Inhibitory Effects of Histidine and their Reversal

THE ROLES OF PYRUVATE CARBOXYLASE AND N^{10} -FORMYLTETRAHYDROFOLATE DEHYDROGENASE

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1. N^{10} -Formyltetrahydrofolate dehydrogenase was purified to homogeneity from rat liver with a specific activity of 0.7–0.8 unit/mg at 25°C. The enzyme is a tetramer ($\bar{M}_w = 413\,000$) composed of four similar, if not identical, subunits ($\bar{M}_w = 108\,000$). 2. Initial-rate studies indicate a sequential mechanism of substrate addition and give the K_m values as $4.5\,\mu\text{M}$ [$(-)-N^{10}$ -formyltetrahydrofolate] and $0.92\,\mu\text{M}$ (NADP^+) at pH 7.0. Tetrahydrofolate acts as a potent product inhibitor [$K_i = 7\,\mu\text{M}$ for the $(-)$ -isomer] which is competitive with respect to N^{10} -formyltetrahydrofolate and non-competitive with respect to NADP^+ . 3. Product inhibition by NADPH could not be demonstrated. This coenzyme activates N^{10} -formyltetrahydrofolate dehydrogenase when added at concentrations, and in a ratio with NADP^+ , consistent with those present in rat liver *in vivo*. No effect of methionine, ethionine or their *S*-adenosyl derivatives could be demonstrated on the activity of the enzyme. 4. Hydrolysis of N^{10} -formyltetrahydrofolate is catalysed by rat liver N^{10} -formyltetrahydrofolate dehydrogenase at 21 % of the rate of CO_2 formation based on comparison of apparent $V_{\max}$ values. The K_m for $(-)-N^{10}$ -formyltetrahydrofolate in the hydrolytic reaction is $23.8\,\mu\text{M}$. Tetrahydrofolate is a non-competitive inhibitor of this reaction with respect to N^{10} -formyltetrahydrofolate, with a mean K_i of $21.5\,\mu\text{M}$ for the $(-)$ -isomer. NAD^+ increases the maximal rate of N^{10} -formyltetrahydrofolate hydrolysis without affecting the K_m for this substrate and decreases inhibition by tetrahydrofolate. The activator constant for NAD^+ is obtained as $0.35\,\text{mM}$. 5. Formiminoglutamate, a product of liver histidine metabolism which accumulates in conditions of excess histidine load, is a potent inhibitor of rat liver pyruvate carboxylase, with 50 % inhibition being observed at a concentration of $2.8\,\text{mM}$, but has no detectable effect on the activity of rat liver cytosol phosphoenolpyruvate carboxykinase measured in the direction of oxaloacetate synthesis. We propose that the observed inhibition of pyruvate carboxylase by formiminoglutamate may account in part for the toxic effect of excess histidine.

Early nutritional studies, which established the necessity for provision of histidine in the normal mammalian diet under certain conditions, also demonstrated that this amino acid is toxic if added in an amount greater than that required for adequate nutrition. This toxic effect of excess histidine can be reversed by concurrent administration of methionine (Greenstein & Winitz, 1961; Harper, 1964). A possible component of this toxic effect was identified when it was shown that histidine, although itself glucogenic (Dakin, 1912), formed no glucose and in fact caused marked inhibition of gluconeogenesis from lactate,

propionate and glutamine in the perfused rat liver (Cornell *et al.*, 1974; Krebs *et al.*, 1976). The properties of the inhibition suggested that a metabolite derived from histidine, rather than the amino acid itself, gave rise to this effect, and it has been demonstrated that formiminoglutamate accumulates in the liver during perfusion with histidine (Tabor & Mehler, 1955; Buehring *et al.*, 1972). Furthermore, both the inhibition of gluconeogenesis and, to a lesser extent, the accumulation of formiminoglutamate could be reversed if methionine was added to the perfusion system. The studies by Buehring *et al.* (1972) indicate that accumulation of formiminoglutamate in livers perfused with histidine and the reversal of this accumulation by addition of methionine to the perfusate can be attributed to changes in the concentration of intermediates in the C_1 pool and

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in particular may be related to the availability of tetrahydrofolate (cf. Scheme 1). Furthermore, Krebs and his co-workers (Krebs *et al.*, 1976) have shown that addition of low concentrations of methionine or ethionine also accelerates the oxidation of formate by isolated hepatocytes and have established the relationship of this effect to that previously observed on the metabolism of histidine.

However, neither the inhibition of gluconeogenesis

by histidine or its metabolites, nor the activation of histidine and formate metabolism by methionine, has been studied at the enzymic level. We have therefore examined the properties of pyruvate carboxylase (EC 6.4.1.1) and *N*¹⁰-formyltetrahydrofolate dehydrogenase (EC 1.5.1.6) which may be important to the effects observed in the isolated tissue preparations. In addition, since little information is currently available on *N*¹⁰-formyltetrahydrofolate dehydro-

Scheme 1. *Interrelationships between histidine and methionine metabolism*

The dotted line and the symbol $\ominus$ indicates regulatory inhibition. The broken line indicates a minor pathway.

genase from mammalian tissues we have purified and partially characterized this enzyme from rat liver.

Methods and Materials

N^5N^{10} -Methenyltetrahydrofolate was synthesized from formic acid and tetrahydrofolate and purified as described by Huennekens *et al.* (1963). The purity of the N^5N^{10} -methenyltetrahydrofolate preparations was established from the ratio of A_{348} to A_{305} , and by paper chromatography, as described by Huennekens *et al.* (1963). N^5N^{10} -Methenyltetrahydrofolate (solid) was stored under N_2 . It was converted into N^{10} -formyltetrahydrofolate as described by Rabinowitz (1963). The working solutions of both N^{10} -formyltetrahydrofolate and tetrahydrofolate were prepared in 0.1M-2-mercaptoethanol and were kept frozen at -10°C in tubes sealed with serum stoppers and covered with aluminium foil. These solutions were gassed at least once a week with O_2 -free N_2 . Such precautions minimized the extent of oxidation of these labile substrates.

N^{10} -Formyltetrahydrofolate dehydrogenase was routinely assayed by measurement of the increase in A_{300} . The assay system contained, in 0.5ml, 50mM-Tris/HCl, pH 7.7, 100mM-2-mercaptoethanol, 0.07mM- N^{10} -formyltetrahydrofolate and 0.2mM- NADP^+ . In order to obtain true initial rates, measurements were made with a model 240 automatic recording spectrophotometer (Gilford Instruments, Oberlin, OH, U.S.A.) equipped with an Omniscribe recorder (Houston Instruments, Bellaire, TX, U.S.A.), which was calibrated such that full-scale recorder deflection corresponded to 0.2 absorbance unit. Control experiments demonstrated that initial rates determined by this assay system did not differ significantly from those obtained by the system described by Kutzbach & Stokstad (1971) in which accumulation of tetrahydrofolate is prevented by addition of N^{10} -formyltetrahydrofolate synthetase. Hydrolysis of N^{10} -formyltetrahydrofolate by N^{10} -formyltetrahydrofolate dehydrogenase was routinely assayed in a similar system but in the absence of NADP^+ .

N^5N^{10} -Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) was assayed in the direction of N^5N^{10} -methenyltetrahydrofolate production by the discontinuous procedure described by Scrimgeour & Huennekens (1963) and with NADP^+ as electron acceptor. This enzyme was assayed in the direction of N^5N^{10} -methylenetetrahydrofolate formation as described by Osborn & Huennekens (1957).

N^5N^{10} -Methenyltetrahydrofolate cyclohydrolase was assayed as described by Greenberg (1971).

Pyruvate carboxylase from rat liver was purified and assayed as described previously (Scrutton *et al.*, 1969; Scrutton & White, 1974). Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) from rat liver

cytosol, which was a gift from Dr. V. L. Schramm, Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA, U.S.A., was assayed in the direction of oxaloacetate synthesis as described by Lane *et al.* (1969). In all cases, enzyme units are expressed as μmol of substrate used (or product formed)/min.

Protein was determined from the A_{280} by assuming $A_{0.1\%}$ at 280nm = 1.0, or by the biuret procedure (Layne, 1957).

Formate was measured by using N^{10} -formyltetrahydrofolate synthetase (EC 6.3.4.3) (a gift from Professor R. H. Himes, Department of Biochemistry, University of Kansas, Lawrence, KS, U.S.A.) as described by Rabinowitz & Pricer (1963).

Blue Dextran-Sephadose 4B was prepared as described by Ryan & Vestling (1974).

Polyacrylamide gels were prepared as described by Gabriel (1971) by using System II and a 3.75–7.5% polyacrylamide gel. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969). After staining for protein with Coomassie Blue (Weber & Osborn, 1969), the destained gels were scanned at 660nm by using a model 240 automatic recording spectrophotometer equipped with a model 2410S linear transport (Gilford Instruments).

Reacting-enzyme-sedimentation studies were performed essentially as described by Taylor *et al.* (1972), but by using the assay system for NADP^+ -dependent N^{10} -formyltetrahydrofolate dehydrogenase activity described above and monitoring the change in A_{300} .

NADP^+ , NAD^+ , NADPH , phosphoenolpyruvate, 2-mercaptoethanol and tetrahydrofolate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.); DEAE-Sephadex A-50 (Cl^- form), Sephadex G-200 and Sepharose 4B were from Pharmacia (London W5 5SS, U.K.). Formiminoglutamate (Na^+ salt) was a kind gift from Mr. R. Hems (Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, U.K.). All other chemicals were prepared or obtained as described previously (Scrutton *et al.*, 1969).

Results

Distribution of some enzymes of C_1 metabolism in rat tissues

Since little information appears to be available on the distribution and amounts of the enzymes of C_1 metabolism in rat tissues (but cf. Lepage *et al.*, 1972), we have measured the maximal activities of three of these enzymes in four different tissues. The results (Table 1) indicate that although significant activities of N^5N^{10} -methylenetetrahydrofolate dehydrogenase and N^5N^{10} -methenyltetrahydrofolate cyclohydrolase are present in all tissues examined, only the liver, and

to a lesser extent the kidney, homogenates exhibit N^{10} -formyltetrahydrofolate dehydrogenase activity, which, even in these tissues, is present in an amount at least an order of magnitude lower than that observed for the other two enzymes.

Purification of N^{10} -formyltetrahydrofolate dehydrogenase from rat liver

All operations were performed at 0–5°C unless otherwise stated.

Rat livers were chopped and homogenized in 5 vol. of 0.3M-sucrose containing 0.05M-potassium phosphate, pH 7.2, 1.0mM-EDTA, 0.1mM-dithioerythritol and 10 μ M-phenylmethanesulphonyl fluoride. Homogenization was performed in a Waring Blendor run at maximal speed for 60s. The pH of the homogenate was adjusted to 6.0 by addition of 4M-acetic acid and the precipitate removed by centrifugation at 45000g for 20min. The pH of the supernatant fraction was then adjusted to 7.0 by addition of 1M-Tris base.

Solid $(\text{NH}_4)_2\text{SO}_4$ (14.4g/100ml) was added slowly to the supernatant fraction with constant stirring to give a final concentration of 25% saturation. The pH was maintained at 7.0 by addition of 1M-Tris base. The suspension was stirred for a further 15min and the precipitate was then removed by centrifugation at 45000g for 15min. Solid $(\text{NH}_4)_2\text{SO}_4$ (15.8g/100ml) was added slowly to the supernatant fraction with constant stirring to give a final concentration of 50% saturation. The pH was maintained, the suspension stirred and the precipitate collected as above. The supernatant fraction was discarded.

The 25–50%-saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in a minimal volume of 0.05M-potassium phosphate, pH 7.2, containing 1mM-EDTA, 0.1mM-dithioerythritol and 10 μ M-phenylmethanesulphonyl fluoride and excess salt was removed by passage over a column (35cm $\times$ 2cm) of Sephadex G-25 equilibrated with this buffer.

The desalted enzyme preparation was applied to a column (30cm $\times$ 2cm) of DEAE-Sephadex A-50 (Cl^- form), which had previously been equilibrated with the same buffer, and the column was washed with this buffer until A_{280} of the effluent decreased to less than 0.1 A unit. The column was further washed with the same phosphate buffer containing in addition 0.1M-KCl until the effluent A_{280} again decreased to 0.1 A unit and was then eluted by application of a 500ml linear gradient of 0.1–0.5M-KCl in this phosphate buffer. Fractions (5ml) were collected and were monitored for protein (A_{280}) and enzymic activity. A typical elution profile is shown in Fig. 1. N^{10} -Formyltetrahydrofolate dehydrogenase is eluted as a single sharp peak at approx. 0.3M-KCl. The most active fractions were pooled as shown on Fig. 1 and the protein was precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ (31.3g/100ml) with the pH maintained at 7.0–7.2 by addition of 1M-Tris base. After stirring for 15min the precipitate was collected and was dissolved in a minimal volume of 0.01M-potassium phosphate, pH 7.2, containing EDTA, dithioerythritol and phenylmethanesulphonyl fluoride as described above. Alternatively, the enzyme may be stored for several weeks at –70°C at this degree of purity if the buffer used to dissolve the precipitate contains 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ and 20% (v/v) glycerol.

Table 1. Distribution of N^{10} -formyltetrahydrofolate dehydrogenase, N^5N^{10} -methylenetetrahydrofolate dehydrogenase and N^5N^{10} -methylenetetrahydrofolate cyclohydrolase in rat tissues

Tissues homogenates (1:10) were prepared in 0.3M-sucrose containing 0.05M-sodium/potassium phosphate, pH 7.6, 1.0mM-EDTA and 0.1mM-dithioerythritol using an all-glass hand homogenizer. Enzymic activities were measured at 25°C as described in the Methods and Materials section. Activities are presented as means $\pm$ S.E.M. with the numbers of samples given in parentheses. Each sample for a given tissue was obtained from a different animal. N.D., Not detectable (< 0.02 unit/g).

Tissue	Activity (units/g)		
	N^5N^{10} -Methylenetetrahydrofolate cyclohydrolase	N^{10} -Formyltetrahydrofolate dehydrogenase*	N^5N^{10} -Methylenetetrahydrofolate dehydrogenase†
Liver	3.5 $\pm$ 0.3 (5)	0.24 $\pm$ 0.02 (5) [0.26 $\pm$ 0.04 (3)]	4.6 $\pm$ 0.6 (5) [0.52 $\pm$ 0.05 (3)]
Kidney	3.8 $\pm$ 0.4 (5)	0.09 $\pm$ 0.02 (5) [0.09 $\pm$ 0.03 (3)]	4.3 $\pm$ 0.4 (5) [0.57 $\pm$ 0.07 (5)]
Adipose tissue	1.1 $\pm$ 0.3 (5)	N.D.	0.6 $\pm$ 0.1 (5)
Intestine	2.0 $\pm$ 0.2 (4)	N.D.	1.8 $\pm$ 0.2 (4)

* Values in square brackets indicate activity observed after 72h starvation. At 37°C the activity of N^{10} -formyltetrahydrofolate dehydrogenase is approx. twice that at 25°C.

† Values in square brackets indicate activities observed when reaction is performed in the direction of N^5N^{10} -methylenetetrahydrofolate formation.

Fig. 1. Chromatography of rat liver N^{10} -formyltetrahydrofolate dehydrogenase on DEAE-Sephadex A-50 (Cl^- form). The column was prepared, loaded with enzyme (Stage II; 15.0 units; specific activity = 0.078 unit/mg) and eluted as described in the text. Fractions were assayed for enzymic activity (■) and protein (A_{280}) (●) as described in the Methods and Materials section. —, Concentration of KCl in the eluting buffer. The fractions pooled for subsequent addition of $(NH_4)_2SO_4$ are indicated by the horizontal double-headed arrow.

Excess salt was removed by passage over a Sephadex G-25 column (30 cm $\times$ 2 cm), but with the column equilibrated with 0.01 M-phosphate, pH 7.2, containing EDTA, dithioerythritol and phenylmethanesulphonyl fluoride as described above. The desalted enzyme was applied to a column (10 cm $\times$ 1 cm) of Blue Dextran-Sephadex 4B equilibrated with this buffer. The column was washed with the same buffer and then with buffer containing 0.1 M-KCl until in each case the A_{280} of the effluent decreased to 0.05 A unit. The column was then eluted by application of a 100 ml linear gradient of 0.1–0.4 M-KCl in the same phosphate buffer. Fractions (2.5 ml) were collected and monitored for protein and enzymic activity. A typical profile is shown in Fig. 2. N^{10} -Formyltetrahydrofolate dehydrogenase activity is eluted as a single symmetrical peak at approx. 0.17 M-KCl, coincident with the major protein peak. Except at the leading and trailing edges the specific activity is constant across the peak. The most active fractions were pooled as shown in Fig. 2 and the protein was precipitated by addition of solid $(NH_4)_2SO_4$ to a final concentration of 50% saturation with the pH main-

tained as above. The precipitate was collected by centrifugation as described above and was dissolved in a minimal volume of 0.05 M-phosphate, pH 7.2, containing 50%-satd. $(NH_4)_2SO_4$, 20% glycerol, 1 mM-EDTA, 0.1 mM-dithioerythritol and 10 μ M-phenylmethanesulphonyl fluoride. If stored frozen at $-70^\circ C$, the enzyme appears stable for several months in this form.

Table 2 summarizes the results of a typical purification by this procedure, which gives a 250-fold purification from the pH 6.0 supernatant fraction with a 33% overall yield. The final specific activity achieved is similar to that obtained for purification of this enzyme from pig liver by Kutzbach & Stokstad (1971), but the procedure is simpler, avoids the potentially denaturing condition of acetone precipitation and gives a better yield. It is notable that a salt gradient was used to elute the Blue Dextran-Sephadex column rather than NADH or NADPH as has been used for many other dehydrogenases (cf. Ryan & Vestling, 1974). For N^{10} -formyltetrahydrofolate dehydrogenase we have observed that NADH is ineffective and tetrahydrofolate is only partially effective

Fig. 2. Chromatography of rat liver N^{10} -formyltetrahydrofolate dehydrogenase on Blue Dextran-Sephadose 4B. The column was prepared, loaded with enzyme (Stage III; 6.8 units; specific activity = 0.16 unit/mg) and eluted as described in the text. Fractions were assayed for protein (A_{280}) (●) and enzymic activity (■) as described in the Methods and Materials section. The broken line indicates the concentration of KCl in the eluting buffer. The fractions pooled for subsequent addition of $(\text{NH}_4)_2\text{SO}_4$ are indicated by the horizontal double-headed arrow.

Table 2. Summary of purification of N^{10} -formyltetrahydrofolate dehydrogenase from rat liver

Stage	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (unit/mg)	Recovery (%)
I. pH 6.0 supernatant*	255	13.7	5610	0.0028	100
II. 25–50% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate	20	14.2	1745	0.0081	103
III. DEAE-Sephadex (Cl^- form) chromatography (pooled fractions)	86	7.4	36	0.204	54
IV. Blue Dextran-Sephadose chromatography (pooled fractions)	27	4.5	6.3	0.71	33

* From 40g of rat liver.

tive in eluting this enzyme. The enzyme can be eluted from Blue Dextran-Sephadose by high concentrations of NADPH (0.5–1.0 mM) as effectively as by 0.2 M-KCl, but the final specific activity obtained (0.76–0.81 unit/mg) is not significantly increased by use of the specific ligand. It is also of interest that treatment of the column with a concentration of KCl that does not itself elute N^{10} -formyltetrahydrofolate dehydro-

genase prevents the subsequent elution with NADPH, although KCl itself is still effective at a higher concentration (Fig. 2).

Molecular properties of rat liver N^{10} -formyltetrahydrofolate dehydrogenase

The homogeneity of Stage-IV preparations of N^{10} -formyltetrahydrofolate dehydrogenase from rat

liver was examined by polyacrylamide-gel electrophoresis in the absence or presence of sodium dodecyl sulphate. Under both conditions a single sharp band is observed when the gels are stained for protein after electrophoresis (Figs. 3 and 4). This band in both cases accounts for at least 95% of the absorbance when the stained gels are scanned as described in the Methods and Materials section, suggesting that an essentially homogeneous preparation of the dehydrogenase is obtained by the purification procedure described here.

Comparison of the extent of migration of N^{10} -formyltetrahydrofolate dehydrogenase on the polyacrylamide gels in the presence of sodium dodecyl sulphate with that observed for six proteins of known subunit molecular weight allows estimation of the subunit molecular weight of rat liver N^{10} -formyltetrahydrofolate dehydrogenase as $108\,000 \pm 4\,000$ (Fig. 3 inset).

The molecular weight of the intact enzyme has been obtained by estimation of $s_{20,w}$ by reacting-enzyme-sedimentation analysis (Taylor *et al.*, 1972) and of the Stokes radius (and hence $D_{20,w}^0$) by calibrated gel filtration (Ackers, 1964). Reacting-enzyme-sedimentation analysis gave a linear plot of $\log r$ versus t for the single boundary observed. The sedimentation coefficient was obtained as 14.2S and is equivalent to $s_{20,w}^0$ owing to the very low protein concentration used ($< 50 \mu\text{g/ml}$). Calibrated gel filtration on Sephadex G-200 gave the Stokes radius of N^{10} -formyltetrahydrofolate dehydrogenase from rat liver as $6.40 \pm 0.05 \text{ nm}$ over a range of protein concentration from 0.1 to 1.0 mg/ml (Fig. 4 inset). No consistent variation of Stokes radius with protein concentration was observed, and hence $D_{20,w}^0$ can be estimated as $3.35 \times 10^{-7} \text{ s}^{-1}$. From these values for $s_{20,w}^0$ and $D_{20,w}^0$ the molecular weight of N^{10} -formyltetrahydrofolate dehydrogenase can be calculated as 413 000, if a value of 0.75 ml/g is assigned for the partial specific volume.

Kinetic properties of the NADP⁺-dependent reaction catalysed by N^{10} -formyltetrahydrofolate dehydrogenase from rat liver

Initial-rate studies as a function of substrate concentration showed that linear Lineweaver-Burk plots were obtained which gave values for the apparent K_m values at pH 7.0 as $12.0 \mu\text{M}$ for $(\pm)$ - N^{10} -formyltetrahydrofolate and $1.0 \mu\text{M}$ for NADP⁺. The apparent K_m for $(\pm)$ - N^{10} -formyltetrahydrofolate showed no consistent variation with pH over the range 6.0–8.0 and the mean value was $10.4 \pm 2.0 \mu\text{M}$ ($\pm \text{S.E.M.}$, $n = 6$). Neither the K_m for this substrate nor that for NADP⁺ was significantly affected by inclusion of 100 mM-KCl in the assay system. Since Kutzbach & Stokstad (1971) have reported that only the (–)-isomer of N^{10} -formyltetrahydrofolate is active as a substrate for pig liver N^{10} -formyltetrahydrofolate dehydrogenase, the cor-

rected value for the rat liver enzyme ($6.0 \mu\text{M}$) is in the same range as that observed in the earlier study on the

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified rat liver N^{10} -formyltetrahydrofolate dehydrogenase and determination of subunit molecular weight

Sodium dodecyl sulphate/polyacrylamide gels ($6 \text{ cm} \times 0.5 \text{ cm}$) were prepared as described in the Methods and Materials section and were loaded with either 5–20 μg of Stage-IV N^{10} -formyltetrahydrofolate dehydrogenase (the trace shown is for the gel loaded with 20 μg) or molecular-weight markers. Electrophoresis was performed at 5 mA/tube until the tracking dye (Pyronin Y) approached the bottom of the gel. The gels were stained for protein by incubation overnight in 0.04% Coomassie Blue in 25% (v/v) propan-2-ol/10% (v/v) acetic acid. The gels were destained initially in 25% (v/v) propan-2-ol/10% (v/v) acetic acid and subsequently in 10% (v/v) acetic acid. The destained gels were scanned as described in the Methods and Materials section. The inset shows the determination of subunit molecular weight by the method of Weber & Osborn (1969). The standard proteins are: A, β -galactosidase; B, bovine serum albumin; C, liver glutamate dehydrogenase; D, alcohol dehydrogenase; E, chymotrypsinogen; F, cytochrome c. The double-headed arrow indicates the range of positions of migration observed for four different preparations of N^{10} -formyltetrahydrofolate dehydrogenase.

Fig. 4. Polyacrylamide-gel electrophoresis of purified rat liver N^{10} -formyltetrahydrofolate dehydrogenase and determination of Stokes radius by calibrated gel filtration on Sephadex G-200

Polyacrylamide gels (5%) (6cm $\times$ 0.5cm) were prepared as described in the Methods and Materials section. Stage-IV N^{10} -formyltetrahydrofolate dehydrogenase (12 μ g; specific activity = 0.75 unit/mg) was loaded on to the gels and electrophoresis performed for 4 h at 5°C. The gels were stained, destained and scanned as described for Fig. 3. Calibrated gel filtration was performed at 5°C on a column (25cm $\times$ 2cm) of Sephadex G-200 equilibrated with 0.01 M-potassium phosphate, pH 7.2, containing 0.1 M-KCl, 1 mM-EDTA, 0.1 mM-dithioerythritol and 10 μ M-phenylmethanesulphonyl fluoride. The void volume (V_0) determined with Blue Dextran 2000 was 22.5 ml. The column was calibrated by passage of proteins of known Stokes radius, which are indicated in the inset as: A, β -galactosidase; B, muscle pyruvate kinase; C, bovine heart lactate dehydrogenase; D, bovine serum albumin; E, ovalbumin. In all cases, after passage of the void volume the eluate was collected as approx. 1 ml fractions in tared tubes and the precise eluate volume determined from the increment in weight. The distribution of N^{10} -formyltetrahydrofolate dehydrogenase in the fractions was determined by assay of enzymic activity as described in the Methods and Materials section. In the inset the mean elution volume observed for runs at four different protein concentrations is shown by an arrow. The

pig liver enzyme (8.2 μ M) (Kutzbach & Stokstad, 1971).

More detailed analyses have been performed by measurement of initial rate as a function of [N^{10} -formyltetrahydrofolate] at a series of fixed concentrations of $NADP^+$ and vice versa. These data, illustrated in part in Fig. 5, demonstrate that rat liver N^{10} -formyltetrahydrofolate dehydrogenase obeys a sequential order of substrate addition, since both slope and intercept are a function of the fixed substrate concentration. Both the primary double-reciprocal plots and the secondary slope and intercept plots are linear, and the latter give values for the true K_m values as 4.5 μ M [$(-)$ - N^{10} -formyltetrahydrofolate] and 0.92 μ M ($NADP^+$) respectively.

In an attempt to elucidate the order of substrate addition, product-inhibition studies were performed. With tetrahydrofolate as product inhibitor competi-

Fig. 5. Double-reciprocal plot of initial rate against N^{10} -formyltetrahydrofolate concentration at a series of different concentrations of $NADP^+$

The assay system contained, in 0.5 ml, 100 mM-Tris/HCl, pH 7.7, 100 mM-2-mercaptoethanol, and the concentrations of $(\pm)\text{-}N^{10}$ -formyltetrahydrofolate as indicated. The concentrations of $NADP^+$ used were 1.0 μ M ($\square$), 2.0 μ M ($\circ$), 2.5 μ M ($\blacksquare$), 5 μ M ($\blacktriangle$) and 20 μ M ($\bullet$). After equilibration to 25°C the reaction was initiated by addition of Stage-IV N^{10} -formyltetrahydrofolate dehydrogenase (specific activity = 0.72 unit/mg) and the initial rate of increase in A_{300} was recorded. v is expressed as $\Delta A_{300}/\text{min}$ per μ g of enzyme. In the inset slope ($\bullet$) and intercept ($\blacksquare$) are plotted against reciprocal $NADP^+$ concentration.

peak protein concentrations in the eluted fractions in these runs ranged from 25 to 200 μ g/ml.

tive inhibition was observed with N^{10} -formyltetrahydrofolate (Fig. 6) and non-competitive inhibition with $NADP^+$ (results not shown) as the variable substrates respectively. From the linear secondary plot of slope versus [tetrahydrofolate] (inset, Fig. 6) the K_i for (-)-tetrahydrofolate is obtained as $7\mu M$ if it is assumed, as shown by Kutzbach & Stokstad (1971) for pig liver N^{10} -formyltetrahydrofolate dehydrogenase, that the (+)-isomer is essentially ineffective.

With $NADPH$, preliminary studies demonstrated that this product was not significantly inhibitory even when non-saturating concentrations of N^{10} -formyltetrahydrofolate and $NADP^+$ were used and when the $NADPH/NADP^+$ concentration ratio exceeded 10^3 . Rather, stimulation of N^{10} -formyltetrahydrofolate oxidation by $NADPH$ is observed. As shown in Fig. 7, the extent of stimulation is maximal at $[NADP^+]/[NADPH]$ ratios in the range 0.01–0.02 and at non-saturating concentrations of N^{10} -formyltetrahydrofolate. However, it is notable that a 10-fold

Fig. 6. Inhibition of rat liver N^{10} -formyltetrahydrofolate dehydrogenase by tetrahydrofolate as a function of N^{10} -formyltetrahydrofolate concentration

The assay system contained, in 0.5 ml, 100 mM-Tris/HCl, pH 7.7, 100 mM-2-mercaptoethanol, 0.1 mM- $NADP^+$ and the concentrations of ($\pm$)- N^{10} -formyltetrahydrofolate as indicated. The concentrations of ($\pm$)-tetrahydrofolate used were 0 ($\bullet$), $11.7\mu M$ ($\square$), $23.4\mu M$ ($\blacksquare$) and $35.8\mu M$ ($\circ$). After equilibration to $25^\circ C$ the reaction was initiated by addition of Stage-IV N^{10} -formyltetrahydrofolate dehydrogenase (specific activity = 0.88 unit/mg), and the rate estimated from the increase in A_{300} . v is expressed as $\Delta A_{300}/\text{min}$ per μg of enzyme. In the inset, slope ($\bullet$) is plotted as a function of tetrahydrofolate concentration.

Fig. 7. Effect of variation of the $[NADP^+]/[NADPH]$ ratio on the catalytic activity of N^{10} -formyltetrahydrofolate dehydrogenase from rat liver

The assay system contained, in 0.5 ml, 100 mM-Tris/HCl, pH 7.7, 100 mM-2-mercaptoethanol, $1.2\mu M$ -(A) or $12.3\mu M$ -(B, C) $NADP^+$, $8.9\mu M$ -(A, B) or $44.3\mu M$ -(C) ($\pm$)- N^{10} -formyltetrahydrofolate, and the concentration of $NADPH$ as indicated. After equilibration to $25^\circ C$ the reaction was initiated by addition of Stage-IV N^{10} -formyltetrahydrofolate dehydrogenase (specific activity = 0.72 unit/mg) and the initial rate of increase in A_{300} recorded. v is expressed as $\Delta A_{300}/\text{min}$ per μg of enzyme. The broken lines indicate the range of the cytosolic $[NADP^+]/[NADPH]$ ratio in fed rat liver (Gumaa *et al.*, 1971).

increase in $[NADP^+]$ to a saturating concentration does not decrease the extent of stimulation. Activation by $NADPH$ causes no significant change in the apparent K_m values for $NADP^+$ and N^{10} -formyltetrahydrofolate, but is associated with a small increase in the K_i for tetrahydrofolate (results not shown).

Other studies have shown that neither methionine nor ethionine when added either as the free amino acid or as the S -adenosyl derivative cause significant activation of rat liver N^{10} -formyltetrahydrofolate dehydrogenase under any conditions tested thus far. Furthermore, these amino acids and their S -adenosyl derivatives fail to cause significant reversal of inhibition by tetrahydrofolate. These experiments have been performed with saturating and non-saturating concentrations of one or both substrates and by using both the homogeneous enzyme and a 45000g liver supernatant fraction which had not been exposed to acid treatment (cf. Table 2).

Hydrolysis of N^{10} -formyltetrahydrofolate catalysed by rat liver N^{10} -formyltetrahydrofolate dehydrogenase

Kutzbach & Stokstad (1971) reported that pig liver N^{10} -formyltetrahydrofolate dehydrogenase catalysed hydrolysis of N^{10} -formyltetrahydrofolate to formate + tetrahydrofolate in the absence of NADP^+ . We have therefore characterized this activity for the rat liver enzyme. Homogeneous rat liver N^{10} -formyltetrahydrofolate dehydrogenase catalyses hydrolysis of N^{10} -formyltetrahydrofolate at 21 % of the rate of the NADP^+ -dependent reaction on the basis of a comparison of apparent V_{max} . The hydrolytic activity is eluted in constant ratio with NADP^+ -dependent N^{10} -formyltetrahydrofolate dehydrogenase from a Blue Dextran–Sephadex column with NADPH as eluent (results not shown). The K_m for (–)- N^{10} -formyltetrahydrofolate in the hydrolytic reaction is obtained as $23.8 \mu\text{M}$ (Fig. 8), if it is assumed that only the (–)-isomer is an active substrate, as is the case for the NADP^+ -dependent reaction.

Hydrolysis of N^{10} -formyltetrahydrofolate by N^{10} -formyltetrahydrofolate dehydrogenase is stimulated by NAD^+ (Fig. 9) and inhibited by tetrahydrofolate

(Fig. 8). Stimulation by NAD^+ is not attributable to this nucleotide acting as an ineffective analogue of NADP^+ , since no NADH is detectable after completion of the reaction on assay with lactate dehydrogenase (Klingenberg, 1963), and assay for formate as described in the Methods and Materials section showed the presence of this metabolite at approximately the concentration predicted from the change in absorbance at 300 nm. The presence of NAD^+ has little effect on the K_m for N^{10} -formyltetrahydrofolate, indicating that the primary effect is on V_{max} , which is increased by 80 % in the presence of 1 mM- NAD^+ . Measurement of initial rate as a function of $[\text{NAD}^+]$ gives the activator constant for this nucleotide as 0.35 mM (Fig. 9).

Tetrahydrofolate acts as a non-competitive inhibitor of the hydrolytic reaction when initial rate is measured as a function of N^{10} -formyltetrahydrofolate concentration (Fig. 8a). The slopes and intercepts are linear functions of tetrahydrofolate concentration and the K_i values are obtained from these secondary plots as $21 \mu\text{M}$ (slope) and $22 \mu\text{M}$ (intercept) respectively, if it is assumed that only the (–)-isomer

Fig. 8. Inhibition of hydrolysis of N^{10} -formyltetrahydrofolate by N^{10} -formyltetrahydrofolate dehydrogenase in the presence or absence of NAD^+ as a function of N^{10} -formyltetrahydrofolate concentration

The assay system contained, in 0.5 ml, 100 mM-Tris/HCl, pH 7.7, 100 mM-2-mercaptoethanol, and the concentrations of of (±)- N^{10} -formyltetrahydrofolate as indicated. The concentrations of (±)-tetrahydrofolate used were 0 (●), 10 μM (□), 20 μM (■) and 30 μM (○). The studies were performed in the absence (—) or presence (---) of 1 mM- NAD^+ . After equilibration to 25°C the reaction was initiated by addition of Stage-IV N^{10} -formyltetrahydrofolate dehydrogenase (specific activity 0.88 unit/mg) and the initial reaction rate estimated from the increase in A_{300} . v is expressed as $\Delta A_{300}/\text{min}$ per 10 μg of enzyme. In (b) the slopes (●) and intercepts (■) of the primary plot for data obtained in the absence of NAD^+ are plotted as a function of (±)-tetrahydrofolate concentration.

Fig. 9. Initial rate of hydrolysis of N^{10} -formyltetrahydrofolate catalysed by N^{10} -formyltetrahydrofolate dehydrogenase as a function of NAD^+ concentration

The assay system contained, in 0.5 ml, 100 mM-Tris/HCl, pH 7.7, 100 mM-2-mercaptoethanol, 33 μ M-($\pm$)- N^{10} -formyltetrahydrofolate and the concentrations of NAD^+ as indicated. After equilibration to 25°C the reaction was initiated by addition of Stage-IV N^{10} -formyltetrahydrofolate dehydrogenase (specific activity 0.88 unit/mg) and the initial reaction rate estimated from the increase in A_{300} . v is expressed as $\Delta A_{300}/\text{min}$ per 10 μ g of enzyme.

is an effective inhibitor (Fig. 8b). Fig. 8 also demonstrates that, in the presence of NAD^+ , tetrahydrofolate is a much less effective inhibitor. For example, at the highest concentration of ($\pm$)- N^{10} -formyltetrahydrofolate used (82.4 μ M), addition of 30 μ M-($\pm$)-tetrahydrofolate causes 53% inhibition in the absence of NAD^+ , but only 17% inhibition in the presence of 1 mM- NAD^+ .

Effect of formiminoglutamate on catalysis by rat liver pyruvate carboxylase and phosphoenolpyruvate carboxykinase

Among the metabolites that accumulate during perfusion of the intact liver, or incubation of isolated hepatocytes, with histidine, formiminoglutamate shows the most striking increase in concentration (Tabor & Mehler, 1955; Buehring *et al.*, 1972). It was therefore decided to determine whether this metabolite caused inhibition of key gluconeogenic enzymes and hence whether its accumulation in the intact liver might give rise to the hypoglycaemia associated with histidine toxicity (Greenstein & Winitz, 1961). Fig. 10 demonstrates that formiminoglutamate is an inhibitor of rat liver pyruvate carboxylase, with 50% inhibition being observed at a concentration of 2.8 mM under the conditions used in this experiment (non-saturating concentrations of pyruvate and acetyl-CoA at pH 7.1). Similar results were obtained when inhibition was studied at pH 8.1 in the presence of saturating concentrations of pyruvate and/or acetyl-CoA.

Fig. 10. Inhibition of rat liver pyruvate carboxylase by formiminoglutamate

The assay system contained, in 0.5 ml, 100 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate (K^+ salt), pH 7.1, 0.2 mM-pyruvate, 2 mM-ATP, 5 mM- $MgCl_2$, 50 mM- $KHCO_3$, 0.07 mM-acetyl-CoA, 2 μ g of malate dehydrogenase, 0.2 mM-NADH and the concentrations of formiminoglutamate as indicated. After equilibration to 25°C the reaction was initiated by addition of rat liver pyruvate carboxylase (specific activity 12.5 units/mg) and the initial reaction rate was estimated from the decrease in A_{340} .

In contrast, 10–15 mM-formiminoglutamate has no significant effect on the catalytic activity of cytosolic rat liver phosphoenolpyruvate carboxykinase in the present of saturating or non-saturating concentrations of the substrates.

Discussion

Our studies demonstrate once again the utility of Blue Dextran-Sepharose in the purification of $NADP^+$ -dependent dehydrogenases (Tormanen *et al.*, 1976), and have resulted in the first description of a homogeneous preparation of N^{10} -formyltetrahydrofolate dehydrogenase, although it appears likely on the basis of the specific activity of their preparation that this state may also have been achieved by Kutzbach & Stokstad (1971) for the pig liver enzyme. Rat liver N^{10} -formyltetrahydrofolate dehydrogenase is

composed of four similar, if not identical, subunits (Figs. 3 and 4), in common with many other dehydrogenases (Rossman *et al.*, 1975). However, despite its oligomeric character no evidence has been obtained for atypical kinetic behaviour by either substrates or products. The pattern of product inhibition is nevertheless of interest in two respects. First, the patterns observed when tetrahydrofolate is added as product inhibitor (Fig. 6) are those expected from the operation of an ordered mechanism in which N^{10} -formyltetrahydrofolate binds before $NADP^+$ (Cleland, 1963). Although the failure to observe inhibition by $NADP^+$ makes it impossible to confirm this postulate in the normal way (Cleland, 1963), the suggested order of substrate addition is consistent with the finding that N^{10} -formyltetrahydrofolate dehydrogenase catalyses hydrolysis of N^{10} -formyltetrahydrofolate in the absence of $NADP^+$ (Figs. 8 and 9), thus indicating involvement of a formyl-enzyme intermediate. Second, the absence of significant inhibition by $NADPH$ appears as an interesting example of metabolic adaptation. The maximal catalytic capacity of rat liver N^{10} -formyltetrahydrofolate dehydrogenase (0.5 unit/g at 37°C) (Table 1) is less than twice the maximal rate of methionine-stimulated histidine or formate oxidation (0.28 $\mu\text{mol/g}$) observed in isolated rat hepatocytes (Krebs *et al.*, 1976). Since cytosolic $NADP^+/NADPH$ ratios in rat liver have been calculated as 0.010–0.015 (Gumaa *et al.*, 1971), it is apparent that a normal pattern of product inhibition would be inconsistent with participation of this enzyme in regeneration of tetrahydrofolate, since it operates in opposition to the prevailing redox state of the $NADP^+/NADPH$ system. We have, however, observed (Fig. 7) that activation, rather than inhibition, of N^{10} -formyltetrahydrofolate dehydrogenase is observed in the presence of $NADP^+/NADPH$ ratios and at $NADPH$ concentrations characteristic of rat liver cytosol (Clark *et al.*, 1965; Gumaa *et al.*, 1971). Since this activation is observed at both saturating and non-saturating concentrations of $NADP^+$ (Fig. 7) and does not influence the K_m for either substrate, the effect does not seem likely to result from occupancy of the $NADP^+$ domain in the catalytic site.

In accord with previous studies (Kutzbach & Stokstad, 1971), rat liver N^{10} -formyltetrahydrofolate dehydrogenase is $NADP^+$ -specific. However, addition of NAD^+ at concentrations within the physiological range (Clark *et al.*, 1965) causes significant activation of N^{10} -formyltetrahydrofolate hydrolysis (Fig. 9) and markedly decreases the effectiveness of inhibition of this reaction by tetrahydrofolate (Fig. 8). If, as seems reasonable, NAD^+ acts as a result of occupancy of the $NADP^+$ domain, the observed effect is suggestive of conformational interaction between this and the tetrahydrofolate domain in the catalytic site. Although it is attractive to suggest that NAD^+ -stimulated hydrolysis of N^{10} -formyltetrahydrofolate

might play a role in recycling tetrahydrofolate, there is no direct evidence for conversion *in vivo* of histidine to formate rather than to CO_2 . Furthermore the maximal capacity of the enzyme in this reaction, which may be calculated as approx. 0.12 unit/g at 37°C, could account for only 40% of the maximal net rate of histidine oxidation by rat hepatocytes (Krebs *et al.*, 1976); and the K_m for N^{10} -formyltetrahydrofolate (23.8 μM) is 4-fold greater than that observed for the oxidative reaction (6 μM) (Figs. 6 and 8).

Our data provide further insight into the mechanisms that may be responsible for development of hypoglycaemia in response to excessive dietary content of histidine and for accumulation of formiminoglutamate in the liver during histidine degradation. The data of Fig. 10 clearly indicate that formiminoglutamate has the potential to act as an effective inhibitor of gluconeogenesis from lactate and alanine, which are the predominant gluconeogenic substrates *in vivo* (Felig, 1975). However, a decrease in intracellular [formiminoglutamate] from 14 mM (for hepatocytes incubated with 2 mM-histidine) to 5 mM (for hepatocytes incubated with 2 mM-histidine + 0.1 mM-methionine) is associated with almost complete reversal of inhibition of gluconeogenesis from lactate (Krebs & Hems, 1976; Krebs *et al.*, 1976), which is not the predicted result based on the data of Fig. 10. Since histidine metabolism is a cytosolic process (Tabor, 1955), operation of the proposed effect *in vivo* requires that formiminoglutamate is translocated into the mitochondrion, probably by the glutamate carrier. This carrier has been shown to have K_m of 4 mM for L-glutamate and a relatively low V_{max} , compared with other mitochondrial transport systems (Bradford & McGivan, 1975). Hence it would not be surprising if effective translocation of formiminoglutamate into the mitochondrion occurred only when the cytosolic concentration of this metabolite is high. The observed inhibition of pyruvate carboxylase by formiminoglutamate is thus able to explain the observed effect of histidine on gluconeogenesis from lactate, but provides no explanation for the inhibition observed with propionate or glutamine as substrates (Cornell *et al.*, 1974; Krebs *et al.*, 1976) unless an effect on cycling at the pyruvate/phosphoenolpyruvate level is invoked. The failure to observe inhibition of phosphoenolpyruvate carboxylase by formiminoglutamate appears to eliminate this enzyme as the inhibitory site. However, since the enzyme activity was measured in the direction of oxaloacetate synthesis in these experiments, we cannot exclude the possibility that inhibition is observed only when the enzyme is assayed in the direction of phosphoenolpyruvate synthesis, as is the case, for example, when quinolinate is used as the inhibitor (Veneziale *et al.*, 1967).

The effect of methionine (and ethionine) in decreasing intracellular accumulation of formimino-

glutamate, and hence presumptively relieving the inhibition of gluconeogenesis, has been attributed to an increase in the availability of tetrahydrofolate (Krebs *et al.*, 1976) which is required for further metabolism of formiminoglutamate (Scheme 1). Such an increase in [tetrahydrofolate] can be demonstrated under these conditions (Buehring *et al.*, 1972). It has been shown by Krebs *et al.* (1976) that the conversion of both [^{14}C]formate and [$2\text{-}^{14}\text{C}$]histidine into $^{14}\text{CO}_2$ is accelerated in isolated hepatocytes by addition of methionine or ethionine. Krebs *et al.* (1976) suggested that these effects could be due either to direct activation of N^{10} -formyltetrahydrofolate dehydrogenase by these amino acids or their *S*-adenosyl derivatives, or alternatively to a less direct effect in which inhibition of N^5N^{10} -methylenetetrahydrofolate reductase by *S*-adenosylmethionine (Kutzbach & Stokstad, 1967) causes an increase in the concentrations of the intermediates of the C_1 pool (Scheme 1) and hence activates N^{10} -formyltetrahydrofolate dehydrogenase. The data presented here allow us to exclude the first of these mechanisms, since the catalytic properties of the rat liver enzyme are unaffected by addition of methionine, ethionine or the *S*-adenosyl derivatives over a wide concentration range and under a variety of conditions.

A simulation experiment based on the kinetic properties of rat liver N^{10} -formyltetrahydrofolate dehydrogenase and the data of Buehring *et al.* (1972) fails to support the second postulate. If the total folate concentration in rat liver is taken as $20\text{ }\mu\text{M}$ (Williams & Spray, 1970), the concentrations of tetrahydrofolate and N^{10} -formyltetrahydrofolate can be calculated as $1\text{ }\mu\text{M}$ each in livers perfused with histidine, and as 9.2 and $4.6\text{ }\mu\text{M}$ in livers perfused with histidine + methionine on the basis of the data of Buehring *et al.* (1972). When the purified enzyme is assayed in the presence of the above concentrations of these metabolites and in the presence of a saturating concentration of NADP^+ , identical rates were observed under both conditions, which corresponded to an expression of 8% of the maximal catalytic activity measured in the presence of saturating concentrations of both substrates and in the absence of tetrahydrofolate. Similar results are obtained when the total folate pool is assumed to be 10 or $40\text{ }\mu\text{M}$, or when these studies are performed by using a liver 45000g supernatant fraction. In this case one can calculate the flux as $0.04\text{ }\mu\text{mol}$ of N^{10} -formyltetrahydrofolate oxidized/min per g of liver at 37°C . This value is, however, inadequate to account for either the basal ($0.09\text{ }\mu\text{mol/min per g}$) or methionine-stimulated ($0.28\text{ }\mu\text{mol/min per g}$) rates of histidine oxidation observed in isolated hepatocytes (Krebs *et al.*, 1976).

These apparent discrepancies could be explained if the analytical data were incorrect (cf. Connors *et al.*, 1977) or if there were extensive compartmentation of tetrahydrofolate and/or N^{10} -formyltetrahydrofolate

such that the apparent concentrations calculated as above do not provide a reliable guide to the effective concentrations *in vivo*. Such compartmentation has been demonstrated with respect to both intracellular organelles and folate-binding proteins (Zamierowski & Wagner, 1977*a,b*) and is also clearly indicated by comparison of the kinetic constants of the various enzymes involved. For example, in histidine degradation 5-formiminotetrahydrofolate-glycine *N*-formiminotransferase (EC 2.1.2.5), the enzyme responsible for the initial step of formiminoglutamate metabolism, has a K_m of $100\text{ }\mu\text{M}$ for (–)-tetrahydrofolate (Tabor & Wyngaarden, 1959), compared with the K_i of $7\text{ }\mu\text{M}$ determined here for the inhibition of N^{10} -formyltetrahydrofolate dehydrogenase by this coenzyme (Fig. 6). If both enzymes are in contact with the same intracellular pool, it would appear unlikely that tetrahydrofolate can be effectively recycled, as has been suggested (Buehring *et al.*, 1972). However, such comparisons, which depend on studies using the monoglutamate coenzymes, could be misleading if, as has often been proposed (Shin *et al.*, 1972), this coenzyme is present in the cell as a polyglutamate derivative. Studies have been performed for several folate-dependent enzymes (Blakeley, 1957; Curthoys & Rabinowitz, 1972; Kisliuk *et al.*, 1974, 1978; Coward *et al.*, 1974, 1975), in which the kinetic properties observed for catalysis by using the monoglutamate and one or more of the polyglutamate forms have been compared. In such comparisons the polyglutamate forms are usually more effective, although the differences in kinetic constants tend in most instances to be in the range 2–3-fold. Few examples exist of cases where the difference is such as to suggest that the monoglutamate species is physiologically inactive (Whitfield *et al.*, 1970). Furthermore, in order to explain our observations for N^{10} -formyltetrahydrofolate dehydrogenase on this basis it would be necessary to postulate that use of the polyglutamate form of the coenzyme had markedly different effects on the K_m for N^{10} -formyltetrahydrofolate and the K_i for tetrahydrofolate. Since both the nature of the reaction and the data of Fig. 6 suggest that these coenzymes combine at the same site on N^{10} -formyltetrahydrofolate dehydrogenase, such a differential effect seems improbable, although it cannot be ruled out. However, no precedent exists in studies of the interaction of the polyglutamate forms with other folate-dependent enzymes.

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