Ion-Dependent Activation of Dihydrofolate Reductase from L1210 Cells*

P. Reyes† and F. M. Huennekens

ABSTRACT: Dihydrofolate reductase from L1210 cells is activated about fivefold by Cl^- or Br^- at 0.8–1.0 M. Thiocyanate produces maximum activation (only about threefold) at 0.4 M, while at higher concentrations the activity drops off sharply. Acetate is relatively ineffective as an activator. Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+ (added as the chlorides) all have similar activation *vs.* concentration curves, suggesting that the effect is due almost entirely to the common anion. Alternatively, Ba^{2+} and Ca^{2+} (added as the chlorides) achieve almost the maximum (*i.e.*, fivefold) degree of activation at 0.2 M, but higher concentrations of these salts are inhibitory. These results are similar to activation by urea.

At 3.5 M, urea activates the enzyme about threefold, but as the concentration is increased above 4 M, the activity falls off rapidly. Activation of the L1210 enzyme by KCl: (a) is reversible; (b) does not change the apparent molecular weight of the protein; (c) converts the double pH optimum (at 4.5 and 7.0) of the nonactivated enzyme to a single optimum at 7.0; (d) increases the K_m value for reduced triphosphopyridine nucleotide from 4.8 to 21 $\times 10^{-6}$ M without increasing the number of binding sites for the pyridine nucleotide; and (e) does not create any new binding sites for amethopterin. Ion- and urea-dependent activation of dihydrofolate reductase is interpreted in terms of changes in protein conformation, most likely involving a transition from a compact structure to a more unfolded form.

Dihydrofolate reductase, a low molecular weight enzyme that catalyzes the TPNH[1]-dependent reduction of dihydrofolate, has now been isolated from a number of sources (reviewed by Huennekens, 1967). Most, but not all, of these enzymes can be activated by a variety of agents, such as salts (Bertino, 1962), urea and formamide (Kaufman, 1963), thiourea and guanidine (Perkins and Bertino, 1965), polyamines (Misra and Adamson, 1963), mercurials (Kaufman, 1964; Perkins and Bertino, 1964, 1965), and iodine (Kaufman, 1966). In addition to providing useful information about the structure of the enzyme and its mechanism of action, these *in vitro* activation effects may also be relevant to the observed increase in the "level" of the enzyme in human leukemic leukocytes that accompanies the development of resistance to folate antagonists (Bertino *et al.*, 1963).

Although activation by urea or mercurials has been studied in detail, much less is known about the mechanism of ion activation. The present communication describes the effect of anions and cations on some of the kinetic and physical properties of the dihydrofolate reductase from L1210 cells. Some comparative experiments with urea are also reported. Activation of the enzyme by these agents is believed to involve conformational changes in the protein. Similar explanations have been advanced previously for the activation of other dihydrofolate reductases by urea (Kaufman, 1963), organic mercurials (Kaufman, 1964; Perkins and Bertino, 1964), and iodine (Kaufman, 1966).

Experimental Section

Materials

Chemicals and materials were obtained from the following sources: folic acid from California Corp. for Biochemical Research; Tris and TPNH from Sigma Chemical Co.; 2-mercaptoethanol from Eastman Chemical Co.; Sephadex from Pharmacia Co.; DEAE-cellulose and the collodian bags for ultrafiltration from Schleicher & Schuell Co.; inorganic salts, sodium hydrosulfite, and urea from J. T. Baker Chemical Co.; rubidium chloride and cesium chloride, K & K Laboratories, Inc.; dialysis tubing from Union Carbide Corp.; and crystalline bovine serum albumin from Armour Pharmaceutical Co.

Hydroxylapatite was prepared by the method of Tiselius *et al.* (1956). Calcium phosphate gel (Keilin and Hartree, 1938) was stored as a suspension (22 mg/ml). Amethopterin (Lederle Laboratories, Division of American Cyanamide Co.) was purified by chromatography on DEAE-cellulose (Silber *et al.*, 1963). The con-

* From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received July 5, 1967.* Paper No. XX in the series Folic Acid Coenzymes and One-Carbon Metabolism. This work was supported by grants from the National Cancer Institute, National Institutes of Health (CA-6522), and the American Cancer Society (P-302C), and by Contract PH 43-65-14 with the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health.

† Special Postdoctoral Fellow of the National Cancer Institute, National Institutes of Health.

[1] Abbreviations used: TPNH, reduced triphosphopyridine nucleotide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

centration of amethopterin solutions was determined spectrophotometrically at pH 13 using an extinction coefficient of 23.0×10^3 M^{-1} cm^{-1} at 257 mμ (Schrecker and Huennekens, 1964). Dihydrofolate was prepared by reduction of folate with hydrosulfite according to the method of Futterman (1957), as modified by Blakley (1960). After being precipitated in acidic solution, the washed pellet of dihydrofolate was dissolved in 10 ml of 0.05 M Tris buffer (pH 7.6) which contained 0.1 M mercaptoethanol. The concentration of dihydrofolate solutions was determined spectrophotometrically at pH 13 using an extinction coefficient of 27.0×10^3 M^{-1} cm^{-1} at 283 mμ (Blakley, 1960). The concentration of dihydrofolate in the stock solution was adjusted to 20 μmoles/ml and 0.3-ml aliquots were stored frozen in stoppered vials. Prior to use, the aliquots were diluted to 3.0 ml with 0.05 M Tris buffer (pH 7.6) containing 0.01 M mercaptoethanol.

Methods

Purification of Dihydrofolate Reductase. Unless otherwise specified, all operations below were carried out at 0–5°.

Preparation of Extract. Male, BDF mice (80–100) (obtained from Simonsen Laboratories) were sacrificed 5–7 days after intraperitoneal injection of lymphoid leukemia (L1210) cells.[2] Cells were obtained by centrifugation of the ascitic fluid for 10 min at 2000 rpm (International refrigerated centrifuge, Model PR-2) and washed three times with 0.15 M NaCl. Contaminating erythrocytes were lysed by adding three volumes of cold water to the cells followed by rapid stirring for 1 min. Isotonicity was then restored by the addition of one volume of 0.6 M KCl. After centrifugation, as described above, the cells were washed twice with 0.15 M NaCl, suspended in two volumes of 0.05 M potassium phosphate buffer (pH 7.6), and homogenized for 60 sec with a Virtis 45 instrument set at maximum speed. The resulting homogenate was then centrifuged twice at 30,000*g* for 40 min in a Servall refrigerated centrifuge, Model RC-2 (rotor SS-34). After each centrifugation, the supernatant fluid was filtered through glass wool.

Acid Precipitation. The pH of the supernatant fluid was slowly adjusted with stirring to 5.4 by the addition of about 2 ml of 6 N acetic acid. The precipitated material was then removed by centrifugation for 20 min at 30,000*g*, and the pH of the supernatant fluid was readjusted to 7.6 with about 10 ml of 1 N KOH.

Fractionation with Solid Ammonium Sulfate. Solid ammonium sulfate was slowly added to the above solution and the material precipitating between 0 and 55% saturation, collected by centrifugation at 30,000*g* for 15 min, was discarded. Additional ammonium sulfate was added to bring the supernatant solution to 85% of saturation and, after stirring for 20 min, the mixture was centrifuged as above and the resulting pellet was dissolved in 0.05 M potassium phosphate buffer (pH 7.6). The solution was dialyzed for 5 hr against 6 l. of 10^{-3} M potassium phosphate buffer (pH 7.6).

Negative Adsorption on Calcium Phosphate Gel. The protein content of the above fraction was adjusted to 2 mg/ml and 1.0 ml of calcium phosphate gel (22 mg/ml) was added dropwise with stirring. After 15 min, the suspension was centrifuged for 10 min at 30,000*g* and the pellet was discarded. This procedure was repeated several times until the yield of total enzyme units in the supernatant fraction began to decrease appreciably; this usually occurred when about one-tenth volume of gel (per volume of the protein solution) had been added.

Filtration through Sephadex G-75. The volume of the above fraction was reduced to about 5 ml by placing it in dialysis tubing packed in solid sucrose for 4 hr, followed by ultrafiltration in collodian bags having a porosity of less than 5 mμ. The concentrated fraction was passed through a 1.8×70 cm column of Sephadex G-75 that had been equilibrated previously with 0.05 M potassium phosphate buffer (pH 7.6), and 3-ml aliquots were collected automatically. Fractions having the highest specific activity were pooled and concentrated with sucrose and ultrafiltration, as described above. Enzyme at this stage of purification (2–3 units/mg of protein, about 1 mg/ml) was used in all experiments. The purified enzyme was stable for several months when stored frozen, according to the procedure of Kaufman and Gardiner (1966), in 1-ml aliquots containing 0.1 mM TPNH.

Enzyme Assay. Dihydrofolate reductase activity was measured spectrophotometrically by the procedure of Osborn and Huennekens (1958). Variations in the assay mixture are given in the legend for each table or figure. Assays were performed at room temperature (approximately 25°) using a Gilford Model 2000 multisample absorbance recorder attached to a Beckman DU monochromator. The reaction was initiated by the addition of dihydrofolate to the other components that had been preincubated for 2–3 min in cuvets placed in the spectrophotometer. Absorbance changes were followed for approximately 5 min and corrected, if necessary, for the blank rate observed prior to addition of dihydrofolate. One unit of enzyme activity is defined as the amount required to oxidize 1 μmole of TPNH/min under these conditions (Mathews and Huennekens, 1963). Specific activity is expressed as units per milligram of protein. Protein concentrations, using crystalline bovine serum albumin as the standard, were determined by the biuret method for crude fractions and by a modification (60-sec color development at 100° instead of the usual 30-min interval at room temperature or 37°) of the method of Lowry *et al.* (1951) for more purified fractions.

Results

Purification of Dihydrofolate Reductase from L1210 Cells. Table I records the details of a typical preparation

[2] This cell line was kindly provided by Dr. Dorris Hutchison, Sloan-Kettering Institute.

TABLE I: Purification of Dihydrofolate Reductase from L1210 Cells.

No.	Fraction	Vol. (ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)	Recov (%)
1	Crude extract	132	1270	13.4	0.01	
2	pH 5.4 supernatant	134	214	15.2	0.07	100
3	55–85% ammonium sulfate precipitate	45	79	10.9	0.14	72
4	Calcium phosphate gel supernatant	51	35.6	10.0	0.28	66
5	Sephadex G-75 eluate	22	1.9	5.1	2.7	34

in which enzyme having a specific activity of about 2–3 units/mg of protein was obtained in about a 30% yield. Enzyme having a specific activity as high as 8 units/mg of protein could be achieved by chromatography of the above preparation on hydroxylapatite (Mathews and Huennekens, 1963), although such a step was accompanied by considerable loss of total activity, presumably owing to dilution of the enzyme.

General Observations on the Activation of Dihydrofolate Reductase by Salts. Stimulation of the L1210 dihydrofolate reductase by monovalent anions, added as the Na^+ salt, is illustrated in Figure 1. Chloride and bromide produced maximum activation (about fivefold) at concentrations of about 0.8–1.0 M. At higher concentrations, the activity with Br^- began to decline. This latter effect was even more noticeable in the case of thiocyanate where inactivation began above 0.4 M. Acetate achieved only about a twofold stimulation and correspondingly little inactivation.

When a number of monovalent cations (Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+, added as the chlorides) were tested, each produced an activation curve similar to that of Cl^- (Figure 1). Thus, these cations are not very effective activators, an assumption borne out by the sodium acetate (Figure 1) curve in which neither the anion nor the cation is able to achieve maximal activation. On the other hand, divalent cations such as Ba^{2+} or Ca^{2+} (added as the chlorides) provide nearly maximum activation at 0.1–0.2 M, the effect declining rapidly at higher concentrations (Figure 2).

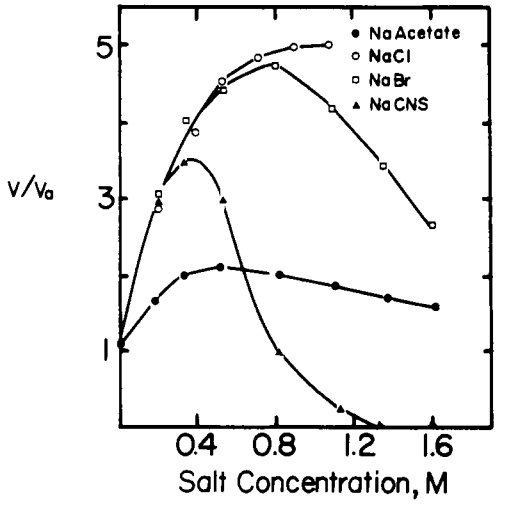

FIGURE 1: Effect of anions on the activity of dihydrofolate reductase. The reaction mixture contained the following components in a total volume of 1.0 ml: enzyme solution, 10 μl; Tris buffer (pH 7.6), 45 μmoles; 2-mercaptoethanol, 9 μmoles; TPNH, 0.12 μmole; and salts at the indicated final concentrations. Dihydrofolate (0.12 μmole) was added to initiate the reaction. Enzyme activity was measured in the presence (v) and absence (v_0) of the respective salts and the ratio v/v_0 is plotted against concentration of the salt.

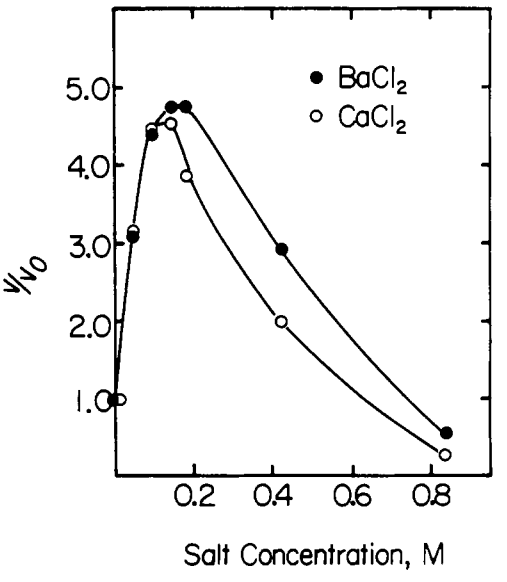

FIGURE 2: Effect of $BaCl_2$ and $CaCl_2$ on dihydrofolate reductase activity. Experimental conditions were the same as those described in Figure 1 except for the use of the indicated salts. Prior to being used in this experiment, the enzyme preparation was passed through an 0.8 × 14 cm column of Sephadex G-25 to remove potassium phosphate.

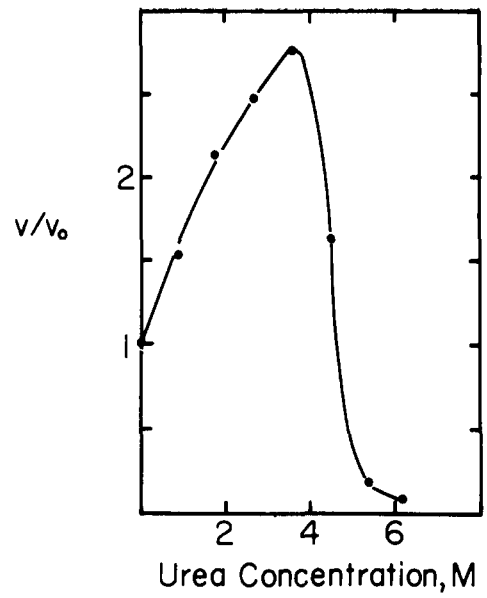

FIGURE 3: Effect of urea on dihydrofolate reductase activity. Experimental conditions were the same as those described in Figure 1 except that urea at the indicated concentrations replaced the salts.

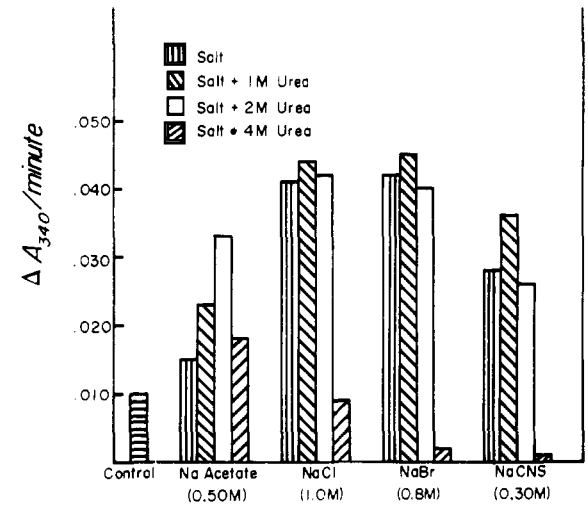

FIGURE 4: Combined effect of urea and anions on dihydrofolate reductase activity. Experimental conditions were the same as those described in Figure 1 except for the presence of salts and urea at the indicated concentrations. The results are expressed as change in absorbancy at 340 mμ (ΔA_{340})/min. Control refers to the activity in the absence of both salts and urea.

Activation of the L1210 dihydrofolate reductase with urea was investigated alone and in combination with ions. The maximum effect (about threefold) occurred with 3.5 M urea[3] in the absence of salts (Figure 3). Similar effects have been observed previously by Kaufman (1963) for the urea-dependent activation of the chicken liver enzyme. At a suboptimal concentration (0.28 M) of NaCl, urea (2.5 M) produced further activation, the combined effect being the same that would have been achieved with 0.8 M NaCl or 3.5 M urea alone. The effects of urea, superimposed upon the anion-activated enzymes, were more complex (Figure 4). Each anion, at its optimal concentration (*cf.* Figure 1), was tested alone and in combination with three different concentrations of urea. Further activation by 1 M urea was observed with the acetate- and thiocyanate-activated enzyme and, to a lesser extent, when Cl^- or Br^- was the activating agent. Raising the concentration of urea to 4 M produced inhibition in all cases, the effect being least with acetate. The inability of urea to produce any further stimulation in the presence of an optimal concentration of Cl^- or Br^- supports the view that the ion- and urea-dependent activated states are similar. Based upon the above observations, KCl was selected for all subsequent experiments involving salt activation.

Speed and Reversibility of Salt Activation. Activation is obviously a rapid process, occurring in less than 15 sec, since the degree of activation was the same whether the enzyme was preincubated with the salt or added to a complete assay mixture containing KCl. The reversibility of salt activation is shown by the results in Table II. In these experiments, the enzyme was preincubated at room temperature in the absence of KCl or in the presence of KCl at a concentration sufficient for complete activation. Aliquots were then

TABLE II: Reversibility of KCl Activation of Dihydrofolate Reductase.[a]

Expt	Preincubn KCl Concn (M)	Assay KCl[b] Concn (M)	Assay Act. (ΔA_{340}/min)
1	0	0.00	0.011
2	1.0	0.10	0.022
3	1.9	0.19	0.028
4	0	0.19	0.031
5	0	0.72	0.048
6	1.0	0.82	0.051

[a] The enzyme solution was mixed with an equal volume (0.6 ml) of 0.05 M Tris buffer (pH 7.6) or with KCl at the indicated concentration in 0.05 M Tris buffer (pH 7.6). After 3 min at room temperature, each mixture was placed in ice and 0.1-ml aliquots were removed and tested for activity in the standard assay mixture (see legend for Figure 1) containing KCl at the indicated final concentration. [b] Owing to KCl initially present in assay medium plus that carried over in the aliquot from the preincubation mixture.

[3] Guanidine is a much more efficient activator, exerting its maximum effect at 0.3 M.

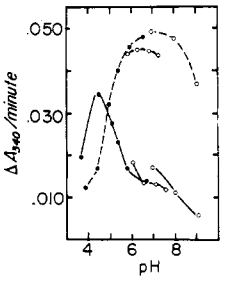

FIGURE 5: Activity of dihydrofolate reductase as a function of pH in the presence and absence of KCl. Experimental conditions were the same as those in Figure 1 except that the mixture contained 90 μmoles of the appropriate buffer (●, citrate-phosphate; □, phosphate; and ○, Tris) at the indicated pH values and 0.80 M KCl where indicated. The dashed lines represent enzyme activity in the presence of KCl while the solid lines represents activity in its absence.

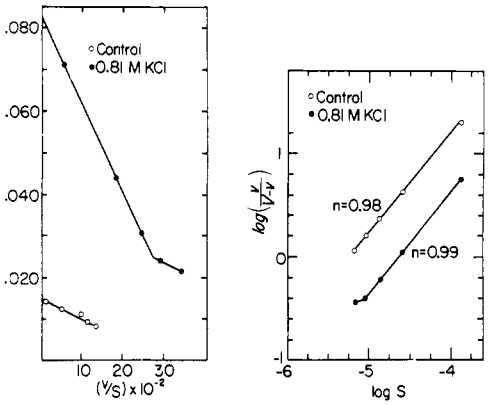

FIGURE 6: Effect of KCl on the Michaelis constant and number of binding sites for TPNH with dihydrofolate reductase. Experimental conditions were the same as those in Figure 1 except that TPNH (S) was varied between 0.63 and 12.6 $\times$ 10^{-2} μmole. Left panel: v (expressed as ΔA_{340}/min) plotted against v/S (where S is the TPNH concentration), according to the method of Dixon and Webb (1960). Right panel, log $(v/(V - v))$ plotted against log S, according to the method of Atkinson *et al.* (1965). V is the maximal velocity obtained from the intercept on the ordinate of the left panel.

removed and assayed in a mixture containing varying amounts of KCl. The base-line activity of 0.011 was observed when KCl was absent from both the preincubation and assay mixtures (expt 1). In expt 2, the enzyme was preincubated with 1 M KCl, and the aliquot was transferred to an assay mixture devoid of KCl. That the higher activity in expt 2 was due to the KCl furnished by the aliquot was proved by expt 3 and 4. In these experiments, the activity was essentially the same whether the enzyme was preincubated in 1.9 M KCl, causing the concentration of KCl in the assay to be 0.19 M, or preincubated without KCl but assayed in a medium containing 0.19 M KCl. Similarly, when the concentration of KCl in the assay medium was optimal, as in expt 5 and 6, it was unnecessary to preincubate the enzyme with KCl.

Effect on Molecular Weight. Molecular weights in the range of 20,000–30,000 have been determined for a number of dihydrofolate reductases (reviewed by Huennekens, 1967). Since these values are rather low for pyridinoproteins, it seemed possible that salt activation might be accounted for by an aggregation of these small protein subunits. However, the approximate molecular weight of the L1210 enzyme, measured by filtration through Sephadex (Whitaker, 1963), was found to be 18,200 and 21,300, respectively, in the absence and presence of 0.5 M KCl. These values are probably not significantly different and may be due to the activated enzyme having the more unfolded conformation which might make it behave like a slightly heavier protein on Sephadex.

Effect on the pH Optimum. The pH–activity profile of dihydrofolate reductase in the presence and absence of KCl is illustrated in Figure 5. In the absence of salt, the enzyme exhibited two optima, a major one at about pH 4.5 and a minor one near pH 7.0. When KCl was present, the double optimum was replaced by a single, broad optimum near pH 7.0. Similar results have been reported for the activation of the chicken liver enzyme by urea (Kaufman, 1963), organic mercurials (Kaufman, 1964), and iodine (Kaufman, 1966). A shift in pH optimum thus appears to be an obligatory aspect of the activation process, regardless of the agent employed. Consistent with this hypothesis is the fact that dihydrofolate reductase from *Lactobacillus leichmanii*, which *cannot* be activated by urea or organic mercurials (Kessel and Roberts, 1965), shows only one broad pH optimum near pH 6.0.

Effect on the K_m Value for TPNH. In the left panel of Figure 6, the activity (v) is plotted against v/S for several levels of TPNH in the absence and presence of 0.8 M KCl. The curve for the KCl-activated enzyme is biphasic, as verified by other experiments (not shown) in which even lower concentrations of TPNH were used. The slope at lower concentrations of TPNH is almost parallel to that of the curve without KCl. From these data, the Michaelis constants for TPNH were calculated to be 21 $\times$ 10^{-6} M (for that portion of the curve corresponding to higher values of TPNH) in the presence of KCl, and 4.8 $\times$ 10^{-6} M in the absence of the salt. Thus, KCl increases both V_{max} for the reaction and the K_m value for TPNH.[4] Similar results (*i.e.*, an increase in V_{max} and K_m) have been observed when aldolase (Kwon and Brown, 1966) and hyaluronic acid synthetase (Ishimoto *et al.*, 1966) are activated by high substrate concentrations. With these enzymes, however, kinetic analyses revealed the existence of

[4] Perkins and Bertino (1965) have also reported that the K_m value for dihydrofolate increases when an L1210 dihydrofolate reductase is activated by *p*-mercuribenzoate.

multiple binding sites accessible at the higher substrate concentrations. The possibility that KCl induces a change in enzyme conformation which allows a second molecule of TPNH to serve as an allosteric effector was checked by replotting (right panel of Figure 6) the previous data according to eq 1 (Changeux, 1963;

$$\log\left(\frac{v}{V-v}\right) = n \log S - \log K \qquad (1)$$

Atkinson *et al.*, 1965; Preiss *et al.*, 1966). v, V, and S have their usual meanings; n, the interaction coefficient, is a function of the total number of binding sites and the strength of their interaction with each other; and K is the product of the dissociation constants of the substrate binding sites. As pointed out by Changeux (1963), n will become the actual number of binding sites when the interactions are strong. Since activation by KCl does not change the slope of the straight line in Figure 6 (n = 0.98 and 0.99 for the control and activated enzymes, respectively), it is evident that, in both cases, only one TPNH is bound per molecule of enzyme.

It was not feasible to carry out a similar study with dihydrofolate since the low concentrations necessary to achieve a linear effect on the rate do not sustain the reaction for more than a few seconds. However, the above fact indicates that the K_m value for dihydrofolate with the L1210 enzyme cannot be greater than *ca.* 10^{-7} M. Perkins and Bertino (1966) have reported a K_m value of 4×10^{-7} M for the enzyme from amethopterin-resistant L1210 cells while Osborn *et al.* (1958) and Kaufman and Gardiner (1966) have found corresponding K_m values with the chicken liver enzyme in the range $1.2\text{–}5.0 \times 10^{-7}$ M.

Additional evidence against the involvement of an allosteric binding site in L1210 dihydrofolate reductase has been provided by thermal inactivation studies. Thus, moderate heating of the enzyme (3 hr at 37°), which is generally capable of "desensitizing" regulatory enzymes to allosteric effectors (Changeux, 1961; Gerhart and Pardee, 1962; Patte *et al.*, 1963; Bauerle *et al.*, 1964), does not alter the degree of activation by KCl even though the absolute level of enzyme activity decreased to about one-half of its original value.[5]

The above K_m value for TPNH in the absence of KCl agrees fairly well with those for other reductases: 5.6×10^{-6} M for the Ehrlich ascites enzyme (Bertino *et al.*, 1965), 1.8×10^{-6} M for the chicken liver enzyme (Kaufman and Gardiner, 1966), and 1.7×10^{-6} M for the sheep liver enzyme (Morales and Greenberg, 1964). Consistent with the previously mentioned fact that it cannot be activated by urea or mercurials, the *L. leichmanii* enzyme has a high K_m value (38×10^{-6} M) for TPNH (Kessel and Roberts, 1965) which is similar to the K_m value for the L1210 enzyme in the activated state.

Effect on Amethopterin Inhibition. Inhibition of a fixed amount of the L1210 enzyme by increasing amounts of amethopterin, in the presence and absence of KCl, is shown in Figure 7A. As noted earlier in this laboratory (Schrecker and Huennekens, 1964), such inhibition plots appear to be biphasic. At the lower concentrations of amethopterin the plots are linear, as would be expected from "stoichiometric" inhibition (Werkheiser, 1961). When the drug concentration is increased (above 1×10^{-8} M in this particular experiment), the plot becomes hyperbolic as though the inhibition were reversible. The linear portion of each curve extrapolates to the same point on the abscissa (*ca.* 1.6×10^{-8} M amethopterin). Thus, in the region of stoichiometric inhibition the *per cent* inhibition produced by a given concentration of the drug is the same whether KCl is present or absent. A similar result has been reported previously for activation of dihydrofolate reductase by $CaCl_2$ (Bertino, 1962) or organic mercurials (Kaufman, 1964; Perkins and Bertino, 1964). Since dihydrofolate and amethopterin very probably compete for the same site on the enzyme, it may be concluded further that salt activation does not lead to an increase in the number of binding sites for dihydrofolate.

The data in Figure 7A were examined further by plotting the reciprocal of enzyme activity against inhibitor concentration (Figure 7B). K_I values for amethopterin, determined by extrapolating the linear portion of each plot to the abscissa, were 1 and 3.6×10^{-8} M in the presence and absence of KCl, respectively. When inhibition is essentially stoichiometric (*i.e.*, at low concentrations of inhibitor), a significant fraction of the inhibitor will be bound to the enzyme (Straus and Goldstein, 1943; Goldstein, 1944), and the inhibitor constants obtained by this graphical method are higher than the true values.

Figure 7C shows the data in Figure 7A replotted according to a modified Hill equation (eq 2) designed

$$\log\left(\frac{v_0 - v}{v}\right) = n \log I - \log K \qquad (2)$$

to study enzyme inhibition (Changeux, 1963; Taketa and Pogell, 1965; Jensen and Nester, 1966; Preiss *et al.*, 1966). v and v_0 are the reaction rates in the presence and absence of inhibitor (I), respectively; n is the interaction coefficient for the inhibitor binding sites; and K is the product of the dissociation constants of these inhibitor binding sites. Biphasic plots are obtained both in the presence or absence of KCl. At lower concentrations of amethopterin, corresponding to the stoichiometric portion of Figure 7A, n = 1.5, but at higher concentrations, n decreases to 1.0 and 0.7 for the salt-activated and control enzyme, respectively. The physical significance of these n values is difficult to interpret in terms of inhibitor-binding sites per enzyme molecule since eq 2 assumes reversible inhibition (Jensen and Nester, 1966), whereas the inhibition in the present case appears to be partially stoichiometric and partially reversible. However, the fact that n does

[5] Unpublished studies of P. Reyes and F. M. Huennekens.

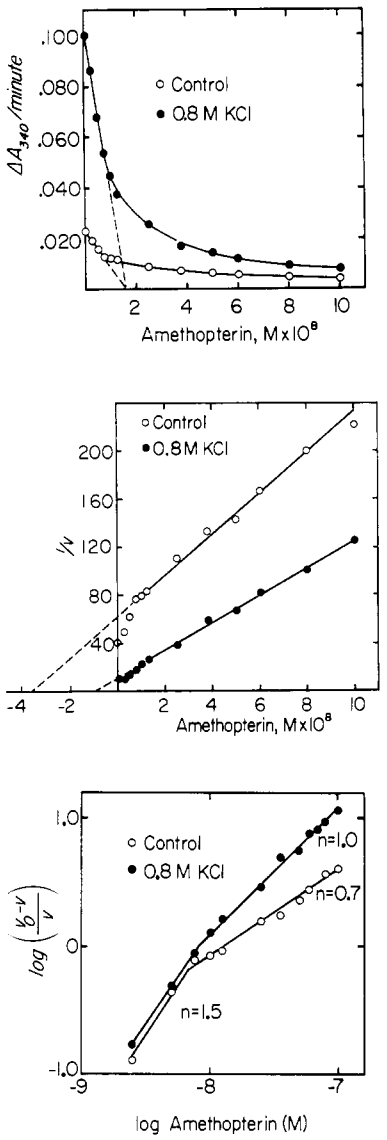

FIGURE 7: Inhibition of dihydrofolate reductase by amethopterin in the presence and absence of KCl. Experimental conditions were the same as Figure 1 except that KCl was 0.8 M (where indicated), TPNH was decreased to 0.11 μmole, and amethopterin was present at the indicated concentrations. After all of the other components had been preincubated for 5 min, 0.12 μmole of dihydrofolate was added to initiate the reaction. (A) top: v (expressed as ΔA_{340}/min) plotted against amethopterin concentration. (B) middle: $1/v$ plotted against amethopterin concentration. (C) bottom: Log $[(v_0 - v)/v]$ plotted against log amethopterin concentration, v_0 and v are the velocities in the absence and presence of the inhibitor.

not increase significantly during activation reinforces the previous argument that no new binding sites for amethopterin (and hence for dihydrofolate) are produced.

The reason for the biphasic nature of the curves in Figure 7A–C is not clear, but one possibility is that the preparation contains at least two forms of the enzyme, which differ in affinity for amethopterin (see Discussion section). If so, activation by KCl does not appear to bring about any interconversion of these forms, since the fraction showing stoichiometric inhibition (about 50%) was approximately the same for both the control and activated enzymes.

Assuming one inhibitor binding site per molecule of enzyme and a molecular weight of about 20,000 for the protein, the common extrapolation point on the abscissa of Figure 7A was used to calculate approximate turnover numbers of 120 and 520 (moles of substrate reduced per minute per mole of enzyme) for the enzyme in the absence and presence of KCl, respectively. Values in the range of 250–350 have been reported previously for the nonactivated enzyme from mammalian (Schrecker and Huennekens, 1964; Bertino *et al.*, 1965) and avian sources (Kaufman and Gardiner, 1966). Conversely, the enzyme from *L. leichmanii* (Kessel and Roberts, 1965) exhibits a turnover number of 1180.

Discussion

The present study demonstrates that dihydrofolate reductase from L1210 cells, like its counterpart from most other tissues (Huennekens, 1967), can be stimulated three- to fivefold by anions, cations, and urea. Activation is rapid and reversible and is generally followed by inactivation at higher concentrations of the agent. In the activated state the enzyme displays a single, broad optimum at pH 7, in contrast to the double optimum (pH 4.5 and 7.0) seen in the absence of salts or urea. Activation does not appreciably change the molecular weight of the protein, thereby ruling out mechanisms involving subunits, nor does it increase the apparent number of binding sites for either of the substrates, TPNH or dihydrofolate. The increased turnover number of the activated enzyme seems to be due, therefore, to one or both of the following possibilities: (1) an increased affinity of the binding sites for the substrates;[6] or (2) a more favorable spatial relationship between the binding sites that facilitates transfer of the hydride ion from TPNH to dihydrofolate. In either instance, the activating agent is envisioned as inducing some type of conformational change in the protein. The same conclusion has been reached previously during studies on inhibition (Perkins and Bertino, 1964), sedimentation (Kaufman, 1964), and gel filtration (Perkins and Bertino, 1965) of the enzyme from other sources.

Urea is generally believed to alter the tertiary structure

[6] Based upon studies of protein fluorescence, there appears to be no change in binding affinity of the protein (at least for the chicken liver enzyme) for substrates following activation by urea or mercurials (G. P. Mell and F. M. Huennekens, unpublished experiments).

of proteins by breaking hydrogen bonds, but the mechanism by which salts induce conformational changes is not entirely clear. von Hipple and Wong (1964) have pointed out that relatively high concentrations (*i.e.*, in the range of 0.1–1.0 M) of the salts can disrupt the organized structure of both proteins and nucleic acids, thereby suggesting a common mechanism of this chaotropic effect (Hamaguchi and Guiduschek, 1962) toward macromolecules. The disruptive ability of anions increases as follows: $CH_3COO^- < Cl^- < Br^- < ClO_4^- < CNS^-$; while for cations the usual order is: $(CH_3)_4N^+ < NH_4^+, K^+, Na^+ < Li^+$. These sequences are similar to the Hofmeister series that relates to the ability of ions to affect such properties as solubility, viscosity, optical rotation, and melting temperature of molecules.

Alternatively, Robinson and Jencks (1965a,b) studied the effect of salts on the solubility of a model peptide and concluded that the solubility of proteins, as well as their denaturation and dissociation into subunits, can be explained largely by the ability of salts to alter the activity coefficient of amide groups. These authors also suggested that urea and guanidinium compounds disrupt protein structure by the same mechanism. Other possibilities, such as electrostatic effects, displacement of bound water, or effects on the "structure" or internal pressure of water were considered to be less likely on theoretical grounds. Still other hypotheses include a proposal that ions alter the distribution of water molecules around nonpolar residues on the protein (von Hipple and Wong, 1965), or that, in addition to a direct interaction with the protein, ions also exert an effect on the solvent (Bello *et al.*, 1966).

In the present investigation, there was no correlation between the degree of activation of dihydrofolate reductase by cations and their position in the Hofmeister series. Thus, Li^+ was not appreciably different than NH_4^+, Na^+, or K^+ (Figure 1), whereas it might have been expected to be the most effective activator. On the other hand, the monovalent anions displayed large differences in their activation–concentration curves (Figure 2), in agreement with other observations that anions, rather than cations, are usually responsible for the effects of salts on proteins (Robinson and Jencks, 1965b; Warren *et al.*, 1966).

Activation of the L1210 reductase by Cl^-, Br^-, or SCN^- is complicated by the fact that higher concentrations of these ions also cause inactivation. In the case of SCN^-, the concentration at which inactivation begins overlaps the concentration range for activation; for this reason, the full fivefold activation is never seen with this ion. In fact, all activators of the salt or urea type produce the same pattern (normal enzyme → active enzyme → inactive enzyme), but the concentrations required for the two transitions differ widely.

Any given dihydrofolate reductase can probably assume several different activated states. Thus, mercurials, iodine, and DTNB (Reyes and Huennekens, 1967) interact stoichiometrically and irreversibly with one or more of the cysteine residues in the protein to produce an activated enzyme containing the covalently bound activator. Salts and urea, which activate reversibly, are effective only at relatively high concentrations. In all cases, however, activation appears to involve conformational changes in the protein,[7] and, since the activated enzymes are more susceptible to proteolytic digestion or to heat denaturation (Mell *et al.*, 1966), it is likely the transition is from a compact to an unfolded form, rather than *vice versa*.

Although the above statements are generally applicable to various dihydrofolate reductases, all of the known enzymes are not uniform in their susceptibility toward different activators (Huennekens, 1967). For example, the chicken liver reductase is activated by mercurials, iodine, and urea but not by salts, while the *L. leichmanii* enzyme appears to be fully activated when isolated and is not stimulated further by any of the above agents.

There is a growing body of evidence that dihydrofolate reductase may also exist in several forms in the normal state. Thus, Hillcoat and Blakley (1966) have encountered two forms of the enzyme from a mutant of *Streptococcus faecalis*, and, similarly, Sirotnak and Hutchison (1966) have observed two separate peaks of activity when aged preparations of the enzyme from *Diplococcus pneumoniae* are filtered through Sephadex. Amethopterin titration curves (*cf.* Figure 7A) also suggest the occurrence of multiple forms of the enzyme. Also, it has been observed occasionally, but not always, during the present investigation, that chromatography of the L1210 enzyme on hydroxylapatite yields separate peaks of enzyme activity. Similar observations[8] have been made when the chicken liver reductase is chromatographed on hydroxylapatite or AE-cellulose (Mell *et al.*, 1966). Studies are in progress to determine whether these forms of the enzyme are similar to the "conformers" of malate dehydrogenase (Kitto *et al.*, 1966) and, if so, whether they are naturally occurring or created artificially during the isolation procedures.

Acknowledgment

The authors are indebted to Mrs. Patricia Rowe for technical assistance and to Drs. G. P. Mell and J. H. Freisheim for helpful discussions of this problem.

References

Atkinson, D. E., Hathaway, J. A., and Smith, E. C. (1965), *J. Biol. Chem. 240*, 2682.

Bauerle, R. H., Freundlich, M., Stormer, F. G., and Umbarger, H. E. (1964), *Biochim. Biophys. Acta 92*, 142.

[7] Fluorescence measurements of the protein (see footnote 6), as well as previous results of Kaufman (1964) and Perkins and Bertino (1964, 1965) in which varying degrees of activation were produced by different mercurials, suggest the existence of an array of activated states.

[8] Following electrophoresis of reductase preparations on starch gel, separate zones of enzyme activity can be seen (unpublished experiments of G. P. Mell, P. Reyes, J. Kirchner, P. Rowe, and F. M. Huennekens).

Bello, J., Haas, D., and Bello, H. R. (1966), *Biochemistry 5*, 2539.
Bertino, J. R. (1962), *Biochim. Biophys. Acta 58*, 377.
Bertino, J. R., Donohue, D. M., Simmons, B., Gabrio, B. W., Silber, R., and Huennekens, F. M. (1963), *J. Clin. Invest. 42*, 466.
Bertino, J. R., Perkins, J. P., and Johns, D. G. (1965), *Biochemistry 4*, 839.
Blakley, R. L. (1960), *Nature 188*, 231.
Changeux, J.-P. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 313.
Changeux, J.-P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 497.
Dixon, M., and Webb, E. C. (1960), Enzymes, New York, N. Y., Academic, p 21.
Futterman, S. (1957), *J. Biol. Chem. 228*, 1031.
Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.
Goldstein, A. (1944), *J. Gen. Physiol. 27*, 529.
Hamaguchi, K., and Guiduschek, E. P. (1962), *J. Am. Chem. Soc. 84*, 1329.
Hillcoat, B. L., and Blakley, R. L. (1966), *J. Biol. Chem. 241*, 2995.
Huennekens, F. M. (1967), *in* Biological Oxidations, Singer, T. P., Ed., New York, N. Y., Wiley.
Ishimoto, N., Temin, H. M., and Strominger, J. L. (1966), *J. Biol. Chem. 241*, 2052.
Jensen, R. A., and Nester, E. W. (1966), *J. Biol. Chem. 241*, 2052.
Kaufman, B. T. (1963), *Biochem. Biophys. Res. Commun. 10*, 449.
Kaufman, B. T. (1964), *J. Biol. Chem. 239*, PC669.
Kaufman, B. T. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 695.
Kaufman, B. T., and Gardiner, R. C. (1966), *J. Biol. Chem. 241*, 1319.
Keilin, D., and Hartree, E. F. (1938), *Proc. Roy. Soc. (London)B124*, 397.
Kessel, D., and Roberts, D. (1965), *Biochemistry 4*, 2631.
Kitto, G. B., Wassarman, P. M., and Kaplan, N. O. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 578.
Kwon, T.-W., and Brown, W. D. (1966), *J. Biol. Chem. 241*, 1509.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mathews, C. K., and Huennekens, F. M. (1963), *J. Biol. Chem. 238*, 3436.
Mell, G. P., Schroeder, G. H., and Huennekens, F. M. (1966), *Federation Proc. 25*, 277.
Misra, D. K., and Adamson, R. H. (1963), *Life Sci. 2*, 858.
Morales, D. R., and Greenberg, D. M. (1964), *Biochim. Biophys. Acta 85*, 360.
Osborn, M. J., Freeman, M., and Huennekens, F. M. (1958), *Proc. Soc. Exptl. Biol. Med. 97*, 429.
Osborn, M. J., and Huennekens, F. M. (1958), *J. Biol. Chem. 233*, 969.
Patte, J. C., LeBras, G., Loviny, T., and Cohen, G. N. (1963), *Biochim. Biophys. Acta 67*, 16.
Perkins, J. P., and Bertino, J. R. (1964), *Biochem. Biophys. Res. Commun. 15*, 121.
Perkins, J. P., and Bertino, J. R. (1965), *Biochemistry 4*, 847.
Perkins, J. P., and Bertino, J. R. (1966), *Biochemistry 5*, 1005.
Preiss, J., Shen, L., Greenberg, E., and Gentner, N. (1966), *Biochemistry 5*, 1833.
Reyes, P., and Huennekens, F. M. (1967), *Biochem. Biophys. Res. Commun. 28*, 833.
Robinson, D. R., and Jencks, W. P. (1965a), *J. Am. Chem. Soc. 87*, 2462.
Robinson, D. R., and Jencks, W. P. (1965b), *J. Am. Chem. Soc. 87*, 2470.
Schrecker, A. W., and Huennekens, F. M. (1964), *Biochem. Pharmacol. 13*, 731.
Silber, R., Huennekens, F. M., and Gabrio, B. W. (1963), *Arch. Biochem. Biophys. 100*, 525.
Sirotnak, F. M., and Hutchison, D. J. (1966), *J. Biol. Chem. 241*, 2900.
Straus, O. H., and Goldstein, A. (1943), *J. Gen. Physiol. 26*, 559.
Taketa, K., and Pogell, B. M. (1965), *J. Biol. Chem. 240*, 651.
Tiselius, A., Hjerten, S., and Levin, O. (1956), *Arch. Biochem. Biophys. 65*, 132.
von Hipple, P. H., and Wong, K.-Y. (1964), *Science 145*, 577.
von Hipple, P. H., and Wong, K.-Y. (1965), *J. Biol. Chem. 240*, 3909.
Warren, J. C., Stowring, L., and Morales, M. F. (1966), *J. Biol. Chem. 241*, 309.
Werkheiser, W. C. (1961), *J. Biol. Chem. 236*, 888.
Whitaker, J. R. (1963), *Anal. Chem. 35*, 1950.