Dihydrofolic Acid Reductase of Calf Thymus*

RAVINDRA NATH AND DAVID M. GREENBERG

From the Department of Biochemistry, University of California School of Medicine, San Francisco, California

Received January 19, 1962

A dihydrofolic acid reductase from calf thymus gland that preferentially utilizes reduced diphosphopyridine nucleotide has been purified about 100-fold. The maximum activity of the enzyme is at *p*H 7.6 with DPNH and at *p*H 5.4 with TPNH. The enzyme can catalyze the reduction of other dihydropteridines to tetrahydropteridines. It is less than one tenth as active in the reduction of pteridines, *e.g.*, folic acid. Like the corresponding liver enzyme, the thymus enzyme has been shown to require sulfhydryl groups for activity. Evidence has been obtained that double-bond tautomerism of the dihydropteridines may play a significant role in the activity of the dihydrofolic acid reductases of different origin. The dihydrofolate formed by an enzyme from *Clostridium sticklandii* is a poorer substrate for liver dihydrofolate reductase than is the chemically prepared compound, and the two are reduced at about the same rate by thymus dihydrofolate reductase. The problem of double-bond tautomerism of the dihydropteridines in relation to enzyme activity and the need for establishing unequivocally the double-bond positions of the different tautomers is discussed.

In addition to their transfer functions in the enzymatic reactions of formate and formaldehyde, hydrogenated pteridines also serve as the coenzymes of certain enzymatic oxidation-reduction systems. This possibility was first suggested by O'Dell *et al.* (1947), and it has now been demonstrated for the biosynthesis of methyl groups from formaldehyde in the formation of thymine-methyl (Greenberg *et al.*, 1961; McDougall and Blakley, 1960; Wahba and Friedkin, 1961) and of methionine-methyl (Hatch *et al.*, 1961).

In methyl group synthesis, a tetrahydropteroyl derivative supplies two hydrogens for reducing "activated" formaldehyde to methyl (Greenberg *et al.*, 1961; McDougall and Blakley, 1960; Wahba and Friedkin, 1961). For the system to keep operating, the dihydropteryl derivative formed has to be reconverted to the tetrahydropteroyl coenzyme. This latter reaction is catalyzed by dihydrofolic acid reductase, which has been discovered in natural materials that catalyze the reduction of dihydropteroyl compounds, with either TPNH or DPNH[1] as the hydrogen donors.

Dihydrofolic acid reductase has been isolated from chicken liver (Futterman, 1957; Osborn and Huennekens, 1958; Zakrzewski, 1960), sheep liver (Peters and Greenberg, 1958a,b), and bacterial sources (Blakley and McDougall, 1961). In the enzyme from these sources, TPNH is a more effective hydrogen donor than DPNH. We have isolated a dihydrofolic acid reductase from calf thymus gland in which the exact opposite is true in the physiologic *p*H range (Nath and Greenberg, 1961).

A study and comparison of the properties of the dihydrofolic acid reductase from calf thymus with that from other sources, particularly liver, is reported here.

MATERIAL AND METHODS

Dihydrofolic acid was prepared by the reduction of folic acid by the method of Futterman (1957) and by the recently described procedure of Blakley (1960). The purity of the product was tested both spectrophotometrically and by paper chromatography; the latter was carried out in 0.1 M K_2HPO_4 under nitrogen according to the procedure of Blakley (1957). Dimethylpteridine and the various pteroyl compounds listed in Tables III and IV were gifts from the Lederle Laboratories Division of the American Cyanamid Company

Enzyme Assay.—Enzyme activity was estimated from the change in absorbancy at 340 mμ measured in a Beckman DU spectrophotometer 5, 15, 30, and 60 minutes after the addition of DPNH or TPNH to the reaction mixture to initiate the reaction. Incubations were performed under nitrogen at 37° in a separate constant-temperature bath. The tubes were quickly withdrawn and read in the Beckman spectrophotometer at the given time intervals. The reaction media in a total volume of 3 ml were composed of folate-H_2 (50–100 μM) (added in 1-ml volume), purified enzyme protein[2] (0.67–1 g/liter) (added

* Aided by research grants from the National Science Foundation (G-12895), the National Cancer Institute (CY 3175), and the National Heart Institute (H 3074), National Institutes of Health.

[1] Abbreviations used are: folate-H_2, dihydrofolic acid; folate-H_4, tetrahydrofolic acid; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DNA, deoxyribose nucleic acid.

[2] In most of the experiments enzyme from acetone fractionation was used (specific activity, 60–85).

in 0.2-ml volume), DPNH or TPNH (67–134 μM) (added in 0.02-ml volume), and Veronal buffer (0.05 M, *p*H 7.6) to adjust to 3 ml. The particular micromolar concentrations of reagents employed in the different experiments are indicated in the tables and figures. Blank runs were performed in the absence of dihydropteridines to correct for DPNH oxidase and in the absence of enzyme to estimate auto-oxidation. After the initial steps in the purification there was little indication of DPNH oxidase activity. The blank corrections amounted to between 5 and 10% of the over-all change in absorbancy.

Protein was determined by the biuret reaction in crude preparation and by absorption in the ultraviolet region in purified preparations (Layne, 1957).

Enzyme Unit.—One unit of enzyme is defined as the quantity that produces a change in absorbancy at 340 mμ of 0.001 per minute[3] under the conditions of the assay. The specific activity is defined as the number of enzyme units per mg protein.

Enzyme Purification

All manipulations in the purification were performed in a cold room at a temperature of about 3° ± 1°.

Calf thymus (fresh or thawed from frozen storage) was sliced into small pieces and homogenized with 4 volumes of 0.05 M Veronal buffer, *p*H 7.6,[4] for $^1/_2$ minute at low speed and 1 minute at high speed in a Waring Blendor. The homogenate was centrifuged and the supernatant fluid collected for further purification.

The homogenate was assayed for activity and then fractionated with successive amounts of solid ammonium sulfate, introduced slowly with stirring. The enzyme activity occurred mainly in the fraction precipitated at 50–75% saturation; this precipitate was dissolved in 0.05 M Veronal buffer, *p*H 7.6, and dialyzed overnight against two changes of the buffer.

The next step was precipitation of nucleic acids with protamine sulfate. A 10% solution of protamine sulfate (usually 2–4 ml per liter of homogenate) was added dropwise with stirring until no more precipitate formed. The precipitate was removed by centrifugation and the supernatant liquid saved for further purification.

Negative adsorption with calcium phosphate gel (36 mg/ml, prepared by the method of Keilin and Hartree, 1938) was performed by adding the gel suspension (2 mg/mg protein) slowly with stirring and then continuing the stirring for a total of 30 minutes. The gel was separated centrifugally and the supernatant liquid saved. Very little activity was detected in the calcium phosphate gel on elution. The supernatant liquid containing the enzyme was refractionated with ammonium sulfate as above and again dialyzed against Veronal buffer in preparation for acetone fractionation.

For acetone fractionation, the enzyme solution was chilled to 0° and the redistilled acetone to −15°. The cold acetone was introduced slowly with stirring, and three different precipitated protein fractions were collected, namely, at 0–20, 20–35, and 35–50 volume % of acetone. The enzyme was found to be confined to the first two acetone fractions, with maximum activity occurring in the 20–35% fraction.

To a solution of the 20–35% acetone fraction in the veronal buffer, a suspension of Alumina Cγ (18 mg/ml) was introduced with stirring to a concentration of 1 mg gel per mg protein. The stirring was continued for 30–40 minutes. The alumina gel was collected by centrifugation and resuspended in 0.05 M phosphate buffer, *p*H 7.6. The suspension was stirred for 30 minutes and again centrifuged.

The eluate contained most of the enzyme activity and was saved.

The purification achieved and the recovery of the enzyme in the isolation procedure are given in Table I. It is to be noted that the degree of purification is about 100 and the over-all recovery 16%. The present enzyme preparation is somewhat more active than the chicken liver dihydrofolate reductase of Osborn and Huennekens (1958) and about 50 times as active as the reductase preparation from *Streptococcus faecalis* R (Blakley and McDougall, 1961). The purified enzyme was shown to be free of thymidylate synthetase and phosphatase (nucleotidase) activity.

Other methods of purification, such as fractionation on DEAE-cellulose, charcoal treatment, and heat treatment, were not effective. Heating caused marked losses of the enzyme; there was an 80% loss at 55° and a complete loss at 60° on 5 minutes' exposure.

Properties of Purified Enzyme

Effect of Enzyme Concentration and Time.—The proportionality between reaction velocity and enzyme concentration is shown in Figure 1. Up to a concentration of 10 mg per ml of enzyme protein the reaction rate is linear. The rate is also linear with respect to time for incubations up to $^1/_4$ to 1 hours.

Effect of pH.—The *p*H-activity curves of the enzyme with DPNH and TPNH as hydrogen donors are shown in Figure 2. DPNH is active in the physiologic *p*H region, with a sharp optimum peak at *p*H 7.6. In acid solution, TPNH increases in effectiveness and DPNH declines.

Substrate Specificity.—The dihydrofolate reduc-

[3] This is equivalent to 88 mμmoles of DPNH oxidized per minute. The factor is based on ϵ_M of DPNH at *p*H 7.4 of 6.22×10^3 and a contribution of absorbancy of DPNH in reduction of folate-H_2 of 55% (Guest and Woods, 1962).

[4] In thymus glands from very young calves, containing large amounts of DNA, the enzyme was extracted with a 0.15 M buffer mixture composed of 0.04 M Veronal buffer and 0.11 M NaCl.

TABLE I
PURIFICATION OF DIHYDROFOLIC ACID REDUCTASE OF CALF THYMUS

	Vol. (ml)	Total Enzyme Units (thousands/l)	Protein (mg/ml)	Specific Activity[a]	Recovery (%)
Crude homogenate	1650	95	10	3	100
50–75% $(NH_4)_2SO_4$	125	40	25	13	80
Protamine-SO_4	135	37	14	20	75
Ca phosphate gel[b]	240	29	3	40	59
Second $(NH_4)_2SO_4$ fractionation	40	26	14.5	45	52
Acetone fraction	25	20	11.1	72	40
Alumina-Cγ[c]	10	8	2.8	287	16

[a] Specific Activity = change in absorbancy of 0.001 per 1 mg of enzyme protein per minute. [b] Ca phosphate gel concentration 36 mg/ml. [c] Alumina gel concentration 18 mg/ml.

tase of thymus gland can catalyze the reduction of dihydropteroyl compounds other than dihydrofolic acid. Some experimental results are shown in Tables II and III. The enzyme can also catalyze the reduction of pteridine derivatives, but at a very low rate compared to the dihydropteridines (Table IV).

Table II shows that enzymatically prepared dihydrofolic acid (line 3) is a good substrate for the thymus dihydrofolate reductase but that it is somewhat less active than the same compound formed chemically. The table also shows that 6,7-dimethyldihydropteridine (line 4) prepared by reduction with zinc is nearly as active a substrate as is dihydrofolic acid. On the other hand, the 6,7-dimethyldihydropteridine prepared by oxidation of the tetrahydropteridine with 2,6-dichlorophenolindophenol (line 6) is only half as active. The true activity is probably even less, since 6,7-dimethyldihydropteridine is rapidly converted spontaneously to the tautomer formed by zinc reduction (Kaufman, 1961). The significance of these results in relation to the structural relationships of the dihydropteroyl derivatives will be discussed below.

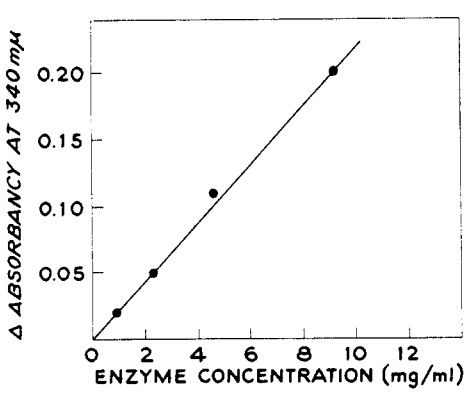

FIG. 1.—Proportionality between enzyme concentration and reaction rate. Incubation mixture contained: DPNH (67 μM), folate-H_2 (65 μM), and varying enzyme concentrations (0.9–9.2 g/liter) in total volume of 3.0 ml. Incubation for 30 minutes at 37°.

Table III contains the data on the comparative rates of reducibility of various dihydropteroyl compounds. Dihydropteropterin is seen to be the most active substrate. Substitution of the L-glutamyl moiety by D-glutamic acid or by aspartic acid does not cause very marked changes in the substrate response of the dihydropteroyl compounds. Loss of the glutamyl side-chain, as in pteroic acid, causes a great drop in the reactivity. Addition of a methyl group at either the N^{10} or the 9 positions decreases the reactivity to about 40% of that of dihydrofolic acid.

Table IV shows the comparative activity of the series of pteridine compounds whose dihydro forms were tested in Table III. In no instance was the activity of any of these compounds greater than 10% of that of dihydropteroyl-(L)-glutamic acid.

ENZYME-SUBSTRATE INTERACTION

Enzyme-substrate interaction was determined for folate-H_2 and dihydroteropterin by the usual procedure of plotting the reciprocals of the

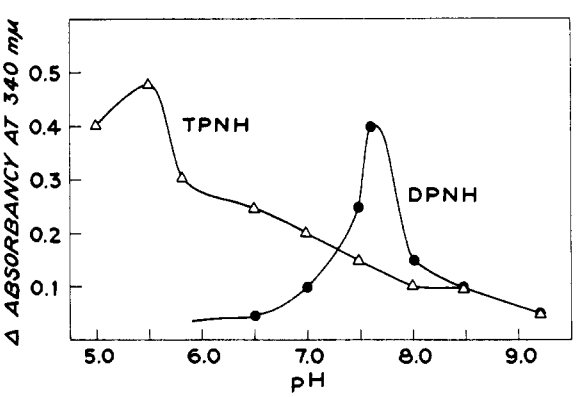

FIG. 2.—*p*H-activity curve of dihydrofolic acid reduction with DPNH and TPNH as hydrogen donors. Buffers used: Acetate from *p*H 5.0–6.0; phosphate from *p*H 6.0–7.5; Veronal from *p*H 7.5–9.2 at ionic strength (μ) 0.05. Substrate concentrations: DPNH or TPNH, 87 μM; folate-H_2, 62 μM; and enzyme 1 g/liter.

TABLE II

ACTIVITY OF DIHYDROFOLATE REDUCTASE ON VARIOUSLY PREPARED SUBSTRATES

The substrates (67–83 μM) were incubated with DPNH (83 μM) and enzyme (0.67 g/liter) in 0.05 M Veronal buffer, *p*H 7.6, for 15 minutes at 37°. Corrections were made for nonenzymatic reduction of substrate as well as for the effect of the enzyme alone on DPNH.

Substrate Tested	Δ Absorbancy$_{340}$	Relative Activity
Folate-H_2[a]	0.55	100
Folate-H_4[b]	0.03	—
Enzymatic folate-H_2[c]	0.45	82
6,7-Dimethyldihydropteridine[d]	0.50	91
6,7-Dimethyltetrahydropteridine[b,e]	0.15	—
6,7-Dimethyl-5,6-dihydropteridine[f]	0.25	45

[a] Prepared by $Na_2S_2O_4$ reduction by the method of Futterman (1957). [b] Prepared by catalytic hydrogenation (O'Dell *et al.*, 1947). [c] Prepared by procedure of Wright and Stadtman (1956). [d] Prepared from the dimethylpteridine by reduction with zinc in alkali (Kaufman, 1959). [e] This sample apparently contained some dihydropteridine. Freshly prepared samples showed less change in absorbancy. [f] Prepared by oxidation of dimethyltetrahydropteridine with 2,6-dichlorophenolindophenol (Kaufman, 1961).

velocity vs. the reciprocal of the substrate concentration (Fig. 3). Extrapolation of the straight lines to the $-1/S$ axis yielded the values of $K_m = 10 \times 10^{-5}$ M for folate-H_2 and 6.25×10^{-5} M for dihydroteropterin. Similar data for the Michaelis constants for DPNH and TPNH were not obtained because of the difficulties in measuring absorbancy when the pyridine nucleotides are in high concentration. At *p*H 7.6, DPNH is three times as active as TPNH, whereas at the *p*H at which TPNH is most active, DPNH is only half as active.

TABLE III

RELATIVE ACTIVITY OF DIHYDROFOLATE REDUCTASE ON DIHYDROPTERIDINE COMPOUNDS

Dihydropteridine compounds (40 μM) were incubated with DPNH (87 μM) and enzyme protein (1 g/liter) in Veronal buffer (*p*H 7.6, 0.05 M) in a total volume of 3 ml for 30 minutes at 37°.

Compound Tested[a]	Δ Absorbancy$_{340}$	Relative Activity (%)
dl-Dihydropteroyl-L-glutamic acid	0.53	100
Dihydroteropterin	0.57	108
Dihydropteroyl aspartic acid	0.55	104
Dihydropteroic acid	0.10	20
N^{10}-Methyldihydrofolic acid	0.21	40
9-Methyldihydrofolic acid	0.19	36
Dihydropteroyl-D-glutamic acid	0.48	91

[a] Generously furnished by Dr. R. B. Angier of the Lederle Laboratories.

TABLE IV

RELATIVE ACTIVITY OF DIHYDROFOLATE REDUCTASE ON PTERIDINE COMPOUNDS

Conditions and concentrations of pteridines are the same as for dihydro compounds except that the time of incubation was 60 minutes.

Compound Tested[a]	Δ Absorbancy$_{340}$	Relative Activity (%)
Dihydropteroyl-L-glutamic acid	0.53	100
dl-Pteroyl-L-glutamic acid	0.04	8
Teropterin[b]	0.05	10
Pteroyl aspartic acid	0.02	4
Pteroic acid	0.00	0
N^{10}-Methylpteroylglutamic acid	0.02	4
9-Methylpteroylglutamic acid	0.03	6
Pteroyl-D-glutamic acid	0.03	6
6,7-Dimethylpteridine	0.00	0

[a] Generously furnished by Dr. R. B. Angier of the Lederle Laboratories. [b] *dl*-Pteroyl-L-triglutamic acid.

INHIBITION

Thymus dihydrofolic acid reductase is inhibited by 4-aminopteridine derivatives, just as is the liver enzyme. The inhibition constant for Amethopterin (2,4-diaminopteroylglutamic acid) was determined with a preparation purified by the method of Nobel (1961). The inhibitory effect with varying concentrations of folate-H_2 and at an Amethopterin concentration of 7.3 mμM is shown graphically in Figure 3, plotted according to the Lineweaver-Burke method. The plot shows that the inhibition is noncompetitive, as has been reported with dihydrofolate reductase from other sources. The inhibition constant, K_i, calculated from the equation for noncompetitive inhibition for Amethopterin, is 2.3×10^{-9} M.

Experiments were also performed to determine the inhibitory effect of Tetrahydroamethopterin (2,4-diamino-5,6,7,8-tetrahydropteroylglutamic acid), prepared by the hydrogenation of purified Amethopterin. Tetrahydroamethopterin proved to be less inhibitory to the enzyme than Amethopterin, although it has been reported to be more toxic to vertebrates and microorganisms (Kisliuk and Fox, 1961). Extrapolation of the straight line obtained by plotting inhibitor concentration (5–100 mμM) against 1/v at the folate-H_2 concentration of 65 μM yielded a value of K_i of 5×10^{-8} M for the inhibition constant of Tetrahydroamethopterin.

The thymus enzyme requires sulfhydryl groups for activity, just as do the liver enzymes (Osborn and Huennekens, 1958; Peters and Greenberg, 1958a,b). This was demonstrated by inhibition

with *p*-chloromercuribenzoate and reversal of the inhibition by glutathione. The inhibition constant, K_i, calculated from a double reciprocal plot, is 3.8×10^{-9} M.

Tautomerism of Dihydropteridines

In our laboratory it was noted that the folate-H_2 formed enzymatically in the thymidylate synthetase reaction is not easily reduced to folate-H_4 with purified sheep liver enzyme, whereas the product formed by reduction with dithionate is an excellent substrate. Other reports have indicated the formation of different double-bond tautomeric forms of dihydropteridines. Thus, Kaufman (1959, 1961) reported the enzymatic and chemical oxidation of 5,6-dimethyltetrahydropteridine to an unknown dihydropteridine that spontaneously changed to what is presumed to be the 6,7-dimethyl-7,8-dihydropteridine. This identification was based on the absorption spectrum of the final product and on its inability to be reduced enzymatically by TPNH and by a sheep liver extract (presumably dihydrofolate reductase.)

Our observations on the thymidylate synthetase reaction led us to study the reducibility of dihydropteridines prepared in different ways by both thymus and liver dihydrofolate reductases. The results are recorded in Tables II and V.

Table II shows that the enzymatically formed folate-H_2 of Wright and Stadtman (1956) (presumed to be the 7,8-compound) is only a little less active than the product prepared chemically according to the method of Futterman (1957). On the other hand, the dimethyldihydropteridine prepared by Kaufman's procedure (postulated to be the 5,6-tautomer) is only about half as active as the dimethyldihydropteridine prepared by reduction with dithionate. In a similar experiment with dihydrofolic acid prepared with dithionate and with dichlorophenolindophenol, the latter reacted about 70% as rapidly as the former with the thymus enzyme during the first 15 minutes. In 30 minutes the degree of reduction was the same for both.

Table V compares the rates of reduction of chemically (Futterman, 1957) and enzymatically (Wright and Stadtman, 1956) prepared folate-H_2 by purified thymus and sheep liver dihydrofolate reductases. The data show that the chemically reduced compound is an excellent substrate for the liver enzyme, about two thirds of the conversion being complete within 5 minutes. The reduction with the thymus enzyme proceeds only about half as rapidly. For the enzymatically prepared folate-H_2 the rate with thymus dihydrofolate reductase is about the same as with the synthetic product, and the reaction proceeds linearly with time. The reaction with the liver enzyme shows a marked time lag for the first 30 minutes and then an increase equal to the value obtained with the thymus enzyme. This may represent a slow tautomeric conversion of the enzymatically prepared folate-H_2.

The folate-H_4 formed from the enzymatically prepared folate-H_2 by reaction with thymus dihydrofolic acid reductase and DPNH responded about 80–85% as well as the chemically prepared folate-H_4 for the synthesis of thymidylic acid in the thymidylate synthetase enzyme system.

Discussion

To facilitate discussion, the structures of the three possible double-bond tautomers of dihydropteridines are presented in Figure 4.

A most significant difference between the dihydrofolate reductase enzymes from liver and from thymus is the differential in the rates of utilization of the different tautomers of dihydropteroyl compounds. The difference in response of the two enzymes is given in Table VI. Experimental data are contained in Tables II and V. Quite clearly,

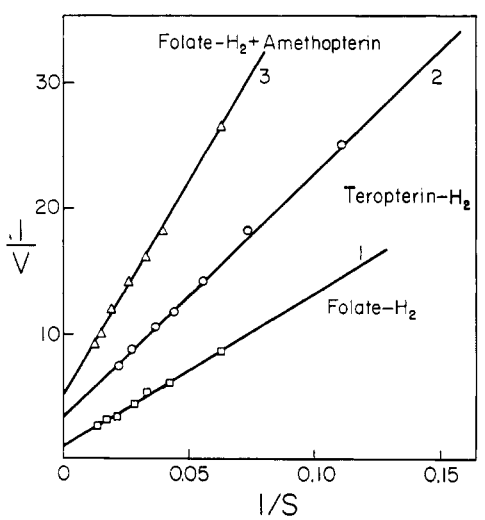

FIG. 3.—Double reciprical plot of activity versus substrate concentration for dihydrofolic acid and dihydroteropterin. Inhibition by Amethopterin. Contents of incubation mixture and conditions: Enzyme (1.2–1.5 g/liter, specific activity 85), Veronal buffer (0.05 M, *p*H 7.6), DPNH (70 μM); Curve 1, folate-H_2 (16–80 μM), incubation time 30 minutes; Curve 2, dihydroteropterin (*dl*-dihydropteroyl-L-triglutamic acid) (9–45 μM), incubation time 5 minutes; Curve 3, folate-H_2 + Amethopterin (7.3 μM), incubation time 30 minutes.

chemically synthesized folate-H_2 is a better substrate for the liver enzyme than is the enzymatically prepared compound. The thymus enzyme acts only a little less well initially on the enzymatic than on the synthetic product (Table V). The liver enzyme reduces the chemically formed folate-H_2 more efficiently than does the thymus enzyme.

There is a good deal of uncertainty about the

TABLE V

ACTIVITY OF THYMUS AND LIVER DIHYDROFOLIC ACID REDUCTASE ON SYNTHETIC AND ENZYMATIC SUBSTRATE

Incubation contents: folate-H_2 (50 μM), DPNH or TPNH (80 μM), and enzyme (1.2 g/liter of thymus or 0.8 g/liter of sheep liver preparations) in 0.05 M Veronal buffer, *p*H 7.6. Incubation times as shown; 37°.

	Δ Absorbancy$_{340}$							
	Synthetic Folate-H_2				Enzymatic Folate-H_2[a]			
	Time (min.)				Time (min.)			
	5	15	30	60	5	15	30	60
Thymus reductase + DPNH	0.06	0.08	0.12	0.13	0.03	0.07	0.09	0.135
Liver reductase[b] + TPNH	0.130	0.150	0.180	0.200	−0.02 −0.01	0 0.02	0.06 0.04	0.14 0.120

[a] Prepared with enzyme from *Cl. sticklandii* (Wright and Stadtman, 1956). The cells were disrupted sonically and enzyme extracted with 0.01 M Tris buffer, *p*H 7.2. Enzyme purified by treatment with protamine sulfate and $(NH_4)_2SO_4$; specific activity = 6.2. Folate-H_2 prepared by reaction in a medium containing folic acid (1 μM), coenzyme A (6.2 μM), serine (10 μM), and 1.5 g/liter of enzyme in phosphate buffer, *p*H 6.5. Incubation for 2 hours under hydrogen. [b] Purified sheep liver preparation prepared by unpublished method of Morales and Greenberg.

TABLE VI

COMPARATIVE SUBSTRATE ACTIVITY OF THYMUS AND LIVER DIHYDROFOLIC ACID REDUCTASE

Source of Folate-H_2	Activity as Substrate	
	Thymus Gland Enzyme + DPNH	Sheep Liver Enzyme + TPNH
Folate-H_2 ($Na_2S_2O_4$ reduced; Futterman)	+++	++++
Folate-H_2 (*Clostridium sticklandii*; Wright *et al.*)	+++ (Tables II, V)	− (0 min., Table V)
Folate-H_2 (from thymus thymidylate synthetase reaction)	+++ with crude thymus (Greenberg *et al.*, 1961) (activity with pure reductase?)	+ with 50–60% $(NH_4)_2SO_4$ thymus fraction (tautomerase?)
Dimethylpteridine (7,8-dihydro) (Kaufman, 1961)	+++ (Table II)	− (sheep liver; Kaufman, 1960; Morales and Greenberg, unpublished)
Dimethylpteridine dye "(5,6-dihydro)" (Kaufman, 1961)	(+)? (Table II)	+ (sheep liver; Kaufman, 1961)

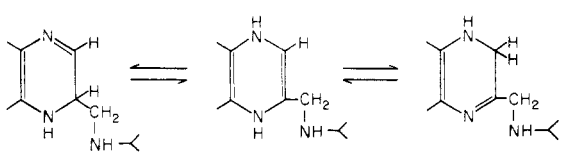

FIG. 4.—Double-bond tautomers of dihydropteridines.

exact structures of the different dihydropteridines formed by the several procedures employed. It is commonly assumed that dihydropteridines prepared by catalytic hydrogenation or by reducing agents (*e.g.*, $Na_2S_2O_4$) from pteridines are 7,8-dihydro compounds (Futterman, 1957; Osborn and Huennekens, 1958). This is based on the observation of O'Dell *et al.* (1947) that the 5,6 double bond in leucopterine and isoxanthopterine is difficult to hydrogenate catalytically in dilute alkali; the 7,8 double bond is easily hydrogenated. Some exception to this conclusion has been expressed by Pfleiderer and Taylor (1960). A confirmation of the accepted 7,8-dihydropteroyl structure by comparison with a compound prepared by unequivocal chemical synthesis would be very valuable, but has yet to be reported. Methods of synthesis yielding authentic 7,8-dihydropteridines that could be tested in the dihydrofolate reductase enzyme reaction have been reported from two laboratories (Elion and Hitchings, 1952; Boon and Leigh, 1951).

The dihydrofolic acid prepared by O'Dell *et al.* (1947) by catalytic hydrogenation has a distinctive absorption spectrum that has been used as the basis of identification by others. Wright and co-workers (Wright and Stadtman, 1956; Wright and Anderson, 1957; Wright *et al.*, 1958) concluded that the compound formed in the *Cl. sticklandii* enzyme system was 7,8-dihydrofolate on the basis of the close agreement in spectra between the compounds formed enzymatically and by hydrogenation. Comparison of these two preparations with dihydrofolate reductases of liver and thymus

(Table V) shows differences in their enzymatic response.

Kaufman (1961) also concluded that the terminal dimethyldihydropteridine compound formed in the phenylalanine hydroxylation system is the 7,8-dihydro tautomer, on the basis of spectral correspondence. This dihydropteridine was found to be inactive in promoting phenylalanine hydroxylation. We have observed that dimethyldihydropteridine prepared by the procedure of Futterman is not reduced by liver dihydrofolic reductase.

In another experiment, Kaufman (1959) tested dimethyldihydropteridine prepared by reduction of 2-amino-4-hydroxy-6,7-dimethylpteridine with zinc and alkali to yield the corresponding dimethyl - 7,8 - dihydropteridine. This compound was inactive in the phenylalanine hydroxylation system.

If the active substrates for dihydrofolate reductase are 7,8-dihydropteridines, then the inactive compounds formed enzymatically in the thymidylate synthetase and phenylalanine hydroxylation systems, and chemically by oxidation of tetrahydropteridines with 2,6-dichlorophenolindophenol, could be either the 5,8- or the 5,6-dihydro tautomers. Perault and Pullman (1960) have concluded, on the basis of quantum chemical calculations, that the 5,8-dihydropteridines would be unusually resistant to hydrogenation.

Pfleiderer and Taylor (1960) have shown experimentally that the 5,8-dihydropteridines possess unusual chemical stability when they contain an ethyl group at position 8, an oxygen function at C-7, and a carboxyl group at C-6. Another feature of these compounds is a high ultraviolet absorption maximum at 405 mμ. No such absorption region was noticed in the dihydropteridines found to be resistant to reduction with dihydrofolate reductase. This unusual absorption peak, too, might be a function of the substituted groups mentioned above.

If the evidence which rules out the 7,8- and 5,8-dihydropteridines as the unknown resistant tautomers is valid, then there is left the 5,6-dihydro compound. This indeed has been suggested by Kaufman (1961) as the structure of his unknown intermediate dihydropteridine. The 5,6-dihydro compound changes spontaneously in an hour or two to what is presumed, on the basis of its inactivity in the phenylalanine hydroxylation system and on its absorption spectrum, to be the 7,8-dihydro compound. This ready tautomerization is rather difficult to understand, since it requires a double shift of the double bond. Furthermore, in the thymidylate synthetase reaction, a C-N bond would appear to have to be broken at position 5. This could readily lead to a double bond at the 5,6 position, but not at the 7,8 position.

If the 5,6-dihydro structure is correct, the C-6 position would be asymmetric, and two diasterioisomers should be formed by chemical synthesis. It should be possible to test this enzymatically, since only one of the diasterioisomers, in all probability, is a substrate for dihydrofolate reductase. However, the observed instability of the compound formed by oxidation of tetrahydropteridines with dichlorophenolindophenol would appear to make this test extremely difficult, if not impossible.

References

Blakley, R. L. (1957), *Biochem. J. 65*, 331.

Blakley, R. L. (1960), *Nature 188*, 231.

Blakley, R. L., and McDougall, B. M. (1961), *J. Biol. Chem. 236*, 1163.

Boon, W. R., and Leigh, T. (1951), *J. Chem. Soc.* 1497.

Elion, G. B., and Hitchings, G. H. (1952), *J. Am. Chem. Soc. 74*, 3877.

Futterman, S. (1957), *J. Biol. Chem. 228*, 1031.

Greenberg, D. M., Nath, R., and Humphreys, G. K. (1961), *J. Biol. Chem. 236*, 2271.

Guest, J. R., and Woods, D. D. (1962), *Biochem. J. 82*, 26.

Hatch, F. T., Larrabee, A. R., Cathou, R. E., and Buchanan, J. M. (1961), *J. Biol. Chem. 236*, 1095.

Kaufman, S. (1959), *J. Biol. Chem. 234*, 2677, 2683.

Kaufman, S. (1961), *J. Biol. Chem. 236*, 804.

Keilin, D. S., and Hartree, E. F. (1938), *Proc. Roy. Soc. (London) B 124*, 397.

Kisliuk, R. L., and Fox, M. H. S. (1961), *Arch. Biochem. Biophys. 93*, 534.

Layne, E. (1957) *in* Methods in Enzymology, vol. III, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc., p. 447.

McDougall, R. M., and Blakley, R. L. (1960), *Nature 188*, 941.

Nath, R., and Greenberg, D. M. (1961), *Fed. Proc. 20*, 227.

Nobel, E. E. (1961), *Biochem. Preps. 8*, 20.

O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S., and Pfiffner, J. J. (1947), *J. Am. Chem. Soc. 69*, 250.

Osborn, M. J., and Huennekens, F. M. (1958), *J. Biol. Chem. 233*, 969.

Perault, A. M., and Pullman, B. (1960), *Biochim. et Biophys. Acta 44*, 251.

Peters, J. M., and Greenberg, D. M. (1958a), *Nature 181, 1669.*

Peters, J. M., and Greenberg, D. M. (1958b), *J. Am. Chem. Soc. 80*, 6679.

Pfleiderer, W., and Taylor, E. C. (1960), *J. Am. Chem. Soc. 82*, 3765.

Wahba, A. J., and Friedkin, M. (1961), *J. Biol. Chem. 236*, PC 11.

Wright, B. E., and Anderson, M. L. (1957), *J. Am. Chem. Soc. 79*, 2027.

Wright, B. E., Anderson, M. L., and Herman, E. C. (1958), *J. Biol. Chem. 230*, 271.

Wright, B. E., and Stadtman, T. C. (1956), *J. Biol. Chem. 219*, 863.

Zakrzewski, S. F. (1960), *J. Biol. Chem. 235*, 1776, 1780.