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PLANT N^5,N^{10}-METHYLENETETRAHYDROFOLATE DEHYDROGENASE: PARTIAL PURIFICATION AND SOME GENERAL PROPERTIES OF THE ENZYME FROM GERMINATING PEA SEEDLINGS

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Abstract—The N^5,N^{10}-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) activity of pea seedlings has been examined in cotyledon extracts during the first 7 days of germination. Specific enzyme activities increased over this period but were not appreciably changed in seeds which had imbibed chloramphenicol, cycloheximide and aminopterin solutions. The general properties of the enyzme from 18-day-old pea shoots were examined using preparations which had been fractionated with $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose. The enzyme was found to be relatively unstable after chromatography on DEAE-cellulose. Enzyme activity was maximal at pH 6·7 and had absolute requirements for formaldehyde and tetrahydrofolic acid. NADP was an effective hydrogen acceptor in the reaction although this coenzyme could be partially replaced by NAD. The enzyme was strongly inhibited by iodoacetate and *p*-chloromercuribenzoate and to a lesser extent by α,α'-dipyridyl and *o*-phenanthroline. The general properties of the plant enzyme are discussed in relation to those of the N^5,N^{10}-methylenetetrahydrofolate dehydrogenases of micro-organisms and animal tissues.

INTRODUCTION

DURING germination of pea seedlings there is a pronounced synthesis of N^{10}-formyl and N^5-methyltetrahydrofolate monoglutamates.[1] This latter folate derivative is the major component of the folate pool present in pea cotyledons and pea leaves. The tetrahydrofolate (THFA) moieties of these molecules appeared to be derived from more oxidized folate derivatives as synthesis was readily inhibited by aminopterin. Furthermore, seeds imbibing [2-^{14}C] folate synthesized labelled N^5-methyl THFA. The origins of the methyl group of N^5-methyl THFA in pea tissues are however still not clear.

Considering the pathways[2] for the biosynthesis of N^5-methyl THFA in micro-organisms and mammalian tissues it is likely that in plants, N^5,N^{10}-methylene THFA is an important precursor as outlined in Scheme 1.

The synthesis of N^{10}-formyl THFA (Scheme 1, reaction a) is catalyzed by formyl THFA synthetase (formate:tetrahydrofolate ligase (ADP), EC 6.3.4.3). This enzyme is widely distributed in plants and is particularly active in germinating pea cotyledons.[3,4] Interconversion of N^{10}-formyl THFA and N^5,N^{10}-methenyl THFA (reaction b) has not been studied in higher plants but is well documented in other organisms.[2] Hydrogenation of N^5,N^{10}-methenyl THFA is catalyzed by an oxidoreductase (5,10-methylenetetrahydrofolate:

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[1] A. J. ROOS, A. M. SPRONK and E. A. COSSINS, *Can. J. Biochem.* **46**, 1533 (1968).
[2] M. FRIEDKIN, *Ann. Rev. Biochem.* **32**, 185 (1963).
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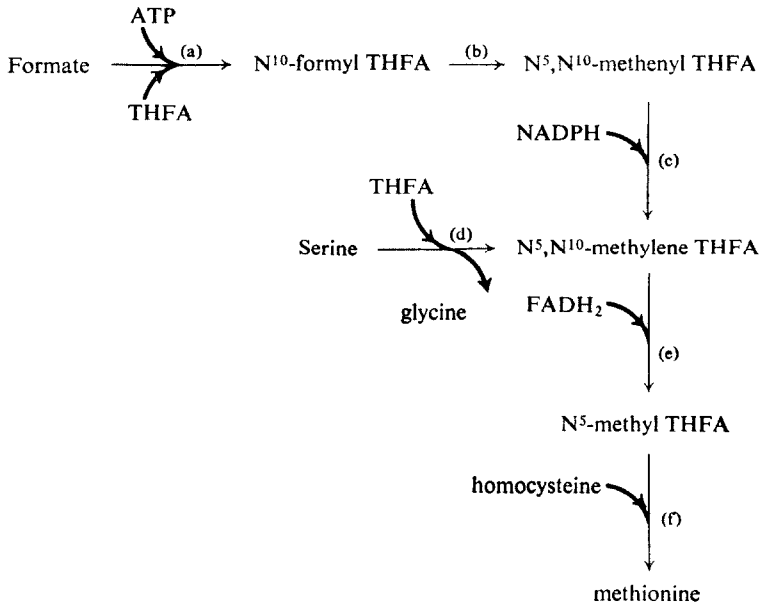

SCHEME 1. THE BIOSYNTHESIS OF N^5-METHYL THFA IN PLANTS.

NADP oxidoreductase EC 1.5.1.5) which has been purified and studied in detail from bacteria and mammalian tissues.[5-12] The enzyme has not however been extensively studied in plants apart from reports of its occurrence in turnip hypocotyls,[13] and several other plant tissues.[14] Synthesis of N^5,N^{10}-methylene THFA from serine in plants is now well documented, the reaction having importance in the interconversion of glycine and serine.[15-17] Production of N^5-methyl THFA from N^5,N^{10}-methylene THFA in plants has not so far been described but a reductase, catalyzing this reaction, has been isolated from other organisms.[2] A recent report from Sakami's laboratory[18] has presented evidence that the mono- and triglutamate derivatives of 5-methyl THFA are utilized by extracts of green beans for methionine biosynthesis (Scheme 1, reaction f). In independent work with cell-free extracts of pea cotyledons, Dodd and Cossins[19] have shown that a transmethylase, catalyzing such a reaction, is present in tissues which are actively synthesizing both 5-methyl THFA (monoglutamate) and S-adenosylmethionine. The reaction was homocysteine-dependent and the enzyme displayed

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a high apparent affinity for the methyl donor.[20] It appears likely that this transmethylase has importance in the *de novo* synthesis of methionine during the early stages of germination.

Although several aspects of the one-carbon metabolism of higher plants have been elucidated, evidence for many of the reactions outlined in Scheme 1 is still fragmentary, and indirect. Clearly, the results of feeding experiments[21-23] are consistent with one-carbon transfer reactions having importance in the biosynthesis of serine and methionine. The details and possible regulation of these syntheses in plants, however, require further examination.

As part of a continuing investigation of the one-carbon metabolism of higher plants, the present studies have concentrated on the properties of N^5,N^{10}-methylene THFA dehydrogenase from pea seedlings.

RESULTS

Changes in Enzyme Activity During Germination

As pea cotyledons display a pronounced biosynthesis of folate derivatives during the early stages of germination,[1] extracts of these organs were examined for possible changes in levels of N^5,N^{10}-methylene THFA dehydrogenase activity. In these investigations, samples of the seeds were initially allowed to imbibe either chloramphenicol, cycloheximide, aminopterin or distilled water for 18 hr at 25°. The seeds were then removed from these solutions, washed thoroughly in distilled water and germinated in moist vermiculite in darkness at 28°. During a 7-day germination period, 10-g samples of the seedlings were removed, the lengths of the radicles and plumules noted and the N^5,N^{10}-methylene THFA dehydrogenase contents of the cotyledons determined (Table 1). It is clear from Table 1 that seeds which had imbibed chloramphenicol (2×10^{-3} M), cycloheximide and aminopterin showed inhibition of seedling development. However, the specific activity of N^5,N^{10}-methylene THFA dehydrogenase, which showed a slight increase as germination proceeded, was not appreciably altered by these treatments. Enzyme activity was detected in the cotyledons, roots and shoots of 6-day-old seedlings, the cotyledons accounting for the greatest activity on both a fresh weight and protein basis.

General Properties of The Enzyme from Pea Shoots

Fractionation of soluble protein from 18-day-old pea shoots by means of $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-cellulose yielded an enzyme preparation with a specific enzyme activity of 91 units/mg protein, approximately 20 times that of the acetone powder extract. Although the enzyme has not been highly purified this specific activity is slightly higher than that of the 100-fold purified enzyme from calf thymus.[10] Attempts to increase the specific activity of these preparations by adsorption and elution from calcium phosphate gel and by further precipitation with $(NH_4)_2SO_4$ were unsuccessful as considerable losses of activity were encountered. The partially purified enzyme was considerably more stable than activity present in the initial tissue homogenate (Fig. 1). However, as losses of activity were appreciable after extended periods of storage at 5°, all subsequent studies were carried out within 48 hr of the DEAE-cellulose treatment. Lyophilization of the enzyme resulted in irreversible losses of activity.

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[22] W. B. McConnell and E. Bilinski, *Can. J. Biochem. Physiol.* **31**, 549 (1959).
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TABLE 1. THE EFFECTS OF CHLORAMPHENICOL, CYCLOHEXIMIDE AND AMINOPTERIN ON SEEDLING GROWTH AND ENZYME ACTIVITY DURING GERMINATION

Seeds imbibed in	Age of seedlings (days) 1	2	3	4	5	6	7
Distilled water							
units/g f.wt.	62·2	69·7	54·1	61·9	60·5	54·9	58·2
units/mg protein	0·8	1·01	0·90	1·29	1·55	2·03	2·53
radicle length (mm)	4·5	11·5	35·0	55·0	70·0	86·0	100·0
plumule length (mm)	<2·0	<2·0	12·0	24·0	35·0	52·0	70·0
Chloramphenicol (10^{-4} M)							
units/g f.wt.	55·0	70·9	60·2	68·7			
units/mg protein	0·7	1·0	1·02	1·53			
radicle length (mm)	4·5	11·0	33·0	51·0			
plumule length (mm)	<2·0	<2·0	13·0	24·0			
Chloramphenicol (2×10^{-3} M)							
units/g f.wt.		74·5		74·2		63·7	
units/mg protein		1·15		1·37		2·28	
radicle length (mm)		6·0		30·0		80·0	
plumule length (mm)		<2·0		11·0		35·0	
Cycloheximide (2×10^{-4} M)							
units/g f.wt.		77·9		69·8		71·0	
units/mg protein		1·11		1·20		2·15	
radicle length (mm)		5·5		14·0		33·0	
plumule length (mm)		<2·0		<2·0		15·0	
Aminopterin (10^{-4} M)							
units/g f.wt.		68·4		75·8			64·9
units/mg protein		0·88		1·40			1·70
radicle length (mm)		4·4		7·0			7·7
plumule length (mm)		<2·0		<2·0			<2·0

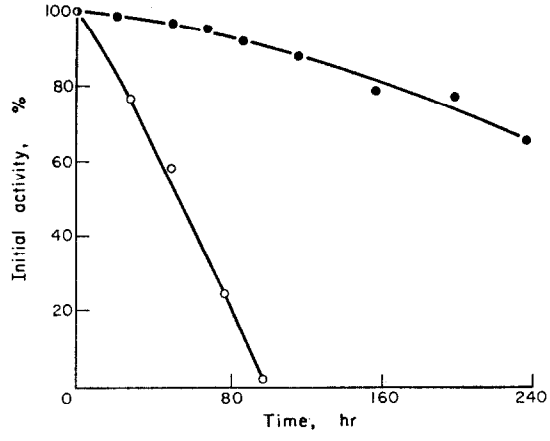

FIG. 1. LOSSES OF ENZYME ACTIVITY AT 5°.

Samples of the initial tissue homogenate (o) and the enzyme after DEAE-cellulose chromatography (●) were assayed for activity after periods of storage at 5°.

As expected, enzyme activity was markedly affected by pH. Although the pH optimum was found to be 6·7 all further assays of activity were conducted at pH 7·5 (*ca.* 55 per cent of maximum activity) in order to minimize dissociation of N^5,N^{10}-methylene THFA.[24]

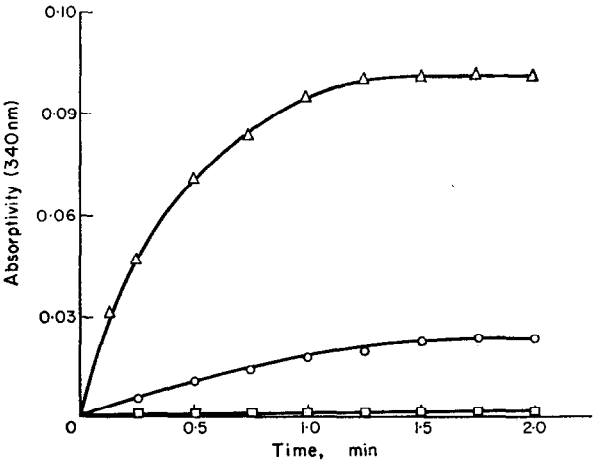

FIG. 2. GENERAL REQUIREMENTS FOR DEHYDROGENASE ACTIVITY.

Assays were carried out spectrophotometrically with complete reaction systems containing 0·6 μmole NADP (△) or 0·6 μmole NAD (○) and with reaction systems omitting formaldehyde and THFA (□).

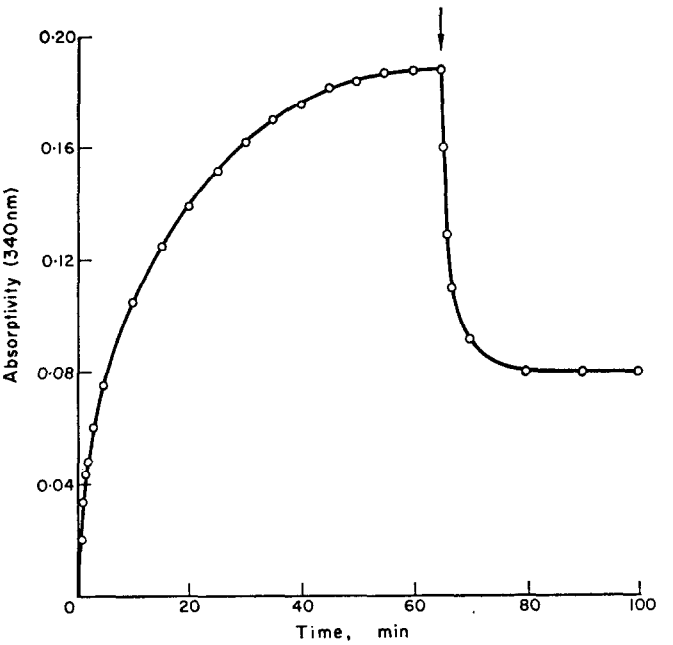

FIG. 3. PRODUCTION OF NADPH BY THE PARTIALLY PURIFIED ENZYME.

At equilibrium (↓) 3 μmoles of oxidized glutathione and 0·04 mg yeast glutathione reductase were added as indicated by the arrow.

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The reduction of NADP by the partially purified enzyme was found to have absolute requirements for formaldehyde and THFA (Fig. 2). No reduction of NADP occurred when formaldehyde was replaced by formate or methanol. Under the standard assay conditions, a smaller reduction of NAD was observed which was approximately 23 per cent of that observed with NADP but which was both formaldehyde- and THFA-dependent. The production of N^5,N^{10}-methenyl THFA as a product of this reaction has already been reported.[14] In the present work the accompanying formation of NADPH was confirmed by addition of glutathione reductase at equilibrium (Fig. 3).

The concentrations of formaldehyde, THFA and NADP were varied and the changes in the initial reaction velocities plotted according to Lineweaver and Burk[25] to give apparent Michaelis constants of $7{\cdot}9 \times 10^{-4}$ M, $2{\cdot}1 \times 10^{-4}$ M and 4×10^{-7} M for formaldehyde, THFA and NADP, respectively. Enzyme activity was also affected by the presence of various metabolic inhibitors as shown in Table 2. The sulphydryl group reagents were particularly inhibitory whereas the chelating agents α,α'-dipyridyl and *o*-phenanthroline produced a smaller but significant inhibition of enzyme activity.

TABLE 2. THE EFFECT OF VARIOUS INHIBITORS ON ENZYME ACTIVITY

Inhibitor	Final concentration (mM)	Inhibition (% of control)
Iodoacetate	23	100
p-CMB	0·23	90
sodium arsenite	1·0	13
α,α'-dipyridyl	12	27
o-phenanthroline	2·3	16

The enzyme (0·35 mg protein) was incubated with 100 μmoles of Tris-HCl (pH 7·8) and the inhibitor for 10 min at 37°. 12·5 μmoles of formaldehyde and 16·5 μmoles of THFA, which had previously been incubated at 37° for 4 min, were then added. Incubation was continued for a further 2 min before initiation of the reaction by addition of 0·6 μmole of NADP.

DISCUSSION

From the present studies it is clear that pea cotyledons contain high levels of N^5,N^{10}-methylene THFA dehydrogenase at a stage of germination when considerable synthesis of folate derivatives is taking place.[1] Considering the established role of this enzyme in the one-carbon metabolism of micro-organisms and animals,[2] it is highly likely that the enzyme in peas has importance in the generation of N^5-methyl THFA from N^{10}-formyl THFA, both major components of the folate pool in these seedlings.[1] Alternatively the enzyme may function in the overall conversion of the β-carbon of serine to N^{10}-formyl THFA which has importance in the biosynthesis of purines.[26] Extracts of pea cotyledons have been found to convert the β-carbon of serine to N^5,N^{10}-methylene THFA (Cossins, Clandinin and Roos, unpublished data) and Iwai *et al.*[27] have presented evidence that purines are synthesized during the early stages of germination in peas.

The marked inhibition of seedling growth following treatments with chloramphenicol (2×10^{-3} M) and cycloheximide (Table 1) suggest that, at the concentrations used, synthesis of new cellular constituents was reduced. Although these compounds are generally known

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[26] S. C. HARTMANN and J. M. BUCHANAN, *J. Biol. Chem.* **234**, 1812 (1959).
[27] K. IWAI, S. NAKAGAWA and O. OKINAKA, *Biochim. Biophys. Acta* **68**, 152 (1963).

to inhibit protein synthesis, recent work[28–30] suggests that, in plants, they may indirectly produce such inhibition by interference in energy transfer reactions. The maintenance of high levels of N^5,N^{10}-methylene THFA dehydrogenase in these treated tissues therefore argues against *de novo* synthesis of the enzyme during the first 6 days of germination. Considering the lack of effect of these protein inhibitors on enzyme activity, the possibility remains that the dehydrogenase is activated during imbibition. This activation cannot be due to the accompanying synthesis of THFA derivatives as the folate antagonist aminopterin, supplied at a concentration sufficient to inhibit synthesis of THFA derivatives,[1] failed to significantly affect the levels of enzyme activity in the cotyledons, although seedling development was markedly curtailed (Table 1).

When the properties of the N^5,N^{10}-methylene THFA dehydrogenase from pea shoots are compared with those of similar enzymes from other organisms (Table 3), it is clear that all

TABLE 3. SOME PROPERTIES OF MAMMALIAN, YEAST AND PLANT N^5,N^{10}-METHYLENETETRAHYDROFOLATE DEHYDROGENASES

Property	Source of enzyme			
	Bovine liver*	Calf thymus†	Yeast‡	Pea shoots
THFA requirement	absolute	absolute	absolute	absolute
Hydrogen acceptor	NADP only	NADP only	NADP only	NADP and NAD
pH optimum	n.r.	6·5	7·45	6·7
Stability at 2–4°	n.r.	100% loss overnight	up to 30% loss in 3–4 days	10% loss in 4 days
K_m values (M)				
Formaldehyde	n.r.	$5{\cdot}2 \times 10^{-5}$		$7{\cdot}9 \times 10^{-4}$
DL-THFA	$2{\cdot}7 \times 10^{-5}$			$2{\cdot}1 \times 10^{-4}$
NADP	n.r.	$2{\cdot}2 \times 10^{-5}$	$3{\cdot}7 \times 10^{-5}$	4×10^{-7}
—SH groups	essential	essential	essential	essential

* Data from Hatefi *et al.*[7]
† Data from Yeh and Greenberg.[10]
‡ Data from Ramasastri and Blakley.[11]
n.r. = not reported.

of the enzymes studied so far have several properties in common. The enzymes all have an absolute requirement for THFA and contain sulphydryl groups which are essential for activity. The enzymes from yeast[11] and calf thymus[10] also display stereospecificity, being active with (+)L-methylene THFA but not with (−)L-methylene THFA. It is likely that this property will be found to be characteristic of this group of oxidoreductases. The small activity displayed by the plant enzyme in the presence of NAD has not been reported for the other enzymes shown in Table 3. The dependence on THFA shows that this NADH formation was not due to a formaldehyde dehydrogenase. This reduction of NAD may indicate the presence in plants of a separate NAD methylene THFA dehydrogenase or a degree of coenzyme specificity which is broader than that of the other enzymes studied to date. Although attempts were not made to determine the presence of a NAD-dependent N^5,N^{10}-methylene

[28] I. R. MACDONALD, J. S. D. BACON, D. VAUGHAN and R. J. ELLIS, *J. Exptl. Botany* **17**, 822 (1966).
[29] R. J. ELLIS and I. R. MACDONALD, *Plant Physiol.* **42**, 1297 (1967).
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THFA dehydrogenase in this tissue, it is of interest to note that Scrimgeour and Huennekens[31] have clearly shown that Ehrlich ascites cells contain both NADP and NAD enzymes.

The N^5,N^{10}-methylene THFA dehydrogenases from calf thymus and yeast, like the enzyme from pea shoots are fairly unstable after partial purification. This instability cannot be entirely attributed to the sulphydryl nature of these enzymes as losses of activity at 0–4° are not appreciably diminished by adding various sulphydryl compounds.[10,11] It is clear that preparations with greater stability will have to be isolated before the mode of action of these oxidoreductases can be fully elucidated.

MATERIALS AND METHODS

Chemicals

All chemicals used in this investigation were purchased from Fisher Scientific Company, Nutritional Biochemicals Corporation, Mann Research Laboratories and Sigma Chemical Company, and were of the highest purity commercially available. Solutions were prepared using de-ionized glass re-distilled H_2O.

Plant Material

Seeds of pea (*Pisum sativum* L. cv. Homesteader) were soaked in H_2O for 18 hr at 25° in darkness. Samples of the imbibed seeds were then germinated in the dark in moist vermiculite for periods up to 7 days at 28°. Other samples, required for extraction of the enzyme from the shoots, were grown in the greenhouse for 18 days.

Extraction and Partial Purification of N^5,N^{10}-*Methylene THFA Dehydrogenase*

Before extraction of the enzyme from germinating cotyledons, the seeds were washed thoroughly in H_2O. After removal of the testas and embryos, the cotyledons (10 g) were ground in 50 ml 0·01 M K phosphate buffer (pH 7·5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol at 2° using a mortar and pestle. All subsequent operations were carried out at 4°. The crude homogenate was passed through fine cheesecloth and immediately centrifuged at 18,000 g for 20 min to remove cellular debris. The supernatant was then used as a source of the enzyme in studies to determine changes in activity during germination and the effects of inhibitors of protein synthesis and folate metabolism.

In studies of the enzyme from pea shoots, the 18-day-old plant material was harvested and homogenized for 1 min in acetone at −15° using a Waring Blendor. The resulting acetone powders were subsequently extracted by stirring for 30 min at 2° in 0·01 M K_2HPO_4 (pH 7·5) containing 0·001 M EDTA and 0·01 M 2-mercaptoethanol. After centrifugation at 16,000 *g* for 20 min the supernatant was fractionated by addition of saturated $(NH_4)_2SO_4$ solution as described previously.[14] Protein precipitating within the 60–80 per cent range of saturation with $(NH_4)_2SO_4$ was dissolved in 25 ml of 5 mM K_2HPO_4 buffer (pH 7·5) containing 0·5 mM EDTA, 5 mM 2-mercaptoethanol and 30 per cent glycerol. The resulting protein solution was dialyzed against this buffer for 15 hr at 2°. A sample (18 ml) of the dialyzed extract, containing approximately 60 mg protein, was then applied to a 20 × 2·7 cm column of DEAE-cellulose which had previously been equilibrated with the same buffer. The column was then washed with this buffer solution at a flow rate of 0·5 ml/min, fractions of 5 ml being collected at 4°. Enzyme activity was present in fractions 19–24 under these conditions. Protein was assayed colorimetrically[32] and spectrophotometrically.[33]

Assay of Enzyme Activity

Enzyme activities were routinely assayed by following the production of NADPH at 340 nm[34] using a Beckman double-beam spectrophotometer equipped with a potentiometric recorder. Initial reaction velocities were calculated from the tangents drawn to the reaction time-course curves. The standard reaction system contained formaldehyde, 12·5 μmoles; 2-mercaptoethanol, 60 μmoles; tetrahydrofolic acid, 16·5 μmoles and K_2HPO_4 (pH 7·5), 10 μmoles. These components were preincubated at 37° for 4 min. The enzyme extract was then added and the system incubated at 37° for a further 2 min period. The reaction was then initiated by addition of 0·6 μmoles of NADP. The final volume was 3 ml. The reference cuvette contained all of the above components with the exception of NADP which was replaced by distilled water. One unit of enzyme activity

[31] K. G. SCRIMGEOUR and F. M. HUENNEKENS, *Biochem. Biophys. Res. Commun.* **2**, 230 (1960).
[32] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
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[34] K. G. SCRIMGEOUR and F. M. HUENNEKENS, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 6, p. 368, Academic Press, New York (1963).

is defined as the amount of extract causing a change in extinction of 0·01 per min[10, 11] under the defined reaction condition. Expression of activity in absorbance units rather than as μmoles of product formed per min was necessary as formation of N^5,N^{10}-methylene THFA in the reference cuvette and the possibility of cyclohydrolase activity in the reaction cuvette would contribute to the changes in extinction at 340 nm.

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