33 mM-bisulphite lowered the extinction at 300 $m\mu$ to a value approaching that for tetrahydroPGA. The absorption spectrum was similar to that of 0.05 mM-tetrahydroPGA. Addition of this amount of bisulphite to tetrahydroPGA in the presence of mM-BAL caused no change in spectrum.

Fig. 9. Absorption spectra of tetrahydroPGA (0.03 mM) in 0.1M-potassium phosphate buffer, pH 7.2, containing mM-BAL. —, No formaldehyde; ---, in the presence of 0.067 mM-formaldehyde; — · —, in the presence of 10 mM-formaldehyde.

Fig. 10. Rate of reaction of tetrahydroPGA with formaldehyde. Curves A, B and C: rate of change of extinction, at 20° with 1 cm. light path, of 0.05 mM-tetrahydroPGA in 0.1M-potassium phosphate buffer, pH 7.2, containing mM-BAL. Formaldehyde was added at point E to give the following final concentrations: curve A, 0.05 mM-formaldehyde; curve B, 0.1 mM-formaldehyde; curve C, 0.2 mM-formaldehyde. Curve D: rate of change of extinction at 20°, with 0.1 cm. light path, of 0.4 mM-tetrahydroPGA in 0.1M-potassium phosphate buffer, pH 7.2, containing mM-BAL. At point F formaldehyde added to give a final concentration of 0.4 mM. Extinction was corrected for dilution by added formaldehyde.

Fig. 11. Absorption spectra of tetrahydroPGA (0.03 mm) in 0.01 N-NaOH containing mM-BAL. —, No formaldehyde; ---, 10 mM-formaldehyde.

Fig. 12. Effect of formaldehyde and subsequent addition of bisulphite on the spectrum of tetrahydroPGA. TetrahydroPGA (0.05 mm) in 0.1 M-potassium phosphate buffer, pH 7.2, containing mM-BAL was treated at 20° with formaldehyde (final concn. 0.15 mm) at point A. At point B bisulphite (final concn. 0.33 mm) was added. Curve is corrected for dilution effect of additions.

The value of the dissociation constant, defined by

$$\frac{[\text{TetrahydroPGA}][\text{H}\cdot\text{CHO}]}{[\text{Complex}]}$$

for the product of the reaction between tetrahydroPGA and formaldehyde was obtained from series of determinations of extinction at the wavelengths 295, 298 or 300 mμ when 0.05 mm-tetrahydroPGA was allowed to equilibrate with formaldehyde at concentrations between 0.05 and 0.15 mm (see Methods section). In an early experiment, with a manual spectrophotometer, the value of the dissociation constant obtained was $4.79 \pm 0.30 \times 10^{-5} \text{ M}$ from reaction in the presence of mM-BAL and potassium phosphate buffer, pH 7.2 (0.1 M), at 20°. Seven more recent determinations made with the Beckman recording spectrophotometer have given a value of $3.12 \pm 0.19 \times 10^{-5} \text{ M}$. The same result is obtained in the presence of 0.15 mM-BAL.

Stability of the product of the reaction of formaldehyde and tetrahydroPGA. At equilibrium 0.1 mM-formaldehyde will convert 0.03 mM-tetrahydroPGA almost entirely into the complex, which is believed to be N^5N^{10} -methylenetetrahydropteroylglutamic acid. The rate of subsequent change of the spectrum will therefore indicate the stability of methylenetetrahydropteroylglutamic acid. It may be seen from Fig. 13 that in 0.1 M-N-ethylmorpholine buffer, pH 7.2, or in 0.01 M-potassium phosphate buffer, pH 7.2, the decomposition of the tetrahydroPGA occurred so rapidly that it was virtually com-

Fig. 13. Rate of change of the spectrum of tetrahydroPGA (0.03 mm) at 20° in the presence of 0.1 mM-formaldehyde and the following additional components, added at zero time: curve A, 0.1 M-N-ethylmorpholine, pH 7.2, and 0.1 mM-8-hydroxyquinoline-5-sulphonic acid; curve B, 0.01 N-NaOH; curve C, 0.1 M-N-ethylmorpholine, pH 7.2, and 0.01 M-EDTA, pH 7.2; curve D, 0.1 M-potassium phosphate, pH 7.2; curve E, 0.1 M-N-ethylmorpholine, pH 7.2; curve F, 0.01 M-potassium phosphate, pH 7.2.

plete before combination with formaldehyde could occur. In the presence of 0.01 M-EDTA, or 0.1 mM-8-hydroxyquinoline-5-sulphonate, the rate of tetrahydroPGA decomposition was slowed sufficiently to allow formation of methylenetetrahydropteroylglutamic acid. The subsequent breakdown of this compound was considerably slower than that of tetrahydroPGA under the same conditions (cf. Fig. 7). Spectrum change was also retarded in the presence of higher concentrations of phosphate (0.1 M), probably due also to the binding of heavy metals. In 0.01 N-sodium hydroxide the spectrum change was small (Fig. 13) and a similar result not shown in the figure was obtained in 0.1 M-N-ethylmorpholine, pH 9.2. As with free tetrahydroPGA, this is more probably due to the smaller spectrum changes that accompany oxidative degradation at high pH rather than to increased stability at high pH, as was at first thought (Blakley, 1958).

Fig. 14. Absorption spectra of dihydroPGA (0.03 mM) in 0.1 M-potassium phosphate, pH 7.2, containing mM-BAL. —, No formaldehyde; ---, 10 mM-formaldehyde; — · —, 0.12 M-formaldehyde.

Reaction of formaldehyde with dihydropteroylglutamic acid and N¹⁰-formyltetrahydropteroylglutamic acid. Dihydropteroylglutamic acid, prepared by the method of Futterman (1957), or by catalytic hydrogenation (Blakley, 1957), showed a bathochromic shift and increase in the extinction maximum (Fig. 14) in the presence of formaldehyde with the formation of a product having a dissociation constant of about 0.01 M, which agrees well with that previously obtained by another method (Blakley, 1959). If the product is the N⁵-hydroxymethyl derivative, the extinction increase associated with its formation constituted an exception to the general rule of decreased extinction accompanying N⁵-hydroxymethyl group formation.

N¹⁰-Formyltetrahydropteroylglutamic acid showed only a slight decrease in the extinction maximum (at 260 mμ) at concentrations of formaldehyde from 0.01 to 0.12 M (Fig. 15) but, in addition, there occurred a change in absorption around the inflexion at 300 mμ. The value obtained for the dissociation constant of the product from extinction changes at 310 mμ where N³-hydroxymethyl formation did not interfere was 16 mM, in good agreement with the previous value (Blakley, 1959).

Fig. 15. Absorption spectra of N¹⁰-formyltetrahydropteroylglutamic acid (0.03 mM) in 0.1 M-potassium phosphate buffer, pH 7.2, containing mM-BAL. —, No formaldehyde; ---, 10 mM-formaldehyde; — · —, 0.12 M-formaldehyde.

DISCUSSION

In a previous paper (Blakley, 1959) data were reported which indicated that formaldehyde combined with 4-hydroxy-6-methyltetrahydropteridine to form a complex with dissociation constant of 0.03M. Since *N*⁵-formyl-4-hydroxy-6-methyltetrahydropteridine was found to bind very small amounts of formaldehyde, the dissociation constant of the product being about 0.4M, the compound formed by 4-hydroxy-6-methyltetrahydropteridine and formaldehyde was considered to be *N*⁵-hydroxymethyl-4-hydroxy-6-methyltetrahydropteridine.

It has now been shown that the spectra of solutions of hydropteridines at pH 7.2 are shifted in the presence of formaldehyde. The evidence indicates the formation of two compounds by the action of formaldehyde on 4-hydroxy-6-methylhydropteridine in the following manner:

The formation of complex 1, observable in 25 mm-formaldehyde, caused a decrease in absorption as well as a bathochromic shift of the spectrum. Complex 2 was formed at concentrations of formaldehyde above 0.1M, as indicated by increased absorption in addition to a further bathochromic shift. The dissociation constant of the second product was calculated as approximately 0.2M.

The high dissociation constant of complex 2 indicated that it was not identical with the complex studied by the chemical method, i.e. *N*⁵-hydroxymethyl-4-hydroxy-6-methyltetrahydropteridine. Complex 1 is therefore presumed identical with the last-named compound. Confirmation that complex 2 is not the *N*⁵-hydroxymethyl derivative was found in the fact that *N*⁵-formyl-4-hydroxy-6-methyltetrahydropteridine also showed increased absorption and a bathochromic shift at high formaldehyde concentrations, and in this case a single product was formed having a dissociation constant of 0.3M. Chemical analysis previously gave a value of approximately 0.4M. Evidence has been presented that this product is the *N*³-hydroxymethyl derivative, and the similarity of the spectrum changes suggests that complex 2 of 4-hydroxy-6-methyltetrahydropteridine is the *N*³*N*⁵-bishydroxymethyl derivative.

It appears from the data on 4-hydroxy-6-methyltetrahydropteridine that formation of the *N*⁵-hydroxymethyl derivative results in decreased absorption. This is also the case when low concentrations of formaldehyde react with *N*¹⁰-formyltetrahydropteroylglutamic acid and with 2-chloro-4-methyl-*N*⁸-benzyltetrahydropteridine. In the last compound only *N*⁵ is available for reaction with formaldehyde, so that the spectrum change when

the solution of this compound is treated with formaldehyde must be due to formation of the *N*⁵-hydroxymethyl derivative.

Tetrahydroquinoxaline, like 4-hydroxy-6-methyltetrahydropteridine, showed a two-stage spectrum change in the presence of formaldehyde. This is interpreted as due to formation firstly of *N*⁵-hydroxymethyltetrahydroquinoxaline and secondly, at high formaldehyde concentration, of *N*⁵*N*⁸-bis(hydroxymethyl)tetrahydroquinoxaline. The spectrophotometric data gave a value of 0.08M for the dissociation constant of the last-named compound. Chemical analysis gave a value of 0.01M, which presumably applies to the *N*⁵-hydroxymethyltetrahydroquinoxaline.

Although the spectrophotometric results thus suggest formation of the bishydroxymethyl derivative of tetrahydroquinoxaline, no definite evidence has been obtained that formaldehyde reacts with *N*⁸ of hydropteridines. In hydropteridines the *N*⁸-amine group is probably affected by amidine-like resonance which confers much higher basic strength to the *N*⁸-amine group than is possessed by the *N*⁵-amine group, and which might cause a difference in the ability of these groups to react with formaldehyde. The evidence for the difference in basic strength of the *N*⁵- and *N*⁸-amine groups of hydropteridines is as follows. Data obtained for 4-hydroxy-6-methyltetrahydropteridine (scheme 2; III) indicated an acidic p*K* of 10.2 and one basic p*K* of 4.1; the other basic p*K* is lower than 4.1. The structurally related 4,5-diamino-6-hydroxypyrimidine (scheme 2; IV) has an acidic p*K* of 9.86 and basic p*K* values of 1.34 and 3.57. To decide which of the basic p*K* values should be assigned to the 5-amino group, reference is made to the fact that this compound acetylates readily on the 5-amino group but not on the 4-amino group, and its basic p*K* values may be compared with those for 4-aminopyrimidine (5.7) and for 5-aminopyrimidine (2.6). This suggests that in 4,5-diamino-6-hydroxypyrimidine 3.57 is the p*K* of the 4-amino group and 1.34 that of the 5-amino group, as shown in scheme 2. By analogy, in 4-hydroxy-6-methyltetrahydropteridine, 4.1 is probably the p*K* of the

Scheme 2

Table 1. *Summary of approximate dissociation constants for the compounds formed by the reaction of various bases with formaldehyde*

Base	Derivative formed	$10^3 \times$ Dissociation constant (M)	Method employed
Tetrahydroquinoxaline	<i>N</i> ⁵ -Hydroxymethyl	10	Chemical
	<i>N</i> ⁵ <i>N</i> ⁸ -Bishydroxymethyl	80	Spectrophotometric
2-Hydroxypyrimidine	<i>N</i> ³ -Hydroxymethyl	400	Spectrophotometric
4-Hydroxy-6-methylpteridine	<i>N</i> ³ -Hydroxymethyl	300	Spectrophotometric
4-Hydroxy-6-methyltetrahydropteridine	<i>N</i> ⁵ -Hydroxymethyl	30	Chemical
	<i>N</i> ⁵ <i>N</i> ⁸ -Bishydroxymethyl	200	Spectrophotometric
<i>N</i> ⁵ -Formyl-4-hydroxy-6-methyltetrahydropteridine	<i>N</i> ³ -Hydroxymethyl	300	Spectrophotometric
		400	Chemical
2-Chloro-4-methyl- <i>N</i> ⁸ -benzyltetrahydropteridine	<i>N</i> ⁵ -Hydroxymethyl	290	Chemical
		500	Spectrophotometric
Pteroylglutamic acid	<i>N</i> ³ -Hydroxymethyl	300	Spectrophotometric
TetrahydroPGA	<i>N</i> ⁵ <i>N</i> ¹⁰ -Methylene	0.03	Spectrophotometric
<i>N</i> ¹⁰ -Formyltetrahydropteroylglutamic acid	<i>N</i> ⁵ -Hydroxymethyl	16	Spectrophotometric
		12	Chemical

*N*⁸-amine group and the p*K* of the *N*⁵-amine group is probably about 1.4, as represented in scheme 2 (the figures in parentheses referring to p*K* values for adjacent groupings). This is a more likely interpretation than that previously given (Blakley, 1958).

The dissociation constants obtained for *N*⁵-hydroxymethyl and *N*³-hydroxymethyl derivatives of hydropteridines were all relatively high (0.01–0.5 M), so that at concentrations of formaldehyde below 10 mM these derivatives would not be formed in significant amounts. Since spectrophotometric studies indicated that 0.05 mM-tetrahydroPGA reacted with formaldehyde at concentrations as low as 0.05 mM and that the reaction was virtually complete in mM-formaldehyde, no interference arose from reaction of formaldehyde at *N*³ or *N*⁸ in studies with tetrahydroPGA. The same value of the dissociation constant was obtained at formaldehyde concentrations ranging from 0.05 to 0.15 M and the spectrum change was reversed by addition of bisulphite to the solution. Together with previous data the spectrophotometric results clearly indicate a reversible reaction which occurs rapidly at neutral pH in the absence of any enzyme.

These results were obtained with mM-BAL used to stabilize the tetrahydroPGA. Since sulphhydryl compounds are known to react with formaldehyde, the possibility that the BAL had some influence on the reaction of tetrahydroPGA and formaldehyde must be considered. The presence of BAL did not appear to alter the product formed by reaction of tetrahydroPGA with formaldehyde, since the spectrum obtained in the presence of 10 mM-formaldehyde was the same whether BAL was present or absent. The possibility remains that the value obtained for the dissociation constant of the product is in error, due to reaction of some of the formaldehyde with BAL. When BAL was present

at a concentration of 5 mM or higher, high values were certainly obtained for the dissociation constant. Since the value obtained in the presence of 0.5 mM-BAL was the same as in the presence of mM-BAL, interference by BAL at these levels is at any rate slight, if not absent. Assuming that the value of the dissociation constant is not greatly in error, the very low value (3.1×10^{-5} M) indicates clearly that it is a compound of a type different from the hydroxymethyl derivatives formed by simple hydropteridines; it is presumably *N*⁵*N*¹⁰-methylene-tetrahydropteroylglutamic acid. The difference between the value obtained for tetrahydroPGA and those obtained with other bases is readily seen from the summary of data in Table 1.

Since a small amount of tetrahydroPGA is always in equilibrium with methylenetetrahydropteroylglutamic acid, the stability of the latter would be expected to be not much greater than that of tetrahydroPGA, and this was found to be the case. Cation-chelating agents, such as EDTA and 8-hydroxyquinoline-5-sulphonic acid, diminished the rate of breakdown and even phosphate at high concentrations (0.1 M) had a similar effect. As with tetrahydroPGA, breakdown occurred at all values of pH, the only difference at high pH being that the spectrum change accompanying breakdown was smaller.

SUMMARY

1. In the presence of increasing concentrations of formaldehyde, the spectrum of 4-hydroxy-6-methyltetrahydropteridine showed a progressive bathochromic shift. At low formaldehyde concentrations (25 mM) the absorption was diminished (believed to correspond to the formation of the *N*⁵-hydroxymethyl derivative), and at higher concentrations of formaldehyde showed an increase.

2. The spectrum shift and increase in the extinction maximum in the presence of high concentrations of formaldehyde was also shown by *N*⁵-formyl-4-hydroxy-6-methyltetrahydropteridine, 4-hydroxy-6-methylpteridine, pteroylglutamic acid and 2-deaminopteroylglutamic acid. The dissociation constants calculated from these spectrum changes ranged from 0.2 to 0.4 M.

3. These data are interpreted as indicating that 4-hydroxypteridines combine with formaldehyde when it is present at a high concentration to form *N*³-hydroxymethyl derivatives. This is confirmed by the fact that 2-hydroxypyrimidine behaves similarly but the spectrum of 2-methoxypyrimidine is unaffected by formaldehyde.

4. At neutral pH solutions of tetrahydropteroylglutamic acid rapidly decomposed as shown by spectrum changes. Chelating agents, thiols (especially 2:3-dimercaptopropan-1-ol) and formaldehyde retarded this oxidative decomposition.

5. In the presence of 2:3-dimercaptopropan-1-ol the reaction of tetrahydropteroylglutamic acid with formaldehyde resulted in an increase in the extinction maximum from 28 400 to 32 000 and shift in the wavelength of the absorption maximum from 298 to 294 mμ. The change was reversed by addition of bisulphite.

6. The dissociation constant for the product of this reaction was calculated to be $3.1 \pm 0.19 \times 10^{-5}$ M.

7. The product of the reaction, believed to be *N*⁵*N*¹⁰-methylenetetrahydropteroylglutamic acid, is also subject to oxidative decomposition at all pH values. Chelating agents retard this decomposition.

8. The changes produced by formaldehyde in the spectra of a number of other hydropteridines are also discussed.

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Nucleoproteins of White Clover

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'Microsomal' ribonucleoproteins have been isolated from several sources, such as liver cells (Petermann & Hamilton, 1957), yeast cells (Chao & Schachman, 1956), pea shoots (Ts'o, Bonner & Vinograd, 1956) and *Escherichia coli* (Tissières & Watson, 1958). Preparations from leaf cells of higher plants have not been reported, although some earlier work has indicated that high-molecular-weight ribonucleoproteins are in fact present. Thus Pirie (1950, 1957) described the preparation from tobacco leaves of a nucleoprotein fraction which was sedimentable by centrifuging at 80 000g for 30 min. With clover the bulk of the ribonucleic acid of leaf extract could be sedimented at 100 000g in 40 min., and, when redissolved, this sediment was shown in the analytical ultracentrifuge to contain a component of sedimentation coefficient 65 s (Lyttleton, 1956).

The studies which have been made on the 'microsomal' ribonucleoprotein particles from the various sources mentioned above have indicated that these

particles are stable in solution only within a limited range of conditions, and it is probable that the lack of success in earlier attempts to isolate high-molecular-weight ribonucleoprotein from clover (Lyttleton, 1956) was due to a failure to recognize this difficulty.

With the knowledge now available of the conditions necessary to prevent degradation of the particles, their isolation has been re-investigated. This paper describes the successful isolation of 'microsomal' ribonucleoproteins from clover leaf. It gives details of their physical and chemical properties, which are shown in many respects to be similar to those of ribonucleoproteins isolated from other sources.

MATERIALS AND METHODS

Plant material. White clover (*Trifolium repens* L.) was grown in boxes in glasshouses. Three or four crops of mature leaves were taken before the plants were discarded for new