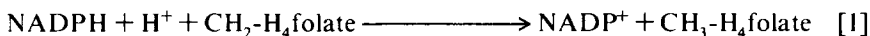# MODULATION OF METHYLENETETRAHYDROFOLATE REDUCTASE ACTIVITY BY S-ADENOSYLMETHIONINE AND BY DIHYDROFOLATE AND ITS POLYGLUTAMATE ANALOGUES

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INTRODUCTION

Methylenetetrahydrofolate reductase (EC 1.1.99.15) catalyzes the reduction of methylenetetrahydrofolate to methyltetrahydrofolate [equation 1]

$$NADPH + H^+ + CH_2\text{-}H_4\text{folate} \longrightarrow NADP^+ + CH_3\text{-}H_4\text{folate} \quad [1]$$

This overall reaction is effectively irreversible *in vivo* (1) and commits one carbon units to the pathways of adenosylmethionine-dependent methylation in mammalian cells. As shown in Figure 1, methylenetetrahydrofolate reductase is located at a branch point in folate metabolism, and can be viewed as competing for tetrahydrofolate-bound one carbon units with thymidylate synthase (EC 2.1.1.45) and with the AICAR* and GAR transformylases (EC 2.1.2.3; EC 2.1.2.2) which catalyze the incorporation of one carbon units into the nascent purine ring system. We have been interested in studying the regulation of flux at this branch point, which has important implications for our understanding of folate metabolism in both normal and neoplastic cells

Mammalian methylenetetrahydrofolate reductase activity was first identified by Donaldson and Keresztesy (2), who showed that FAD was required for maximal activity. The enzyme was purified about 500-fold from pig and rat liver by Kutzbach and Stokstad (3). They demonstrated that the enzyme was inhibited by AdoMet and that the inhibition could be reversed by adenosylhomocysteine, and they provided evidence that these effectors were bound at an allosteric site on the enzyme Thus regulation of methylenetetra-

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*Abbreviations used are AICAR, 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide, GAR, glycineamide ribonucleotide, $CH_3\text{-}H_4\text{folate}$, methyltetrahydrofolate, $CH_2\text{-}H_4\text{folate}$, methylenetetrahydrofolate, $CH_2\text{-}H_4PteGlu_n$, methylenetetrahydropteroyl-polyglutamate with n glutamyl residues, $H_2PteGlu_n$, dihydropteroylpolyglutamate with n glutamyl residues, AdoMet, adenosylmethionine
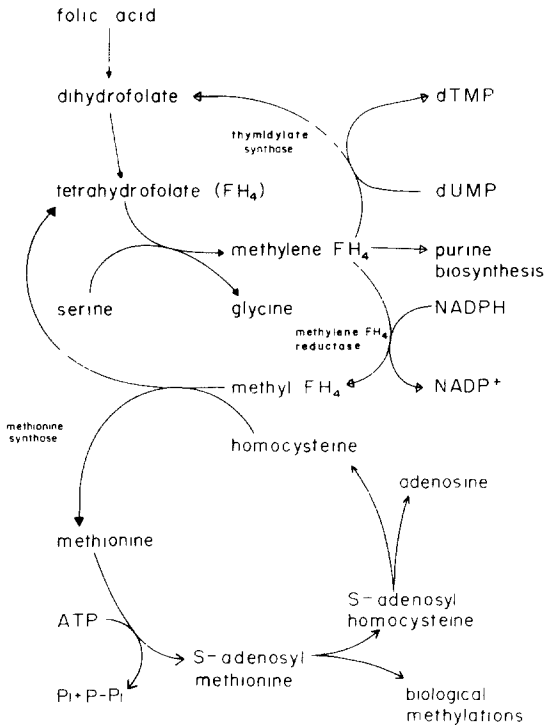
FIG 1 Pathways of mammalian folate metabolism

hydrofolate reductase activity appeared to conform to the pattern of feedback inhibition by the final common product of a reaction pathway

Regulation of methylenetetrahydrofolate reductase activity by the AdoMet/adenosylhomocysteine ratio is responsive only to the demands for one carbon units imposed by AdoMet-dependent biological methylations, and not to demands of the competing pathways of thymidylate and purine biosynthesis Thus, *a priori*, one might expect that methylenetetrahydrofolate reductase activity would also be modulated by cellular requirements for *de novo* biosynthesis of purines and pyrimidines as well as by cellular levels of AdoMet

We have now purified pig liver methylenetetrahydrofolate reductase to homogeneity and demonstrated that the enzyme contains non-covalently bound FAD as a prosthetic group (4, 5). In this paper we shall review our studies on the inhibition of the enzyme by dihydrofolate and its polyglutamate analogues (6, 7) and report the results of more recent studies on the modulation of enzymatic activity by AdoMet

MATERIALS AND METHODS

Homogeneous pig liver methylenetetrahydrofolate reductase was prepared as described by Daubner and Matthews (4, 5) and was used for studies of AdoMet inhibition AdoMet was purchased from Sigma or from Boehringer-Mannheim (both sources isolate AdoMet from yeast so that the metabolite is the natural stereoisomer both at the α-carbon of methionine and at the sulfonium pole). The purchased AdoMet was further purified by high performance liquid chromatography on an Altex Ultrasphere ODS column (8). Adenosylhomocysteine was purchased from Sigma and used without further purification For measurements of the effects of AdoMet and/or adenosyl-homocysteine on methylenetetrahydrofolate reductase activity, the assay methods used were those described previously (5, 6) except that all assays were performed in 50 mM potassium phosphate buffer, pH 6.7, containing 2 μM FAD For measurements of NADPH-menadione or NADPH-CH_2-H_4folate oxidoreductase activities in the presence of AdoMet or adenosylhomocysteine, the enzyme and buffer and any AdoMet or adenosylhomocysteine were preincubated in the assay cuvette for 10 min at 25° and then substrates were added to start the reaction For CH_3-H_4folate-menadione oxidoreductase assays the enzyme was preincubated with AdoMet and then diluted into an assay mixture containing the same concentration of AdoMet. Assays were teminated after incubation for 8 min at 25°

RESULTS AND DISCUSSION

Inhibition of Methylenetetrahydrofolate Reductase by Dihydropteroylpolyglutamates

Methylenetetrahydrofolate reductase exhibits parallel line kinetics during catalysis of NADPH-CH_2-H_4folate oxidoreduction. Dihydrofolate is an inhibitor of the reaction and is competitive with respect to CH_2-H_4Pte Glu_1 and uncompetitive with respect to NADPH. Dihydrofolate is a more potent inhibitor than similar concentrations of the natural stereoisomers of tetrahydrofolate, methyltetrahydrofolate, folic acid or methenyltetrahydro-folate (6). The K_i values for inhibition of NADPH-CH_2-$H_4$$PteGlu_1$ oxidoreductase activity by $H_2PteGlu_n$ derivatives with one to seven glutamyl residues have been measured (7) and are shown in Figure 2 In all cases the observed inhibition was linearly competitive with respect to CH_2-$H_4$$PteGlu_1$. The K_i values decreased from 6 5 μM for $H_2PteGlu_1$ to 0.013 μM for $H_2PteGlu_6$, and then increased to 0.065 μM for $H_2PteGlu_7$. These data indicate a free energy decrease of 0.75 kcal per glutamyl residue associated with the binding of the five terminal residues of $H_2PteGlu_6$. Methylenetetrahydro-pteroylpolyglutamates are substrates for methylenetetrahydrofolate reductase and show increased values for $k_{cat}/K_{CH_2\text{-}H_4PteGlu_n}$ relative to the monoglutamate
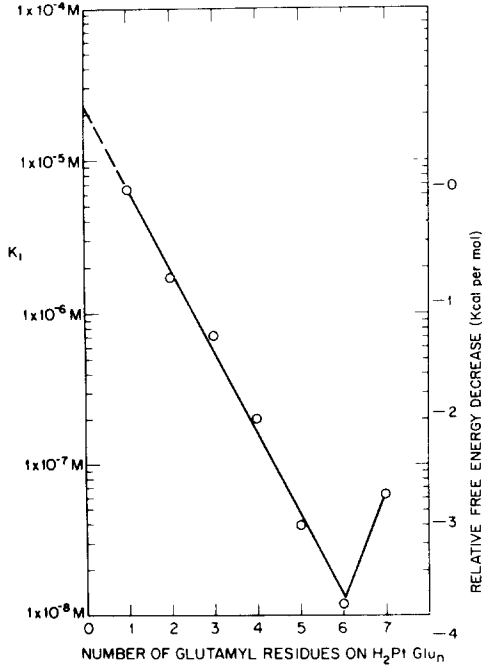
FIG 2 Variation of the K_i values for dihydropteroylpolyglutamate inhibitors with the number of glutamyl residues Individual K_i values were determined for the NADPH-CH_2-H_4PteGlu$_1$ oxidoreductase assay as described in reference (7) The K_i values represent dissociation constants for the interaction of H_2PteGlu$_n$ with that enzyme form which is binding the inhibitor, and are thus measures of the free energy decrease associated with binding of inhibitor to enzyme On the right hand border of the figure are shown the free energy decreases corresponding to each K_i value

substrate, with the maximum k_{cat}/K_m value being observed for the hexaglutamyl substrate (Table 1)

Intracellular CH_2-H_4PteGlu$_n$ is present mainly as the hexaglutamate in pig liver (9) and although the concentration of this metabolite has not been determined it is probably in the range from 0 5 to 5 μM in the interphase cell Thus, the free energy decrease associated with the binding of the polyglutamyl side chain of CH_2-H_4PteGlu$_6$ is used to lower the K_m for this substrate well below the probable concentration of this metabolite in the cell While we have no information on the polyglutamyl chain length of H_2PteGlu$_n$ in pig liver, we may perhaps assume that this too is largely present as the hexaglutamyl derivative, since it is generated primarily, if not exclusively, from CH_2-H_4PteGlu$_n$ by the action of thymidylate synthase The cellular concentration of H_2PteGlu$_n$ is too low to measure ($<$ 1 μM) Its concentration in cells has been estimated from the rate of thymidylate incorporation into DNA and

TABLE I KINETIC PARAMETERS ASSOCIATED WITH NADPH-CH_2-H_4PteGlu$_n$ OXIDOREDUCTION

Substrate	k_{cat} (min)$^{-1}$	$K_{CH_2-H_4PteGlu_n}$ (μM)	K_{NADPH} (μM)	$k_{cat}/K_{CH_2-H_4PteGlu_n}$ (μM)$^{-1}$(min)$^{-1}$
CH_2-H_4PteGlu$_1$	1600	7 1	16	225
CH_2-H_4PteGlu$_2$	2820	5 2	—	542
CH_2-H_4PteGlu$_3$	2740	1 7	15	1610
CH_2-H_4PteGlu$_4$	2770	0 62	76	4470
CH_2-H_4PteGlu$_5$	1020	0 26	125	3920
CH_2-H_4PteGlu$_6$	1090	0.10	185	10900
CH_2-H_4PteGlu$_7$	1090	0.51	176	2140

from the concentration and kinetic parameters of dihydrofolate reductase in these cells. The estimated value obtained by Jackson and Harrap was 0.02–0.06 μM (10) while the data of White and Goldman (11) suggest steady state concentrations in the range of 2–5 nm (Courtland White, personal communication). Values estimated in this manner are based on mean values of the rate of thymidylate incorporation into DNA for an entire population of logarithmically growing cells. Since only a small portion of the cells are actually synthesizing DNA at any given time, these values probably underestimate the rate of thymidylate biosynthesis during DNA synthesis. At present, we do not have sufficiently accurate data to assess the quantitative significance of H_2PteGlu$_6$ inhibition of methylenetetrahydrofolate reductase.

We have demonstrated that the activities of thymidylate synthase, methylenetetrahydrofolate reductase and methionine synthase are all associated with the cytoplasmic fraction of rat liver cells (Figure 3). In the case of thymidylate synthase, this is true whether the activity is measured in normal adult rat liver or 24 hr after partial hepatectomy, where thymidylate synthase activity is increased about 10-fold. Studies of Werkheiser (12) have shown that dihydrofolate reductase activity is also associated with the cytoplasmic fraction of rat liver cells Thus, in the rat, there is no evidence for differential metabolic compartmentation of thymidylate synthase, dihydrofolate reductase and methylenetetrahydrofolate reductase activities

As the data in Table 2 show, the methylenetetrahydrofolate reductase reaction velocity should be extremely sensitive to variations in the ratio of CH_2-H_4PteGlu$_6$ to H_2PteGlu$_6$ in cells The K_m of dihydrofolate reductase for its dihydropteroylpolyglutamate substrates lies between 0 1 and 5 μM, depending on the source of the mammalian dihydrofolate reductase and on the conditions under which the K_m values are measured (13, 14) In general, the K_m of the mammalian enzyme for H_2PteGlu$_n$ derivatives is rather independent of the number of glutamyl residues (14) Since H_2PteGlu$_n$ levels in the cell are generally lower than the K_m for this enzyme, elevations in the
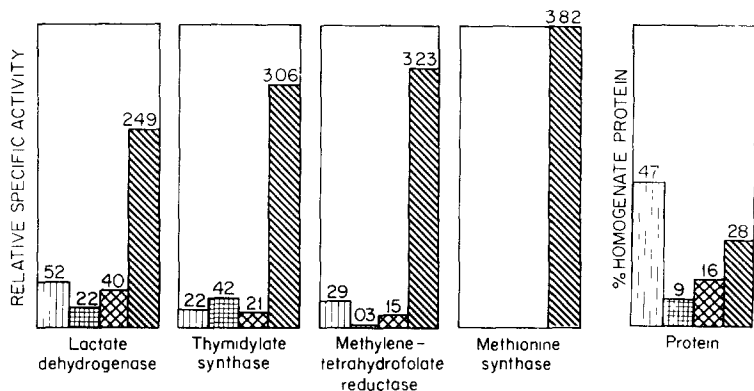
FIG 3 Cellular compartmentation of methylenetetrahydrofolate reductase, methionine synthase and thymidylate synthase from rat liver Adult male Sprague-Dawley rats were fasted for 24 hr prior to sacrifice The livers were excised and subjected to cellular fractionation as described by Hogeboom (16) Particulate fractions were solubilized by sonication in the presence of 0 05% Triton X-100 and 0 5% 2-mercaptoethanol From left to right are shown the relative specific activities of the nuclear, mitochondrial, microsomal and cytoplasmic fractions The enzyme assays used have been previously described thymidylate synthase (17), methylenetetrahydrofolate reductase (6), methionine synthase (18) and lactate dehydrogenase (19) The data shown here for thymidylate synthase were obtained using rat livers removed 24 hr after partial hepatectomy, but similar results were obtained from normal adult rat liver The protein content of the cell fractions was determined using Bio-Rad protein assay mix and bovine serum albumin as the protein standard The results shown here represent the averages of 3 separate cell fractionation studies
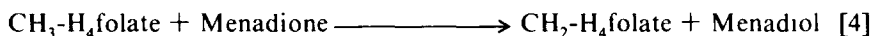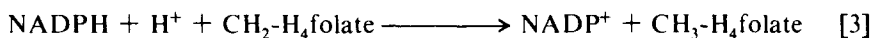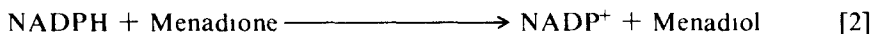
TABLE 2 VALUES OF I_{50} FOR $H_2PteGlu_n$
INHIBITION OF $CH_2-H_4PteGlu_n$ REDUCTION

Inhibitor/substrate	I_{50} at 5 μM substrate (μM)	I_{50} at 0 5 μM substrate (μM)
$H_2PteGlu_1/CH_2-H_4PteGlu_1$	11 1	6 95
$H_2PteGlu_2/CH_2-H_4PteGlu_2$	3 3	1 85
$H_2PteGlu_3/CH_2-H_4PteGlu_3$	2 8	0 89
$H_2PteGlu_4/CH_2-H_4PteGlu_4$	1 8	0 36
$H_2PteGlu_5/CH_2-H_4PteGlu_5$	0 81	0 11
$H_2PteGlu_6/CH_2-H_4PteGlu_6$	0 61	0 07
$H_2PteGlu_7/CH_2-H_4PteGlu_7$	0 69	0 13

rate of thymidylate biosynthesis will produce elevated steady state levels of dihydrofolate We suggest that the consequent decrease in the $CH_2-H_4folate/H_2folate$ ratio will lead to inhibition of methylenetetrahydrofolate reductase, sparing $CH_2-H_4folate$ for purine and pyrimidine biosynthesis Certainly, methylenetetrahydrofolate reductase activity will be decreased in cells which have been exposed to methotrexate, where the $H_2folate$ concentration may approach 20 μM (11)

Inhibition of Methylenetetrahydrofolate Reductase by Adenosylmethionine

We have investigated the inhibition of methylenetetrahydrofolate reductase activity by AdoMet by measuring three different activities of the enzyme.

$$NADPH + Menadione \longrightarrow NADP^+ + Menadiol \qquad [2]$$

$$NADPH + H^+ + CH_2\text{-}H_4folate \longrightarrow NADP^+ + CH_3\text{-}H_4folate \quad [3]$$

$$CH_3\text{-}H_4folate + Menadione \longrightarrow CH_2\text{-}H_4folate + Menadiol \quad [4]$$

If these reactions are all studied under the same conditions, *viz.* in 50 mM phosphate buffer, pH 6 7, 25°, AdoMet inhibits all three reactions

As reported by Kutzbach and Stokstad (3), we find that adenosylhomocysteine reverses the inhibition of the NADPH-menadione oxidoreductase activity exerted by AdoMet, and we obtain an apparent K_D of 3 μM for adenosylhomocysteine. In contrast, methylthioadenosine (150 μM) and methionine (560 μM) neither reverse the inhibition exerted by AdoMet nor have any inhibitory effect themselves The transitions between uninhibited and inhibited forms of the enzyme are slow, as previously observed by Kutzbach and Stokstad (3). Since all assays reported here were performed after a 10-min preincubation of the enzyme with AdoMet and/or adenosylhomocysteine in the absence of NADPH, we have assumed that the equilibrium between inhibited and uninhibited forms of the enzyme established during preincubation did not change during the time required for initial velocity measurements Kinetics of this type can not be described by the conventional equations for competitive or noncompetitive inhibition, which assume rapid equilibrium binding of inhibitor and varied substrate to the enzyme Rather, the enzyme behaves in the presence of non-saturating inhibitor as if it were a mixture of two forms of enzyme, not in equilibrium, each of which is described by its own kinetic equation. The proportion of the two forms of enzyme is a function only of I/K_i.

SUMMARY

Methylenetetrahydrofolate reductase catalyzes the reduction of methylenetetrahydrofolate to methyltetrahydrofolate This reaction commits one carbon units to the pathways of adenosylmethionine-dependent methylation in mammalian cells We have purified the pig liver enzyme to homogeneity and shown that it contains FAD as a non-covalently bound prosthetic group Methylenetetrahydrofolate is not only a substrate for the reductase, but also for thymidylate synthase and for methylenetetrahydrofolate dehydrogenase. The latter reaction leads to utilization of one carbon units in *de novo* purine biosynthesis. *A priori*, one might expect that methylenetetrahydrofolate

reductase activity would be modulated by cellular requirements for *de novo* biosynthesis of purines and pyrimidines, as well as by cellular levels of adenosylmethionine Methylenetetrahydrofolate reductase is inhibited by dihydrofolate and its polyglutamate analogues The K_i is 6 5 μM for dihydrofolate and decreases with each additional glutamyl residue to a minimum value of 0 013 μM for dihydropteroylhexaglutamate The I_{50} for dihydropteroylhexaglutamate inhibition of reductase activity in the presence of 0 5 μM methylenetetrahydropteroylhexaglutamate is 0 07 μM We propose that stimulation of thymidylate synthase activity (as in the replicating cell) may lead to elevations in the steady state levels of cellular dihydrofolate derivatives and to resultant inhibition of methylenetetrahydrofolate reductase activity Thus methylenetetrahydrofolate derivatives would be spared for purine and pyrimidine biosynthesis.

We have also examined the inhibition of methylenetetrahydrofolate reductase by adenosylmethionine, which serves as an allosteric effector of the enzymatic activity Adenosylmethionine induces a slow transition in the enzyme, and leads to the inhibition of NADPH-menadione, NADPH-methylenetetrahydrofolate and methyltetrahydrofolate-menadione oxido-reductase activities

ACKNOWLEDGEMENTS

REFERENCES

1   H M KATZEN and J M BUCHANAN, Enzymatic synthesis of the methyl group of methionine VIII Repression-depression, purification and properties of 5,10-methylene-tetrahydrofolate reductase from *Escherichia coli, J Biol Chem* **240**, 825–835 (1965)

2   K O DONALDSON and J C KERESZTESY, Naturally occurring forms of folic acid 1 "Prefolic A" Preparation of concentrate and enzymatic conversion to citrovorum factor, *J Biol Chem* **234**, 3235–3240 (1959)

3   C KUTZBACH and E L R STOKSTAD, Mammalian methylenetetrahydrofolate reductase Partial purification, properties, and inhibition by S-adenosylmethionine, *Biochim Biophys Acta* **250**, 459–477 (1971)

4   S C DAUBNER and R G MATTHEWS, Purification and properties of methylenetetra-hydrofolate reductase from pig liver, pp 165–172 in *Flavins and Flavoproteins* (V MASSEY and C H WILLIAMS, JR , eds), Elsevier/North Holland, New York (1982)

5   S C DAUBNER and R G MATTHEWS, Purification and properties of methylenetetra-hydrofolate reductase from pig liver, *J Biol Chem* **257**, 140–145

6   R G MATTHEWS and B J HAYWOOD, Inhibition of pig liver methylenetetrahydro-folate reductase by dihydrofolate Some mechanistic and regulatory implications, *Biochemistry* **18**, 4845–4851 (1979)

7   R G MATTHEWS and C M BAUGH, Interactions of pig liver methylenetetrahydro-
    folate reductase with methylenetetrahydropteroylpolyglutamate substrates and with
    dihydropteroylpolyglutamate inhibitors, *Biochemistry* **19**, 2040–2045 (1980)

8   S C DAUBNER, E KRAPF and R G MATTHEWS, Interactions of adenosyl-
    methionine with methylenetetrahydrofolate reductase, in *Transmethylation* (R
    BORCHARDT, R CREVELING and E USDIN, eds) Macmillan, New York (in press)

9   D G PRIEST and M T DOIG, Evaluation of folylpolyglutamates by electrophoretic
    separation of fluorodeoxyuridylate-thymidylate synthase-methylenetetrahydrofolate com-
    plexes, in *Folyl- and Antifolylpolyglutamates* (I D GOLDMAN, B CHABNER and J J
    BERTINO, eds) Advances in Experimental Biology, Plenum Press, New York (in press)

10 R C JACKSON and K R HARRAP, Studies with a mathematical model of folate
    metabolism, *Arch Biochem Biophys* **158**, 827–841 (1973)

11 J C WHITE and I D GOLDMAN, Methotrexate resistance in an L1210 cell line resulting
    from increased dihydrofolate reductase, decreased thymidylate synthetase activity and
    normal membrane transport Computer simulations based on network thermodynamics, *J
    Biol. Chem* **256**, 5722–5727 (1981)

12 W C WERKHEISER, Specific binding of 4-amino folic acid analogues by folic acid
    reductase, *J Biol Chem* **236**, 888–893 (1961)

13 R C JACKSON, L I HART and K R HARRAP, Intrinsic resistance to methotrexate of
    cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate
    reductases, *Cancer Res* **36**, 1991–1997 (1976)

14 J K COWARD, K N PARAMESWARAN, A R CASHMORE and J R BERTINO,
    7,8-Dihydropteroyl oligo-γ-L-glutamates Synthesis and kinetic studies with purified
    dihydrofolate reductase from mammalian sources, *Biochemistry* **13**, 3899–3903 (1974)

15 I H SEGEL, *Enzyme Kinetics*, Wiley, New York (1975)

16 G H HOGEBOOM, Fractionation of cell components of animal tissues, pp 16–19 in
    Methods in Enzymology, **1**, (S P COLOWICK and N O KAPLAN, eds), Academic
    Press, New York (1955)

17 S T ROODMAN and G R GREENBERG, A temperature-sensitive *thr* mutant blocked
    in the synthesis of thymidylate synthetase, *J Biol Chem* **246**, 2609–2617 (1971)

18 R T TAYLOR and H WEISSBACH, N^5-Methyltetrahydrofolate-homocysteine methyl
    transferases, pp 121–165 in *The Enzymes*, 3rd Edition (P BOYER, ed) **9**, Academic Press,
    New York (1973)

19 L A DECKER, *Worthington Enzyme Manual*, Worthington Biochemical, Freehold, New
    Jersey (1977)