PROSPECTIVE INHIBITORS OF SERINE HYDROXYMETHYLTRANSFERASE

BY

SAUL JONATHAN BENJAMIN TENDLER

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The biochemical role of serine and serine hydroxymethyltransferase (SHMT) are discussed, and the significance of this enzyme in tumour cells is reviewed. The activities of SHMT were examined in various tumour cell lines and in a variety of murine organs. The enzyme was partially purified from murine L1210 leukaemia cells.

DL-α-Vinyl-, DL-α-allyl- and DL-α-propargylserine were synthesised as potential irreversible enzyme activated inhibitors of SHMT. The preparative routes involved alkylations of appropriate stabilised anions.

The testing of the α-substituted compounds against total K562 cell SHMT showed DL-α-vinylserine to be a competitive inhibitor, with a Ki of 15.2 mM. DL-α-Allyl- and DL-α-propargylserine caused no inhibition of the enzyme from this source.

No dehydroxymethylation of the α-substituted compounds could be detected using the partially purified L1210 SHMT enzyme. The compounds were also not dehydroxymethylated by the homogeneous rabbit liver enzyme, nor was there any evidence for quinonoid formation, or any change in the absorbance of the enzyme.

The crystal structure of DL-α-vinylserine was determined by direct methods. Molecular modelling studies showed that for a modelled α-vinylserine-PLP conjugate there is no reason why the correct orientation for α-β bond cleavage could not be achieved.

Circular dichroism spectroscopy indicated that the lack of activity of these compounds was due to them not binding to the active site PLP in the enzyme from homogeneous rabbit liver.

The compounds were shown not to have antibacterial properties, nor did they cause growth inhibition in the K562 or GM0621 human leukaemia cell lines. In conclusion, the α-substituted serine analogues were not effective inhibitors of SHMT.

Key words: Serine hydroxymethyltransferase, EC 2.1.2.1, Inhibitor, Serine, Antitumour.

To my parents

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ABBREVIATIONS

ADH	Alcohol dehydrogenase.
BES	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulphonic acid.
CH-THF	5,10-Methenyltetrahydrofolate.
CH2-THF	5,10-Methylenetetrahydrofolate.
CD	Circular dichroism.
d	Doublet.
dd	Double doublet.
DCAS	N-Dichloroacetylserine.
DMSO	Dimethylsulphoxide.
DNA	Deoxyribonucleic acid.
DTNB	5,5'-Dithiobis(nitrobenzoic acid).
EDTA	Ethylenediaminetetraacetic acid.
FT	Fourier transform.
ID_{50}	Concentration to produce 50% inhibition of cell growth.
Ki	Inhibitor dissociation constant.
Km	Michaelis constant.
LD_{50}	Lethal dose for 50% of test population.
m	Multiplet.
Mer-	O6-guanine-DNA-methyltransferase deficient.
Mer+	O6-guanine-DNA-methyltransferase proficient.
m.p.	Melting point.
NAD	Nicotinamide adenine dinucleotide.
NADH	Reduced nicotinamide adenine dinucleotide.
NADP	Nicotinamide adenine dinucleotide phosphate.
NADPH	Reduced nicotinamide adenine dinucleotide phosphate.
NMF	N-Methylformamide.
NMR	Nuclear Magnetic Resonance
ODCAS	O-Dichloroacetylserine.

PAGE	Polyacrylamide gel electrophoresis.
PHA	Phytohaemagglutinin.
PLP	Pyridoxal-5'-phosphate.
q	Quartet.
RNA	Ribonucleic acid.
RPMI	Roswell Park Memorial Institute.
s	singlet.
SDS	Sodium dodecylsulphate.
t	Triplet.
TEMED	N,N,N',-Tetramethylethylenediamine.
THF	Tetrahydrofolate.
TLC	Thin layer chromatography.
Tris	2-Amino-2(hydroxymethyl)-1,3-proprandiol.
UV	Ultra Violet.
Vmax	Maximum reaction velocity.

'Unimportant, of course I meant,'

the king hastily said and went on

to himself in an undertone

'important-unimportant-unimportant-important '

as if he were trying which word sounded best.

ALICE'S ADVENTURES IN WONDERLAND
Lewis Carroll 1831-1898

CHAPTER 1

INTRODUCTION

1.1. Serine hydroxymethyltransferase

The enzyme serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1) catalyses the interconversion of L-serine and glycine, in the presence of DL-L-tetrahydrofolic acid (THF), generating a one-carbon unit in the form of N-5,N-10-methylenetetrahydrofolate (CH2-THF) (figure 1.1). This one-carbon unit may be used either for the biosynthesis of purines and pyrimidines or as N-5-methyltetrahydrofolate (CH3-THF) for the biosynthesis of methionine[1].

The enzyme was first discovered by Shemin[2] in 1946. It is found in a large variety of cells and organisms, including plants, bacteria, yeast, insects and mammals[3].

Eukaryotic SHMT has been purified to homogeneity from beef[4], rabbit[5], rat[6] and lamb liver[7]. It has also been partially purified from human leukaemic leukocytes[8], pig kidney[9] and monkey liver[10]. Mitochondrial and cytosolic forms of the enzyme are known, but the action of mitochondrial SHMT is still in question[1]. Homogeneous SHMT is a tetramer with a subunit molecular weight of about 53,000. Each subunit has a pyridoxal 5'-phosphate (PLP) bound as a Schiff's base to the terminal amine of a lysine residue[5]. The purified enzymes do not require any metal ions. In the chicken liver SHMT has been found in a folate-requiring enzyme complex[11].

1.2. The biochemical role of serine.

L-Serine is a non-essential amino acid; it is synthesised by a phosphorylated pathway as shown in figure 1.2. This utilises glucose

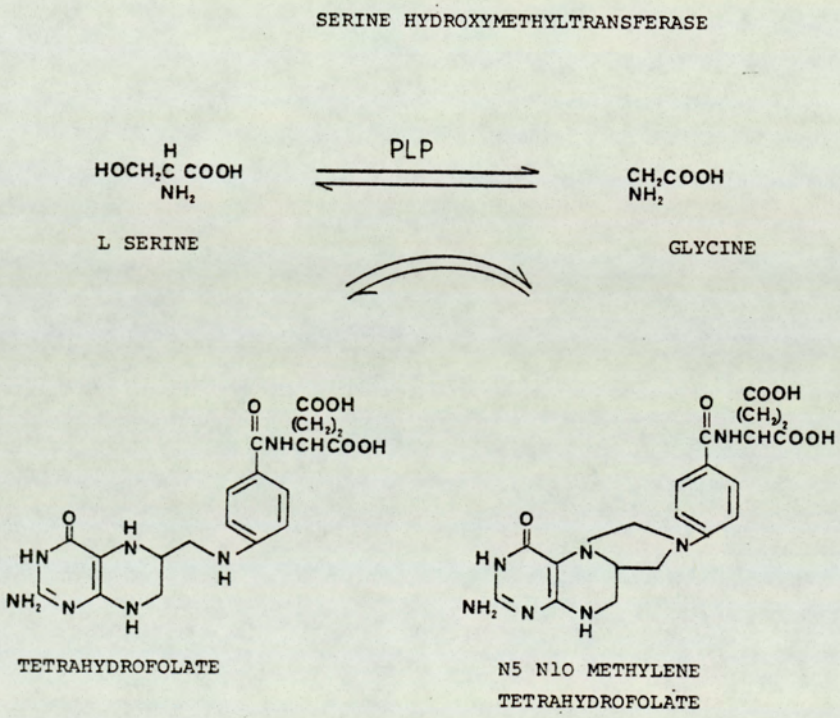


Figure 1.1. Serine hydroxymethyltransferase.

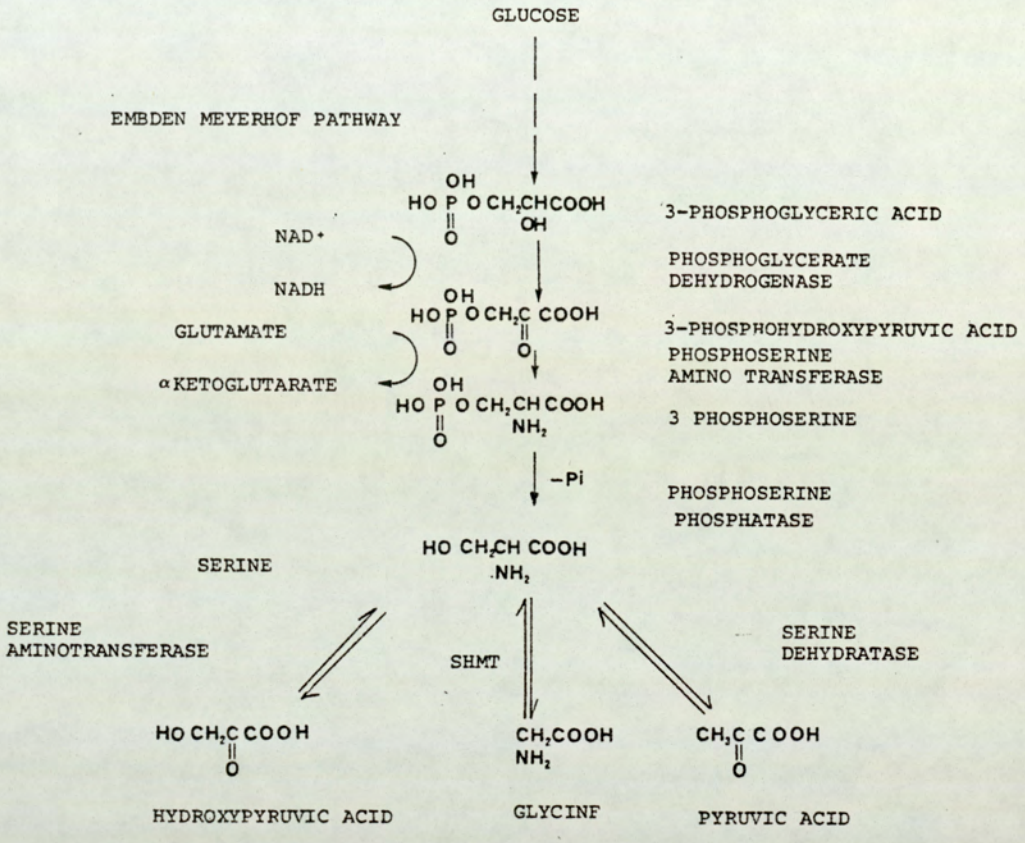


Figure 1.2. The biosynthesis of L-serine.

as its starting material which is then converted via the Embden-Meyerhof pathway to 3-phosphoglycerate[12]. Once synthesised, the majority of L-serine is used for the maintenance of the one-carbon pool by the enzyme SHMT[12]. L-Serine may also be converted by serine dehydratase to pyruvate, or serine aminotransferase to hydroxy-pyruvate which may then be utilised for gluconeogenesis.

Jenson sarcoma[13], neoplastic mast cells[14], chronic granulocytic leukaemia[15], HeLa cell lines[16] and Novikoff hepatoma[17] all have a nutritional requirement for L-serine, although it is normally a non-essential amino-acid. Conflicting evidence has been produced for the requirement of human bone marrow cells for L-serine[15,18]. When deprived of L-serine, BHK21 cells arrest in the G1 phase of cell growth[19]. This requirement for L-serine in cell proliferation may be due to the lack of the enzyme phosphoglycerate dehydrogenase as found in leukocytes from patients with granulocytic leukaemias[20]. Morris hepatomas have abnormally high levels of this enzyme, but are unable to vary the level in protein deprivation[21]. This increased requirement for L-serine in proliferating cells is also indicated by the elevated levels of phosphoserine phosphatase that have been found in neoplastic rat tissues[22].

Snell and Weber have recently investigated the levels of enzymes of serine synthesis and metabolism in rat hepatomas[23]. They found that the enzyme 3-phosphoglycerate dehydrogenase was present at elevated levels in the hepatomas. Unlike the other enzymes of serine utilisation, only SHMT was present in the hepatomas. This enzyme imbalance they suggest, is due to L-serine being biosynthesised preferentially for nucleoside formation in the tumour cells[23].

The activity of SHMT and other enzymes in the serine pathway during the neonatal development period of the rat[24], and of SHMT in

the chick embryo[25] indicates endogenous L-serine is required in periods of cellular proliferation. Two siblings with non-ketotic hyperglycinaemia with an inability to convert glycine to L-serine, were developmentally retarded[26].

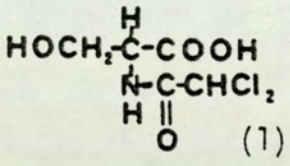
SHMT has been found in several tumour cells[27], with 5-10 fold elevated levels being reported in chronic lymphocytic leukaemia[28,29]. Following the mitogenic stimulation of human lymphocytes with phytohaemagglutinin (PHA) a 3-12 fold increase in the levels of SHMT was obtained[30,31]. A similar result was obtained when chronic lymphocytic leukaemia cells were stimulated with PHA[28]. The rapid increase in the level of SHMT on mitogenic stimulation, indicates that the enzyme has an important role in supplying one-carbon units for nucleotide synthesis in proliferating cells. This has been confirmed by Burkin and Draudin-Krylenko who found a relationship between the levels of SHMT in Friend's mouse leukaemia or Pliss' rat leukaemia with the rate of the tumours' development[32].

SHMT appears to have an essential role in rapidly proliferating tumour cells which have a high requirement for L-serine. In these cells it is found at elevated levels and serves to channel one-carbon units into nucleoside biosynthesis. If SHMT could be inhibited using analogues of L-serine, then it may be possible to inhibit the growth of these cells.

1.3. Serine analogues in the literature.

1.3.1. (DL)-N-Dichloroacetylserine.

Levi et al first reported in 1960 that the compound (DL)-N-dichloroacetylserine; Blevidon (DCAS) (1) showed promise as an antitumour agent[33]. The rationale for the molecule's synthesis was



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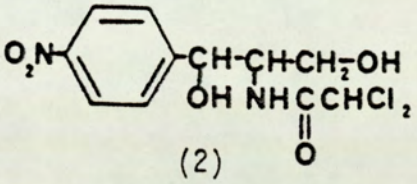
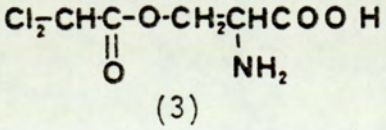


(2)

that several antibiotics have anticancer activity, therefore an amino-acid modified to contain a portion of an antibiotic may have antimetabolite properties. The addition of chloramphenicol's (2) N-dichloroacetyl group to DL-serine produced a compound that caused complete regression of sarcoma 37 in mice at a dose of 1 g Kg body weight^{-1} day^{-1} with the only toxic effect being a diuresis[34,35]. The LD_{50} for this compound in mice was shown to be[35] between 15 and 20 g Kg body weight^{-1}. DCAS when used in combination with cyclophosphamide showed an enhanced effect against Walker 256 tumour in rats. This synergy was also observed in sarcoma 37 in mice, as was the synergistic effect of DCAS with cyclophosphamide. The workers later proposed[36] that the activity of DCAS was due to an enzymatic N to O haloacyl shift producing DL-O-dichloroacetylserine (3) (ODCAS). ODCAS they reported to



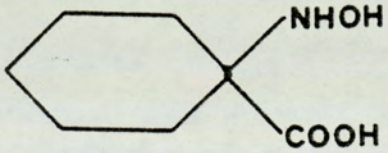
(3)

be active against sarcoma 37 at 1/40th the dose and sarcoma 180 at 1/20th the dose[36].

In clinical trial, DCAS at 50 g, three times weekly, showed little effect[35]. All patients experienced a diuresis with an induction of thirst and a slight drop in blood pressure. In combination therapy with X-irradiation or cyclophosphamide, DCAS showed more promise, with positive results in 5 out of 21 cases. The DCAS was given at a dose of 5-12 g day^{-1} orally, with the same side effects being noted. In addition 19 out of 21 cases developed a neuropathy which was associated with a pyridoxine deficiency[35]. It appeared that this toxicity prevented this compound proceeding to further evaluation.

1.3.2. Cyclohexylserine.

1-(Hydroxylamino)cyclohexanecarboxylic acid; Cyclohexylserine (4) was synthesised by Pazmiño et al as a cyclic serine analogue[37], although it shows very little structural resemblance to serine. The molecule inhibited RNA and DNA synthesis at $1x10^{-3}M$ in human chronic granulocytic cells, with L-serine present at normal levels ($1x10^{-4}M$). The reversal of the DNA synthesis inhibition by the addition of formate, allowed the authors to postulate that the compound may be an inhibitor of SHMT.



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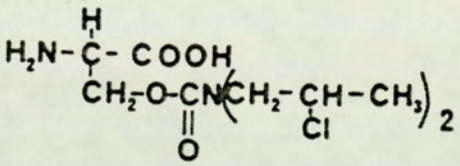
1.3.3. CB-3210.

CB-3210; Camosin, DL-Serine bis(chlorpropyl)carbamate ester (5), NSC-37023, was first synthesised in 1959[38]. It was shown to be active against a number of tumour models including Walker 256, Dunning leukaemia and Ehrlich ascites, but was inactive against L1210 leukaemia, sarcoma 180 and adenocarcinoma 755. At elevated doses CB-3210 had some influence on the central nervous system and the endocrine system in C57/Bl mice bearing adenocarcinoma 755 [39]. In clinical trials CB-3210 given orally (0.5-1.5 g day^{-1}) was evaluated in 24 patients. Nine of seventeen patients with stage IV breast cancer and 1 of 5 with prostatic cancer showed improvement[40]. The side effects for this compound were severe, including nausea, vomiting, diarrhoea, leukopaenia, a drop in haemoglobin levels, and a neurological syndrome. The neurotoxicity was present as malaise and muscle ache[40].



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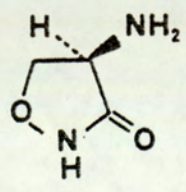
1.3.4. D-Cylcloserine and related compounds.

D-Cycloserine; oxamycin, 4-amino-3-isoxazolinone (6), is used clinically as an antibacterial. The action of this compound is due to



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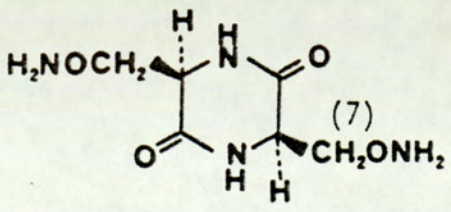


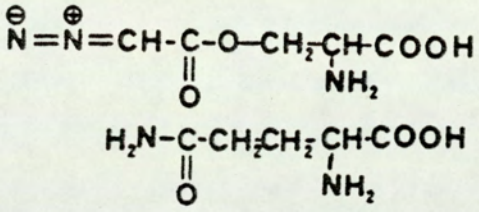
(7)

the competitive inhibition of the enzyme D-alanylalanine synthetase (EC 6.3.2.4), which is involved in bacterial cell wall synthesis[41]. Burkin and Draudin-Krylenko found both D-cycloserine and its dimer 3,6-bis(amino-hydroxymethyl)-2,5-diketopiperazine (7), to be effective inhibitors of the enzyme SHMT[32]. The inhibition of SHMT was shown to be short lasting in Friend's leukaemia bearing mice, with the rate of recovery of normal enzyme levels being dependent on the level of PLP present[42]. Potentiation of the inhibition of SHMT was found using the PLP antimetabolite 4-vinylpyridoxal[43]. The interaction of D-cycloserine with SHMT has been recently investigated by Manohar et al[44]. They found that 2.5 mM D-cycloserine irreversibly inhibited the enzyme, causing the disassociation from the active site of a PLP-D-cycloserine complex. When the PLP was replaced the enzyme was fully reactivated. The mechanism of this action was not due to the abstraction of the proton analogous to the α-position in serine, as there was no evidence of quinonoid formation.

Little evidence of antitumour activity was found by Weaver et al for D-cycloserine against nasopharyngeal epidermoid carcinoma cells in cell culture[45].

1.3.5. Azaserine.

The identification of an active antitumour agent in a Streptomyces culture[46] led to the discovery of O-diazoacetyl-L-serine; azaserine (8)[47]. The L-form of this compound was shown to be the most effective, with activity at 1 mg Kg^{-1} day^{-1} against Crocker mouse sarcoma 180[48].



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(9)

Azaserine, an analogue of L-glutamine (9), blocks the enzymatic transfer of amide groupings in the synthesis of purines, inhibiting the enzymes phosphoribosylformyl-glycinamide synthetase and amido-phosphoribosyl transferase[49].

Azaserine induced single stranded DNA breaks in several strains of Esherichia Coli. The damage induced in DNA could not be induced by the compound itself, indicating that some type of activation was required[50]. This activation may be via the production of the reactive diazoacetic acid from azaserine. Longnecker and Snell showed that pyridoxal in the presence of metal ions catalyses the de-amination of azaserine to give pyruvate, ammonia and diazoacetic acid[51]. This deamination was also shown to occur with serine-3-phosphate and O-carbamylserine[51] and follows an $\alpha\beta$ elimination, figure 1.3.

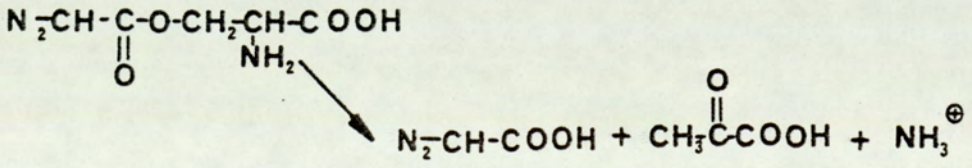


Figure 1.3. The activation of azaserine.

1.4. Mechanism of action of SHMT.

1.4.1. The role of pyridoxal phosphate.

PLP is bound at the active site of the enzyme SHMT[52] in the form of a Schiff's base[5]. The mode of action[1] of the PLP in the bond cleavage of L-serine at the enzyme's active site is as shown in figure 1.4. The spectral species were determined by comparing the absorbance bands with those of known model compounds[53].

L-Serine attacks the PLP bound to an active site lysine, to form a geminal diamine II, with the transfer of a proton from the L-serine onto the terminal amine group of the lysine. This is then followed by a second nucleophilic attack by the L-serine to give the L-serine Schiff's base III. In the presence of THF the hydroxymethyl group is then cleaved to give a resonance-stabilised carbanion, which may be represented by quinonoid canonical forms. For convenience this will be referred to as the quinonoid intermediate IV. The protonation of the carbanion, followed by a reversal of the transimination produces glycine and CH2-THF. The role of THF is discussed in section 1.5.1. A ^{31}P nuclear magnetic resonance (NMR) study showed that the pKa of the PLP was 6.45[54]. Upon the addition of glycine, L-serine and THF no change in the chemical shift or the pKa of the phosphate group was obtained, indicating that no change occurs in the environment of the phosphate group on catalysis[54].

1.4.2. Structure activity relationships and stereochemistry of bond cleavage.

SHMT will also catalyse the interconversions listed in table 1.1.

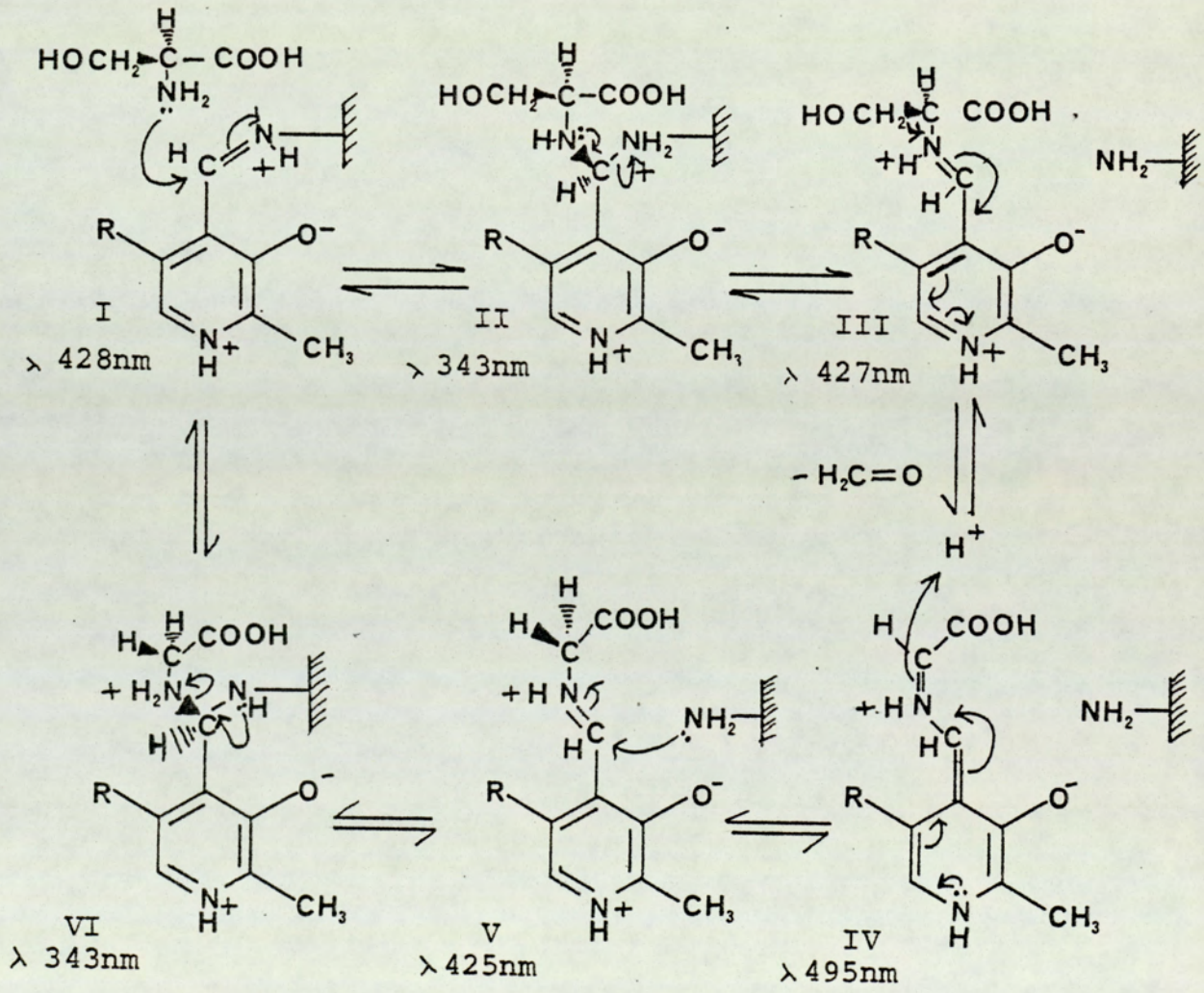


Figure 1.4. The mode of action of the active site PLP in bond cleavage.

Table 1.1. Reactions catalysed by SHMT.

	Reaction	Reference
1.	L-serine + THF ⟶ glycine + CH2-THF	(3)
2.	α-methyl-serine + THF ⟶ D-alanine + CH2-THF	(55)
3.	allothreonine ⟶ glycine + acetaldehyde	(56)
4.	L-threonine ⟶ glycine + acetaldehyde	(56)
5.	erythro-β-phenylserine ⟶ glycine + benzaldehyde	(7)
6.	threo-β-phenylserine ⟶ glycine + benzaldehyde	(7)
7.	D-alanine + PLP ⟶ pyruvate + pyridoxamine P	(57)
8.	aminomalonate ⟶ glycine + CO_2	(58)
9.	glycine ⟶ methylamine + CO_2	(59)
10.	β-hydroxytrimethyllysine ⟶ glycine + butyrobetaine aldehyde	(60)

indicating that it is able to accept a wide range of substrates. The enzymatic dealdolisation of L-threonine and allothreonine[56], and erythro- and threo- β -phenyl serines[7], both proceed with the erythro isomer having a higher Vmax and lower Km values. Ulevitch and Kallen[61] studied the requirements for the dealdolisation of DL-erythro-β-phenylserines. They found that in cleavage by SHMT there was an ordered release of first benzaldehyde and then glycine, figure 1.5. If the reaction was carried out in deuterium oxide, a kinetic isotope effect of 6.25 was obtained indicating that the quinonoid was quenched by the solvent.

For β-aryl substituted L-erythro-β-phenylserines the Hammett substitution values for dealdolisation were k2 = -0.94 (±) 0.21, k3 = 0 and 1/K1 =+0.96 (±) 0.21.

$$E + S \underset{k-1}{\overset{k1}{\rightleftharpoons}} ES \underset{k-2}{\overset{k2}{\rightleftharpoons}} B + EQ_{500} \xrightarrow{k3} G + E$$

$K1$

Abbreviations: B, benzaldehyde;

EQ_{500}, Enzyme quinonoid complex.

Figure 1.5. The cleavage of substituted β-phenylserines.

The Hammett ρ value for k2 suggests that for α-β bond cleavage stabilisation is given by electron donating species. The Hammett substitution value for 1/K1 suggests that prior to cleavage alkoxide formation occurs. The enzyme is specific for L-amino-acids although D-alanine is a substrate[57]. It is transaminated to give pyruvate and pyridoxamine-5'-phosphate which then diffuses from the active site, inactivating the enzyme[57].

The stereo specificity is as predicted by Dunathan[62]. He proposed that the loss of a group from the α-carbon of an amino-acid bound to PLP as a Schiff's base is accompanied by an increase in the system's delocalisation energy. For this gain in energy to cleave the σ bond, this bond must be held in a plane perpendicular to the delocalised system. The overlap allows the electrons of the σ bond to fall into the PLP imine, as shown in figure 1.6.

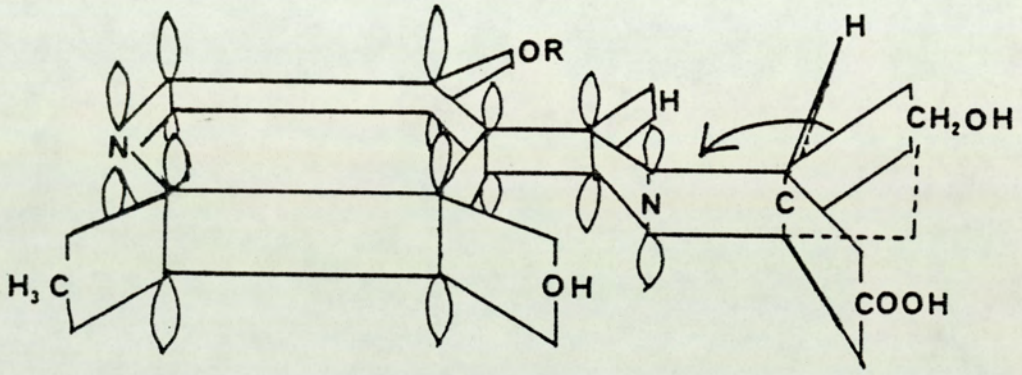


Figure 1.6. The labilisation of the α-β bond.

This conformation may be achieved by electrostatic bonding via the carboxylate, fixing the amino-acid in the correct orientation. It follows that if D-serine is presented to the enzyme, the hydroxymethyl group is in the wrong conformation for bond cleavage. D-Alanine is transaminated as its proton is in the correct conformation for bond cleavage[62].

Glycine, as predicted by Dunathan, when processed by the enzyme is exclusively hydroxymethylated by abstraction of the pro(S) proton[63]. All of the enzyme's bond breaking is stereospecific, with the bond equivalent to the pro(S) proton being cleaved with two exceptions; (3-^{14}C)-aminomalonate is not decarboxylated with stereospecificity[58] and the enzyme removes the pro(R) proton from L-

phenylalanine[64]. These results indicate that the enzyme may also be able to achieve the conformation required to cleave the pro(R) bond in some molecules.

1.4.3. The active site of SHMT.

It is at present unknown which nucleophile is responsible for the α-β bond cleavage in SHMT. Three possibilities exist in theory, as shown in figure 1.7.

Scheme C is unlikely, since when ^{18}O-threonine is cleaved by SHMT all of the label is found in acetaldehyde[63], which is inconsistent with this mechanism.

While scheme B is possible, scheme A is the most likely, with the nucleophilic thiol trapping the aldehyde as a thiohemiacetal. In the case of formaldehyde, this is stable and will not be readily removed, which may account for the role of THF. This is further discussed in section 1.5.1. Both benzaldehyde and acetaldehyde will be spontaneously eliminated from the active site.

Schirch has shown that two thiols are present near the active site of the cytosolic enzyme. These react rapidly with 5,5'-dithio-bis(2-nitrobenzoic acid) DTNB, inactivating the enzyme and causing the loss of PLP[65]. On removal of the active site PLP, one of these groups is inactivated by iodoacetate which is bound to an adjacent cationic site[66].

Methyl methanethiosulphonate, which reacts rapidly with thiols to give a methyl disulphide, was used as an active site probe to look at the eight active site thiols identified by tryptic digestion. Five of these thiols reacted with this reagent. Of these thiols, the two that reacted with DTNB are not critical for catalysis, but hold the PLP amino-acid Schiff's base in position in the enzyme's active site.

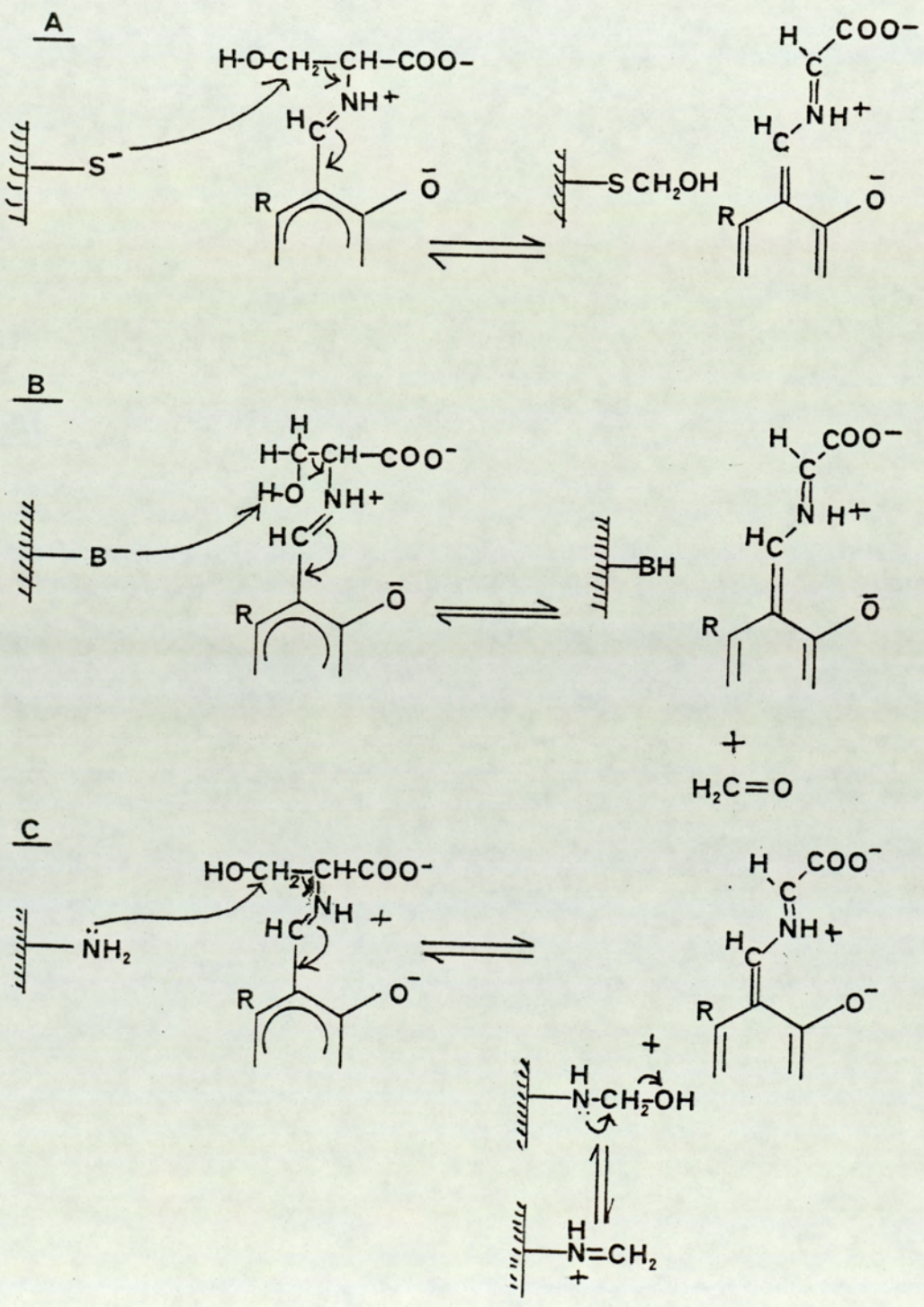


Figure 1.7. The three possible nucleophiles at the active site of SHMT.

It is one of these thiols that is blocked when the enzyme is inhibited by D-fluoroalanine (section 1.7.2). Two thiols are essential for the catalytical activity of SHMT. The remaining thiol only reacted on the treatment of the apo-SHMT with methyl methanethiosulphonate[67].

The nonapeptide that binds the PLP of cytosolic rabbit liver SHMT has been shown[68] to be Val-Val-Thr-Thr-Thr-His-Lys(PLP)-Thr-Leu. This was later shown to contain the (-Thr-)4 and not (-Thr-)3 sequence, and it is identical to that of the mitochondrial enzyme [69]. The histidine is present adjacent to the lysine on the N-terminal side as in many PLP enzymes.

The active site of sheep liver SHMT has been shown to contain at least one residue each of arginine, cysteine and histidine[70]. However the two cysteine residues found near the active site of the cytosolic enzyme are missing in the mitochondrial form[69].

1.4.4. Mitochondrial SHMT.

Mitochondrial SHMT is found in the matrix of the mitochondrial membrane. Due to the lack of THF in the membrane the enzyme shows little activity. It has been proposed to act as a one-carbon shuttle across the mitochondrial membrane[71]. Both enzymes have similar structural properties and similar affinities for amino-acids, except that the mitochondrial enzyme has a four-fold affinity for glycine[72].

Kikuchi has suggested that the small proportion of SHMT in association with the mitochondrial inner membrane[73] is coupled to the glycine dehydrogenase complex[74]. Snell has proposed that the stoichiometry[12] is as follows, figure 1.8.

Glycine + NAD^+ + THF $\rightleftharpoons$ CO_2 + NH_4^+ + NADH + H^+ + CH2-THF

Glycine + CH2-THF $\rightleftharpoons$ L-serine + THF

Giving a net;

2 Glycine + $NAD^+ \rightleftharpoons$ L-serine + CO_2 + NH^+_4 + NADH + H^+.

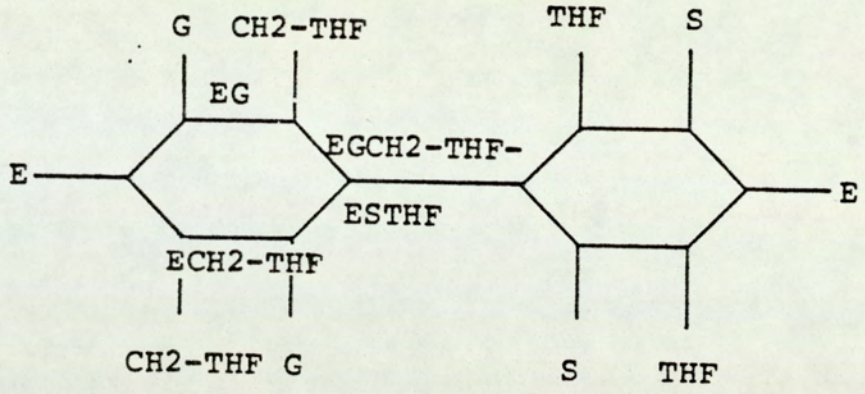
Figure 1.8. Proposed stoichiometry of SHMT-glycine dehydrogenase complex

1.5.1. The role of THF.

The finding that THF is required for the cleavage of L-serine and α-methyl serine suggests that its role is to transport formaldehyde in the active site.

Schirch and Jenkins[75] showed that the addition of THF to enzyme-glycine complexes caused an increase in the absorbance of the 500 nm band, and a stimulation of the exchange of the α-proton. This they interpreted as THF acting as a base abstracting the proton. Evidence now suggests that the role of THF is the reverse, that is to slow down the re-protonisation of the quinonoid. In temperature-jump experiments the rate of formation of the quinonoid is twice that of the rate of α-proton exchange in glycine with no THF present. When THF is added the rate of formation of the quinonoid increases by about four-fold, while the rate of proton removal is only increased by a factor of two[76]. In the case of allothreonine, the synthesis is much faster than the rate of exchange of the α-proton in the absence of acetaldehyde, and so THF is not required to inhibit the re-protonation of the quinonoid[77]. THF is not an absolute requirement for the conversion of L-serine to glycine, as in its absence the conversion occurs, albeit slowly[77].

The enzyme SHMT has shown to operate via a sequential random mechanism[78], as shown in figure 1.9, with THF adding and removing formaldehyde from the active site of the enzyme.



Abbreviations:G, glycine;S, serine ;E, SHMT.

Figure 1.9. The sequential random mechanism of SHMT.

THF reacts rapidly at near neutral pH with formaldehyde to form N-5-hydroxymethyl-THF. figure 1.10. This carbinolamine then slowly dehydrates, to give the cationic imine. This imine is very reactive and is nucleophilically attacked by the N-10 position of the molecule to give CH2-THF. The rate limiting step in the pathway is the dehydration of the carbinolamine[79].

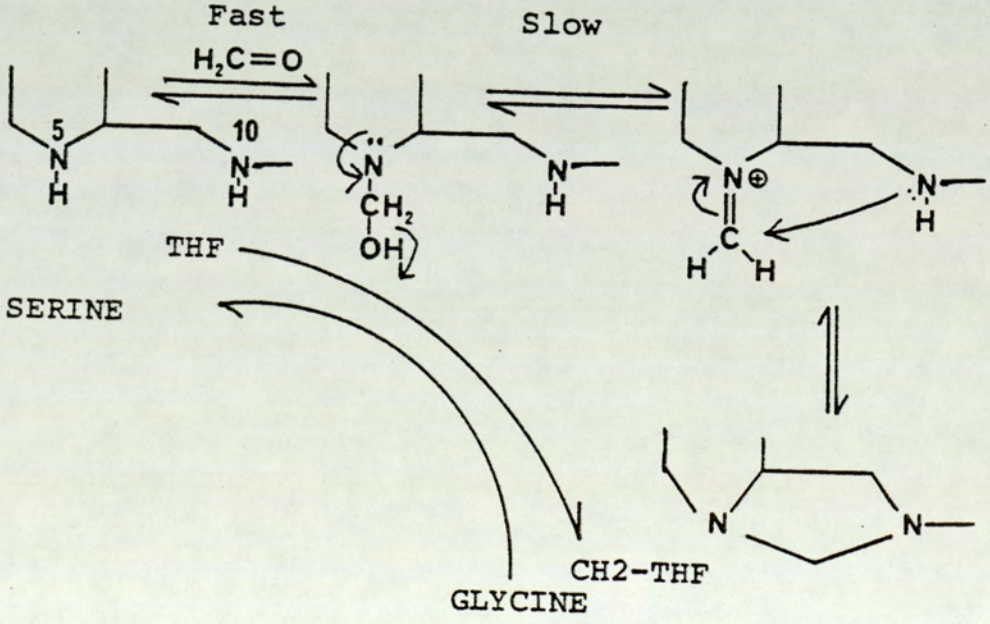


Figure 1.10. The formation of CH2-THF.

If THF and excess formaldehyde are mixed in a cuvette, CH2-THF is produced. On the addition of glycine and SHMT, L-serine and THF are formed. The THF then reacts with the excess formaldehyde to give CH2-THF which allows the catalysis to proceed. At low enzyme concentrations the enzymatic rate of catalysis is rate limiting, however at high levels of enzyme the attack on the carbinolamine becomes limiting[80].

The stereochemistry of the addition of the formaldehyde equivalent to THF has been determined by Slieker and Benkovic. They found that the enzymatic cleavage of (3-S)- and (3-R)-(3-^{1}H,^{2}H)serine produced (6R-11S)- and (6R,11R)-5,10-methylene(11-^{1}H,^{2}H)tetrahydrofolate respectively[81].

It is still unknown how the formaldehyde is transferred from L-serine to the THF. Whether this occurs directly or via an intermediate such as a thiohemiacetal remains to be elucidated.

1.5.2. The NAD binding site and the regulation of THF binding.

The role of homotropic binding of THF to SHMT has been quite controversial. Cooperation between THF and SHMT has been reported by Kumar *et al*[9]. It was found in the pig kidney enzyme and in normal mouse liver, but not in mouse L1210 leukaemia[9]. The cooperation was also found to be abolished in the livers of mice bearing the L1210 leukaemia, suggesting the formation in the tumour of a biochemical mediator inhibiting the cooperation[9]. Ramesh and Rao[10] found that positive homotropic binding of THF to monkey liver SHMT was inhibited on the addition of reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) but only at a concentration of 10-50 mM, and that heating the enzyme inhibited the allosteric property.

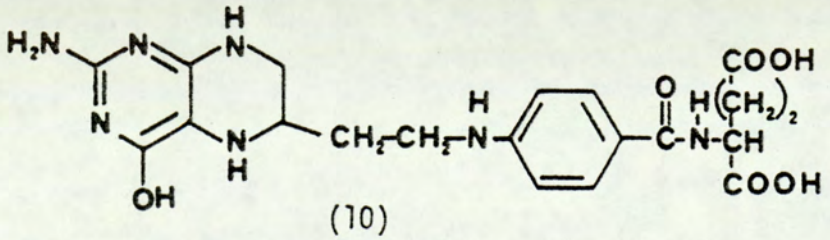
Schirch has presented evidence, however, that this cooperation was an artefact of their experimental conditions, with the THF being oxidised by molecular oxygen in the pre-incubation stage of the assay[82]. He also could find no evidence that nicotinamide nucleotides bind to the enzyme.

1.5.3. Cibacron blue 3G-A.

Following their finding of the nicotinamide nucleotide binding site[10] Ramesh and Rao[83] investigated with the dye cibacron blue, which being a structural analogue of NAD^+, has been used when bound to inert supports to purify many enzymes by binding to their dinucleotide fold. They found that the dye was inhibiting SHMT competitively with respect to THF, and that L-serine, NADH and NADPH had no effect on this inhibition. These results indicate that the dye binds at the THF binding site, and not the NADH binding domain. Thresher and Swaisgood have immobilised this dye on glycerolpropyl-glass beads, which may be useful in the long term purification of the enzyme[84]. A Cibacron blue column has been utilised in the assay of porcine liver SHMT[85].

1.5.4. Tetrahydrohomofolate.

Tetrahydrohomofolate (10); NSC 89473, a homologue of THF, was shown to be an inhibitor of L1210 SHMT with an ID_{50} of 6.25 μM. In whole cell studies (3-^{14}C) serine incorporation into nucleic acids and methionine was inhibited by this compound[86].



(10)

1.6. Mechanism of control of SHMT.

The affinity of the enzyme SHMT from pig liver for glycine has been shown to be dependent on the number of glutamyl residues attached to methyl tetrahydropteroyl-(glutamate)$_n$. An increase in the number of residues increases the enzyme's binding of the amino-acid, with n>3 causing effective inhibition[87] at concentrations of glycine higher than 20 μM. Polyglutamation may provide a method for the cell of controlling the enzyme. Nitrous oxide, an inducer of elevated levels of folate polyglutamates[87], does not, however, inhibit the enzyme SHMT[88].

Both E. coli and S. Typhimurium have methionine and purine levels implicated in the control of the SHMT levels[89,90]. In E. coli methionine auxotrophs[90], a relationship between the ratio of homocysteine to S-adenosylmethionine and SHMT activity was found. Homocysteine was found to be an inducer and S-adenosylmethionine a repressor molecule, but only during methionine depletion. SHMT in the rat liver has been shown to be increased significantly on methionine loading[91]. Upon this treatment, an inhibition of CH_2-THF-reductase was induced due to the production of elevated levels of S-adenosylmethionine.

The methionine requirement for the control of eukaryotic cells is also shown by the compound L-2-amino-4-methoxy-trans-3-butenoic acid. This molecule, a methionine antagonist, was found to increase the levels of SHMT in Walker carcinoma cells[92] (see section 1.7.3).

1.7. Enzyme activated irreversible inhibitors.

For an enzyme to be irreversibly inhibited the inhibitor normally has to bind covalently, which may be achieved in one of two ways[93]. The first is by the use of alkylating agents: these

molecules are chemically reactive and their selectivity, if any, is due to their structural similarity with the normal substrate of the enzymes or due to their distribution. Carboxymethylation of an active site thiol of SHMT by iodoacetate, results in a loss of activity[66]. The compound, however, shows no selectivity.

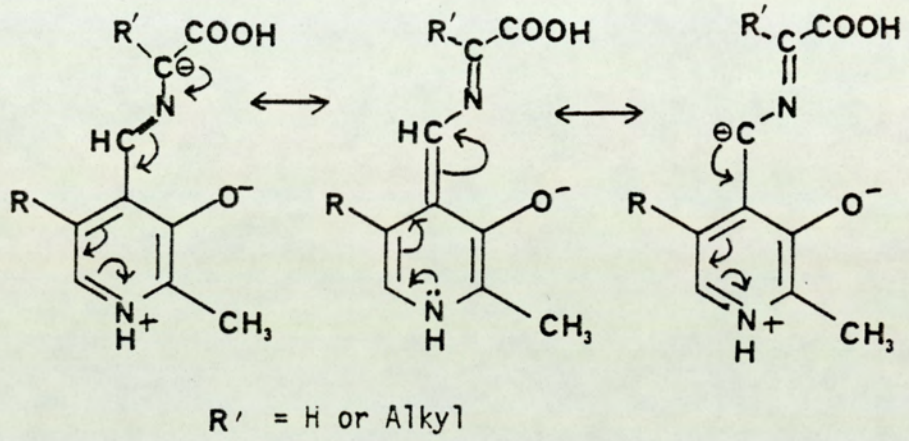
The second and more selective method of alkylation is by the enzyme producing the reactive species in situ. Molecules which resemble the normal substrate and are chemically inactive may, upon catalytic activation, produce a chemically reactive species. This intermediate-enzyme complex may either react covalently with the enzyme, irreversibly inhibiting it, or dissociate.

These compounds are known as kcat inhibitors[94], suicide enzyme inactivators[95], suicide substrates[96] or enzyme activated irreversible inhibitors[97]. The latter title is the most accurate as there is little analogy between the taking of one's own life and activated inhibition, the anthropomorphism is unjustifiable.

1.7.1. The design of enzyme activated irreversible inhibitors.

Using the knowledge of the mechanism of action of an enzyme, selective inhibitors may be rationally designed. Many enzyme activated inhibitors of PLP enzymes have been shown to be active, as will be discussed later. The activity of these molecules is based upon them being isosteres of the normal substrates, and binding to the PLP in the normal manner.

Upon the α-β bond cleavage an intermediate that can be represented by quinonoid and carbanionic canonical forms is produced, figure 1.11.



R′ = H or Alkyl

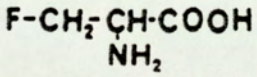
Figure 1.11. The quinonoid and carbanionic canonical forms produced on bond cleavage.

For convenience this will be referred to as the quinonoid intermediate, but for activity the carbanion formation is essential. The fate of this intermediate may involve the protonation of the 4′-carbon position of the co-enzyme or the α-carbon of the amino-acid. This protonation will determine the position of the double bond of the imine, and so determines whether or not transamination occurs following hydrolysis.

If, however, the α-position of the amino-acid also contains a group capable of elimination or rearrangement then a Michael acceptor may be formed. This is shown using the examples of known enzyme activated inhibitors of PLP enzymes.

1.7.2. D-Fluoroalanine.

D-Fluoroalanine, (11), has been shown by Wang et al[98] to be a mechanism based inhibitor of SHMT. It falls into the group of PLP enzyme inhibitors with an electronegative substituent on the β-carbon. On the formation of the quinonoid intermediates, elimination of hydrogen fluoride occurs to give an α-β unsaturated amino-acrylate.



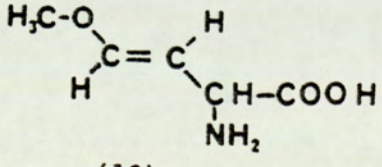
(11)

These intermediates partition between the hydrolysis to pyruvate, ammonia and PLP, and nucleophilic attack from an active site thiol, irreversibly inhibiting the enzyme as shown in figure 1.12. The ratio of partition of the amino-acrylate compound was found to be one inactivation per 40-60 turnovers.

The turnover of the elimination is about 120 min^{-1}, but the Km of the compound is high at 10-60 mM, suggesting that the compound is of no use for the in vivo inhibition of SHMT.

1.7.3. α-Vinyl substituted amino-acids.

α-Olefin substituted α-amino-acids prove to be efficient inhibitors of PLP enzymes. The first compound of this class studied was the bacterial toxin L-2-amino-4-methoxy-trans-3-butenoic acid (12), a potent inhibitor of pig heart aspartate aminotransferase[99,100].



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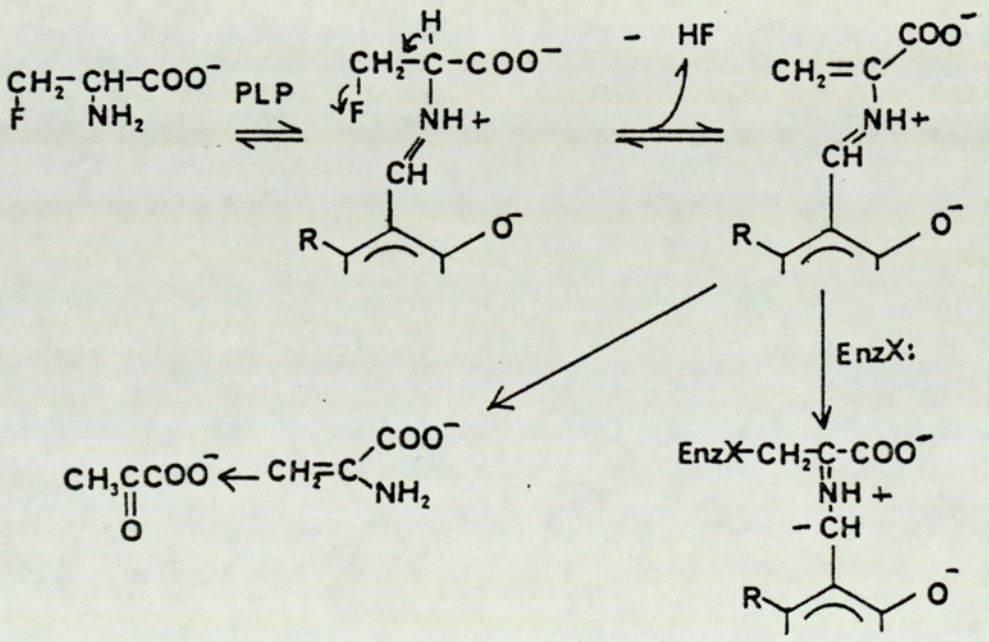
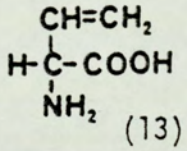


Figure 1.12. The mechanism of action of D-fluoroalanine.

It is also an inhibitor of bacterial tryptophan synthetase[101]. The mechanism of action of this molecule is shown in figure 1.13, with nucleophilic attack by an active site base with the concomitant loss of methoxide resulting in the inhibition of the enzyme. This inhibition is highly efficient, with each turnover of the compound resulting in irreversible inhibition[100]. Tisdale has shown that this compound is an enzyme activated non-competitive inhibitor with respect to L-serine of SHMT from cytosolic extracts of Walker carcinoma cells, with a similar Km to that of L-serine. When the Walker carcinoma cells were incubated with 12 an increase in the enzyme activity of SHMT was obtained[92] (see section 1.6.).


(13)

The simplest α-vinyl-amino-acid is vinylglycine (13). This was found to be an aspartate aminotransferase inhibitor[102] and it was also found to inhibit aspartate transaminase[103]. The general route of inactivation is shown in figure 1.14.

Active site nucleophiles will attack the β-carbon site resulting in enzymatic inactivation. Vinylglycine is 90% efficient in inhibiting the transaminase. It will also transaminate and inhibit D-amino-acid transaminases with a k trans ki =450 and ki =6 x 10^{-3} s^{-1}.

α-Vinylornithine has been shown to inactivate ornithine decarboxylase, probably by a similar mechanism[104]. Many other α-vinyl-amino-acids are known to inhibit PLP enzymes[103] indicating that this general class of compound may be used as irreversible inhibitors of PLP enzymes.

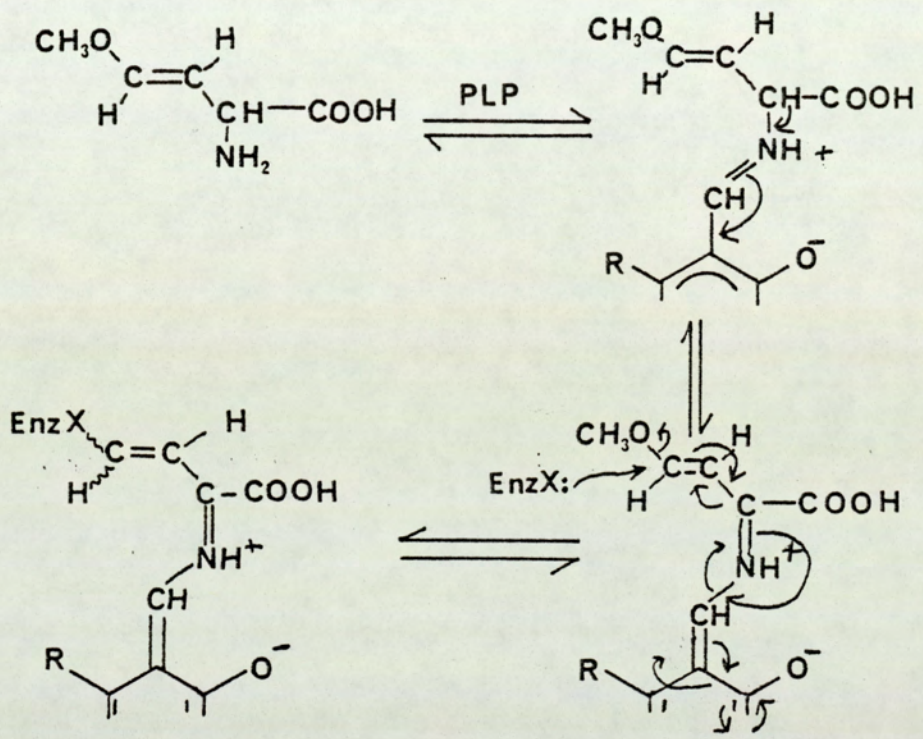


Figure 1.13. The mode of action of 2-amino-4-methoxy-trans-3-butenoic acid.

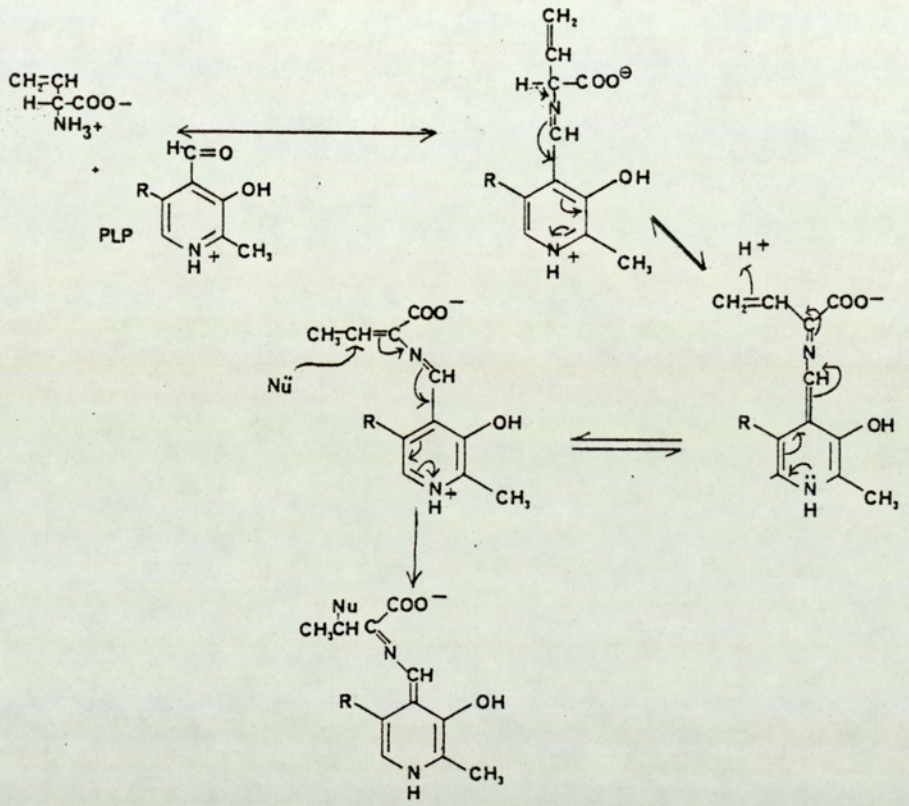


Figure 1.14. The mode of action of vinylglycine.

1.7.4. α-Propargyl substituted amino-acids.

Propargyl-glycine has been shown to be an effective inhibitor of α-cystathionase[105], cystathionine-γ- synthetase[106], alanine transaminase[107] and aspartate transaminase[108]. In the case of α-cystathionase and cystathionine-γ-synthase the inhibitory activity is due to the enzyme abstracting the α- and β- protons forming a conjugated allene, figure 1.15. In the cases of alanine and aspartate transaminase no β-proton abstraction has been found[106].

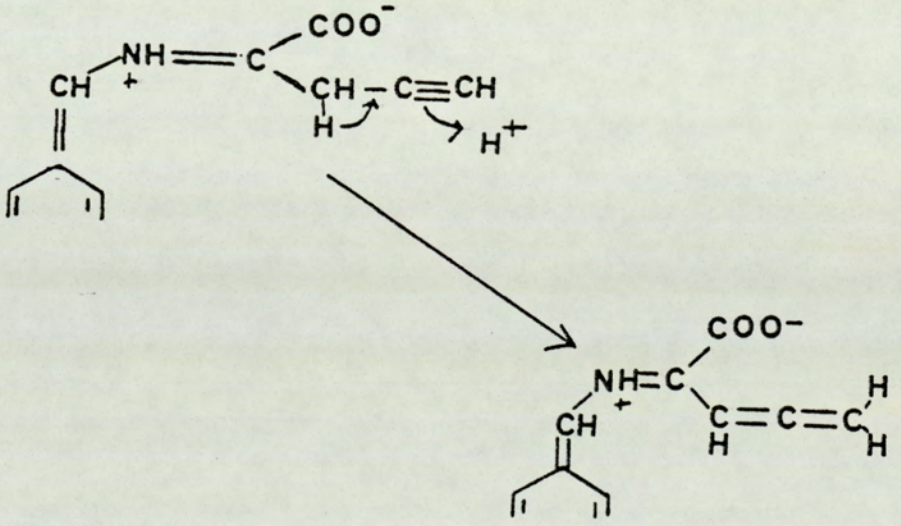


Figure 1.15. The activation of α-propargyl amino-acids

1.7.5. α-Nitrile substituted amino-acids.

These compounds, on the formation of the carbanion, rearrange to give the reactive α-keteneimine compound as shown in figure 1.16. Tryptophan synthetase is inhibited by cyanoglycine[109].

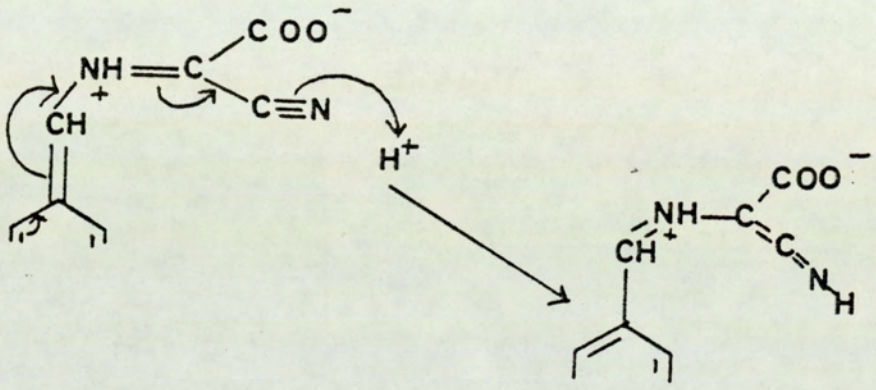


Figure 1.16. The activation of α-nitrile amino-acids.

1.7.6. Design of inhibitors of SHMT.

SHMT can catalyse the α-β bond cleavage of a variety of β-substituted L-serine analogues, but the extent that it can suffer α-substituted analogues of L-serine is unknown with the exception of α-methyl-, α-ethyl- and α-hydroxymethyl serine. If the enzyme were able to tolerate such substituents, then, via the introduction of suitable groups on the serine framework, such as α-vinyl, inhibition of the enzyme may be achieved, figure 1.17.

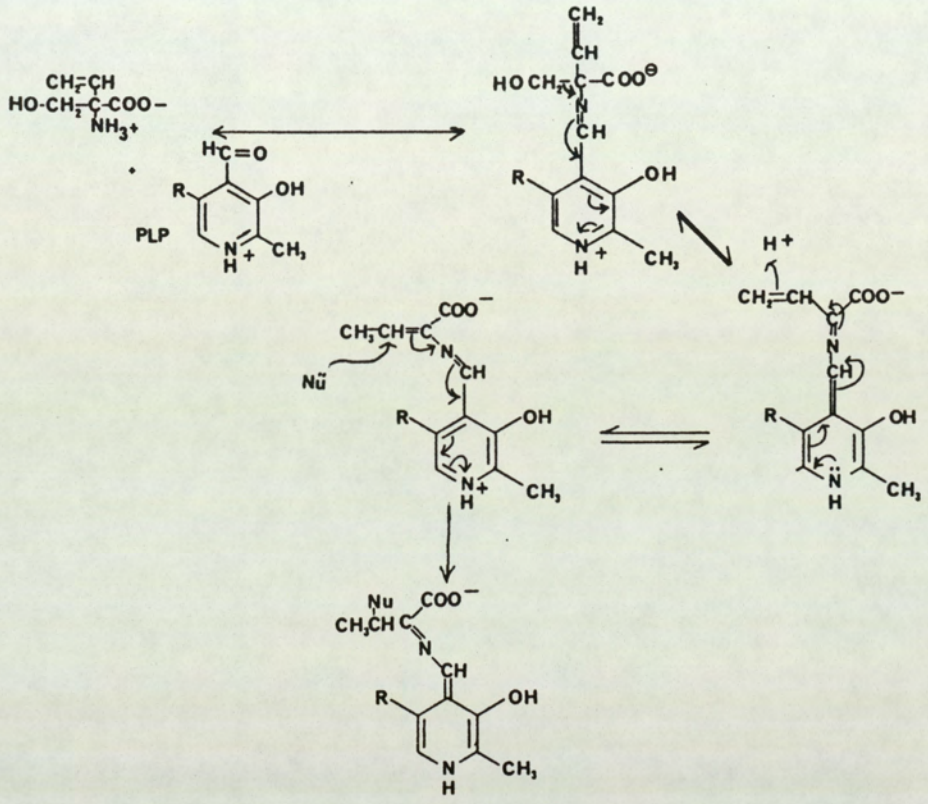


Figure 1.17. The proposed mechanism of inhibition of SHMT by α-vinylserine.

CHAPTER 2

SYNTHETIC CHEMISTRY

2.1. Introduction.

Serine was isolated as early as 1865 when Cramer undertook an investigation into raw silk[110]. He found that the acid hydrolysis of sericine, a protein from silk, produced a sweet crystalline material that he called serine. The structure of serine was clarified by Fischer and Leuchs[111] in 1902. They synthesised DL-serine through a Strecker type reaction with glycolaldehyde, figure 2.1.

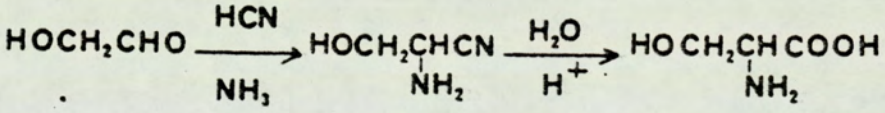


Figure 2.1. The synthesis of DL-serine by the method of Fischer and Leuchs.

The methods of synthesis of serine can be classified into three main groups.

The first is the synthetic route from alkoxyacetaldehydes, modifying the original route of synthesis of serine. The route of Fischer and Leuchs[111] to DL-serine resulted in a 9% yield, since the starting material, glycolaldehyde, is easily polymerised. Using methoxyacetaldehyde as the starting material, Nadea and Gaudry[112] were able to prepare DL-serine in 38% yield, after the demethylation of O-methylserine with hydrobromic acid.

The second major route of synthesis of serine involves the use

of condensation reactions. A hydroxymethyl group is introduced onto the activated methylene groups of hippuric acid, aminomalonate derivatives, or copper glycinate. Hydrolysis, following reduction where necessary, yields racemic serine.

Erlenmeyer and Stoop[113] obtained DL-serine in 48% yield from ethyl hippurate, figure 2.2.

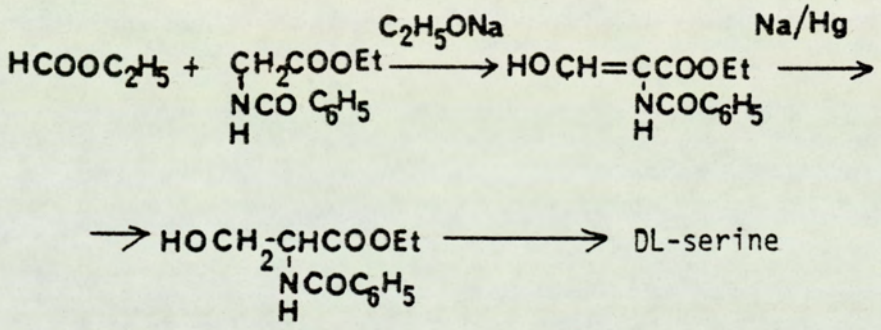


Figure 2.2. The synthesis of DL-serine by the method of Erlenmeyer and Stoop.

Ethyl hippurate was condensed with ethyl formate to form ethyl hydroxymethylene hippurate. This ester was then reduced with sodium amalgam and hydrolysed to give DL-serine.

For the small scale preparation of DL-serine, the method of King[114] may be used, figure 2.3. Formaldehyde was condensed with diethyl acetamidomalonate in the presence of a basic catalyst.

The ester was hydrolysed with base and, upon heating, the compound was decarboxylated to give N-acetylserine. Acid hydrolysis gave DL-serine in 65% yield.

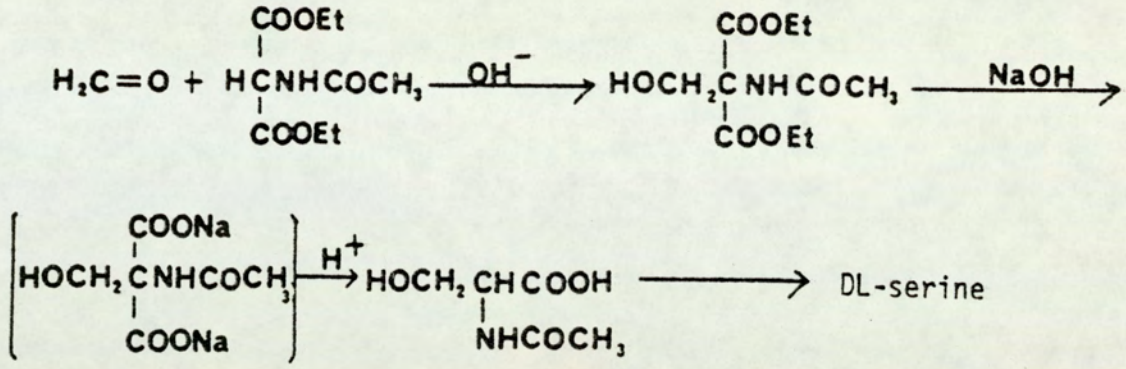


Figure 2.3. The synthesis of DL-serine by the method of King.

Copper has the ability to co-ordinate the amine and carboxylate groups of amino-acids. Copper glycinate and one equivalent of formaldehyde in the presence of catalytic amounts of base can be used to synthesise DL-serine[115], as shown in figure 2.4.

The third method of synthesis of serine uses vinylic compounds such as acrylonitrile and acrylic acid esters as the starting materials. The compounds, following halogenation, have one halide replaced by a hydroxyl group and the other replaced by an amine. An example is shown by the synthetic route devised by Mattocks and Hartung[116], figure 2.5. Methyl 3-hydroxy-2-bromopropanoate was prepared by the action of hypobromite upon methyl acrylate. This was then aminated to give the methyl 2-benzylamino-3-hydroxypropanoate. The ester was hydrolysed and the product reduced with palladium-hydrogen to give DL-serine in 32% yield.

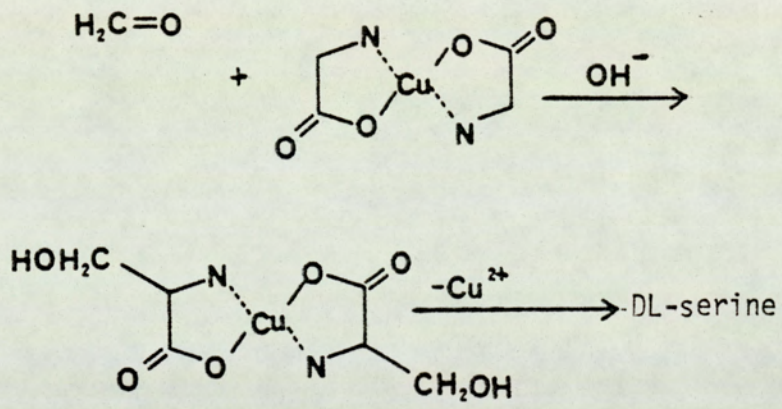


Figure 2.4. The synthesis of DL-serine from copper glycinate.

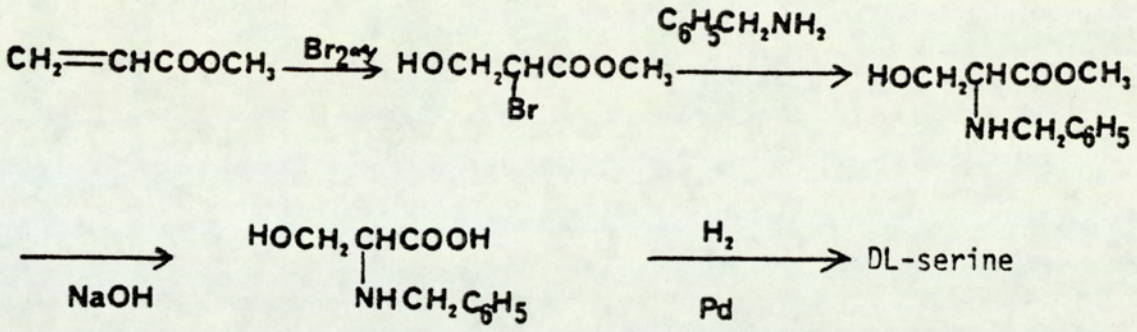


Figure 2.5. The synthesis of DL-serine by the method of Mattocks and Hartung.

A novel high yield synthetic route to DL-serine was developed by Berlinquet[117]. Ethyl acetamidocyanoacetate was reduced with sodium borohydride. The resulting alcohol, upon acid hydrolysis, gave DL-serine in 70% yield, figure 2.6. The reduction of the ester with sodium borohydride was undertaken in ethanol at room temperature. This is surprising as this reagent does not normally reduce ester groups in the absence of Lewis acids[118].

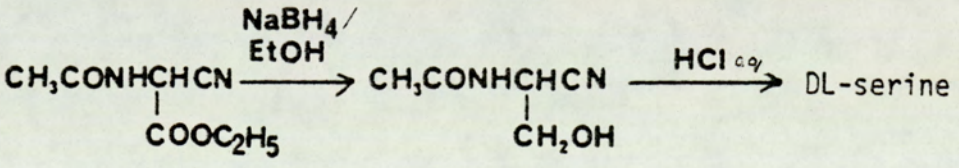


Figure 2.6. The synthesis of DL-serine by the method of Berlinquet.

The asymmetric synthesis of substituted serines has recently been investigated by Schöllkopf et al[119], who have utilised L-alanine for the asymmetric synthesis of (2R)-α-methyl serines. The synthetic route is dependent on the preparation of the bislactim-ether from cyclo-(L-Ala-L-Ala) (14), as shown in figure 2.7. The compound (15), when lithiated, was alkylated stereospecifically (16), with the alkyl group entering trans to the bulky methyl group, inducing the (R)-conformation at C-3. This route has been used for the synthesis of (R)-amino-acids via the regioselective alkylation of the bislactim ether prepared from cyclo-(L-Val-Gly)[120]. Schöllkopf and Groth[121] have extended the bislactim-ether method to the synthesis of (R)-α-vinyl amino-acids, figure 2.8. The bislactim-ether lithium derivative (17) of cyclo-(L-Val-Gly) reacts with acetophenone with high diastereoselectivity. The carbonyl electrophile enters trans to the C-6 isopropyl group inducing the (R)-configuration at the 3-position (18). The treatment of the product with thionyl chloride and 2,6-lutidine gave a 4:1 mixture of the two olefins (19) and (20). Acid hydrolysis gave the (R)-α-vinyl amino-acid (21), and the α-keto ester (22), along with methyl valinate.

The preparation of L-vinylglycine from L-glutamic acid has recently been described by Hanessian and Sahoo[122]. N-(Benzyloxy-carbonyl)-L-glutamic acid α-methyl ester (23) undergoes a decarboxy-lative elimination with lead tetraacetate in the presence of catalytic cupric acetate, figure 2.9. Acid hydrolysis of N-(Benzyl-oxycarbonyl)-L-vinylglycine methyl ester (24), gave L-vinylglycine in 82% yield.

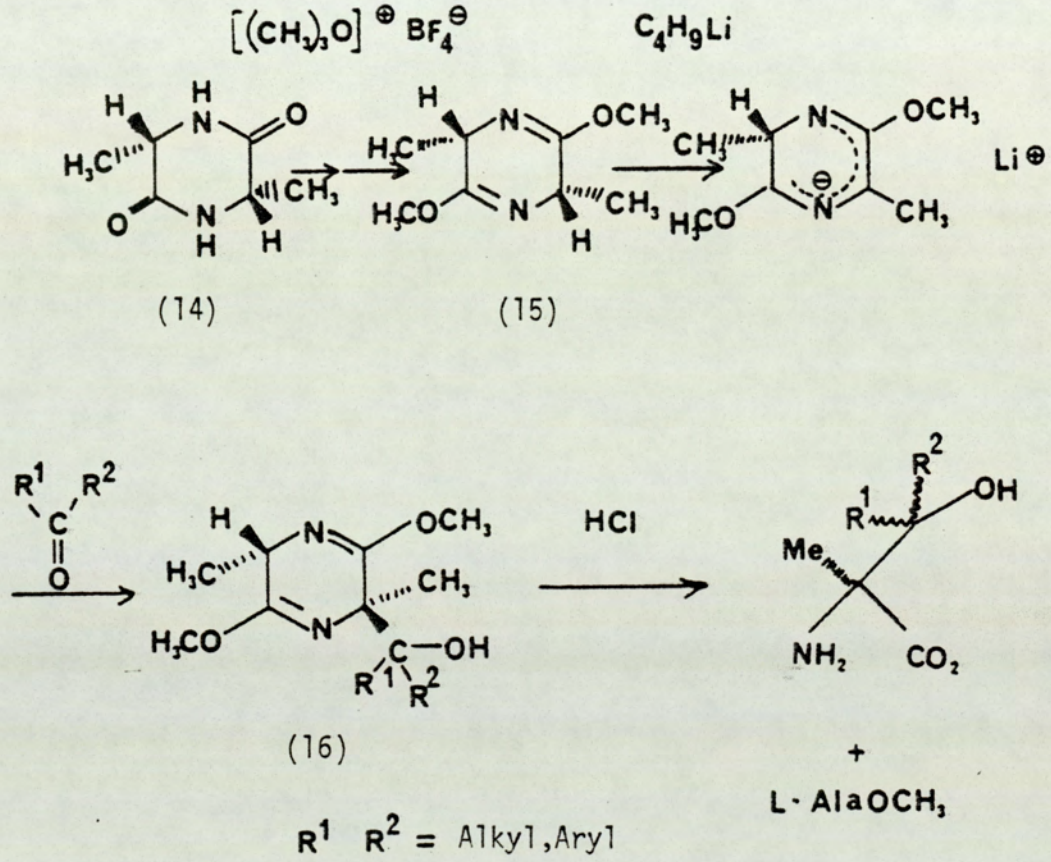


Figure 2.7. The asymmetric synthesis of (2R)-α-methyl serines.

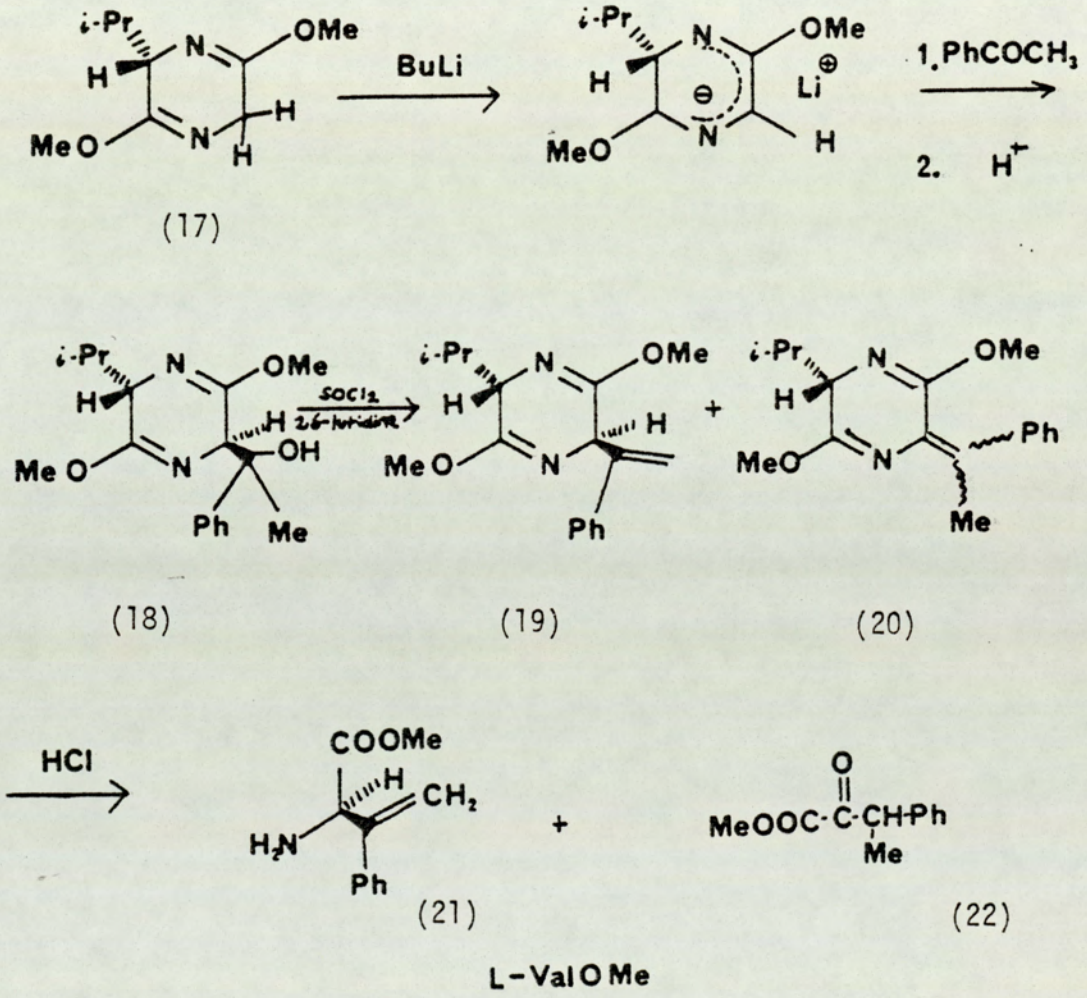


Figure 2.8. The asymmetric synthesis of (R)-α-vinyl amino-acids.

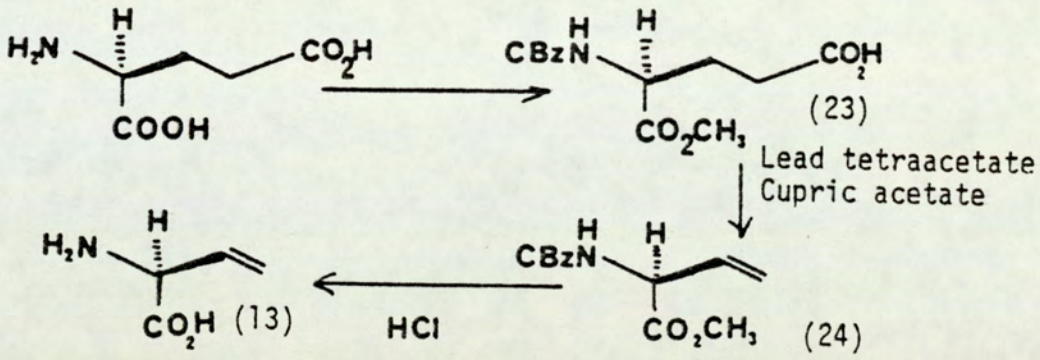


Figure 2.9. The route of synthesis of vinylglycine.

A number of methods are known for the production of α-substituted amino-acids. The use of glycine alkyl ester Schiff's bases for the synthesis of substituted amino-acids has been explored by Stork et al[123]. Alkylation of the benzylidene derivative of glycine ethyl ester produced α-mono and disubstituted amino-acids. O'Donnell et al[124] have used the same procedures to alkylate the benzophenone Schiff's base of glycine alkyl esters. The major limitation of the method has been the synthesis of the starting amino acid imine[125]. Recently Seebach and Aebi[126] have α-alkylated serine with the retention of the centre of chirality. L-Serine methyl ester, when heated with pivalaldehyde and triethylamine, produces a 1:1 mixture of diastereomers (25:26a), figure 2.10. The formylation of the diastereomers gives a 95:5 mixture of the N-formyl substituted compounds (25:26b), with the major diastereomer being isolated by crystallisation. Addition of lithium diisopropylamide to the oxazolidine produces the enolate (27) which upon methylation with

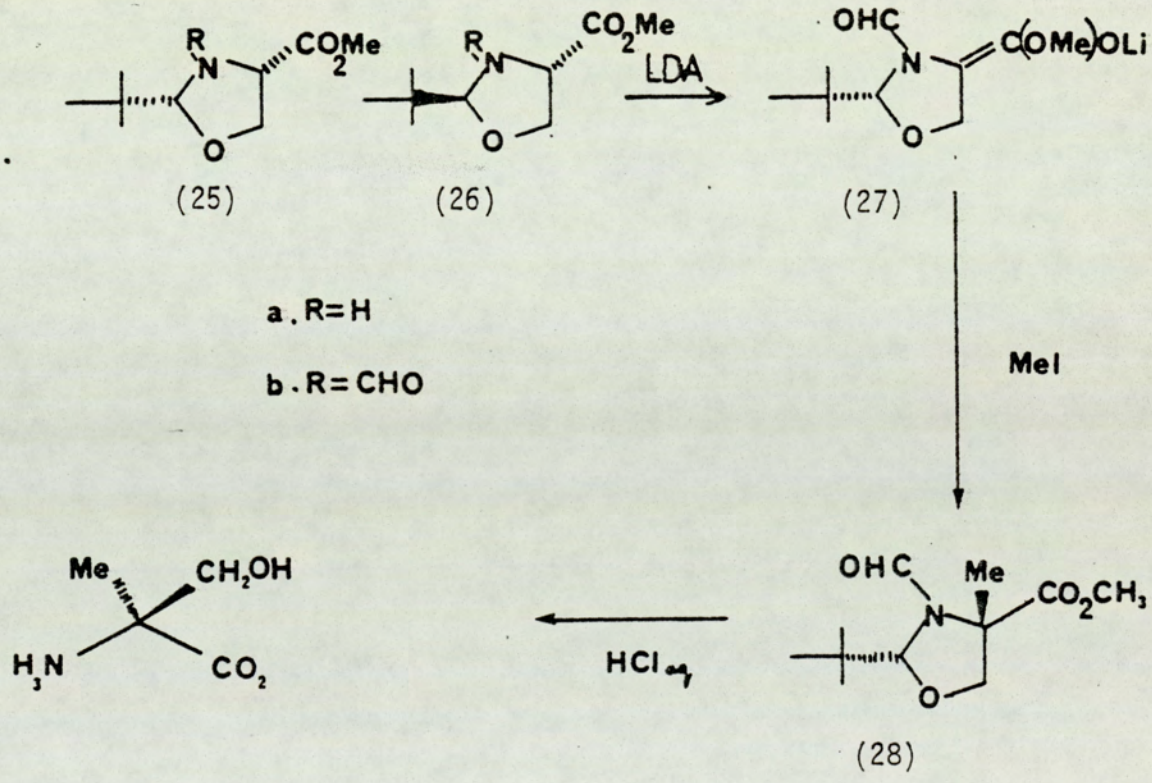


Figure 2.10. α-Alkylation of serine with retension of the centre of chirality.

iodomethane produces diastereomerically pure 4-methyl oxazolidine (28). Acid hydrolysis yields L-α-methyl serine. This synthetic method was used for the production of a number of L-α-substituted serine compounds.

The aim of the work presented in this chapter is the synthesis of novel α-substituted serine analogues that are capable of being irreversible enzyme activated inhibitors of the enzyme SHMT. N-Haloacyl serine compounds were also synthesised to re-investigate the claims of the antitumour activity of these molecules.

2.2. Results and discussion.

2.2.1. N-, and O-haloacyl serine derivatives.

DL-N-Chloroacetylserine[127], DL-N-dichloroacetylserine[34] and DL-N-trichloroacetylserine[36] were synthesised by direct Schotten-Baumann acetylation of serine. DL-O-Chloroacetylserine[36] and DL-O-dichloroacetylserine[36] were synthesised by the acetylation of DL-serine in acidic conditions.

DL-O-Dichloroacetylserine was also synthesised from DL-N-dichloroacetylserine[36]. The compound, when suspended in dry diethyl ether that was saturated with dry hydrogen chloride and left stirring at room temperature for 72 hours undergoes a N→O haloacyl shift. The proposed mechanism for the shift via the hydroxyoxazolidine (29) is shown in figure 2.11. A N⟶O acyl migration has been previously reported for phenyl_serine[128].

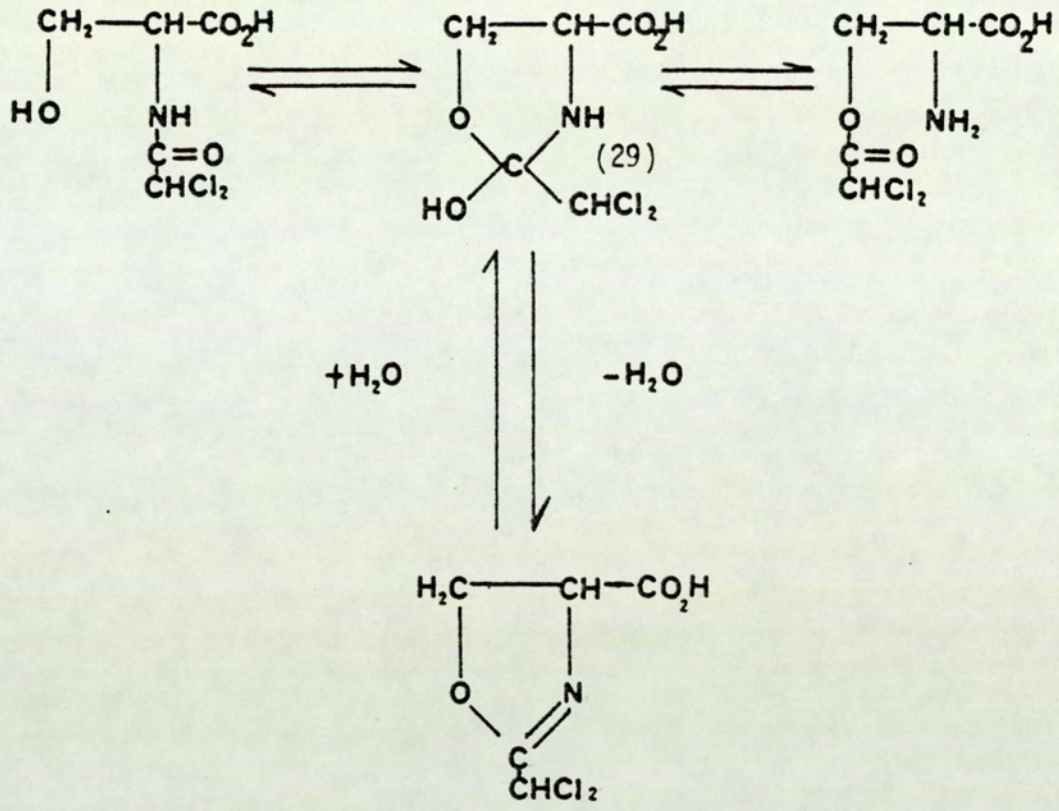


Figure 2.11. The proposed mechanism for the N⟶O shift.

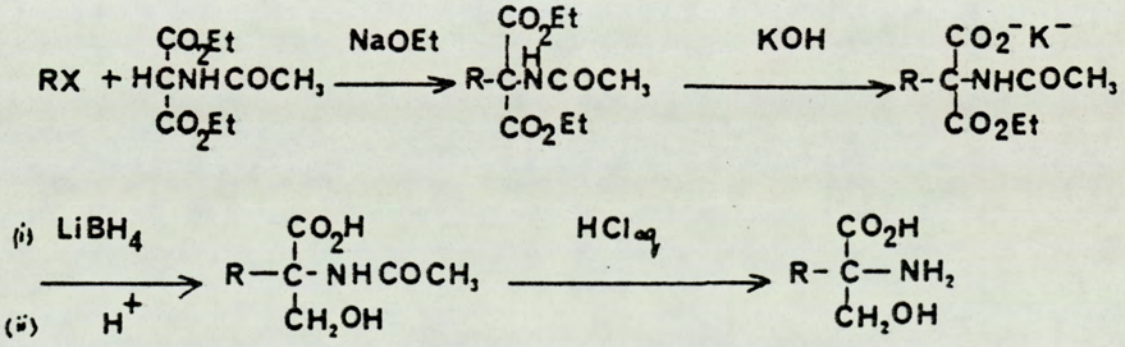
For compounds that undergo a N→O haloacyl shift, in every case the N-acyl derivative is much more stable than the O-acyl compound under neutral or basic conditions[36]. In the course of dissolving DL-O-chloro- and DL-O-dichloroacetylserine hydrochloride in deuterium oxide for NMR spectroscopy, both compounds hydrolysed to DL-serine hydrochloride and chloro- and dichloroacetic acid respectively. The lability of DL-O-dichloroacetylserine in these conditions makes its reported antitumour activity appear dubious. It appears that the stability of the O-haloacyl compounds decreases as the number of halogens increases, due to the increased lability of the ester bond.

2.2.2. The use in synthetic routes of copper glycine and copper serine.

∝-Hydroxymethylserine was synthesised by the method of Otani and Winitz[129]. Glycine, in the presence of copper sulphate, formaldehyde and aqueous sodium carbonate solution was dihydroxymethylated to produce ∝-hydroxymethylserine. ∝-Benzyloxymethylserine was prepared by the hydroxymethylation of O-benzylserine with aqueous formaldehyde in the presence of copper sulphate and sodium carbonate.

2.2.3. A general route of synthesis for ∝-substituted serine derivatives.

Initial attempts at the synthesis of ∝-substituted serine analogues used a modification of the synthetic route to serine devised by King[114], figure 2.12.



R= Alkyl

Figure 2.12. The synthesis of DL ∝-substituted serine using diethyl acetamidomalonate as starting material.

The route involves the alkylation of diethyl acetamidomalonate by a variety of alkyl halides using the method of Albertson[130]. One ester group is then removed by base hydrolysis, and a selective reduction introduces the hydroxyl group into the molecule. This produces, upon acid hydrolysis and neutralisation, a α-substituted serine analogue.

In the presence of a catalytic amount of base, diethyl acetamidomalonate was hydroxymethylated by formaldehyde[114]. The sodium derivative of diethyl acetamidomalonate was treated with allyl bromide, ethyl bromide and 4-chlorobenzyl chloride, to produce the expected alkylated derivatives. The sodium salt of diethyl acetamidomalonate failed to react with 1,2-dibromoethane. The 2-bromoethyl derivative was required for the proposed synthesis of the vinyl compound via the elimination of hydrogen bromide in the presence of triethylamine.

Diethyl acetamidomalonate did not react with acetaldehyde in the presence of base. The expected 1-hydroxymethyl derivative would have produced the vinyl substituted compound on dehydration.

The vinyl malonate was eventually prepared by the catalytic addition of ethylene to the diester. Ethylene was added to diethyl acetamidosodiomalonate in the presence of triethylamine, using dichlorobis(acetonitrile) palladium (II) as the catalyst. The proposed mechanism for the addition is as shown in figure 2.13. β-Elimination in the presence of air results in the vinyl compound. This addition is known for dimethyl malonate[131] but not for diethyl acetamidomalonate. The vinylic compound was found to decompose rapidly.

One ester group of diethyl ethylacetamidomalonate was hydrolysed with one equivalent of potassium hydroxide in absolute ethanol. The monoesters were invariably oils that resisted all attempts at

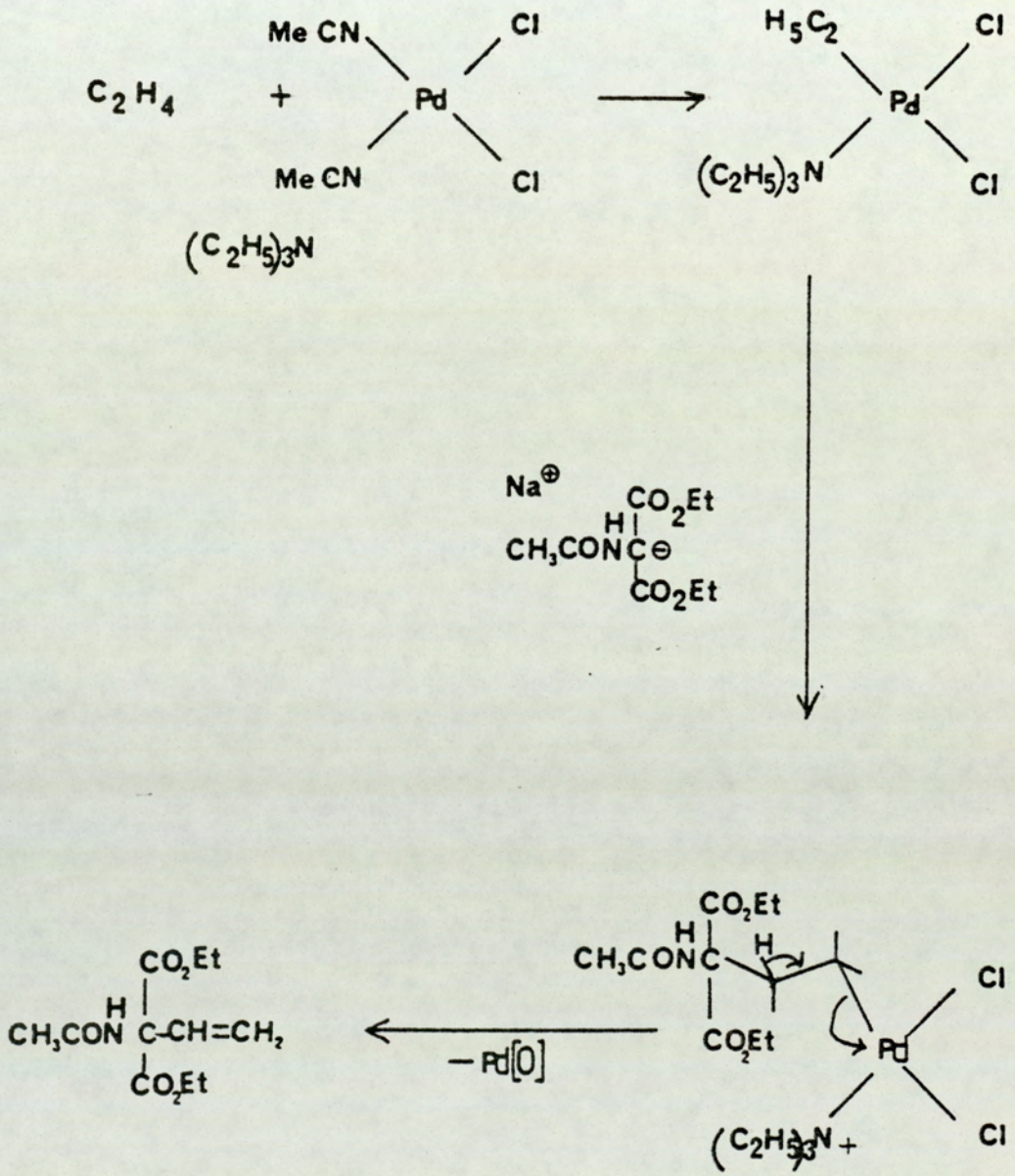


Figure 2.13. The synthesis of diethyl vinylacetamidomalonate.

crystallisation.

The selective reduction of the ester in the presence of an amide was attempted with sodium borohydride. Although this complex hydride has previously been used to reduce ethyl acetamidocyanoacetate[117], the ethyl ethylacetamidomalonate was not reduced with sodium borohydride. It was therefore decided to prepare a derivative with an ester that was susceptible to reduction. The chloromethyleneiminium ester was prepared[132] using N,N-dimethylchloromethyleneiminium chloride (30), figure 2.14. The activated ester makes the carbonyl group more susceptible to attack by nucleophiles such as hydride ions.

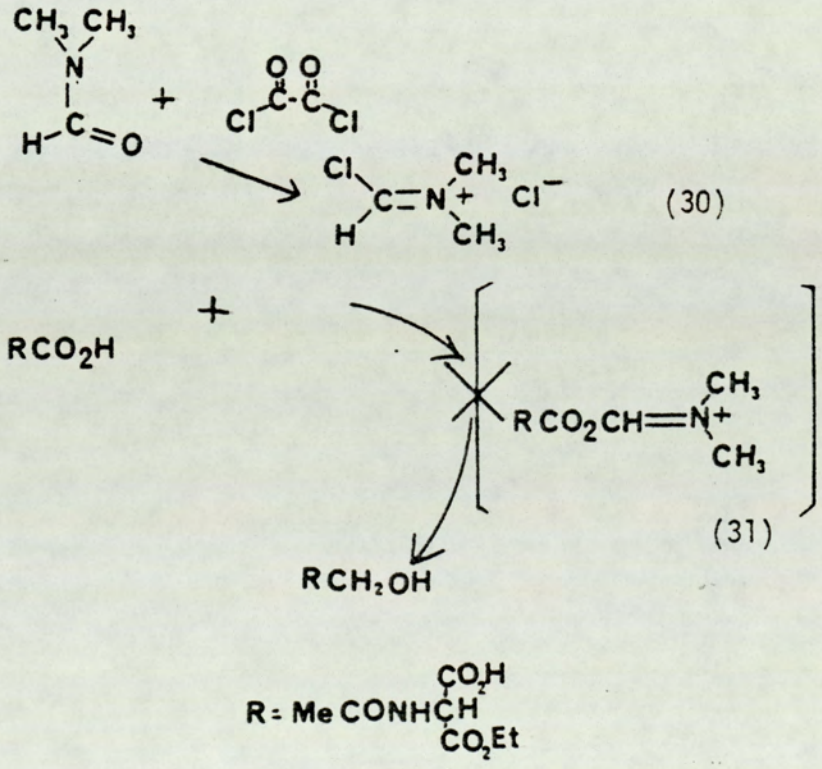


Figure 2.14. The preparation of an ester activated to reduction.

The chloromethyleneiminium ester (31) on treatment with sodium borohydride gave a quantitative return of starting material.

The failure of this reaction is probably due to the steric bulk surrounding the ester group. Liu *et al* have reported similar difficulty when trying to reduce sterically congested thiol esters with sodium borohydride[133].

Kayser and McMahon[134] have found that borohydride reductions are dependent on counter ion participation. Reduction with the more active lithium borohydride[135] proved to be more successful. However the production of borate complexes from which the product could not be isolated was common. This has been reported previously in the literature[135].

In order to remove the need for the mono hydrolysis of the ester of diethyl acetamidomalonate, ethyl acetamidocyanoacetate was used as the starting material. The route of synthesis of DL-α-substituted serine derivatives from this substrate is shown in figure 2.15.

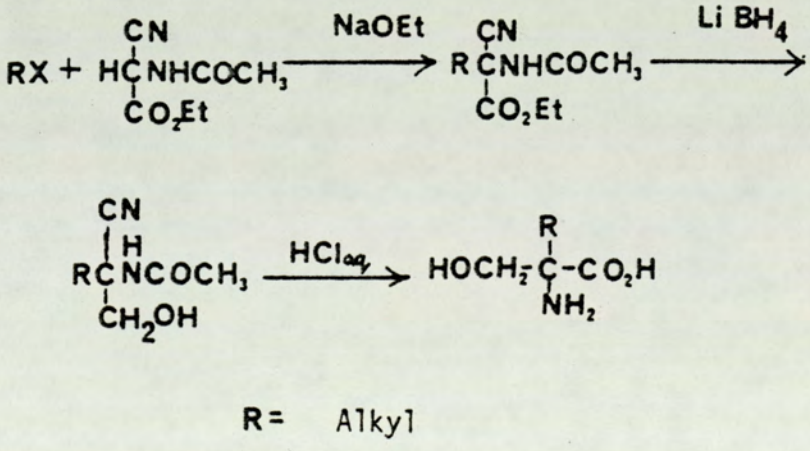


Figure 2.15. The synthesis of DL-α-substituted serine from ethyl acetamidocyanoacetate.

The sodium derivative of ethyl acetamidomalonate was reacted with allyl bromide[130]. This was then successfully reduced with lithium borohydride in dry tetrahydrofuran and the intermediate ester was hydrolysed with 6M hydrochloric acid. Neutralisation with aqueous ammonia gave DL-α-allyl serine.

The sodium derivative of ethyl acetamidocyanoacetate, prepared in absolute ethanol, failed to react with propargyl bromide. However the use of sodium hydride in toluene[136] to generate the anion produced the required compound. This was then reduced with lithium borohydride and hydrolysed with 6M hydrochloric acid, to give a yellow tar. DL-α-propargyl serine was isolated from the tar using a Dowex 50-X8 cation exchange column. The sodium derivative of ethyl acetamidocyanoacetate, failed to react with chlorobromomethane or chloroiodomethane, in an attempt to prepare α-halomethyl serine derivatives. Bey et al[137] have investigated the halomethylation of amino-acid Schiff's bases. They introduced the difluoromethyl group by treating the anion with chlorodifluoromethane (Freon 22). This alkylation, they suggest, proceeds via a chain process involving the formation of the reactive difluorocarbene. They went on to alkylate malonate ester anions with dichlorofluoromethane (Freon 21) in anhydrous tetrahydrofuran[138]. The sodium salt of ethyl acetamidocyanoacetate failed to react with chlorodifluoromethane using the conditions specified[137].

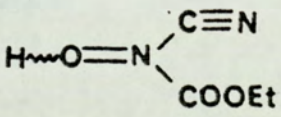
2.2.4. Attempted routes to DL-α-cyanoserine.

Ethyl acetamidocyanoacetate once hydroxymethylated is an attractive starting material for the synthesis of DL-α-cyanoserine. However the hydrolysis required to cleave the amide would hydrolyse the nitrile group. Therefore it was decided to prepare a derivative

with a nitrogen protecting group which could be removed easily in basic conditions. The N-formyl group can be used to protect amines and it may be removed under basic conditions[139] that do not cause the hydrolysis of nitriles. Various attempts were made at the synthesis of ethyl formamidocyanoacetate.

The commercially available, but expensive, ethyl cyanoglyoxylate oxime (32) was initially utilised as the starting material. Catalytic hydrogenation with palladium on charcoal in ethyl formate by the method of Liau et al[140] produced a black glass on purification.

In order to follow the reductive formylation by thin layer chromatography, the benzyl ester of the oxime (32) was prepared[141].



(32)

The sodium dithionite reduction of the oxime to the amine, followed by formylation with formic acetic anhydride[142] failed to produce the expected compound[143]. An unidentifiable yellow/orange material was produced.

Pojer[144] has investigated the ability of sodium dithionite to reduce oximes. He found that aqueous dithionite was able to reduce oximes to imines. In acidic or basic conditions, the imines were hydrolysed to the carbonyl compound. If benzyl 2-oxo-2-cyano-ethanoate was formed by this reduction, then it would have been highly unstable. The reduction of this oxime with aluminium amalgam produced the desired amine[141], however this reagent proved to be over vigorous. The method of Rusting et al[145] was finally used to prepare

the formylated compound. The oxime was reduced with zinc and formic acid with the temperature maintained between 60-63°C.

Once prepared, the benzyl formamidocyanoacetate could not be hydroxymethylated by formaldehyde in the presence of catalytic base. The compound, when added to sodium ethoxide in ethanol, rapidly went orange then black and no starting material could be isolated.

2.2.5. The synthesis of DL-α-vinylserine.

DL-α-Vinylserine was synthesised by the general route for the preparation of α-substituted amino-acids developed by Greenlee et al[146].

The route, as shown in figure 2.16., utilises the readily available amino-acid DL-threonine (33) as the starting material. This was then converted to the methyl ester hydrochloride (34) by refluxing in dry methanol saturated with hydrogen chloride. Phosphorus pentachloride was used to replace the hydroxyl group with a chlorine (35) by the method of Plattner et al[147]. The Schiff's base (36) was prepared using benzaldehyde in dichloromethane, with dried magnesium sulphate present as a drying agent. 1,5-Diazabicyclo-[5.4.0]undec-7-ene (DBU), a bicyclic amidine base, was used to eliminate hydrogen chloride to give methyl N-benzylidine-2-amino-crotonate (37). This dehydroamino-acid represents the carbon framework for α-vinyl substituted amino-acids. The lithiated compound (38) was prepared using lithium hexamethyldisilazide and the anion was acetoxymethylated with chloromethyl acetate (39) in the presence of hexamethylphosphoramide. The acetoxymethylation provides a convenient method of introducing a protected hydroxymethyl group into the compound, hydrolysis with 6M hydrochloric acid removes the O-acyl group. The previous use of bromomethylmethyl ether resulted in the

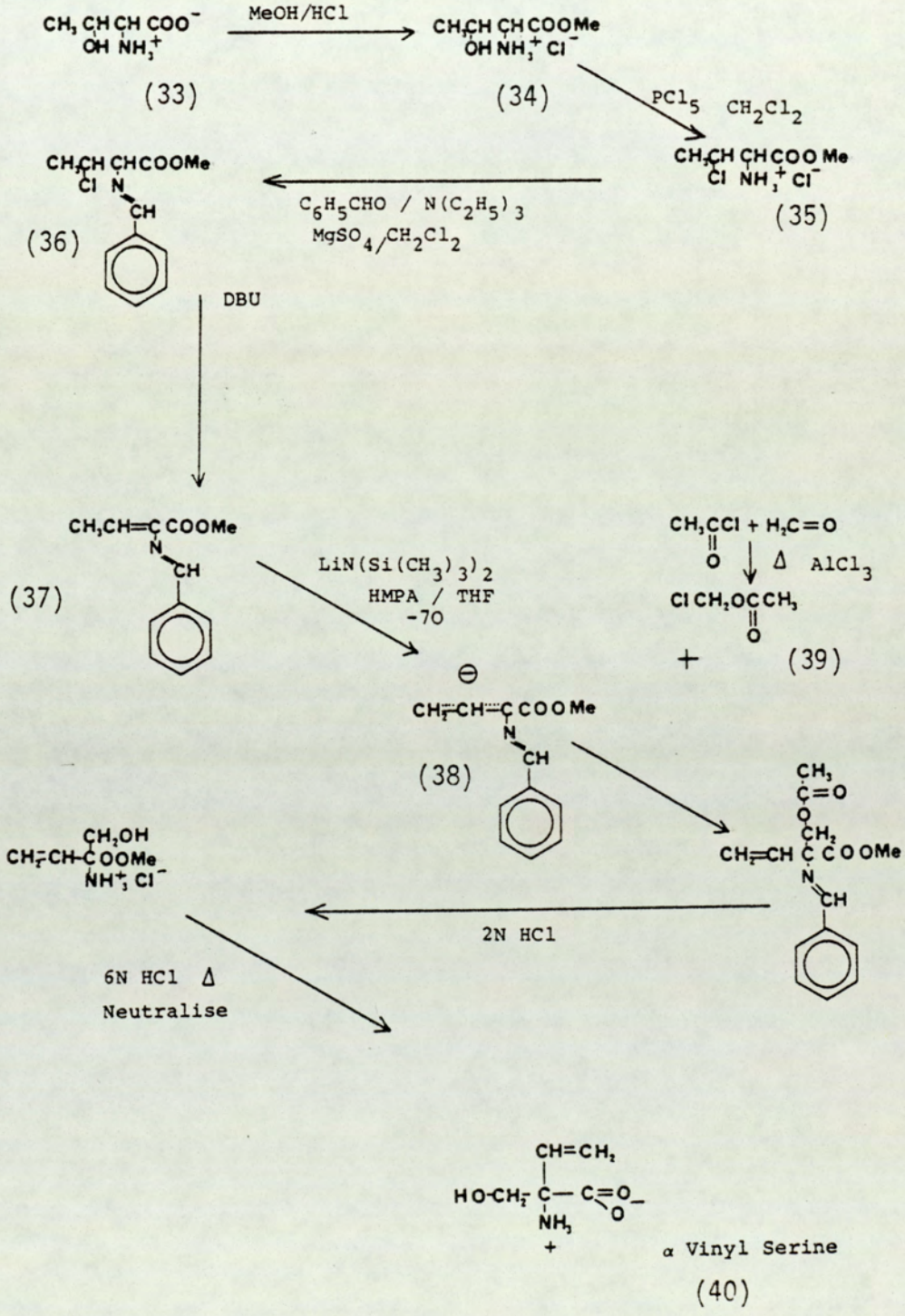


Figure 2.16. The route of synthesis of α-vinylserine

compound being methyloxymethylated. This ether was however resistant to cleavage using boiling hydrobromic acid[148]. The chloromethyl acetate (39) had been previously prepared from acetyl chloride and paraformaldehyde, by the method of Ulich and Adams[149].

2M Hydrochloric acid was used to remove the Schiff's base. This was then followed by boiling the compound in 6M hydrochloric acid and neutralisation with aqueous ammonia to give the unprotected DL-α-vinylserine (40).

CHAPTER 3

SHMT LEVELS IN TISSUES AND TUMOURS

3.1. Introduction.

The distribution of SHMT in rat tissues has been extensively investigated by Snell[12, 23, 150]. He found the enzyme to be fairly ubiquitous, with the highest levels in the liver and kidney, and lower levels in the spleen, testis, intestine and heart. These results are in agreement with the values obtained by Yoshida and Kikuchi for rat tissue soluble extracts[151], and reaffirms the role of SHMT in nucleotide biosynthesis[12]. As discussed in section 1.2., SHMT has been found in many tumour cells.

The work described in this chapter investigates the levels of SHMT in different murine organs and tissues. In order to find a suitable tumour test system for assessing the effect of potential inhibitors on tumour SHMT, the levels of activity of the enzyme in different tumour cells were quantitatively evaluated.

3.2. Materials and Methods.

3.2.1. Materials.

All reagents and chemicals were of Analar grade and were purchased from BDH Chemicals Limited (Atherstone) or Sigma Ltd (Poole).

3.2.2. Radiochemicals.

L-[3-^{14}C]Serine 53mCi mmol^{-1} was obtained from Amersham International plc.

3.2.3. Erythrocyte cell lysis buffer.

Ammonium chloride (7.5 g l^{-1}) was added to a solution of Tris-HCl (0.016M) and the pH adjusted to 7.2 [152].

3.2.4. Tumour cells used in this study.

3.2.4.1. TLX5 ascites.

The TLX5 lymphoma is an invasive, fast growing murine tumour. It was originally induced by X-irradiation of the thymus of a CBA mouse[153]. The ascites fluid was obtained from CBA mice supplied by Mr D Chubb, Aston University.

3.2.4.2. Lewis lung carcinoma.

This tumour was discoverd by Dr M Lewis in 1951. It originated spontaneously as a carcinoma of the lung of a C57B1/6 mouse [154]. The cells were obtained from C57B1/6 mice, supplied by Dr R Fenton, Glaxo Group Research, Greenford.

3.2.4.3. GM0621.

The GM0621 cell line is a human lymphoblastoma cell line derived from normal individuals[155]. The cells, classified as O6-guanine-DNA-methyltransferase deficient (mer-), were obtained from Dr M Tisdale, Aston University.

3.2.4.4. Raji.

The Raji cell line is derived from Burkitt lymphoma. The cells, classified as O6-guanine-DNA-methyltransferase proficient (mer+), or deficient (mer-)[155] were obtained from Dr M Tisdale.

3.2.4.5. K562.

The human erythroid leukaemia K562 cells were obtained from Dr M Tisdale.

3.2.4.6. HL60.

The human promyelocyte leukaemia cell line derives from a patient with acute promyelocytic leukaemia[156]. The cells were obtained from Dr M Tisdale.

3.2.4.7. L1210.

Murine L1210 leukaemia cells were first isolated by Law et al, in DBA/2 mice following exposure to 3-methylcholanthrene[157]. The L1210 cells obtained derive from a number of different sources, namely:

L1210 cells, originally obtained from Flow laboratories;

L1210 MTX cells, originally obtained from Flow laboratories, and made resistant to methotrexate.

These cells were supplied by Dr M Tisdale.

L1210 (Glaxo) cells, originally obtained from Flow laboratories;

L1210 CB cells, derived from the Chester Beatty Institute L1210 cell line. The cells were obtained from BD2F1 mice, and routinely passaged in DBA mice;

L1210 NCI, derived from the National Cancer Institute L1210 cell line. The cells were obtained from BD2F1 mice, and routinely passaged in DBA mice.

These cells were supplied by Dr R Fenton.

3.2.4.8. PC6 Ascites.

The murine plasmacytoma ascites were obtained from BALB/c mice supplied by Mr D Chubb.

3.2.4.9. HT29.

The human colon carcinoma cells were obtained from Dr A Baxter, Glaxo Group Research, Greenford.

3.2.4.10. Walker Ascites.

The Walker carcinosarcoma ascites cells were obtained from Wistar rats supplied by Dr R Fenton.

3.2.4.11. Walker 256.

The Walker carcinosarcoma cells were obtained from Wistar rats supplied by Dr R Fenton.

3.2.4.12. Sarcoma 180.

The sarcoma 180 cells were obtained from CRH mice supplied by Dr R Fenton.

3.2.4.13. Colon 26.

The Colon carcinoma cells were obtained from Dr A Baxter.

3.2.5. Protein determination.

Protein was determined by the method of Lowry et al using bovine serum albumin as standard[158].

3.2.6. Taylor and Weissbach assay for SHMT activity.

The SHMT activity was assayed using the method of Taylor and Weissbach[159]. Ascites tumours were removed from the peritoneal cavity of mice or rats, washed with 0.9% sodium chloride and resuspended in erythrocyte cell lysis buffer. The suspension was sedimented at 4000 g for 10 min, washed twice with 0.9% sodium chloride, and the cells were disrupted as below.

Internal organs and solid tumours were freshly removed, homogenised and treated as for ascites fluid. Murine bone marrow was removed by the method of Oliver and Goldstein[160].

The cells, once washed with 0.9% sodium chloride, were disrupted in a minimum volume of 100 mM Tris-HCl pH 7.1, at 10kcs for 3 by 10 sec using a MSC sonic oscillator. The suspension was centrifuged at 3000 g for 1 hr and the pellet discarded.

Each assay contained 0.1 μmol L-[3-^{14}C] serine, 0.1 μ mol PLP, 0.8 μ mol THF, 4 μmol 2-mercaptoethanol, 30 μ mol potassium phosphate pH 7.4 and supernatant in a total volume 0.2 ml.

Reactions were initiated by the addition of enzyme and were terminated with 1M sodium acetate, pH 4.5 (0.3 ml), 0.1M formaldehyde (0.2 ml) and 0.4M 5,5-dimethyl-1,3-cyclohexanedione (0.3 ml) (in 50% ethanol). Heating for 5 min to accelerate the reaction was followed

by 5 min cooling in an ice bath. Scintillation grade toluene (5 ml) was added and the 5,5-dimethyl-1,3-cyclohexanedione-[^{14}C]formaldehyde compound was extracted into the organic phase by vigorous shaking for one min. Following centrifugation, the radioactivity of the organic layer was determined by mixing 3 ml of the upper layer with Beckman E.P. scintillation fluid (10 ml) (Aston University) or Perkin Elmer non aqueous scintillation fluid (Glaxo Group Research). The number of counts per minute were recorded on a Beckman LS-230 scintillation counter.

3.3. Results.

The production of [^{14}C]formaldehyde from [3-^{14}C]serine proceeds linearly for 20 min, with enzyme concentrations of up to 0.3 mg of protein. The SHMT activity for the tumour cells investigated was determined by measuring the rate of formation of [^{14}C]formaldehyde over a 20 min period. Enzyme activity of the tissues was measured by determining the quantity of [^{14}C]formaldehyde produced during a 10 min incubation. Duplicate determinations agreed within 5%.

The relative rate of formation of [^{14}C]formaldehyde by tumour cells is presented in the form of a histogram in figure 3.1. The data from which this is summarised is presented in a tabulated form in appendix 1. The kinetic constants for the two Raji and the GM0621 tumour cell lines were evaluated using varying serine concentrations and a fixed concentration of THF (4 mM). This results in saturation kinetics giving Lineweaver-Burk plots which appear to be bi-phasic (figures 3.2-3.4). The tabulated kinetic constants for these tumour cells are given in table 3.1.

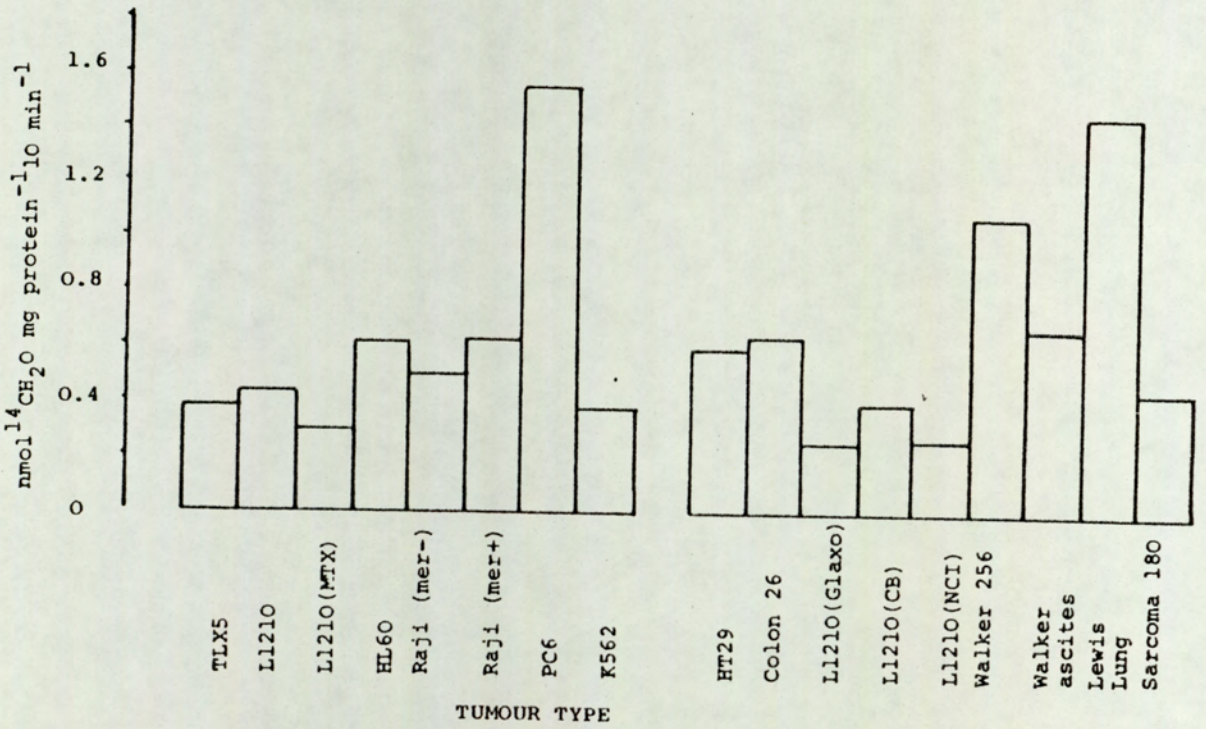


Figure 3.1.The relative rate of formation of(^{14}C)formaldehyde by tumour cells.

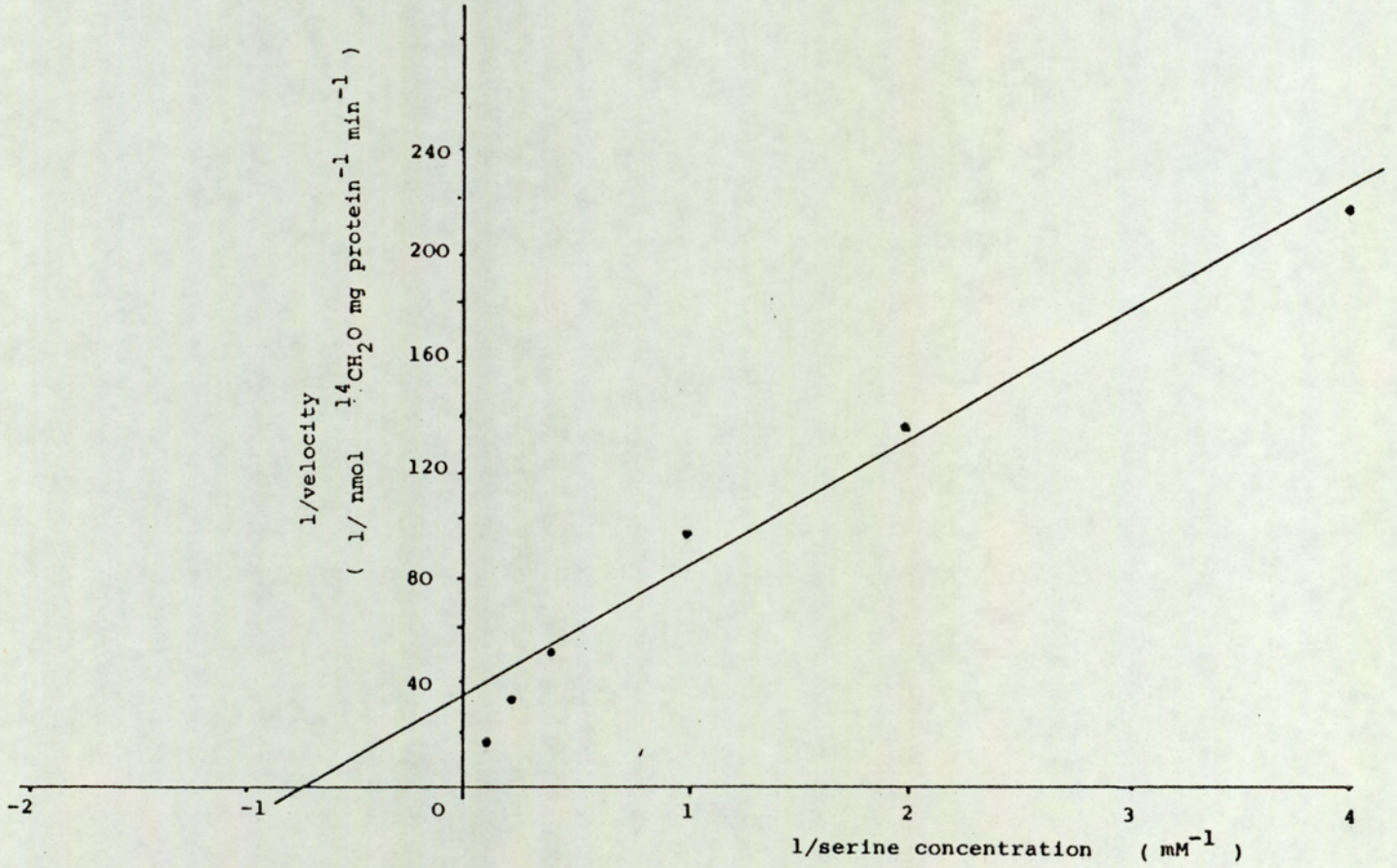


Figure 3.2. Lineweaver-Burk plot for Raji mer- cells.

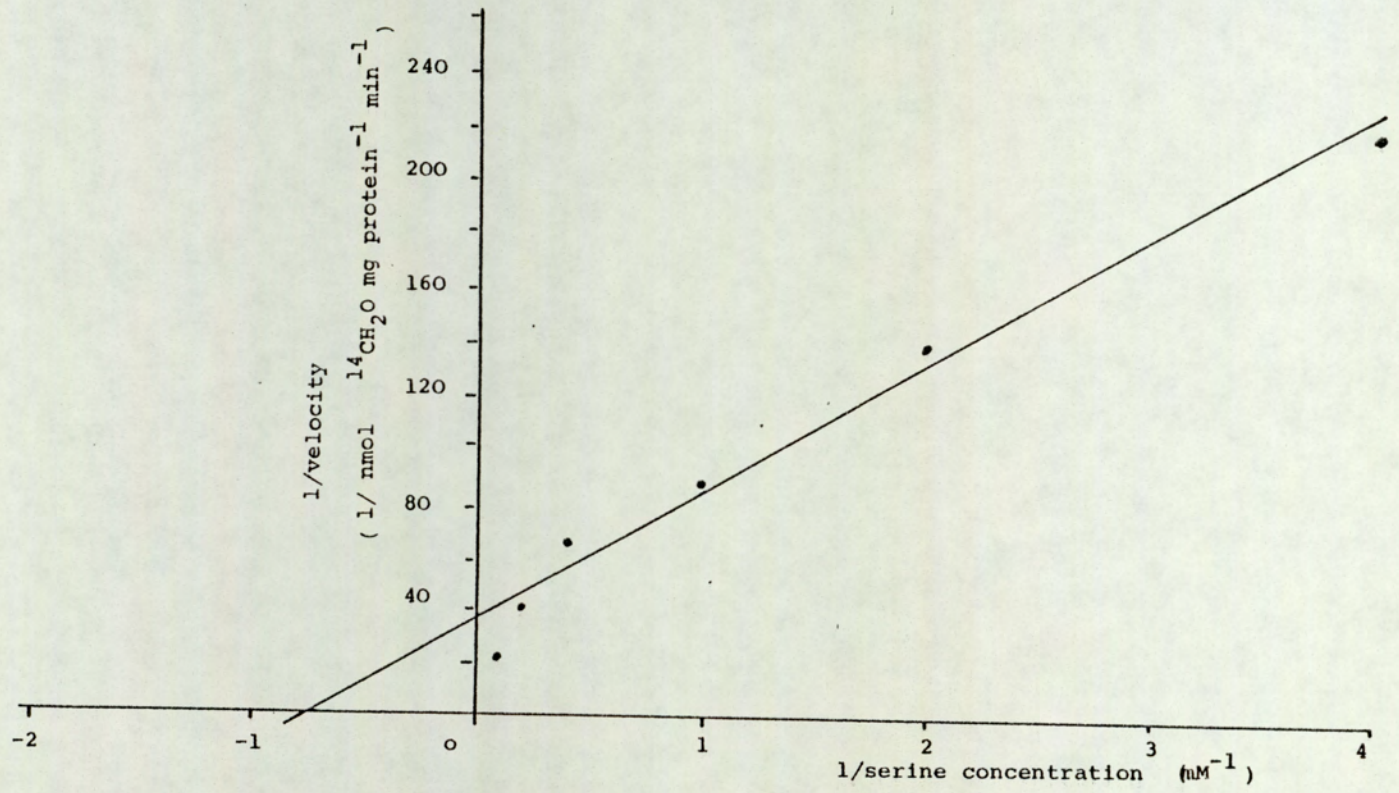


Figure 3.3. Lineweaver-Burk plot for Raji mer+ cells.

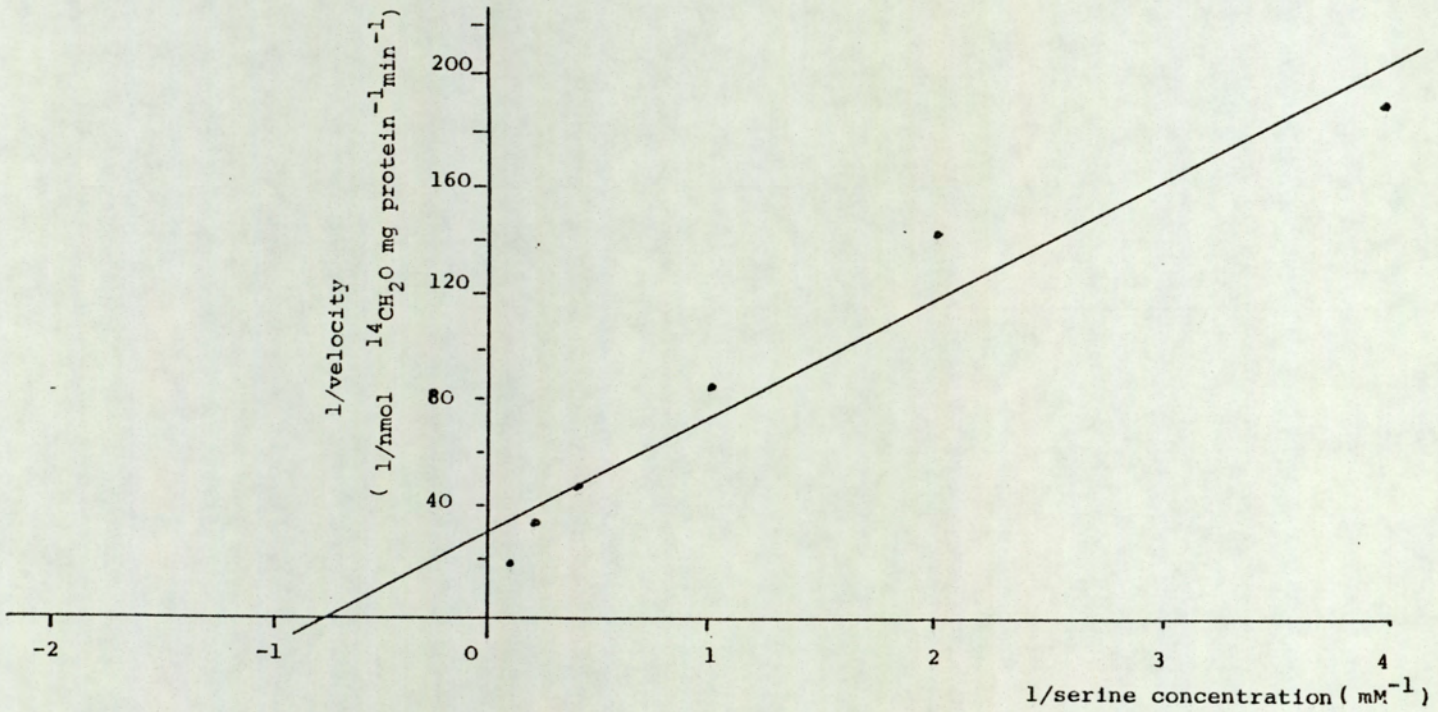


Figure 3.4. Lineweaver-Burk plot for GM0621 cells.

Table 3.1. The kinetic constants for Raji mer$^+$, Raji mer$^-$ and GM0621 tumour cell lines.

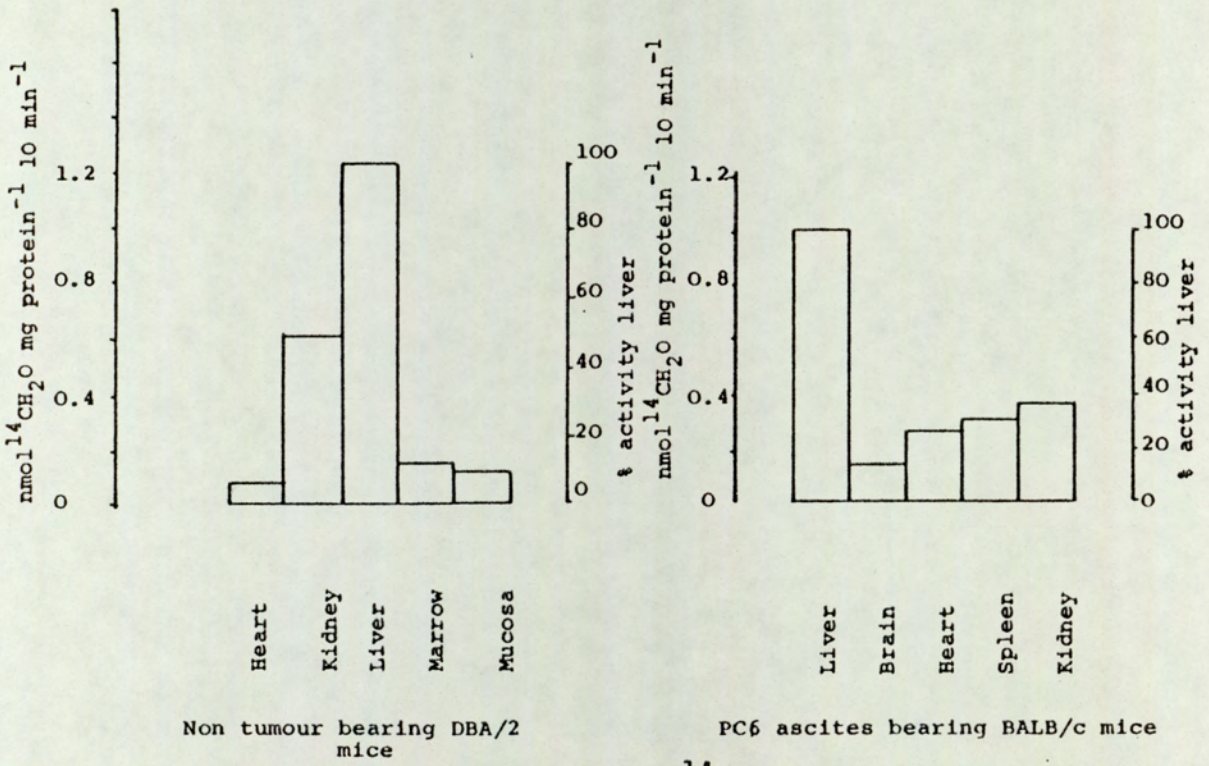
Cell line	Km (mM)	Vmax nmole($^{14}CH_2O$ mg protein^{-1} min^{-1})
Raji mer$^+$	1.33	0.028
Raji mer$^-$	1.38	0.029
GM0621	1.43	0.035

The levels of SHMT activity for the different tissues of non tumour bearing DBA/2 and BALB/c (bearing PC6 ascites) mice are shown in the form of histograms in figure 3.5.

3.4. Discussion.

The enzyme SHMT appears to be present in all of the tumour cells assayed in this study. Similar levels were obtained for all the cell types investigated with the exception of the PC6 ascites, Walker 256 and Lewis lung cells, which appear to have elevated levels of activity. The five L1210 cell lines obtained appear to have similar levels of activity, with little difference being observed between the enzyme activity of the L1210 cell lines sensitive or resistant to methotrexate.

The presence of the O6-guanine-DNA-methyltransferase phenotype[161], does not appear to influence the activity of the enzyme SHMT. It is interesting to note the bi-phasic nature of the Lineweaver-Burk plots for the Raji mer$^+$, Raji mer$^-$ and GM0621 cells. This may be due to an increase in the rate of formation of [^{14}C]formaldehyde at lower concentrations of L-serine, or a decrease at high concentrations of serine. This phenomenon however has not



3.5.The relative rate of formation of formation of (^{14}C)formaldehyde by murine tissues.

been previously observed for this enzyme[162]. The rate constants are in agreement with the literature values for purified enzyme from a variety of sources (as shown in section 4.3).

The levels of SHMT found in the different organs of the BALB/c bearing PC6 ascites and the non tumour bearing DBA/2 mice are similar to those previously reported[12, 151]. The highest levels of the enzyme are found in the liver, followed by the kidneys. SHMT was found to be present in all the tissues assayed. The levels of SHMT activity found in the murine liver appears to be higher than the level of activity of many of the tumour cells assayed. The other murine tissues have comparable levels to the majority of the tumour cells.

The high activity of SHMT in the kidneys is to be expected as the rat kidney has been shown to have significant levels of the enzyme in the proximal segments of the nephron[163], allowing the independent synthesis of serine from glycine[164]. This is further supported by the finding that rats, following bilateral nephrectomy have decreased blood serine levels[165].

The aim of evaluating the levels of SHMT in different tumour cells was to find a suitable source of SHMT to test the activity of potential inhibitors. The levels of enzyme activity found in the tumour cells investigated indicates that any of the systems tested will serve for this purpose. There may be an advantage in using one of the three cell types with elevated levels of SHMT activity, as any induced inhibition may be more apparent.

CHAPTER 4

THE PURIFICATION AND PROPERTIES OF L1210 LEUKAEMIA SERINE HYDROXYMETHYLTRANSFERASE.

4.1. Introduction.

Most systems for the purification of the enzyme SHMT follow the methods developed by Schirch and co-workers[5]. They have purified to homogeneity mitochondrial and cytosolic rabbit liver enzyme[72], as well as the enzyme from Escherichia coli containing a high copy plasmid of the glyA gene[166].

4.1.1. SHMT preparation.

The eukaryotic enzyme is normally purified following the initial homogenising of the tissue, by a heat denaturing step. The rabbit, lamb, bovine and rat liver enzymes are all stable to a heat step in the purification procedures with no loss in activity[4,6,7,72].

Ammonium sulphate fractionation of the homogenate then yields further purification. Levels of 35% saturation ammonium sulphate will not precipitate the mammalian enzyme, whereas 50% saturation and above will cause precipitation. Unlike the mammalian enzymes, the E.coli enzyme may be precipitated at levels of 70% saturation ammonium sulphate, while it is soluble in 50% saturation ammonium sulphate[166].

All forms of the enzyme are stable to dialysis at 4°C[7,72]. CM-Sephadex has a high affinity for the mammalian enzyme and has been used for further purification[4,7,72], as has hydroxylapatite[56]. The finding by Schirch and Peterson[72] that DEAE-Sephadex can be used to separate the mitochondrial and cytosolic forms of the rabbit liver

enzyme has also been used by Ogawa and Fujioka to separate the two forms of the rat liver enzyme[6]. Monkey and rabbit liver SHMT binds to both free cibacron blue 3G-A dye and to the dye bound as blue sepharose CL-6B gel[83]. However, the E.coli enzyme has no affinity for this dye nor will it bind to Amicon orange-A columns[166].

4.1.2. Properties of SHMT.

The mitochondrial and cytosolic enzymes of the rabbit liver are homotetramers with a molecular weight of about 215,000 with the mitochondrial enzyme having a slightly larger molecular weight by about 1,000 per subunit[69]. Similarly the tetramers of mitochondrial and cytosolic rat liver SHMT are 200,000 and 230,000 Daltons respectively[6]. The E.coli enzyme normally exists as a homodimer of molecular weight 95,000[166].

Kinetic constants have been determined for L-serine, THF and allothreonine for the homogeneous rabbit, rat and bovine liver enzymes[6,7,166]. The rabbit liver iso-enzymes are stable over many months if stored frozen, and are stable over several hours at 30°C in a pH range of 5.8-8.5[1].

4.1.3. The preparation of SHMT from a tumour source.

With the exception of the ammonium sulphate precipitation of human leukaemic leukocytes[8], no work has been done to purify and characterise SHMT from tumour cell lines. Consequently in the search for effective inhibitors of the enzyme, many groups have had to assess the effect of compounds on sonicated tumour cells total SHMT.

The aim of this work was to try to provide a simple but effective method of purifying SHMT from tumour cell lines, in order firstly to be able to test potential inhibitors in a system that is

relatively free from artefacts and secondly, to examine the properties of the enzyme derived from a tumour source, looking for differences in physical and biochemical properties.

4.2. Materials and Methods.

4.2.1. Materials.

Hydroxylapatite was obtained from Biorad (California, USA). The rabbit liver enzyme and CH2-THF dehydrogenase were the kind gift of Dr V Schirch. Chemicals and reagents were obtained from Sigma (St. Louis).

4.2.2. Preparation of the folinic acid Sepharose 4-B column.

The folinic acid column was prepared using the general method of Jones and Priest[4].

Sepharose 4-B (66 ml) was suspended in distilled water (15 ml) and cooled to 4°C with stirring. The pH was adjusted to 10 using 20% sodium hydroxide. Cyanogen bromide (20 g) in dimethylformamide (20 ml) was then added and the pH maintained between 9-11 with the sodium hydroxide. After about 45 min the pH had stabilised, and the suspension was stirred for a further 2.5 hr.

1,6-Diaminohexane (30g) was added and the suspension was stirred overnight at 4°C. The 1,6-diaminohexane Sepharose 4-B was washed with 1M sodium hydroxide (1.2l) and then distilled water (1l).

To 1,6-diaminohexane Sepharose 4-B (25ml) which had been de-gassed, folinic acid (0.0625 mM 31.9 mg) in sodium hydrogen carbonate (6.25ml 0.5 M) and dimethylformamide (6.25ml) was added. The pH was adjusted to 5.5-6 and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (130 mg) was added. The suspension was bubbled with

argon and stirred overnight in the dark at room temperature. The folinic sepharose 4-B column was then washed with sodium chloride (500 ml 1M) and then distilled water (500 ml) and the absorbance monitored at 285 nm. Folinic acid ϵ^{285} = 37,200, the absorbance at 285 nm = 0.191.

The column had 2.1 μM folinic acid bound ml^{-1} gel.

Unreacted amino groups of the 1,6-diaminohexane were capped by the addition of sodium acetate (62.5 mg) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (62.5 mg). The pH was adjusted to 5.5 and the gel was saturated with argon gas, and left to stir overnight in the dark. The gel was washed with sodium chloride (300 ml 1 M) and then distilled water (300 ml) containing 0.1% 2-mercaptoethanol.

The column was always used in the dark using degassed solvents saturated with argon containing 0.1% 2-mercaptoethanol. Methanol (10%) was used as a bactericide for storage.

4.2.3. Purification of L1210 SHMT.

4.2.3.1. L1210 leukaemia cells.

Murine L1210 leukaemia cells that had been grown in a humidified 37°C atmosphere were used in all experiments. They were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, L-glutamine (2mM), penicillin (100u ml^{-1}), streptomycin (100 $\mu g ml^{-1}$) and 2-mercaptoethanol (20 μM).

Typical cell density used for the purification of the enzyme was 152 x 10^4 cells per ml with a total volume of 3150 ml.

4.2.3.2. Cellular disruption.

The cells were centrifuged at 3000g for 6 min, washed with 0.9% sodium chloride, and then resuspended in about 3ml 50mM potassium phosphate, 50 mM L-serine, 1mM PLP, pH 7.3. Cellular disruption was undertaken on a Branson sonifier setting 3.5 for 3 by 10 sec. The creamy suspension was centrifuged at 25,000g for 30 min and the pellet was discarded.

4.2.3.3. Ammonium sulphate precipitation.

Solid ammonium sulphate was added to the supernatant to give a concentration of $176gl^{-1}$ (30% saturation) with the pH being maintained between 7-8. After stirring for 10 min at 4°C, the suspension was centrifuged for 15 min at 20,080 g, and the pellet was discarded.

Solid ammonium sulphate ($127gl^{-1}$) was then added to the supernatant to give a final concentration of 50% saturation. After stirring for 15 min at 4°C, with the pH maintained between 7-8, the suspension was centrifuged at 20,080 g for 15 min. The supernatant was discarded and the pellet was re-dissolved in 50 mM potassium phosphate, 1 mM PLP, 50 mM L-serine, pH 7.3.

4.2.3.4. De-salting.

The supernatant was de-salted by passing through a G-75 Sephadex column, equilibrated with 20 mM BES, 50 mM glycine, pH 7.0. Protein was detected by monitoring the absorbance at 280 nm. The 280 nm positive eluate was pooled.

4.2.3.5. Folinic acid Sepharose 4-B.

Protein from the G-75 Sephadex column was passed through a folinic acid Sepharose 4-B column (1.5 x 2 cm) previously equilibrated with 20 mM BES, 20 mM glycine, 0.1% 2-mercaptoethanol, pH 7.0, (degassed and in an argon atmosphere). Protein was detected by recording the absorbance at 280 nm. When all the 280 nm positive eluate was removed from the column, the enzyme was eluted with 300 mM potassium phosphate, 0.1% 2-mercaptoethanol, pH 7.25 (degassed and saturated with argon). The protein collected was pooled and concentrated using an Amicon concentrator, and stored overnight with 1 mM PLP and 50 mM L-serine.

4.2.3.6. Further purification.

Protein from the folinic acid column was then dialysed against 20 mM BES, 1 mM EDTA, pH 7.0 for 3 hr at 4°C. The tubing was pre-cleaned by boiling in 5 mM EDTA and then boiling it in distilled water. The enzyme solution was then concentrated in an Amicon concentrator, and passed through either a hydroxylapatite or a CM-Sephadex column.

4.2.3.6.1. Hydroxylapatite.

The pooled protein was added to a hydroxylapatite column (0.5 x 2 cm) that had been previously equilibrated with 20 mM BES, pH 7.0 (buffer A). The enzyme was eluted by passing a linear gradient of buffer A and buffer B (200 mM sodium phosphate, pH 7.0). Fractions were collected in 0.6 ml aliquots. Fractions 4-6 had high SHMT activity, and were pooled and concentrated. The enzyme, when filtered through a 0.2 μm Millipore Millex filter, remained stable for greater than 2 weeks if stored at 2°C.

4.2.3.6.2. CM-Sephadex.

The pooled protein was added to a 3 x 0.5 cm column of CM-Sephadex which had been previously equilibrated with buffer (10 mM potassium phosphate, 1 mM EDTA, 0.1% 2-mercaptoethanol, pH 6.7). The column was washed with buffer A until no 280 nm positive eluate could be detected. A linear gradient between buffer A and buffer B (200 mM sodium phosphate, 1 mM EDTA, 0.1% 2-mercaptoethanol, pH 7.3) was then applied to the column, and fractions of 1 ml were collected. Fractions 8-10 had SHMT activity and were pooled. The enzyme, when filtered through a 0.2μm Millipore Millex filter, remained stable for greater than 2 weeks if stored at 2°C.

4.2.4. Preparation of stock solution of tetrahydrofolic acid.

To 25 mg dl-L-tetrahydrofolic acid was added 2 ml distilled water, 0.5 ml 1 M dipotassium hydrogen phosphate, 20 μl 2-mercaptoethanol and a trowel point of ascorbic acid. The solution was allowed to stir in the dark on ice for 20 min and was then saturated with argon gas. Once prepared, the solution is stable if stored at -20°C and may be assayed by procedure 4.2.5.

4.2.5. Coupled assay for SHMT activity.

All absorbances were recorded on a Cary 210 spectrophotometer. The rate of formation of CH2-THF was determined by oxidation to 5,10-methenyltetrahydrofolate (CH-THF) and NADPH with CH2-THF dehydrogenase and $NADP^+$. The formation of NADPH was continuously monitored at 340 nm. A typical 1 ml cuvette contained: 50 mM potassium phosphate, pH 7.3; 30 mM 2-mercaptoethanol; 0.24 mM NADP; 0.5-4 mM L-serine, and was allowed to incubate at 30°C for 5 min. THF (18-146 μM) and CH2-THF dehydrogenase (8 μg) were then added and a base

line established at 340 nm. Sufficient SHMT was then added to give a velocity that was recordable at 340 nm. Using an excess of SHMT the concentration of THF may be determined from the total change in absorbance at 340 nm.

$$\text{Total activity} = \frac{\Delta \text{ absorbance 340 nm min}^{-1}}{\text{assay volume}} \text{ total volume}$$

4.2.6. Coupled SHMT assay for determining aldolase activity.

Allothreonine and threonine were assayed against SHMT by an alcohol dehydrogenase (ADH) coupled assay. The acetaldehyde produced was reduced to ethanol with ADH and NADH. The disappearance of the NADH was used as a measure of the appearance of acetaldehyde, and was monitored continuously at 340 nm.

A 1 ml cuvette containing 0.02 ml ADH-NADH solution, 0.9 ml 10 mM potassium phosphate pH 7.3; EDTA 0.001 M and either 10-6.25 mM allothreonine or 20-4 mM threonine was allowed to incubate for 5 min at 30°C. After a base line was recorded the reaction was initiated by adding sufficient SHMT to give a decrease in absorbance at 340 nm. ADH-NADH solution was prepared by dissolving 6 mg of NADH and 4 mg ADH in 1 ml of buffer. The solution was stable for 1-2 days if stored at 4°C.

4.2.7. Sodium dodecylsulphate-polyacrylamide gel electrophoresis.

The size and purity of the L1210 SHMT were determined by 12% sodium dodecylsulphate-polyacrylamide mini-gel electrophoresis (SDS-PAGE). The gels were made according to the following formulae;

	Running gel	Stacking gel
Water	1.373 ml	1.157 ml
Buffer	0.980 ml	0.482 ml
30% Acrylamide	1.60 ml	0.33 ml
10% Ammonium persulphate	12 µl	6 µl
TEMED	5 µl	5 µl

Stock solutions

Running gel buffer

1.5 M Tris-HCl pH 8.8

0.4% SDS

30% acrylamide

30 g acrylamide

0.81g bisacrylamide

water to 100 ml

Electrode buffer

Dilute 10 fold to use

30.3 g Tris-HCl pH 8.3

144.2 g glycine

1 g SDS

Water to 1l

Stacking gel buffer

0.5 M Tris-HCl pH 6.8

0.4% SDS

10% Ammonium persulphate

0.05 g ammonium persulphate

water to 0.5 ml

Sample buffer

1.45 g Tris-HCl pH 6.8

6 g SDS

15 ml 2-mercaptoethanol

0.015 g Bromphenol blue

water to 100 ml

Samples of approximately 5-10 µg were loaded in 50% sample buffer

Gel stain

1.25 g of Coomassie blue was dissolved in 250 ml of methanol. To this 200 ml of water and 50 ml of acetic acid were added.

Gel de-stain

The medium contained 300 ml acetic acid, 300 ml methanol and water to 3l. Gels were run at 25 mA for 1.5 hr or until the running dye travels the length of the plate.

The following molecular weight markers were used; phosphorylase B from rabbit muscle, bovine serum albumin, egg albumin, carbonic anhydrase from bovine erythrocytes, soya bean trypsin inhibitor and α-lactalbumin.

4.2.8. Polyacrylamide gel.

To determine the native molecular weight of the enzyme, Pharmacia pre-made polyacrylamide 4-30% gradient gel PAA 4/30 plates were used.

Stacking gel

2 ml 30% acrylamide stock solution

2.5 ml 0.5M TRIS pH 8.5

0.1 ml Ammonium persulphate stock solution

5.4 ml water

5 μl TEMED

Stain

40 g Coomassie blue was added to 100 ml of freshly prepared 3.5% perchloric acid solution. The solution was stirred for 1 hr, filtered through a Whatman number 1 filter paper, and then through a 0.2 μm Millipore Millex filter. The stain was stable at room temperature indefinitely.

Running conditions

Pre-run	15 min	125V constant
Pre-electrophoresis	20 min	70V constant
Electrophoresis	15 hr	125V constant

Buffer TRIS 0.09 M, borate 0.08 M, EDTA 0.003 M, pH 8.5

Samples of approximately 100-150 μg were loaded in 50% running buffer containing 10% glycerol and a trowel point of bromphenol blue.

4.2.9. Protein determination.

Protein was determined by the method of Lowry et al[158] using bovine serum albumin as standard.

4.3. Results.

4.3.1. Enzyme purification.

A method was developed for the partial purification of L1210 leukaemia cells SHMT, as shown in Table 4.1. Ammonium sulphate precipitation, and de-salting on a G-75 Sephadex column were used in the initial stages of purification.

Unlike the rabbit liver enzyme, the L1210 enzyme would not bind to either the blue Sepharose or Amicon orange A columns. The knowledge that rabbit liver SHMT has a high affinity for folinic acid, especially in the presence of glycine[162], prompted the preparation of a folinic acid affinity column. The column was prepared and evaluated using the general method of Jones and Priest[4]. 1,6-Diaminohexane was bound to cyanogen bromide activated Sepharose 4-B, and folinic acid was then bound to this spacer group, to give a concentration of 2.1 μM folinic acid per ml of gel. Once prepared, the column, if used in the dark with degassed solvents in the presence of 0.1% 2-mercaptoethanol, remained stable for several weeks.

The L1210 and E.coli enzyme were both bound by this column efficiently, but it had no affinity for the rabbit liver enzyme. The L1210 enzyme had a good affinity for CM-Sephadex and hydroxylapatite and either could be used for the further purification of the enzyme, although the CM-Sephadex was normally used because the enzyme was eluted from this column at a far greater rate.

The enzyme, when prepared by the above method, was greater than 56% pure (by SDS-PAGE electrophoresis) and was stable if stored at 4°C for a period exceeding two weeks.

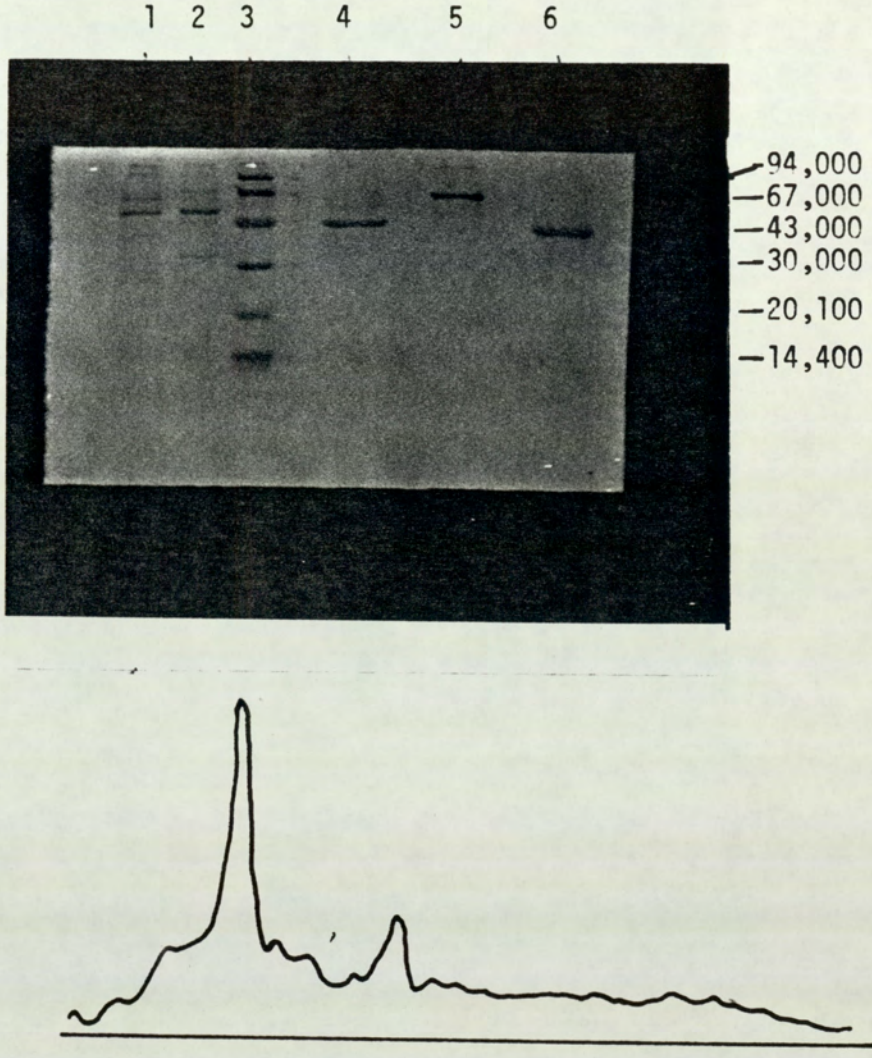
Table 4.1. Purification of L1210 SHMT.

Step	Total protein (mg)	Total activity U(min^{-1})	Specific activity (mg min^{-1})	Yield (%)	Purification (fold)
Supernatant	271.6	2800	103	100	1
30/50% Amm.Sulp.	54.2	16800	310	60	3
G75 sephadex	31.2	14050	446	50	4.3
Folinic column	6.1	8600	1387	31	13.4
CM Sephadex	3.5	5200	1485	19	14.4

4.3.2. Physical properties of L1210 leukaemia SHMT.

The denatured enzyme has a monomer molecular weight of 52,000 ± 5,000 by SDS-PAGE electrophoresis as shown in figure 4.1. The major contaminant band of the L1210 SHMT eluted from the CM sephadex may be a breakdown product from the enzyme. It is not present in the enzyme eluted from the folinic acid column.

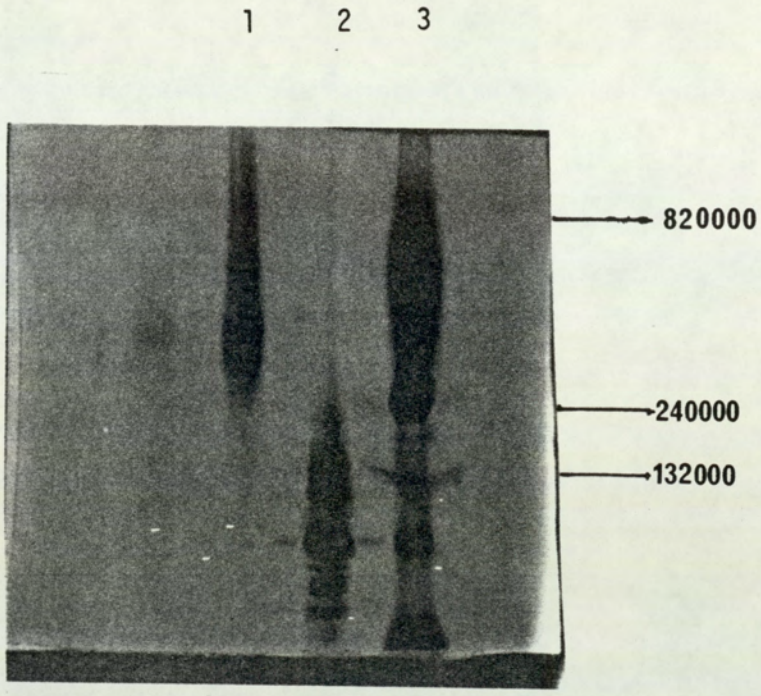
The native form of the enzyme was determined by pore exclusion polyacrylamide electrophoresis (figure 4.2.). This shows the tumour enzyme to be a tetramer, with an equilibrium existing between this state and two tetramers being associated. This is similar to the



Densitometer scan of lane 2.

Lane	Contents
1.	L1210 SHMT ex-folinic column.
2.	L1210 SHMT ex-CM-Sephadex column
3.	Molecular weight standards.
4.	*E. coli* SHMT.
5.	Bovine serum albumin
6.	Egg albumin.

Figure 4.1. SDS-PAGE of L1210 leukaemia SHMT.



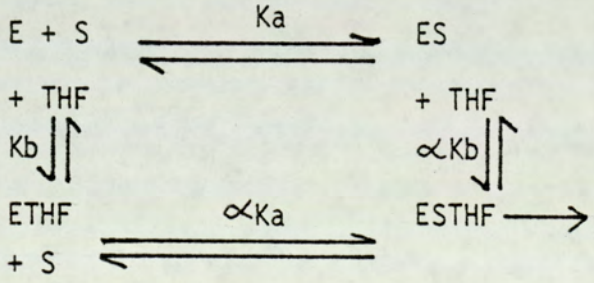
Lane	Contents.
1.	Cytosolic rabbit liver SHMT.
2.	*E. coli* SHMT.
3.	L1210 SHMT.

Figure 4.2. Pore exclusion PAGE of SHMT from various sources.

results obtained in this gel for the cytosolic rabbit liver enzyme. The E.coli enzyme is shown to be a dimer. The multiple bands obtained for the E.coli enzyme may be due to instability of the enzyme at the running pH, or sub-forms of the enzyme.

4.3.3. Substrate specificity and kinetic constants for L1210 leukaemia SHMT.

Kinetic constants for L-serine and THF were determined by initial velocity studies and were shown to obey Michaelis-Menten kinetics. Double reciprocal plots of velocity versus substrate concentration, figures 4.3 and 4.4, give converging lines, indicating a sequential-random addition of substrates. The kinetic constants in table 4.2 were evaluated in accordance with figure 4.5 where αKa and αKb' represent Km values at an infinite concentration of the co-substrate. Literature values for the rabbit liver and E.coli enzymes[166] are also given. Synergy in binding of the compounds is shown by the decrease in the Km values with increasing concentration of the co-substrate.



Abbreviations; S,serine; E, SHMT.

Figure 4.5. Scheme for the determination of the kinetic constants of L1210 SHMT.

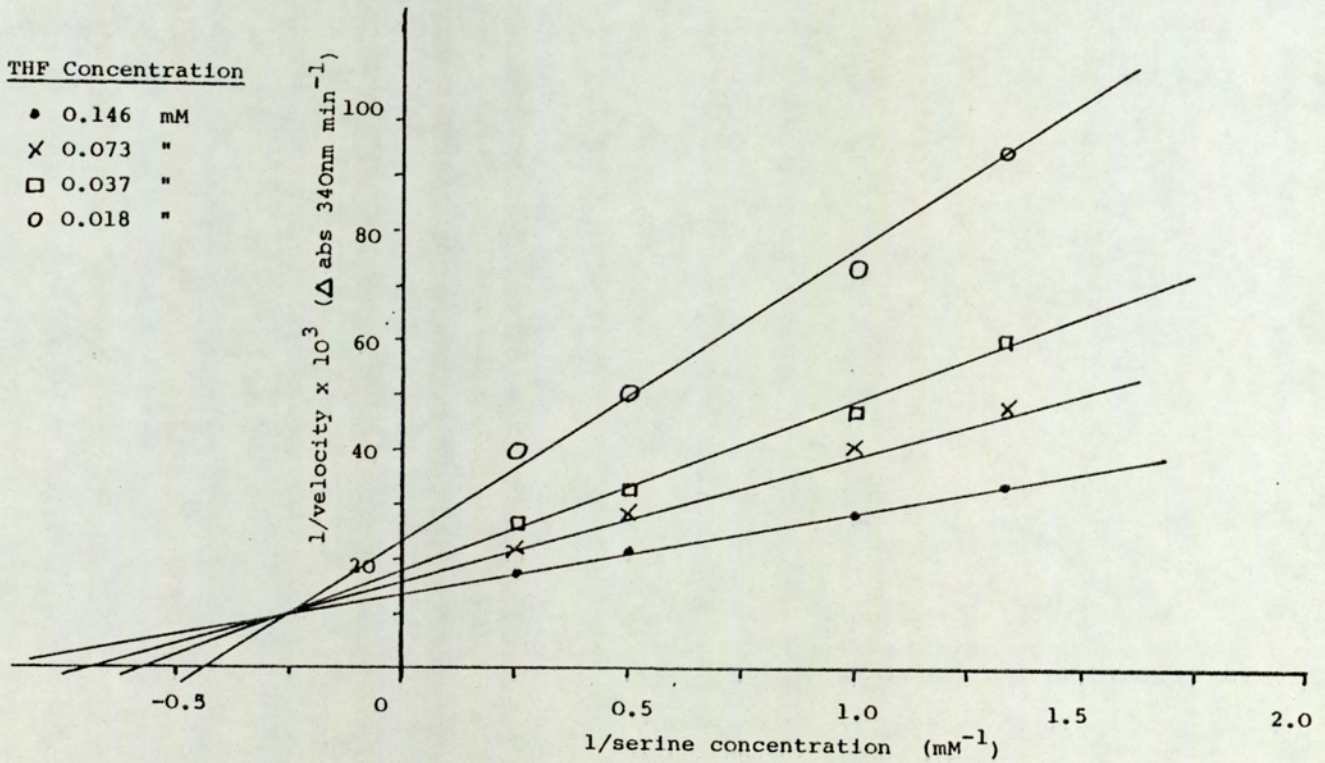


Figure 4.3. Lineweaver-Burk plot for serine with varying concentration of THF for L1210 SHMT.

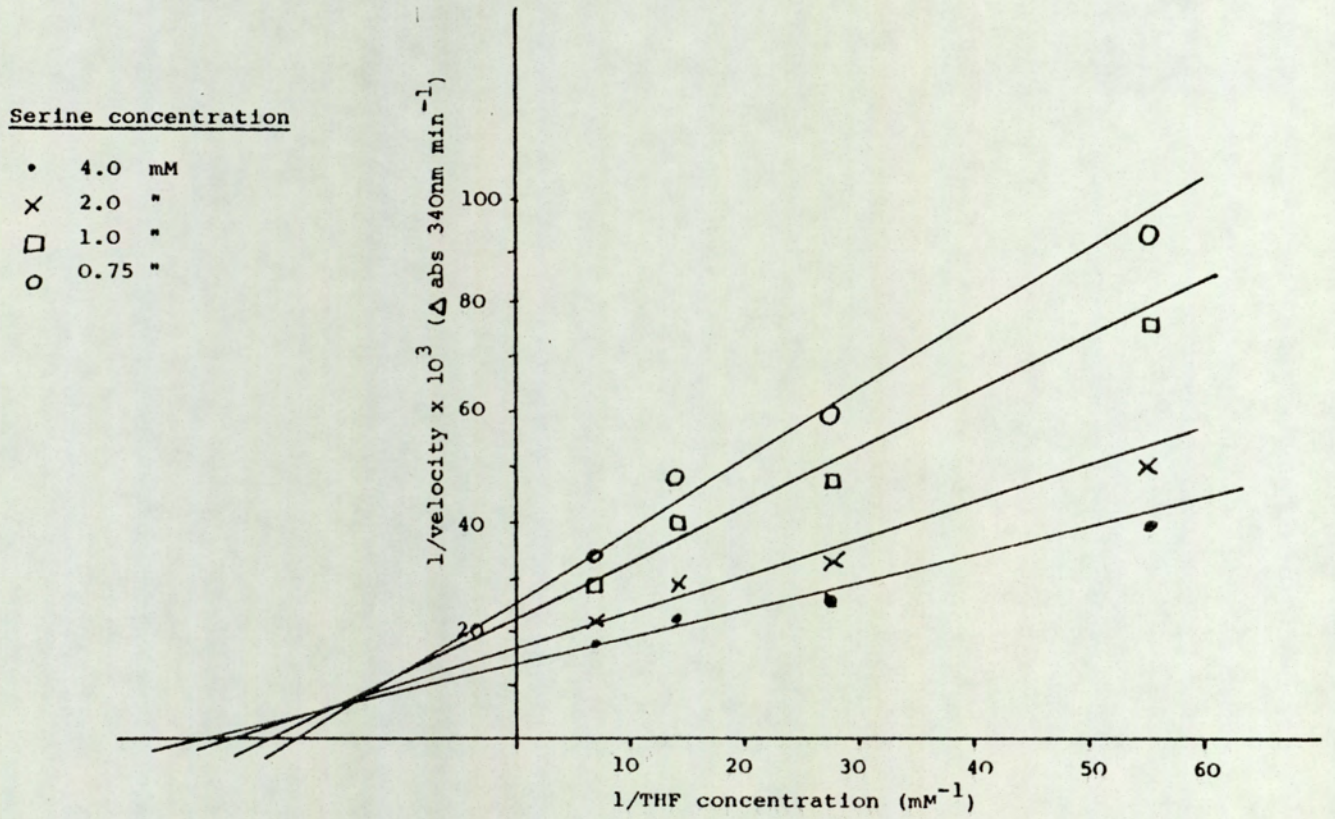


Figure 4.4.Lineweaver-Burk plot for THF with varying concentration of serine for L1210 SHMT.

Table 4.2. Kinetic binding constants for SHMT.

Substrate	Km (mM)	L1210	cSHMT[1]	mSHMT[2]	E.SHMT[3]
L-serine	Ka	4.32	1.3	1.4	0.8
	αKa	0.94	0.4	0.6	0.3
THF	Kb	0.083	0.06	0.045	0.08
	αKb	0.014	0.02	0.015	0.025
L-allothreonine		2.7	1.5	2.5	1.5
L-threonine		40	40	33	-
α-Hydroxymethylserine		20			

1 = cytosolic rabbit liver, 2 = mitochondrial rabbit liver 3 = E.coli

The kinetic constants for the cleavage of threonine and allothreonine were determined in the absence of THF (figures 4.6 and 4.7). The Km for α-hydroxymethylserine was determined with a THF concentration of 0.07 mM (figure 4.8).

4.3.4. Spectral properties of L1210 leukaemia SHMT.

The ultraviolet spectrum of the partially purified L1210 leukaemia SHMT is shown in figure 4.9. It exhibits absorbance maxima at 410 and 320 nm.

4.4. Discussion.

The L1210 SHMT appears to be similar to the mammalian and E.coli enzyme, although some subtle differences do exist. The L1210 and E.coli enzymes have, unlike the rabbit liver enzymes, a high affinity

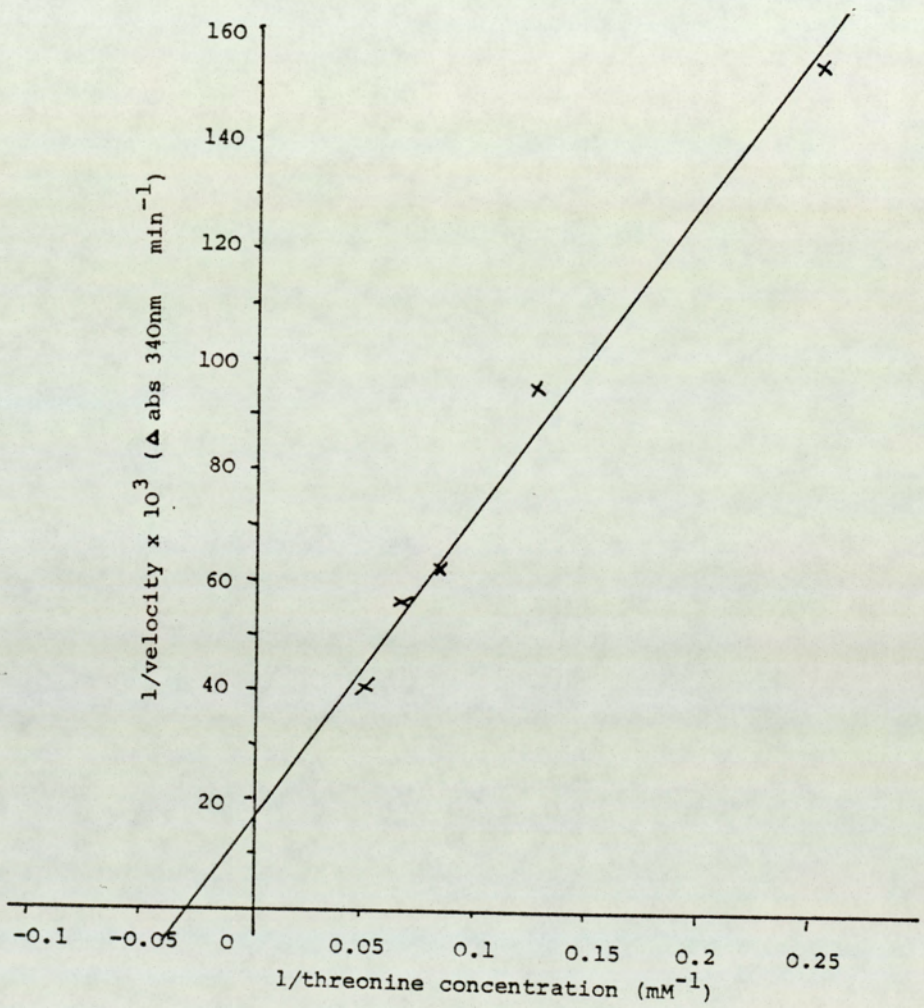


Figure 4.6.Lineweaver-Burk plot for threonine using L1210 SHMT,

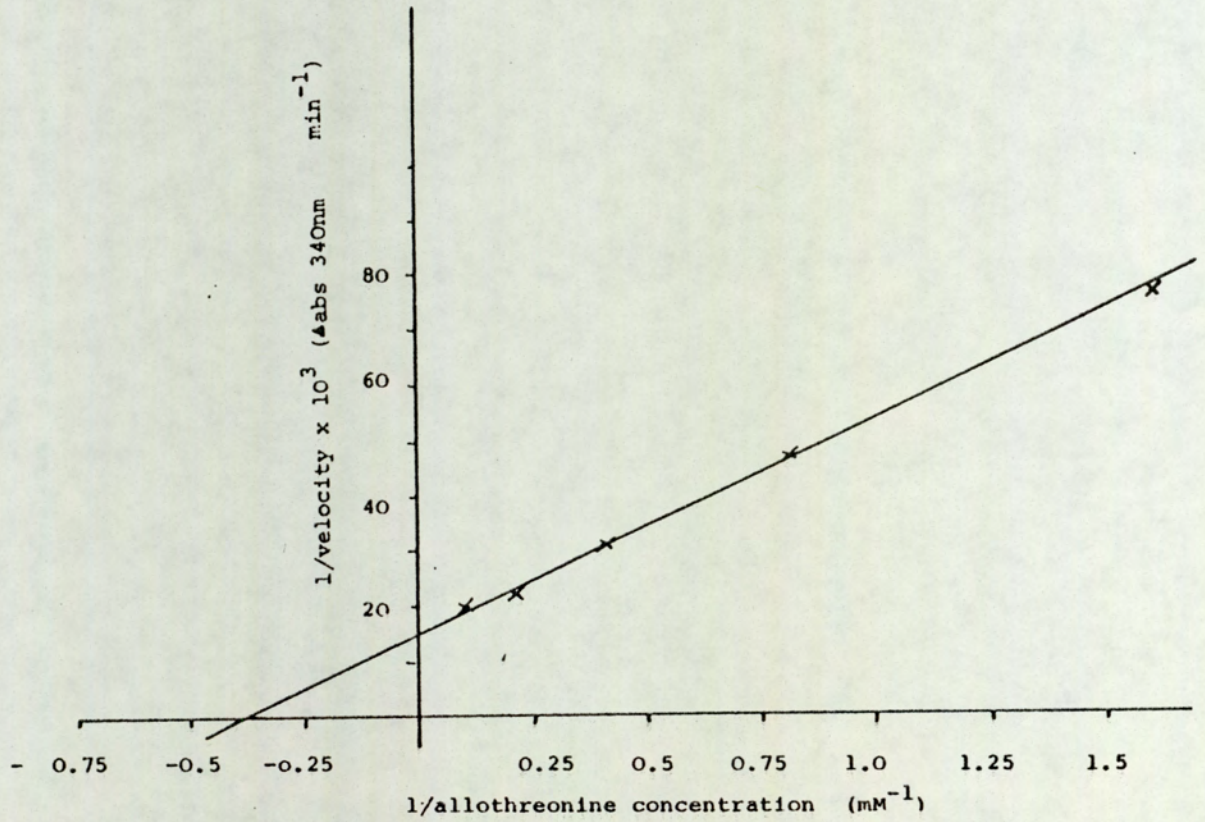


Figure 4.7. Lineweaver-Burk plot for allothreonine using L1210 SHMT.

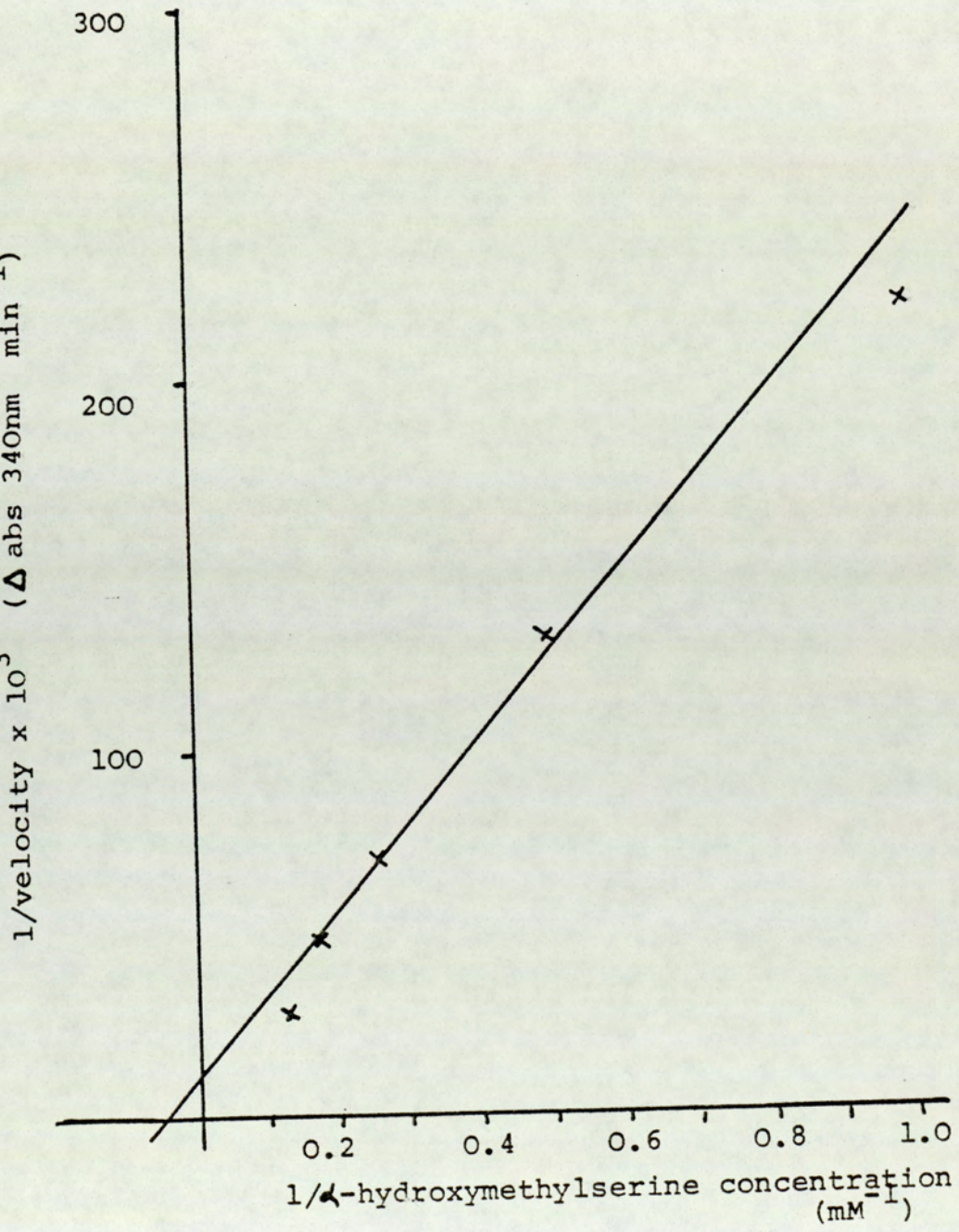


Figure 4.8. Lineweaver-Burk plot for α-hydroxymethylserine using L1210 SHMT.

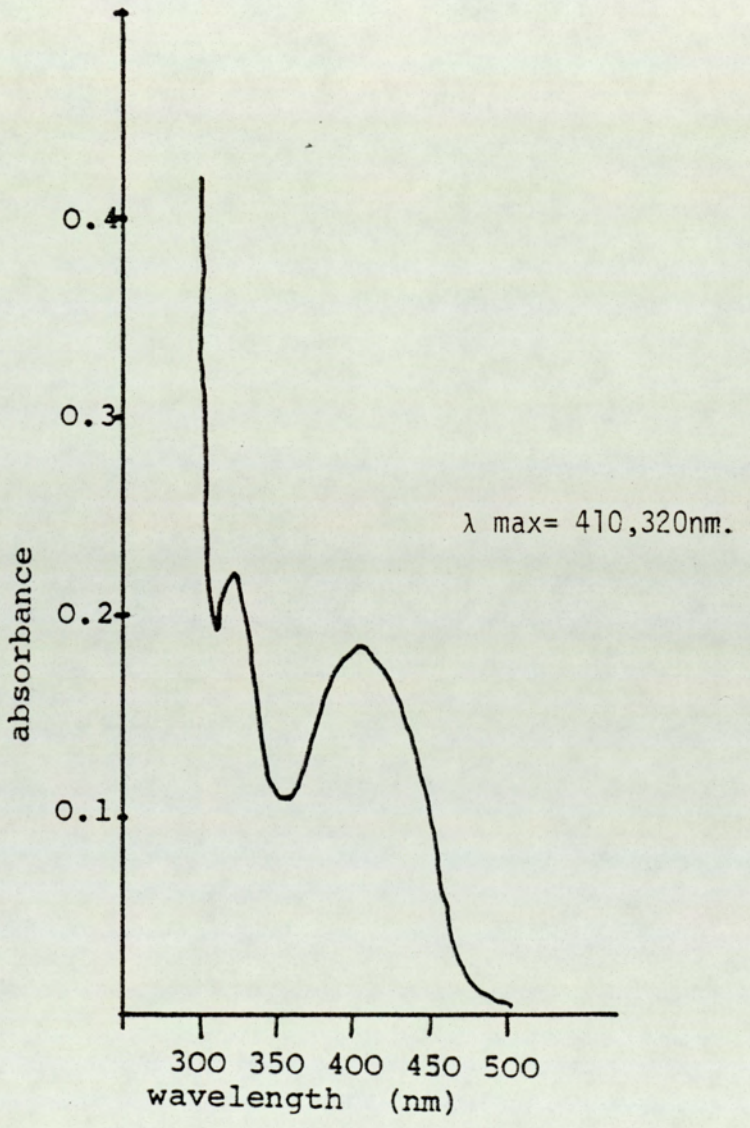


Figure 4.9. UV absorbance sprectrum of the partially purified L1210 SHMT enzyme.

for the folinic acid column, and no affinity for the blue Sepharose column. While it is tempting to propose that the tumour and the E.coli enzyme are related to one another and are different to the mammalian enzymes, it is unlikely that these minor differences are significant.

Pore exclusion polyacrylamide electrophoresis indicates that the L1210 enzyme, like the rabbit liver enzyme, exists naturally as a tetramer. This is unlike the E.coli enzyme which appears to be a dimer. All forms of the enzyme in digesting SDS-PAGE electrophoresis have similar molecular weights.

The kinetic constants obtained for the L1210 enzyme suggest that all forms of the enzyme compared are similar. The values obtained for the synergistic binding of L-serine and THF are similar to values in the literature[166]. The L1210 enzyme will catalyse the de-aldolisation of L-threonine and L-allothreonine in the absence of folate. In this cleavage, as with other forms of the enzyme, the erythro conformation is preferred. The finding that the L1210 enzyme cleaves α-hydroxymethyl serine, indicates that the enzyme is capable of catalysing the dehydroxymethylation of α-substituted analogues.

Kumar et al[9] have previously found that with L1210 solid tumours SHMT there was no positive homotropic co-operation between the L-serine and THF. They had found this co-operation in non tumour bearing mouse liver and kidney, but it was abolished on the implantation of the L1210 tumour.

The results obtained for the cell culture L1210 SHMT agrees with their finding that there is no homotropic cooperativity. However the finding by Schirch and Quashnock[82], that the cooperativity obtained by Kumar et al was an artefact of their procedures, brings doubt on the validity of their findings.

This work presents a simple method of partially purifying the enzyme SHMT from a tumour cell line. From 4.8×10^9 cells, 1.5 mg of enzyme may be prepared in under two days. This preparation retains its activity over a period of greater than two weeks if stored at 4°C.

The use of partially purified SHMT, which has been characterised kinetically and physically, for the evaluation of inhibitors of the enzyme, should provide more information on the true nature of the interactions of molecules with this enzyme.

CHAPTER 5

THE TESTING OF POTENTIAL INHIBITORS OF SHMT

5.1. Introduction.

The α-substituted serine analogues described in Chapter 2 were rationally designed to be irreversible enzyme activated inhibitors of the enzyme SHMT.

In addition to the testing of these, and other serine analogues of interest, the work presented in this chapter describes the screening of a number of other related compounds against the enzyme. Many molecules that are structurally related to serine and THF have been previously synthesised for a variety of reasons. The selection of these compounds for testing was normally based upon the availability of suitable chemically characterised samples.

Initially the effect of the potential inhibitors on the activity of the enzyme SHMT was assayed using the method of Taylor and Weissbach[159]. In order for these compounds to be bioactivated, they must firstly bind to the active site PLP and then be dehydroxymethylated, producing CH2-THF. The coupled assay of Schirch et al[78] was used to examine the rate of production of CH2-THF when the serine analogues were incubated with the partially purified L1210 leukaemia and homogeneous cytosolic rabbit liver SHMT.

The spectroscopic properties of the enzyme SHMT have been well characterised by Schirch and Mason[52]. The changes in the absorption of the enzyme upon the serine and glycine interconversion are shown in section 1.4.1.

Upon the addition of THF to an enzyme-glycine complex, a rapid increase in the absorbance at 495nm is observed[5]. This characteristic peak is due to the THF stabilising the quinonoid by inhibiting

its reprotonation[76] (section 1.5.1.). In the case of enzyme-serine complexes the 495nm absorption peak is not observed. This is due to the rapid production of CH2-THF[1].

Substrates for the enzyme which form stabilised quinonoids may therefore be detected spectrophotometrically by monitoring the absorption of the enzyme-substrate complex at 495 nm, in the presence of THF. The effect of the α-substituted serine analogues on the absorption spectrum of the homogeneous rabbit liver enzyme was investigated both in the absence and in the presence of THF.

Circular dichroism (CD) is normally used to examine the conformation of the tertiary structure of enzymes. The CD absorption measured between 180-240nm yields valuable information on the α-helix and β-sheet content of an enzyme[167].

Although PLP is achiral it has an induced optical activity when bound to peptides[168]. This is due to the asymmetric environment of enzymes. The active site PLP bound to aspartate aminotransferase is optically inactive if the polypeptide chain, in the presence of urea, is unfolded[169]. In the native enzyme, a strong extrinsic Cotton effect in the $\pi \rightarrow \pi^*$ transition of the aldimine chromophore is observed[170]. This positive CD absorption in the 320-500nm region is found in most PLP dependent enzymes[168]. The CD spectrum of a mutated E.coli SHMT has this absorption band present[171].

When the PLP bound to the lysine residue in the active site of the enzyme binds substrates, the formation of a new aldimine bond induces a change in the CD absorption in the 320-500nm region [171]. CD spectroscopy was utilised in an attempt to see whether or not the potential inhibitors bind to the PLP in the active site of the rabbit liver enzyme.

In order to investigate the effects of the α-substituted serine analogues on whole cells, the cytotoxicity of the molecules was evaluated against the GM0621 and K562 cell lines.

5.2. Materials and Methods.

5.2.1. Materials.

The 24-well plastic plates were obtained from Flow Laboratories, Scotland. The compounds listed in figure 5.2 were the kind gift of Glaxo Group Research, Greenford. RPMI 1640 Media and Fetal Calf Serum were obtained from Gibco U.K. Ltd. The rabbit liver SHMT and CH2-THF were the kind gift of Dr V Schirch. For general chemicals see section 3.2.

5.2.2. Total cell SHMT inhibition studies.

The effect of potential inhibitors on total cell SHMT was investigated using the assay method of Taylor and Weissbach[159]. The assay procedure is described in section 3.2.6.

5.2.3. Inhibition studies utilising partially purified L1210 SHMT.

The coupled assay method, as described in section 4.2.5., was used to detect the enzymatic production of CH2-THF from the α-substituted analogues under test. The L1210 SHMT was prepared by the method given in section 4.2.

5.2.4. Absorption studies utilising purified rabbit liver SHMT.

The absorption spectra were recorded on a Cary 210 spectrophotometer, using degassed solvents, with the cuvette temperature maintained at 30°C.

The spectrum of homogeneous cytosolic rabbit liver SHMT (2 mg ml^{-1}) in 50 mM potassium phosphate, pH 7.3, was recorded between 300-530nm against buffer alone. The amino-acid under test, dissolved in the phosphate buffer, was added to the cuvette and the absorption spectrum of the enzyme was re-recorded over a period of 100 min.

In order to amplify the production of a stabilised quinonoid intermediate, 0.7mM THF and 30mM 2-mercaptoethanol was added, and the cuvette was sealed. The absorption spectrum between 460-500 nm was re-recorded over a time period of 30 min.

This was then followed by the addition of 0.24mM $NADP^+$ and an absorption base line was established at 340 nm. CH2-THF dehydrogenase (8 μg) was then added and the change in the absorbance at 340 nm was recorded. This change in the absorption is due to the production of CH2-THF by the enzyme SHMT.

5.2.5. Circular dichroism spectroscopy.

CD spectra were recorded on a Jasco J-500C recording spectropolarimeter. A 1cm path length cell was used, with all buffers degassed prior to use. The CD spectrum of homogeneous cytosolic rabbit liver SHMT (3.1mg ml^{-1}) in 50mM potassium phosphate pH 7.3, was recorded between 300-530nm at a scan rate of 50nm min^{-1}. The amino-acid under test (50mM in 20mM potassium phosphate pH 7.3) was added to the cell and the spectrum was re-recorded.

5.2.6. Growth inhibition assays.

The growth inhibitory effects of the compounds tested were determined against the GM0621 (section 3.2.4.3) and K562 (section 3.2.4.5) cells, plated out into duplicate 1ml wells of a 24-well

plastic plate at a density of 5×10^4 cells ml^{-1}. RPMI 1640 media supplemented with 10% fetal calf serum was used as culture medium, with drug solutions added as appropriate. The drugs tested were previously dissolved in the culture medium. The cells were maintained in an atmosphere of 10% CO_2 in air. Cell numbers were enumerated using a Coulter-counter over a five day incubation period and the percentage inhibition determined over the linear part of the growth curve.

5.3. Results.

5.3.1. The effect of solvents on the activity of SHMT.

Many THF analogues are poorly soluble in water. In order for them to be assayed, they are normally dissolved in a variety of solvents. The effect of various solvents on Flow L1210 (Glaxo) total cell SHMT was investigated. N-Methylformamide (NMF), dimethyl-sulphoxide (DMSO) and absolute ethanol, at concentrations of 0.3-30% v/v, were included in the assay prior to the addition of the enzyme. The effect of the inclusion of the solvents in the assay, is presented in figure 5.1.

Ethanol and NMF both cause an initial increase in activity at concentrations from 0.5-2.5% v/v, which is then followed by inhibition at levels of up to 30% v/v solvent. All concentrations of DMSO cause a decrease in the activity of the enzyme.

In order to investigate the effect of solvents on the assay itself, a modified method for determining total cell SHMT activity was followed. Upon the quenching of the incubation with sodium acetate the solvents were added, and the SHMT activity was evaluated by the normal method. No variation from control was observed for any of the solvents at the stated concentrations.

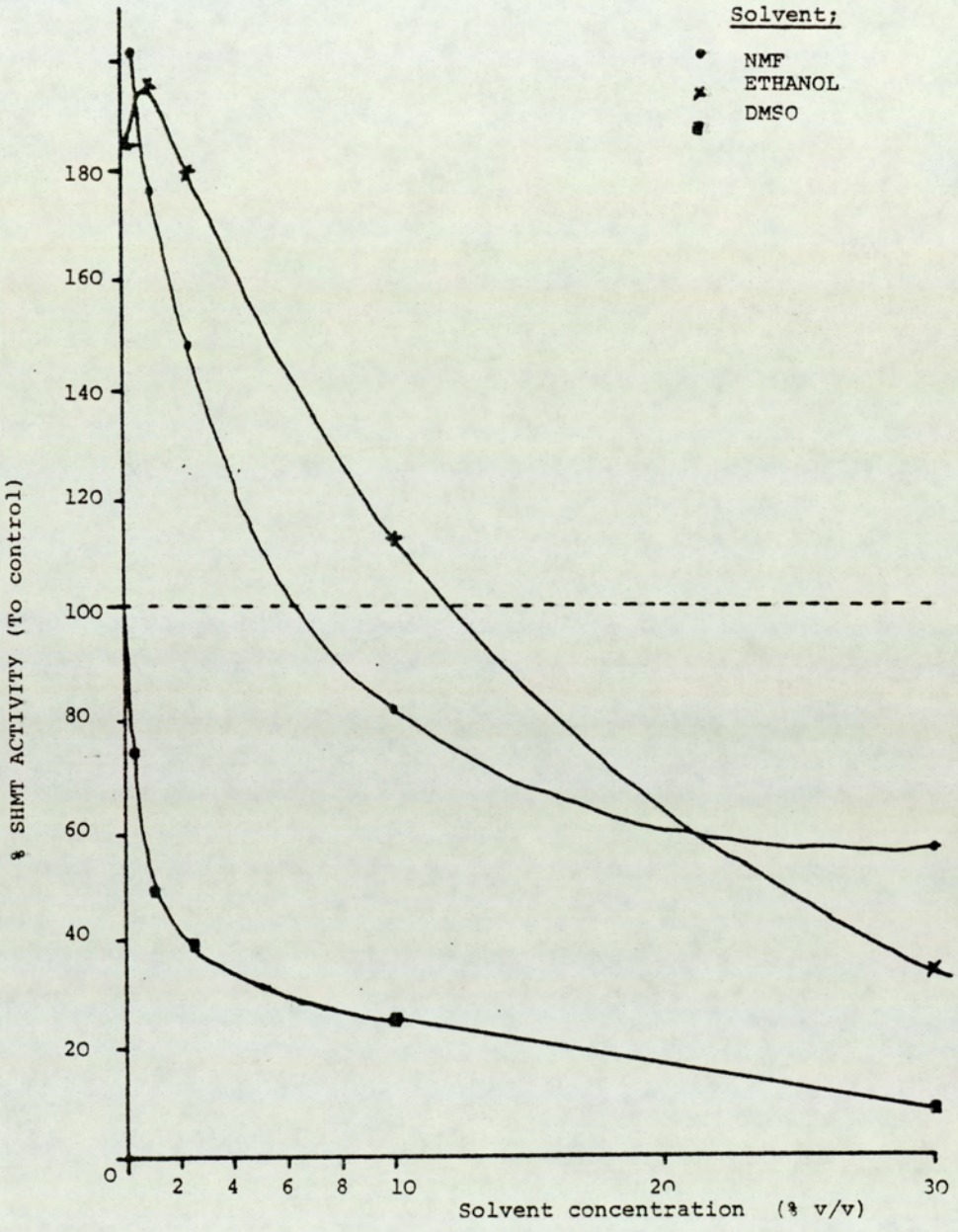


Figure 5.1.The effect of various solvents on L1210 (Glaxo) total cell SHMT activity (30min incubation).

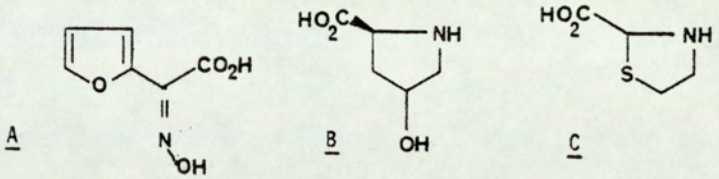
The change in activity with different concentrations of solvents was taken into account when the solvents were used to dissolve compounds under test.

5.3.2. The effect of the compounds against total cell SHMT.

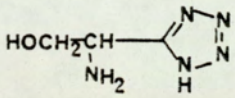
The compounds shown in figure 5.2 were tested as potential inhibitors of Flow L1210 (Glaxo) total cell SHMT at concentrations of 0.5-10 mM. The results obtained are presented graphically in figures 5.3-5.4. Duplicate determinations for each point agreed within 5%. The effect of N-mono-, N-di- and N-trichloroacetylserine on TLX5 total cell SHMT activity was investigated using concentrations of 0.5-2 mM. The compounds appeared to have little inhibitory activity at these concentrations, as shown in figure 5.5.

α-Allyl-, α-hydroxymethyl-, α-propargyl-, and α-vinylserine were tested as inhibitors of K562 total cell SHMT. The compounds, at concentrations of 0.25-4 mM, have little inhibitory effect on the enzyme activity, figure 5.6.

To establish whether or not α-allyl-, α-propargyl- and α-vinyl-serine are effective inhibitors of total cell SHMT, and to classify the type of inhibition occurring, the kinetics of the inhibition caused by each of the compounds was evaluated. The rate of reaction was determined with serine present at concentrations of 0.25- $\frac{3}{4}$ mM, and the α-substituted amino-acids being present at concentrations of 2.5-10 mM. Only α-vinylserine deviated from control. Lineweaver-Burk plots, figure 5.7, show this compound to be a competitive inhibitor of total K562 cell SHMT with a Ki of 15.2 mM (figure 5.8).



A B C



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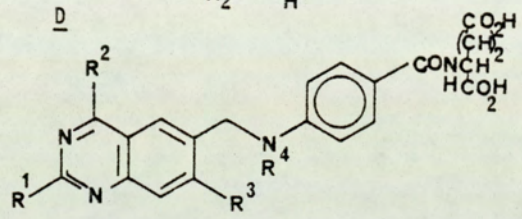


F-I

Compound	R^1	R^2	R^3	R^4
F.	NH_2	OH	H	CH_2CCH
G.	NH_2	OH	CH_3	CH_2CCH
H.	H	H	H	CH_2CCH
I.	NH_2	OH	H	H

A . Furanyl oximinoacetic acid.

B. 4-Hydroxy-2-pyrrolidine carboxylic acid.

C. Thiazolidine-2-carboxylic acid.

D. 2-Amino-2-tetrazolyl-5H-ethanol.

E. Vinylglycine .

F. N-(4-(N-((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynylamino)benzoyl)L-glutamic acid.

G. N-(4-(N-((2-amino-4-hydroxy-6-quinazolinyl-7-methyl)methyl)prop-2-ynylamino)benzoyl) L-glutamic acid.

H. N-(4-(N-((6-quinazolinyl)methyl)prop-2-ynylamino)benzoyl)L-glutamic acid.

I. N-(4-(N-((2-amino-4-hydroxy-6-quinazolinyl)methyl)amino)benzoyl)L-glutamic acid.

Figure 5.2. The compounds that were randomly screened.

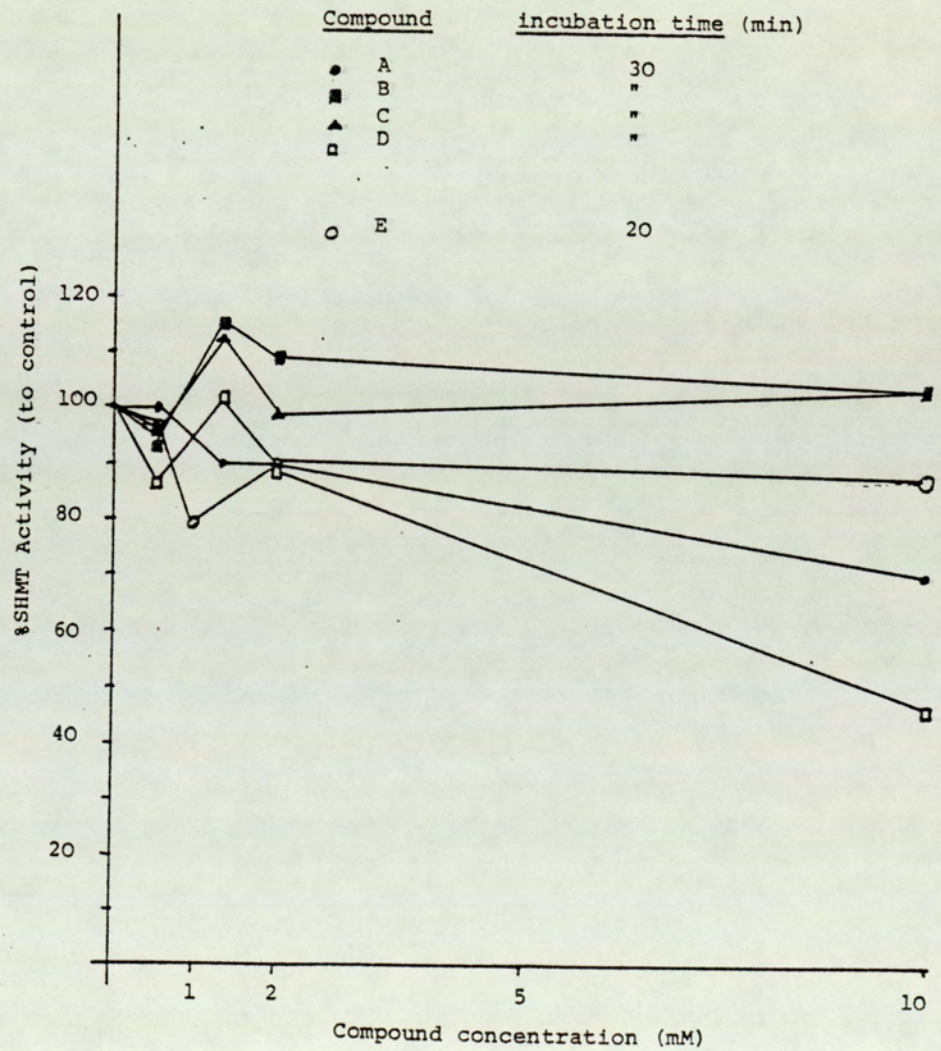


Figure 5.3. Effect of compounds on L1210 (Glaxo) total cell SHMT activity.

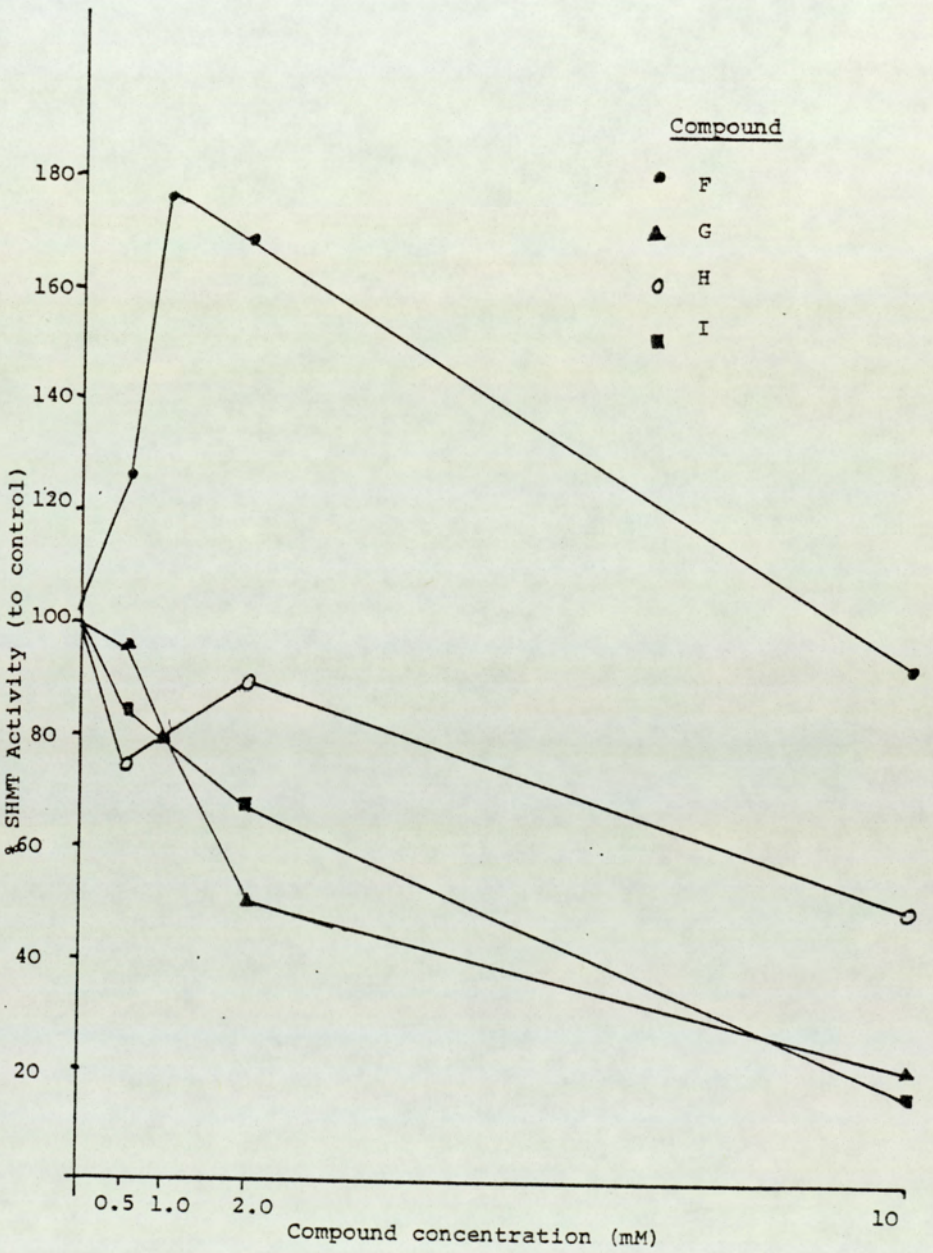


Figure 5.4. Effect of compounds on L1210 (Glaxo) total cell SHMT activity, following a 30min incubation.

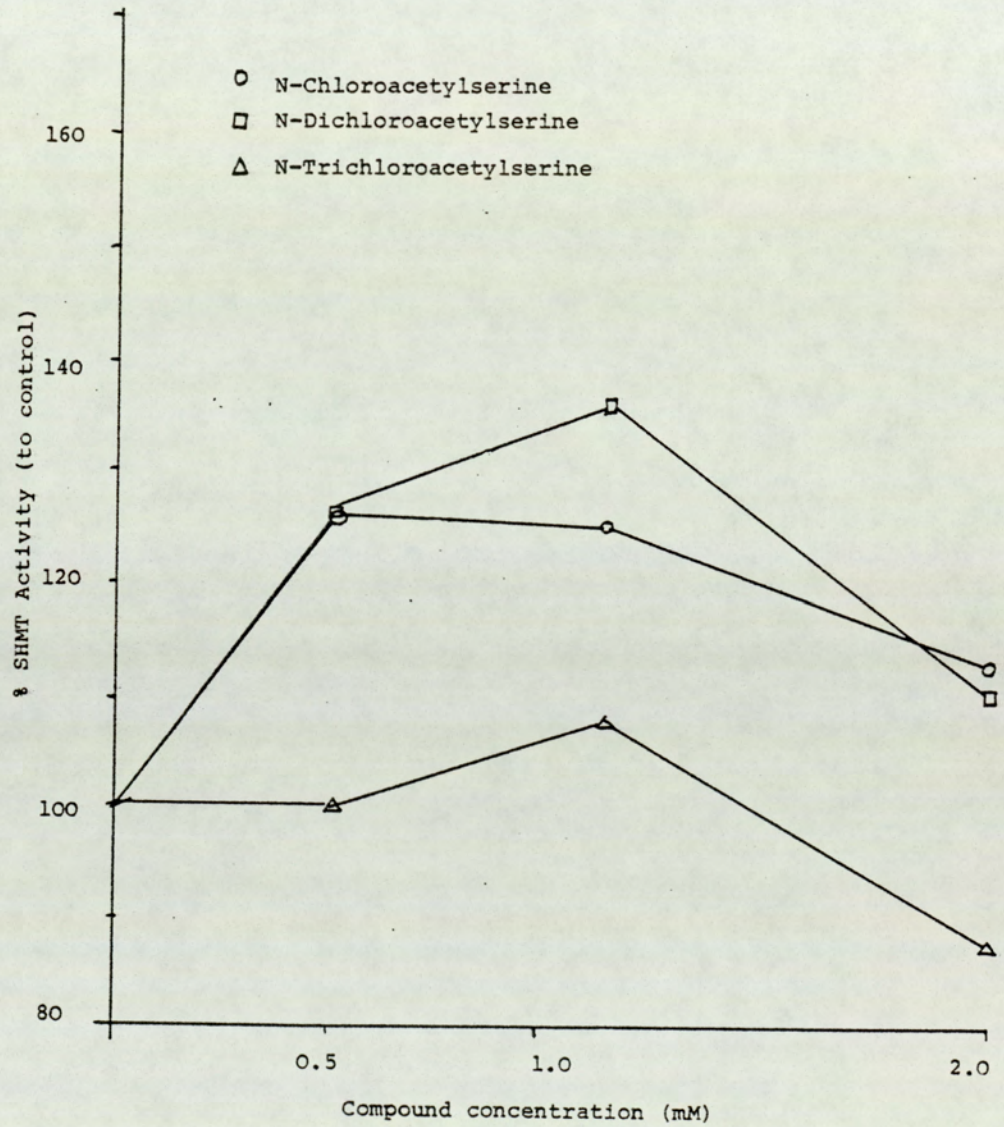


Figure 5.5. Effect of N-haloacetylserine compounds on total cell TLX5 SHMT, following a 10 min incubation.

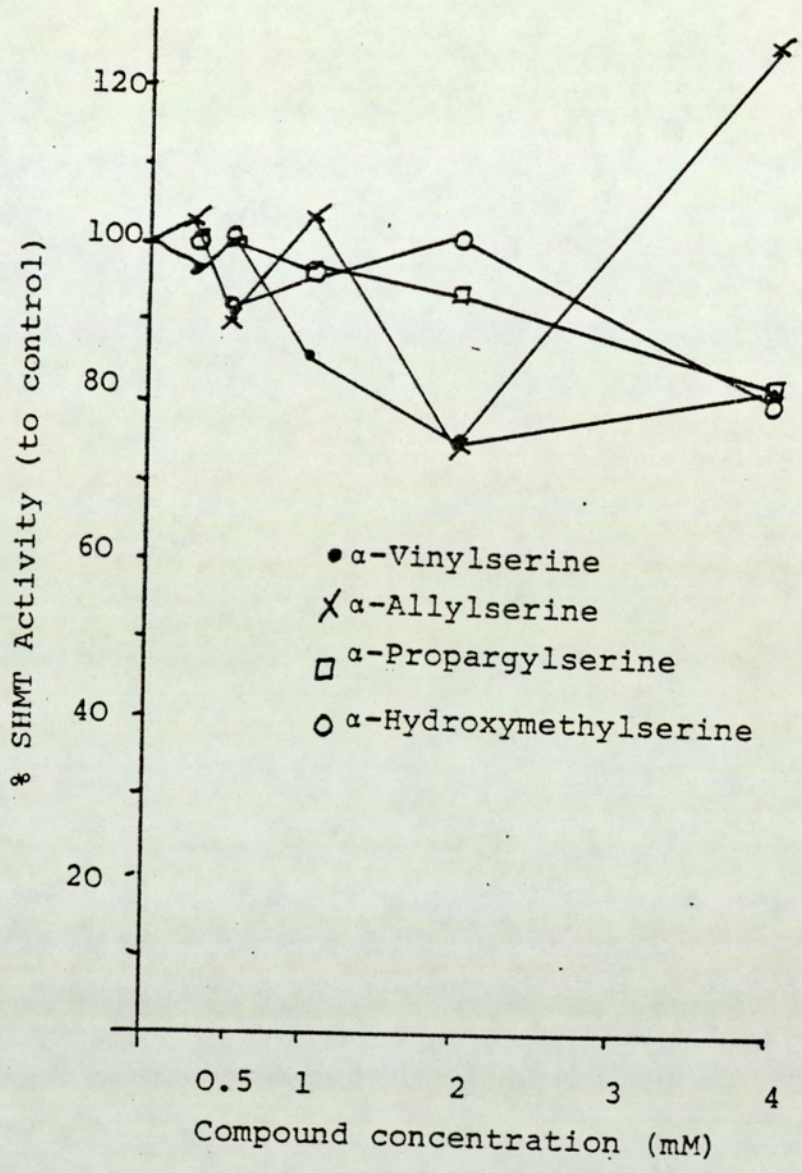


Figure 5.6. Effect of α-substituted serine compounds on total cell TLX5 SHMT, following a 10 min incubation.

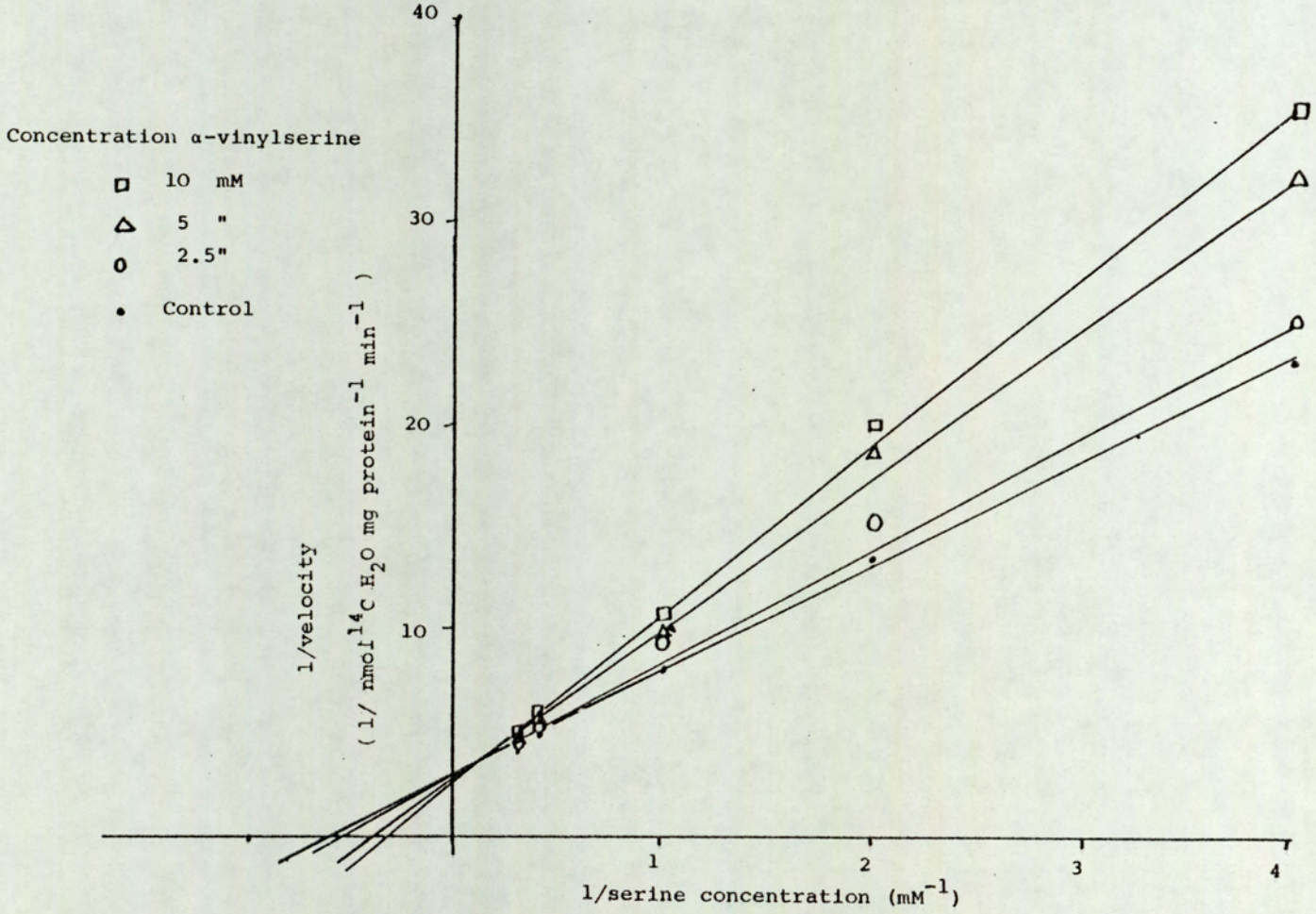


Figure 5.7. Lineweaver-Burk plot for serine in the presence of varying concentrations of α-vinylserine (For K562 total cell SHMT).

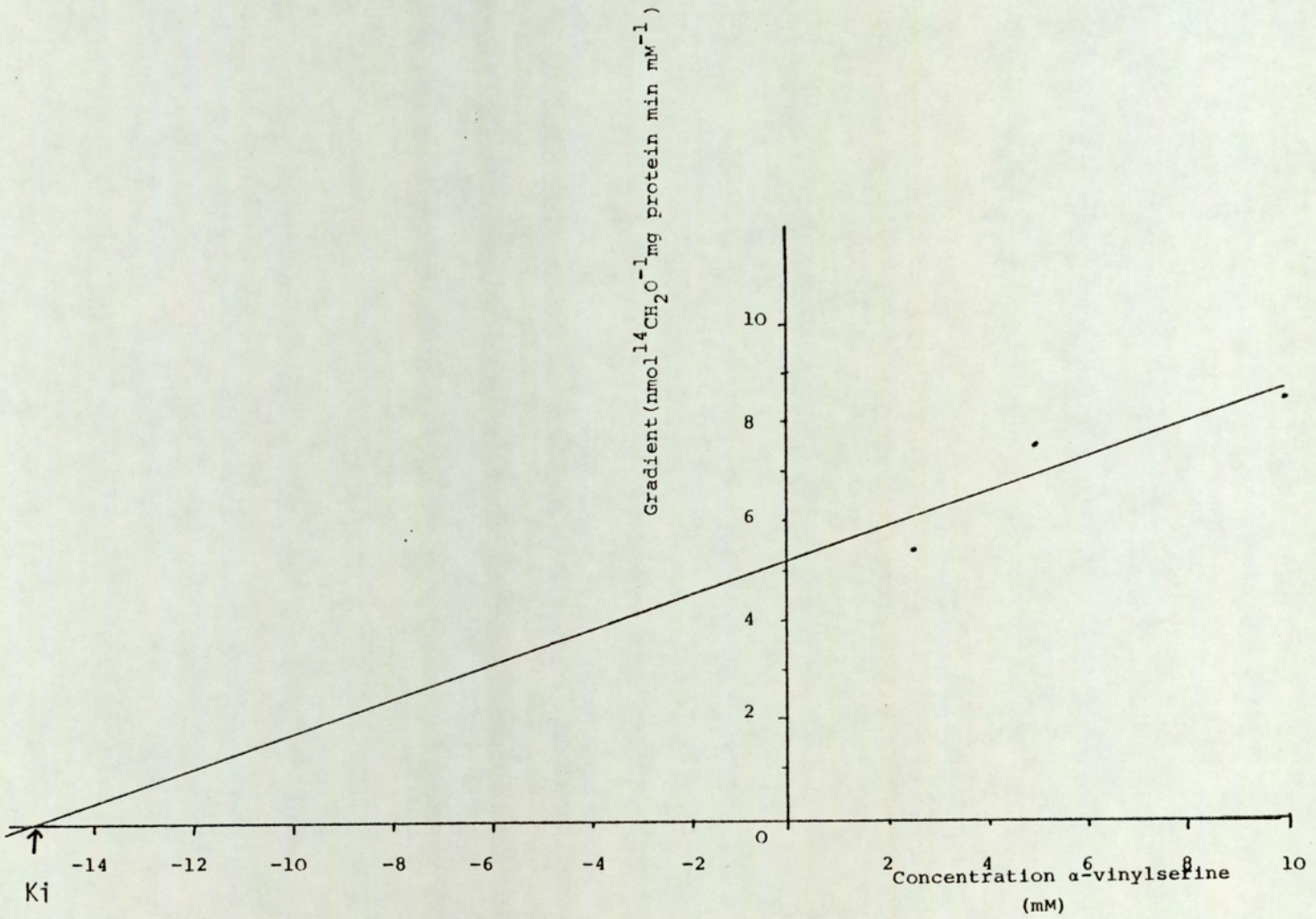


Figure 5.8. Plot of gradient (figure 5.7) against concentration α-vinylserine present.

5.3.3. The effect of methotrexate on L1210 SHMT.

Methotrexate caused no inhibition of partially purified L1210 SHMT at concentrations of up to 0.1 mM. The methotrexate was assayed in the presence of 4 mM L-serine and 0.068 mM THF.

5.3.4. Bioactivation of α-substituted serine analogues by L1210 SHMT.

The production of CH2-THF by partially purified L1210 SHMT from α-allyl-, α-propargyl- and α-vinylserine was investigated. No CH2-THF could be detected for each of the compounds at concentrations of up to 20 mM.

5.3.5. The effect of α-substituted serine analogues on cytosolic rabbit liver SHMT.

The action of α-allyl-, α-propargyl- and α-vinylserine on homogeneous cytosolic rabbit liver SHMT was investigated spectrophotometrically.

The effect of incubating the enzyme with 20 mM α-vinylserine is shown in figure 5.9. Similar results were obtained for the other compounds under test and for the control. This indicates that the compounds do not alter the absorption properties of the enzyme. The absorbance between 300-360 nm is an artefact due to the previous decomposition of the enzyme[162].

For the three compounds tested, the addition of THF caused no change in the absorption of the enzyme between 490-530 nm.

The addition of $NADP^+$ and CH2-THF dehydrogenase caused no elevation above the control of the absorbance at 340 nm, indicating that there is no production of CH2-THF.

The effect of incubating the enzyme with 5 mM glycine is shown in figure 5.10. An increase in the absorption at 500 nm and a

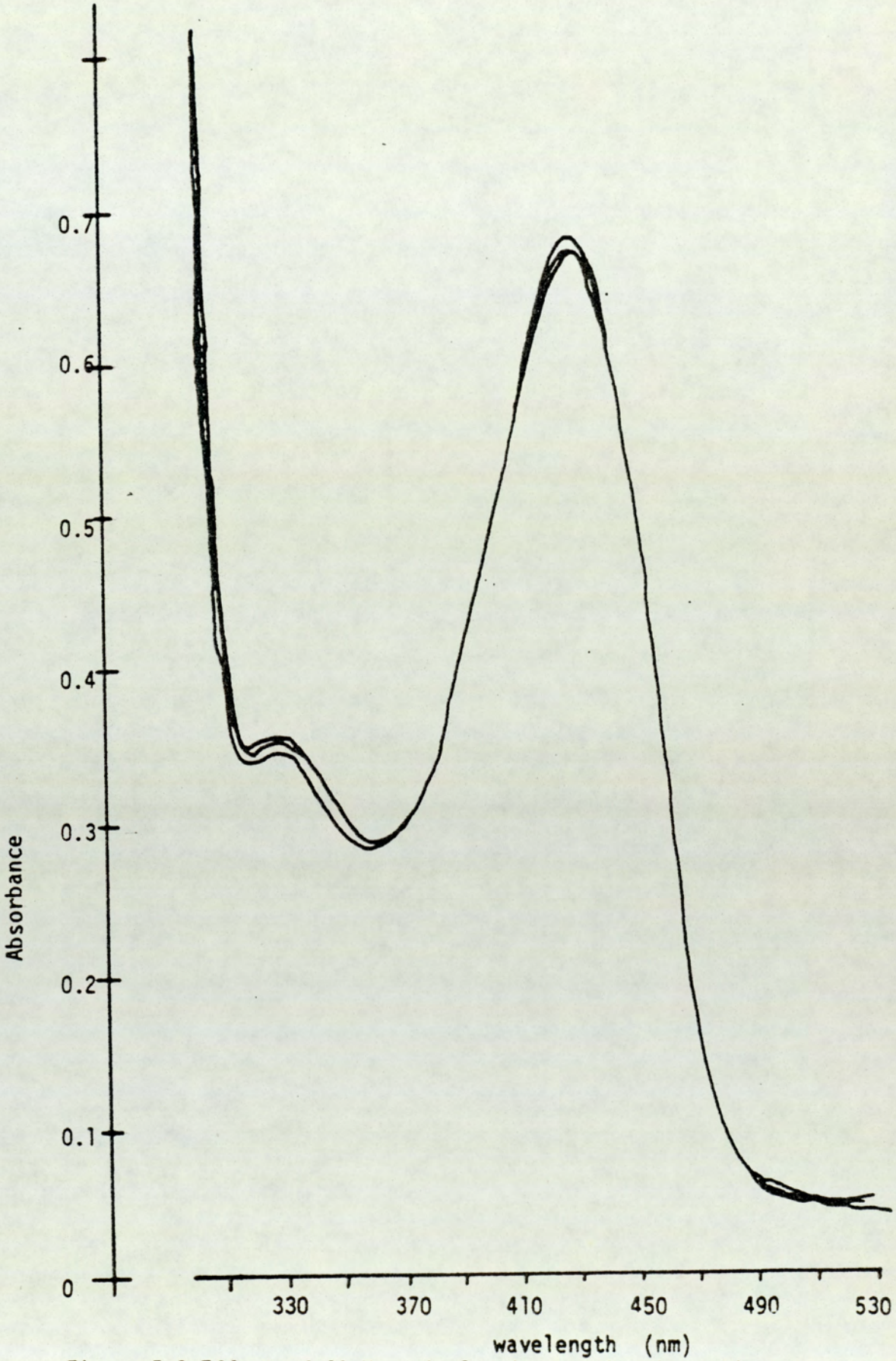


Figure 5.9. Effect of 20mM α-vinylserine on the absorption spectrum of purified rabbit liver enzyme.

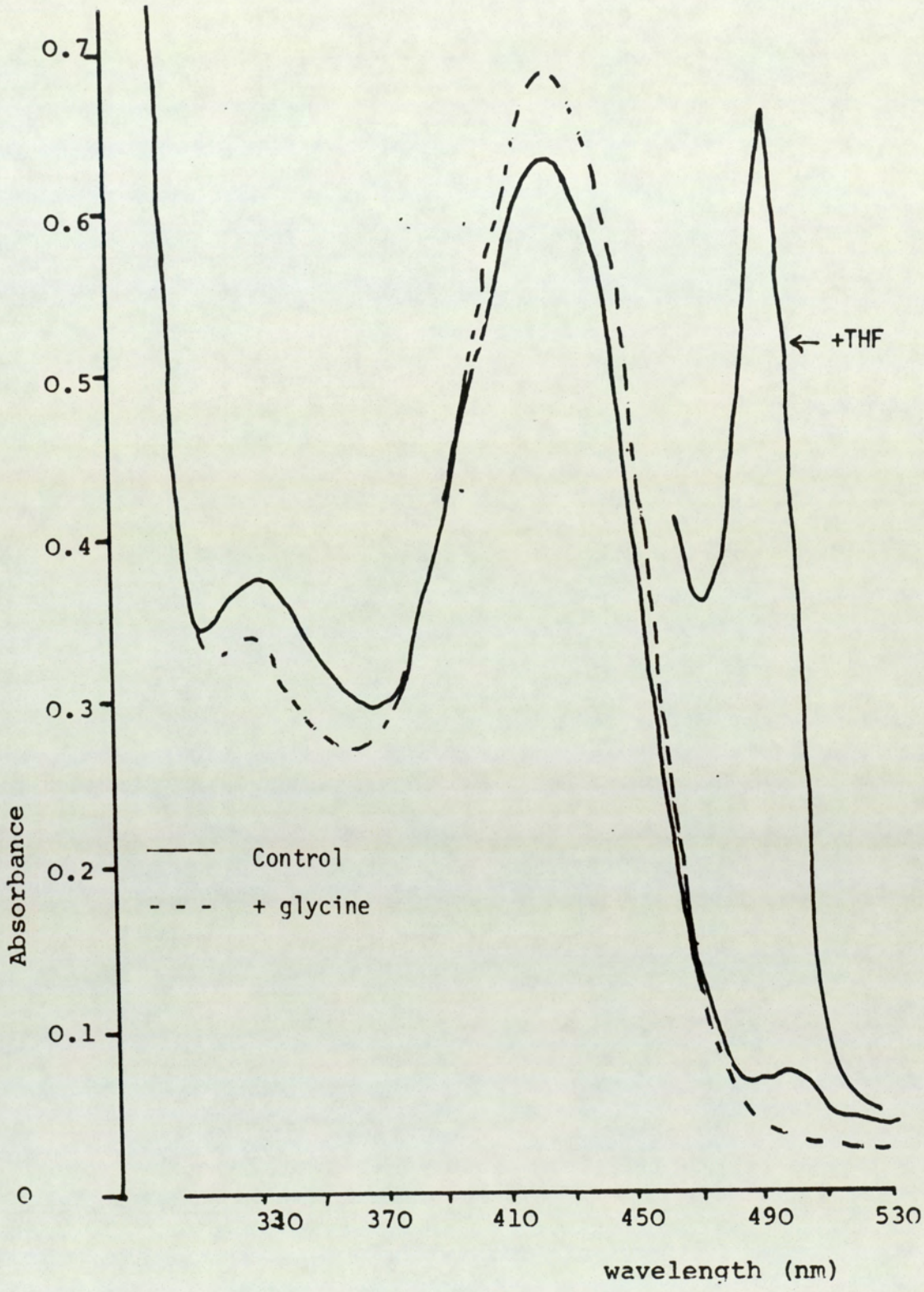


Figure 5.10. Effect of 5mM glycine on the absorption spectrum of purified rabbit liver enzyme.

decrease in the 420 nm band is observed. On the addition of THF a large increase in the absorbance at 495 nm is observed.

5.3.6. CD spectroscopy.

The effect of the addition of 50 mM α-vinylserine on the CD spectrum of the rabbit liver enzyme is shown in figure 5.11. Similar results were obtained for α-allyl- and α-propargylserine and for the control. The drop in the θ_{max} on the addition of the compound in buffer is due to a dilution effect. These results indicate that no change in the CD spectrum of the enzyme is induced by these compounds. The effect of the addition of 50 mM serine and 50 mM glycine are shown in figures 5.12 and 5.13 respectively. These two amino-acids cause a change in the CD absorption of the enzyme relative to the control. This is due to the amino-acids forming new aldimine bonds with the active site PLP.

5.3.7. Cytotoxicity assays.

N-Dichloroacetylserine, α-allyl-, α-propargyl- and α-vinyl-serine are not toxic to K562 and GM0621 cells at concentrations of 0.005-10 mM. α-Hydroxymethylserine is not cytotoxic to the GM0621 cells but shows some toxicity to the K562 cell line at elevated concentrations, figure 5.14.

5.4. Discussion.

The increased activity of the enzyme SHMT at low concentrations of NMF and ethanol may be accounted for by these solvents inducing a conformational change in the active site of the enzyme. A small change in the conformation may increase the activity of the enzyme. However, if the concentration of solvent induces a large change in

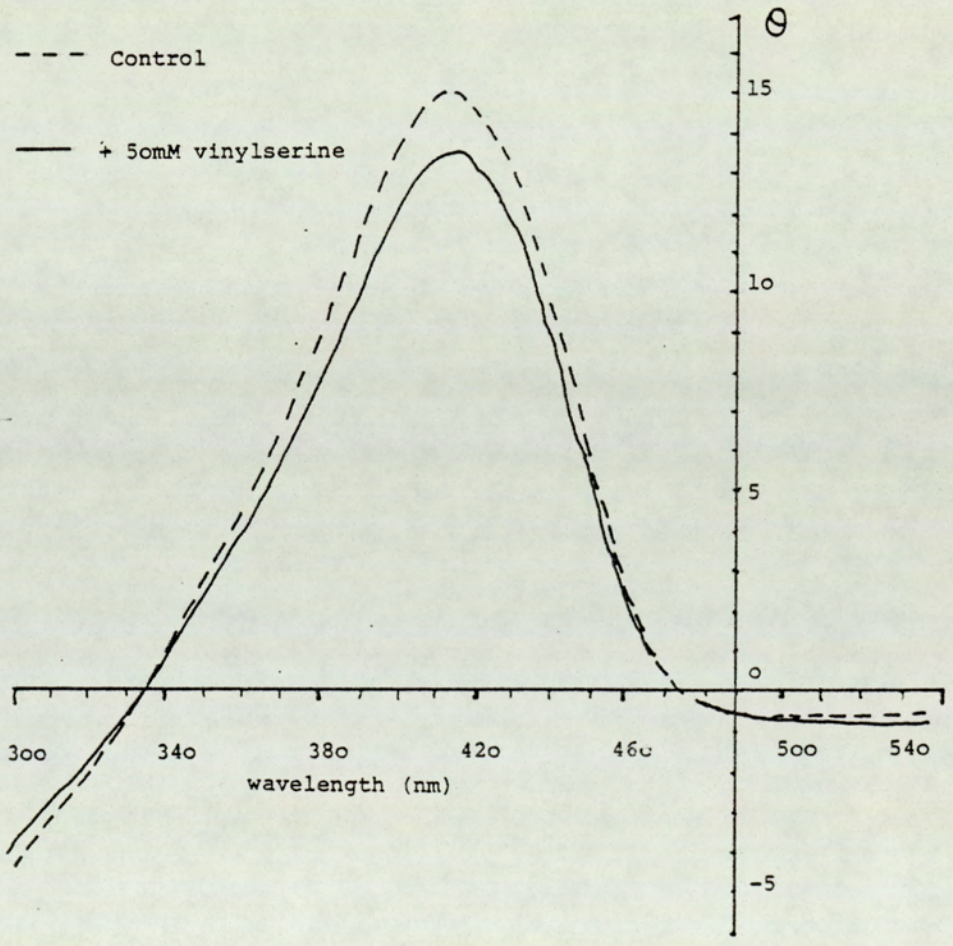


Figure 5.11. Effect of 50mM α-vinylserine on the CD absorption spectrum of the purified rabbit liver enzyme.

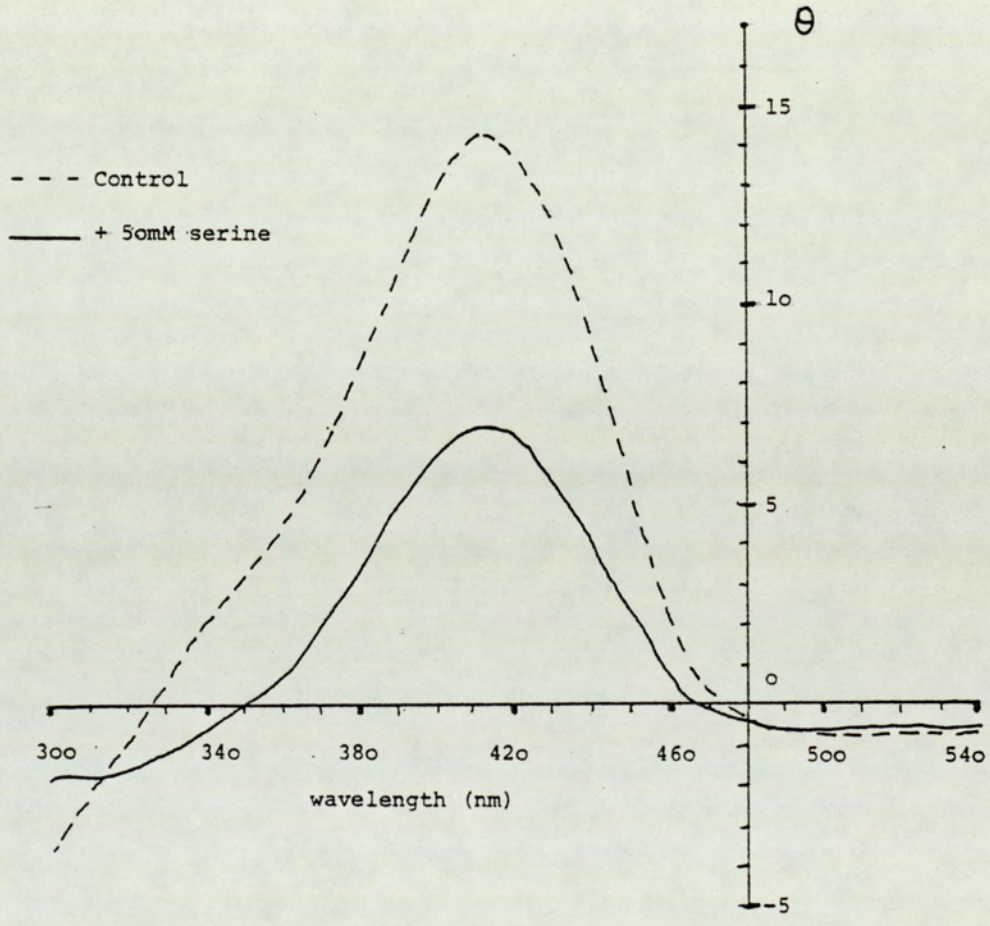


Figure 5.12. Effect of 50mM L-serine on the CD absorption spectrum of the purified rabbit liver enzyme.

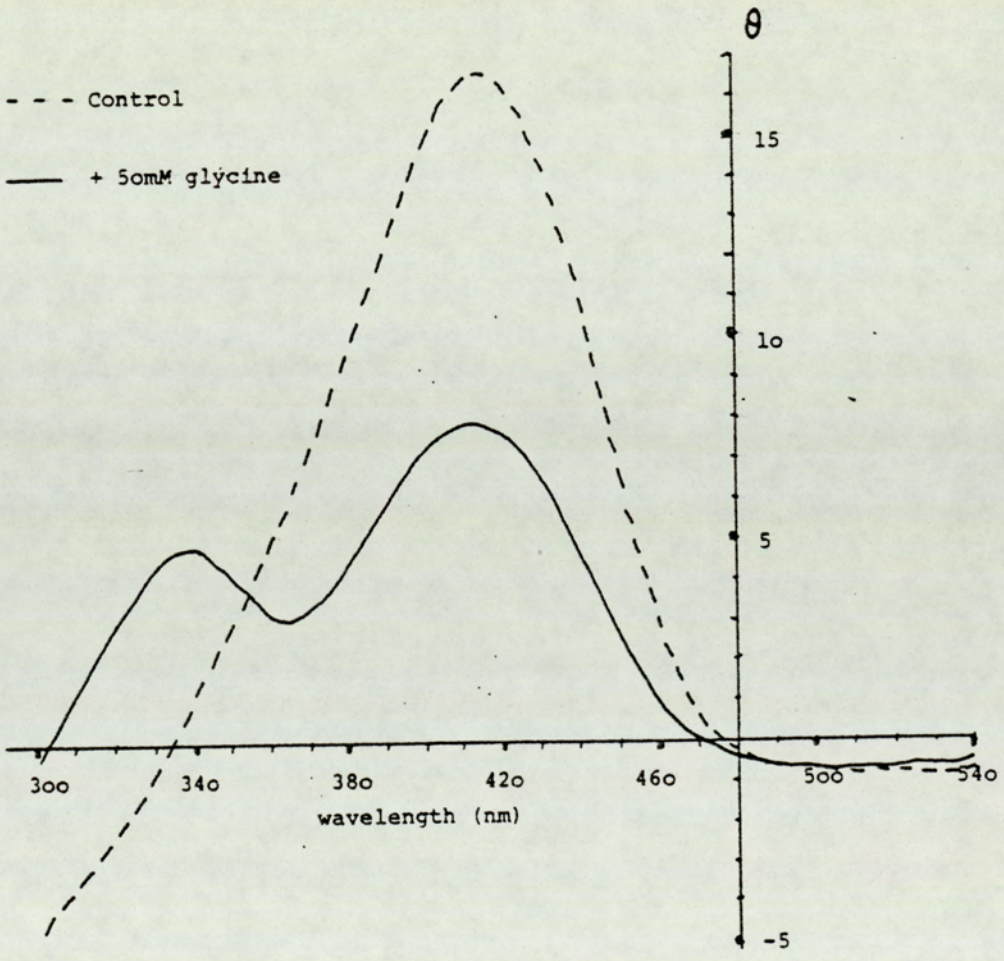


Figure 5.13. Effect of 50mM glycine on the CD absorption spectrum of the purified rabbit liver enzyme.

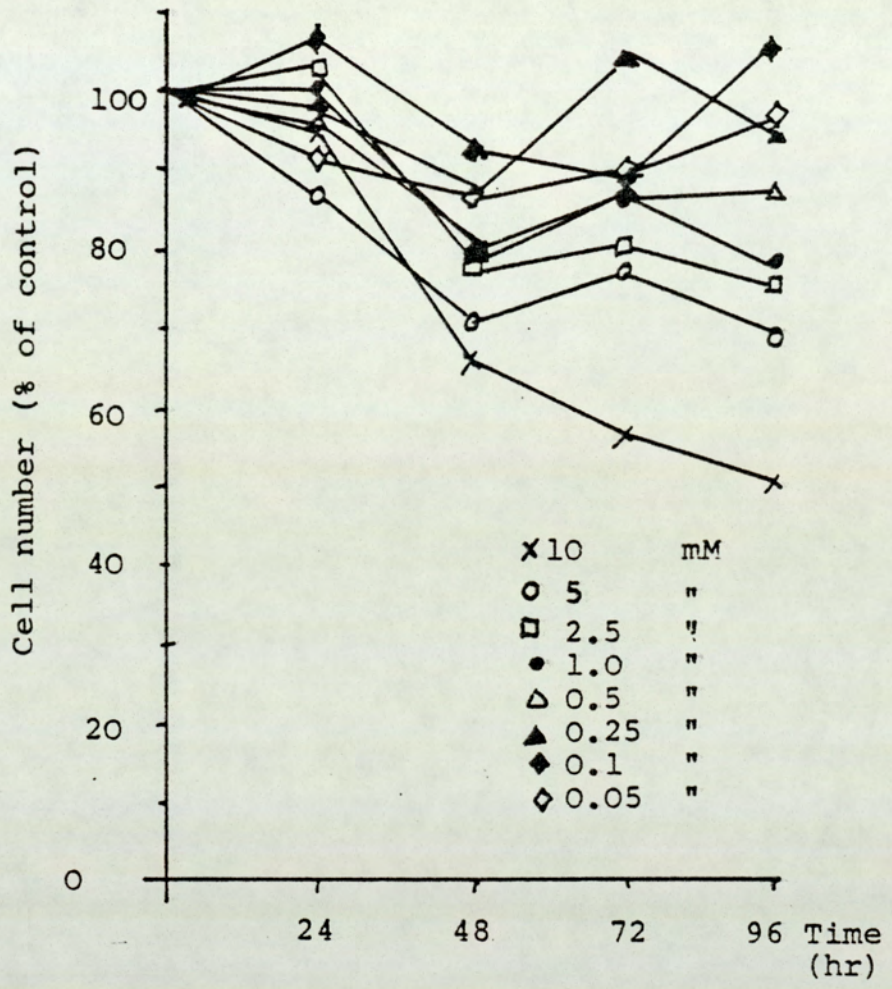


Figure 5.14. Growth inhibitory effect of α-hydroxymethylserine on K562 cells.

conformation then inhibition of catalytic activity may result.

The finding that the addition of the solvents after the assay was quenched caused no effect on enzyme activity indicates that the change in activity cannot be accounted for by a change in the partitioning into toluene of [3-^{14}C]serine, [^{14}C]formaldehyde or the [^{14}C]formaldehyde-5,5-dimethyl-1,3-cyclohexanedione compound.

Random screening of serine and the THF analogues failed to reveal any potent inhibitors of SHMT. Only the tetrazole analogue of serine (D) showed significant activity at a 10 mM concentration. The tetrazole group has a similar charge distribution to the carboxylate group making the compound isoelectronic with serine. The folate analogues showed very little activity especially when it is considered that they are competing with THF which has a Km of 0.083mM (section 4.3.1). Slavikova and Slavik[172], in 1961, claimed that methotrexate caused the inhibition of the enzyme SHMT obtained from pigeon liver. Ramesh and Rao have recently supported this claim. They found methotrexate to be a partial inhibitor of monkey liver SHMT[173]. Many other workers have however failed to show methotrexate to be active against this enzyme[174,175]. This supports the present finding that the compound is not an inhibitor of the enzyme.

The N-haloacetylserine compounds show no activity as inhibitors of the enzyme. This suggests that the antitumour activity originally discovered for DL-N-dichloroacetylserine[33] was not due to the compound inhibiting SHMT. The results for the previous testing of this compound against the M5076 tumour in BDF_1 mice are shown in appendix 2. DL-N-Dichloroacetylserine was remarkably non toxic at doses of up to 8 g Kg^{-1}. This dose gave a weight difference between the treated and control animals of 67%. The compound was shown by Levi *et al*[33] to cause complete regression of sarcoma 37 in Connaught

mice at a dose of 1 g Kg^{-1}.

The investigation into the activity of the novel α-substituted analogues of serine shows these compounds not to be substrates for this enzyme.

The compounds caused no change in the absorption spectra of the enzyme nor could the production of CH2-THF be detected. This suggests that the enzyme SHMT is not capable of dehydroxymethylating the α-substituted serine analogues.

CD spectroscopy suggests that the reason why the compounds are not bioactivated is that they do not bind to the PLP in the enzymes active site. The formation of a Schiff's base between the compounds and the PLP, in the absence of THF, could not be observed.

There is no reason why the compounds under test should not form Schiff's bases with PLP. Transimination has been extensively reviewed by Malekia and Korpela[176]. α-Vinylserine is synthesised in the form of a protected benzaldehyde Schiff's base. Amino acids with a tertiary α-carbon and a primary amine may form PLP Schiff's bases. This is shown by the fact that α-methyl-, α-ethyl- and α-hydroxymethylserine are all substrates for the enzyme SHMT.

The finding that α-vinylserine is a competitive inhibitor of SHMT and yet does not bind to the active site PLP, suggests that the inhibition may be caused by the compound competing with serine for the amino-acid binding site, without the α-vinylserine itself being able to bind.

The lack of toxicity of these compounds in the cytotoxicity assays suggests that the compounds do not inhibit any vital cellular enzymes at the drug concentrations tested.

CHAPTER 6

ASSESSMENT OF ANTIBACTERIAL ACTIVITY

6.1. Introduction.

SHMT has been found to be widely distributed in prokaryotes[3]. The enzyme has been partially purified and characterised from Clostridium cylindrosporum[177] and has been purified to homogeneity from Escherichia coli containing a high copy plasmid of the gly A gene[166]. The E. coli enzyme has similar properties to the rabbit liver iso-enzymes[166], with the enzyme being synthesised in response to the demand for one-carbon units[90]. The premise that the synthesised α-substituted serine analogues may irreversibly inhibit eukaryotic SHMT should be applicable to prokaryotic cells. DL-N-Dichloroacetylserine, DL-α-vinylserine, DL-α-allylserine and DL-α-propargylserine were assessed for antimicrobial activity against four species of bacteria by an agar diffusion method. The strains were selected to represent a typical Gram -ve organism (E.coli), and a typical Gram +ve organism (Staphylococcus aureus). Two strains of Pseudomonas aeruginosa (Gram -ve) were chosen as a permeability pair to detect antimicrobial activity in compounds which have poor uptake characteristics in bacteria[178].

6.2. Methods.

Plates of nutrient agar (Lab M, Salford, U.K.) were prepared in Petri dishes (5 mm deep) containing wells (5 mm diameter) for the addition of solutions of the test compounds. The plates were surface seeded with 1 x 10^6 cells of: E. coli strain W3110, S. aureus (Oxford) NCTC 6751, P.aeruginosa K799 and a hypersensitive P. aeruginosa mutant strain Z61, derived[178] from K799.

Test compounds dissolved in sterile water (25 μl of a 4 mg ml^{-1} solution) were placed in the wells and allowed to diffuse at room temperature for 1 hr. The plates were then incubated for 16 hr at 37°C and examined for zones of growth inhibition around the wells. Control active compounds (trimethoprim and chloramphenicol) were also included on the plates in the form of antibiotic impregnated tablets (Neo Sensitabs, A.S. Rosco, Denmark). Approximate minimum inhibitory concentrations in the antibiotics were determined from regression lines supplied by the manufacturer.

6.3. Results.

No growth inhibition was detected for DL-N-dichloroacetylserine, DL-α-vinylserine, DL-α-allylserine and DL-α-propargylserine against E.coli, S.aureus or P.aeruginosa K799 bacteria. Some activity, however, was detected against the permeability mutant P.aeruginosa Z61. The diameters of the zones of inhibition are listed in table 6.1. (including well diameter).

The compounds therefore have a weak antimicrobial activity, which is only apparent in a freely permeable highly sensitive mutant. The minimum inhibitory concentration for these compounds in the hypersensitive permeability mutant is over 100 fold greater than that of trimethoprim (based on the activity of trimethoprim in this assay).

Table 6.1. Diameters of zones of inhibition for P.aeruginosa Z61.
(Compound concentration 25 μl of a 4mg ml^{-1} solution).

Compound	Zone of inhibition[1] (mm)
DL-N-Dichloroacetylserine	10.5
DL-α-allylserine	9.0
DL-α-propargylserine	8.0
DL-α-vinylserine	11.5
DL-α-hydroxymethylserine	10.5
Controls	
Trimethoprim[2]	25.0
Chloramphenicol[3]	32.0

1. Mean of duplicate determinations
2. Trimethoprim concentration equivalent to a minimum inhibitory concentration (MIC) of 20 μg ml^{-1}
3. Chloramphenicol concentration equivalent to a MIC of 4 μg ml^{-1}

6.4. Discussion.

The lack of antibacterial activity exhibited by the compounds against bacteria with normal antibiotic permeability properties and the slight activity against the hypersensitive permeability mutant Z61 shows two things. The first is that the compounds do not appear to be readily taken up by bacteria. Secondly, they do not appear to have a useful antibacterial activity.

CHAPTER 7

CRYSTALLOGRAPHIC AND MOLECULAR MODELLING STUDIES

7.1. Introduction.

The enzyme SHMT has low substrate specificity for the cleavage of β-substituted analogues of L-serine[1]. It will also catalyse the dehydroxymethylation of α-methyl-, α-hydroxymethyl-[5,55], and α-ethyl-serine[55]. However, there is no evidence for the dehydroxymethylation of α-vinyl-, α-allyl-, or α-propargylserine by the enzyme. This activity is an obligate requirement for the bioactivation of these molecules.

The lack of activation of these α-substituted serines suggests that, when these compounds are used as substrates, structural requirements for the enzyme are not achievable.

Little is known about the tertiary structure of the enzyme. The nucleotide sequence of the *E. coli glyA* gene has been determined[179], as has the corresponding peptide sequence for the SHMT produced from it. Extensive homology was found between this sequence, and that of chymotryptic and tryptic digested peptides from cytosolic and mitochondrial rabbit liver SHMT by Barra *et al*[180].

Schirch *et al*[182] have published methods for the crystallisation of the enzyme in forms suitable for X-ray diffraction studies. The crystals, unfortunately, once prepared, are unstable in the X-ray beam[162].

The predictions by Dunathon on the conformation and reaction specificities of PLP enzymes[62], section 1.4.2 indicate that, for α-β bond cleavage, the bond to be broken must be held in a plane perpendicular to the plane of the pyridoxal imine system, i.e. that

there is overlap between the σ bonding orbitals of the α-β bond and the π orbitals of the imine function.

It is possible that the reason why these α-substituted analogues of serine are not active is that they cannot achieve this conformation in the active site of the enzyme. This work, with the use of the crystal structure of α-vinyl-serine and molecular modelling studies, looks at the energy levels associated with this and other conformations.

7.2. Materials and methods.

7.2.1. Preparation of α-vinylserine crystals.

DL-α-vinylserine (10 mg) was dissolved in distilled water (10 ml) and the solution was warmed gently. To this was added hot 95% ethanol (20 ml). The solution was then left undisturbed for a period of two weeks, wrapped in a cotton wool insulating jacket.

The crystalline florets of needles were separated by sonicating the suspension in a sonic bath for 2 by 5 seconds. The suspension was filtered and the crystals were dried *in vacuo* for 3 days. The density of α-vinylserine was measured to be 1.42 g cm^{-3} by flotation. A specimen 0.7 x 0.07 x 0.055 mm was mounted on a glass fibre.

7.2.2. Data collection.

The unit cell dimensions were determined by least squares analysis of setting angles of 25 reflections on an Enraf-Nonius four circle CAD-4 diffractometer, using a graphite Mo$K\alpha$ (λ= 0.71069 Å) source. The data were collected using the Needle option by ω-2θ scans, with a scan range in Θ of $(1.00 + 0.35 \tan\Theta)^{\circ}$ at a scan rate of 0.5 to 5 $^{\circ}$ min^{-1} depending on intensity. Three intensity and three orien-

tation standards were utilised, with negligible variation observed.

4612 Reflections were measured with $2< \Theta <25^{\circ}$ of which 2177 were unique (R_{int} = 0.0561). 1037 Reflections were deemed observed with Fo > 3σ. Standard deviations were calculated on the basis of counting statistics and instrument instability; no correction was made for absorption. Maximum $(\sin\Theta)/\lambda$ reached in intensity measurements was 0.63, with range, L,H,K -12 to 12, 0 to 12, and -14 to 14 respectively.

7.2.3. Molecular studies.

Molecular mechanics conformational energy calculations were performed on the Glaxo Group Research molecular modelling system. The structure of L-serine was taken from fragments constructed from the system's internal database. The crystal structure of PLP was derived by Fujiwara[183] and taken from the Cambridge Crystallographic Database [184]. The variation in molecular potential energy with changes in torsion angle was calculated by the summation of the separate non-bonded, torsional and electrostatic energies[185].

7.2.4. Molecular modelling.

The molecular modelling studies were performed on an Evans and Sutherland PS-300 terminal using the Vax mainframe of the Medical College of Virginia. The crystal structure of aspartate amino-transferase[182] was manipulated using the MOGLI computer programme also by Evans and Sutherland. The photographic plates were taken directly from the screen using Kodak Ektachrome 200 ASA film.

7.3. Results and discussion.

7.3.1. Solution of structure.

The crystal structure of ∝-vinylserine was solved by direct phasing with SHELX[186]. Molecular drawings in accordance with the scheme in figure 7.1, were obtained using the PLUTO programme developed by Motherwell[187] figures 7.2-7.5. All non-hydrogen atoms were determined by direct electron density synthesis, with the hydrogen atoms then being located by difference Fourier analysis. In the final least-squares full matrix refinement the coordinates and anisotropic thermal parameters were adjusted, together with the isotropic temperature factors of hydrogens (using the same factors for structurally equivalent hydrogen atoms in the two independent molecules comprising the asymmetric unit).

The Fo factors were weighted by w=1 $[\sigma^2$ (Fo) + 0.008 $Fo^2]$; this gave satisfactory agreement analysis. The final discrepancy indices were R=0.0648, Rw=0.0693, with a goodness-of-fit ratio of S=1.35.

7.3.2. Structural features observed for ∝-vinylserine.

The monoclinic crystals have four pairs of molecules (Z=8), in a unit cell of dimensions a=12.346(3)Å b=10.442(4)Å and c=10.763(5)Å with ∝=90.066(28) , β =116.694(30) and γ =89.8960(34) . The calculated unit cell volume =1239.74$Å^3$ gives a calculated density of 1.446 g cm^{-3} (mw=135 and F(000)=560). The linear absorption coefficient was 0.75 cm^{-1}, and the space group was $P2_1/c$[188]. The bond angles and distances of the non-hydrogen atoms are as shown in table 7.1 and 7.2 respectively. The fractional coordinates and isotropic temperature factors are given in table 7.4.

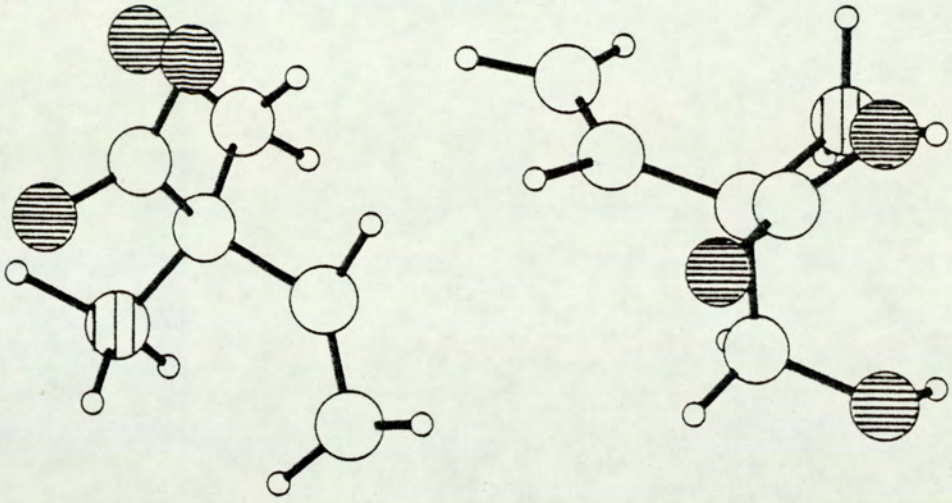


Figure 7.2. PLUTO drawing of the two structurally independent molecules of α-vinylserine in the asymmetric unit.

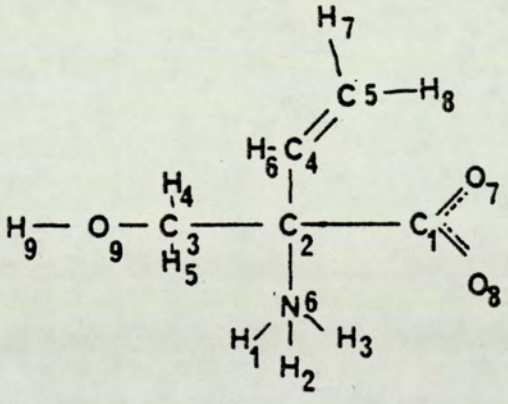


Figure 7.1. α-Vinylserine.

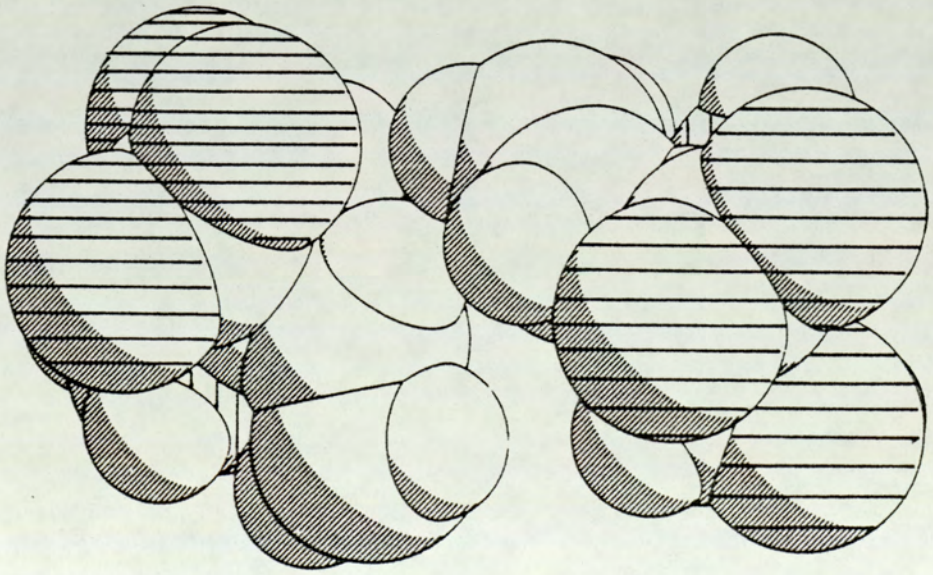


Figure 7.3. PLUTO space filling drawing of the two structurally independent molecules of α-vinylserine in the asymmetric unit.

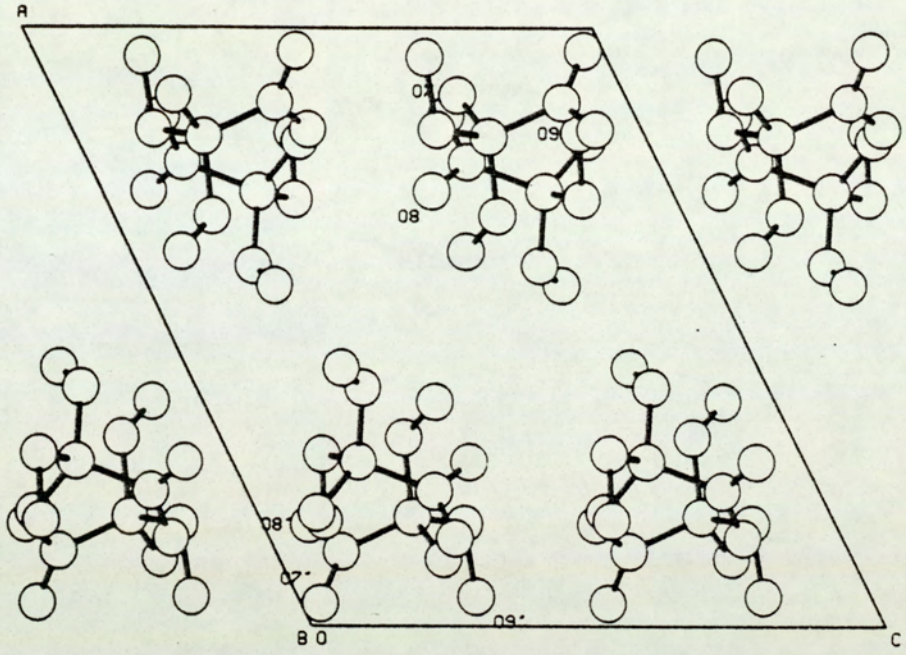


Figure 7.4. The unit cell of α-vinylserine.

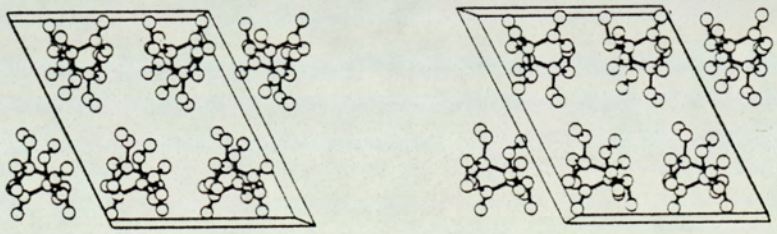


Figure 7.5. Stereo drawing of the unit cell of α-vinylserine.

Table 7.1. Bond angles (°) for non-hydrogen atoms in α-vinylserine with the estimated standard deviations given in parentheses.

C2-C1-O7	117.8(5)	C2-C1-O8	114.5(5)	O7-C1-O8	127.7(6)
C1-C2-N6	109.3(4)	C3-C2-C4	108.9(5)	C3-C2-N6	109.1(5)
C4-C2-N6	110.3(5)	C2-C3-O9	111.0(5)	C2-C4-C5	129.0(7)
C1-C2-C3	110.9(5)	C1-C2-C4	108.3(5)		
C2'-C1'-O7'	117.6(5)	C2'-C1'-O8'	117.1(5)	O7'-C1'-O8'	125.3(6)
C1'-C2'-C3'	109.7(5)	C1'-C2'-C4'	110.9(5)	C1'-C2'-N6'	107.9(5)
C3'-C2'-C4'	108.9(5)	C3'-C2'-N6'	108.0(5)	C4'-C2'-N6'	111.5(5)
C2'-C3'-O9'	113.1(5)	C2'-C4'-C5'	127.6(7)		

Table 7.2. Bond lengths (Å) for non-hydrogen atoms in α-vinylserine with estimated standard deviations given in parentheses

C1-C2	1.545(8)	C1-O7	1.238(7)	C2-C3	1.528(9)
C2-C4	1.513(9)	C2-N6	1.496(8)	C3-O9	1.423(8)
C4-C5	1.271(11)	C1-O8	1.242(7)		
C1'-C2'	1.533(8)	C1'-O7'	1.249(7)	C1'-O8'	1.252(7)
C2'-C3'	1.533(9)	C2'-C4'	1.501(9)	C2'-N6'	1.515(7)
C3'-O9'	1.412(8)	C4'-C5'	1.287(10)		

Table 7.3. Torsion angles for all non-hydrogen atoms in α-vinyl-serine, with the estimated standard deviations given in parentheses[1]. (°)

O7-C1-C2-C3	137.7	O7-C1-C2-C4	-102.9
O7-C1-C2-N6	17.4 (9)	O8-C1-C2-C3	- 45.0
O8-C1-C2-C4	74.4 (7)	O8-C1-C2-N6	-165.3 (8)
C1-C2-C4-C5	114.1 (10)	N6-C2-C3-O9	67.0
C3-C2-C4-C5	-125.3	N6-C2-C4-C5	-5.6
O7'-C1'-C2'-C3'	119.5	O7-C1'-C2'-C4'	-120.2
O7'-C1'-C2'-N6'	2.2	O8'-C1'-C2'-C3'	- 61.7
O8'-C1'-C2'-C4'	58.6	O8'-C1'-C2'-N6'	-179.1
N6'-C2'-C3'-O9'	58.9	C1'-C2'-C4'-C5'	139.8
C3'-C2'-C4'-C5'	-99.4	N6'-C2'-C4'-C5'	19.6

1 The estimated standard deviations were calculated using the formula given in appendix 3.

Table 7.4. Fractional coordinates and isotropic temperature factors ($Å^2$) with estimated standard deviations in parentheses.

$$Ueq=(U_{11}+U_{22}+U_{33} + 2U_{13} \cos)$$

Atom	X	Y	Z	Ueq/Uiso
C1	.7753(6)	.2572(6)	.6692(6)	.0226(19)
C2	.7258(5)	.2411(6)	.7771(6)	.0222(19)
C3	.7133(6)	.3708(7)	.8352(7)	.0316(23)
C4	.6020(6)	.1792(8)	.7055(8)	.0399(27)
C5	.5688(7)	.0695(9)	.7262(10)	.0569(33)
N6	.8107(5)	.1582(4)	.8939(5)	.0219(16)
O7	8534(4)	.1805(4)	.6733(4)	.0281(15)
O8	.7268(5)	.3449(5)	.5841(5)	.0392(18)
O9	.8243(5)	.400(4)	.8879(5)	.0400(18)
C1'	.1246(5)	.2091(6)	.1158(6)	.0232(20)
C2'	.1823(5)	.2438(6)	.2707(6)	.0210(18)
C3'	.1704(6)	.1306(6)	.3544(6)	.0275(21)
C4'	.3142(6)	.2753(7)	.3232(7)	.0332(23)
C5'	.3743(7)	.3649(7)	.4083(8)	.0398(26)
N6'	.1123(5)	.3557(5)	.2888(5)	.0247(17)
O7'	.0380(4)	.2764(4)	.0345(4)	.0293(15)
O8'	.1697(4)	.1168(4)	.0809(4)	.0324(17)
O9'	.0490(4)	.0936(4)	.3125(5)	.0349(17)

Table 7.4. continued ...

H1	.9167	.2042	.9616	.062(10)
H2	.8183	.0672	.8611	.062(10)
H3	.7725	.1551	.9749	.062(10)
H4	.6630	.3707	.9039	.036(9)
H5	.6591	.4046	.7577	.036(9)
H6	.5589	.2390	.6230	.067(10)
H7	.4942	.0625	.6402	.067(10)
H8	.6379	.0284	.8331	.067(10)
H9	.8540	.4551	.9680	.097(26)
H1'	.0206	.3370	.1988	.062(10)
H2'	.1425	.3502	.4013	.062(10)
H3'	.1167	.4439	.2469	.062(10)
H4'	.2174	.0529	.3563	.036(9)
H5'	.2098	.1565	.4380	.036(9)
H6'	.3672	.2160	.2612	.067(10)
H7'	.4686	.3714	.4429	.067(10)
H8'	.3383	.4259	.4591	.067(10)
89'	.0155	.1365	.3619	.097(26)

This crystal structure of DL-α-vinylserine confirms the spectroscopic data obtained, with all the bond lengths being essentially as predicted. The enantiomers are present as zwitterions. The two molecules in the asymmetric unit have the carboxyl group and the C2-N6 bond in the same plane, with one hydrogen atom of the ammonium group being in a near eclipsed conformation.

A precision neutron diffraction study of the crystal structure of DL-serine has been undertaken by Frey et al[189], based on the coordinates proposed by Shoemaker et al[190]. These studies show that this eclipsed conformation is also present in the unsubstituted amino-acid, with all the DL-serine molecules being symmetry related[189,190]. Shoemaker et al[190] described the composition of the unit cell to be sheets of serine stacked parallel to the [100] face. A sheet of L-serine molecules is bound to a sheet of D-serine molecules by H1...O bonds. The sheets of serine are created by the two other amine protons and the hydroxyl proton bonding to three other serine molecules[190].

The introduction of a vinyl group onto the α-position of serine changes the way in which the amino-acid is packed. The unit cell of α-vinylserine contains four pairs of R and S enantiomers which are bound together by hydrogen bonds of which four have a H - O distance of less than 2.3Å as shown in table 7.5. The bonds between the protons of the ammonium group and the oxygen atoms of the carboxylate and between the H3' proton of the quaternary ammonium and O9 tightly bind the molecules together. It is tempting to propose that the N6-H1..O7' bond with its short bond length of 1.541Å and bond angle of 171.5° may represent the H1 proton being shared betwen the N6 and O7' atoms of the two molecules. A neutron diffraction study, however, would be required to establish the validity of this proposal.

Table 7.5. Hydrogen bonds present in the binding of the α-vinylserine in the unit cell.

Bond	Distance(Å)			Angle (°)
a-b..c	a-b	b-c	a-c	
N6-H1..07'	1.27	1.54	2.81	171
N6-H2..08'	1.04	2.00	2.89	141
N6'-H1'..07	1.13	1.96	2.89	136
N6'-H3'..09	1.04	2.25	3.18	149
09'-H9'..09	0.92	2.62	3.24	125
09-H9..07'	0.79	2.77	2.93	94

In the crystalline state, α-vinylserine is orientated so that the polar carboxylate, alcohol and ammonium groups point towards the edge of the unit cell. The vinyl groups point into the centre of the unit cell, creating alternate polar and non-polar planes extending throughout the crystal.

The observed and calculated structure factors for α-vinylserine are given in appendix 4.

7.3.3. Studies on the energy levels associated with PLP amino-acid Schiff's bases.

With the aid of molecular modelling the L-serine-PLP conjugated imine was constructed, figure 7.6. The effect of rotation of the imine nitrogen-serine-C_α bond shows two energy minima, figure 7.7; the one at a C(8)-N(6)-C(2)-H torsion angle of 320° corresponds almost exactly to the preferred conformation, as predicted by Dunathon[62]. The interaction occurring when the torsion angle is in the region of 130° is due to the fixed position of the phosphate group of the PLP. NMR studies show that in the active site of SHMT, when the PLP already bound to the lysine residue, binds L-serine and glycine, no change in the environment of the phosphate group occurs [54]. This suggests that in the active site of the enzyme the negatively-charged phosphate group may act to anchor this imine. The correct orientation of the phosphate group in the enzyme is not known; in this study it was positioned arbitrarily.

For α-vinylserine, figure 7.8 shows that there are no significant potential barriers for the rotation of the vinyl group.

Similar calculations were then carried out for the modelled α-vinylserine-PLP conjugate, figure 7.9. The C2-C4 and N6-PLP bonds were rotated simultaneously and the potential energy calculated. The data in the form of a contour map are presented in figure 7.10. Minima of 1 kcal mol^{-1} are observed at 300°, 330° and 70°, 20°. The former corresponds to the required conformation for α-β bond cleavage. Rotation of the PLP pyridine group about 180° to bring about hydrogen bonding between the alcohol group of the PLP and the hydrogen of the imine, figure 7.11, causes no significant alteration in the energy levels associated with these torsions, figure 7.12. This work indicates that for the α-vinylserine-PLP conjugate, there

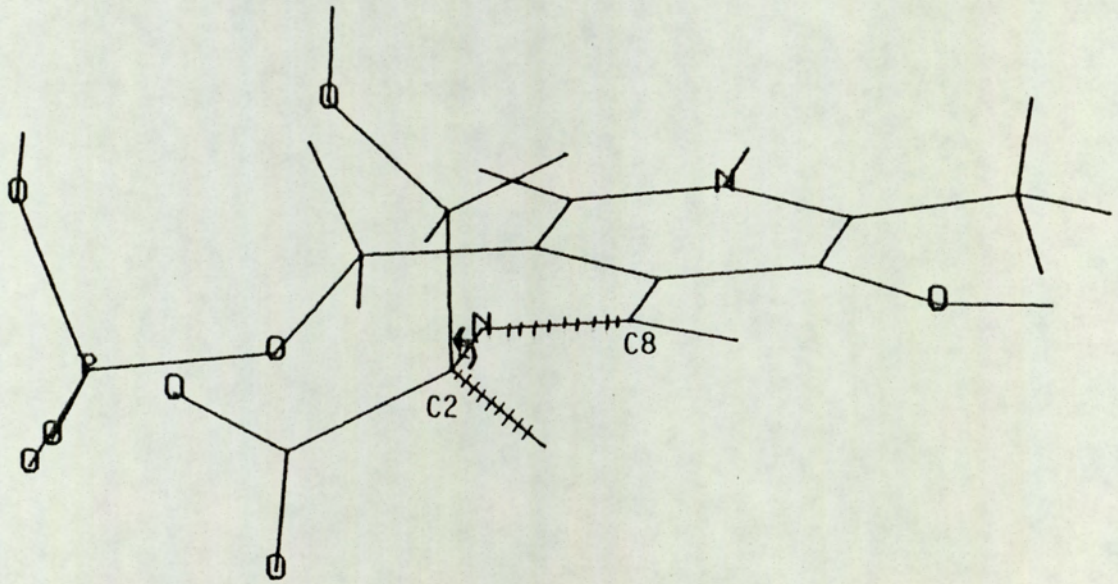


Figure 7.6. The modelled L-serine-PLP Schiff's base.

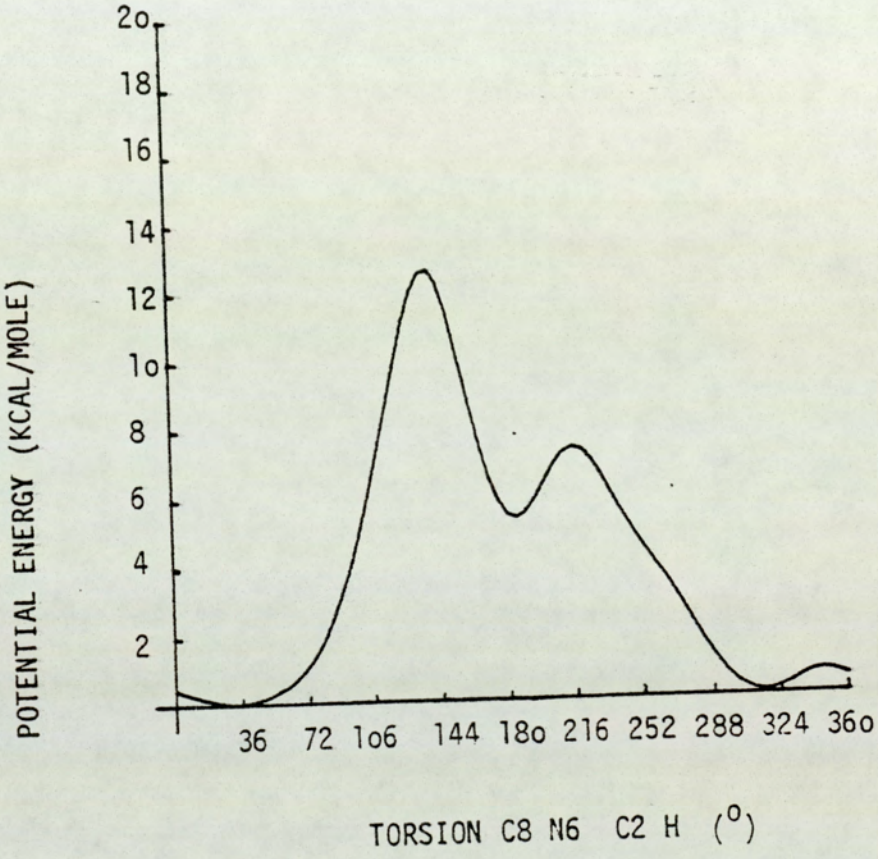


Figure 7.7. Effect of the rotation of the N6-C2 bond of the modelled L-serine-PLP Schiff's base on the potential energy of the model.

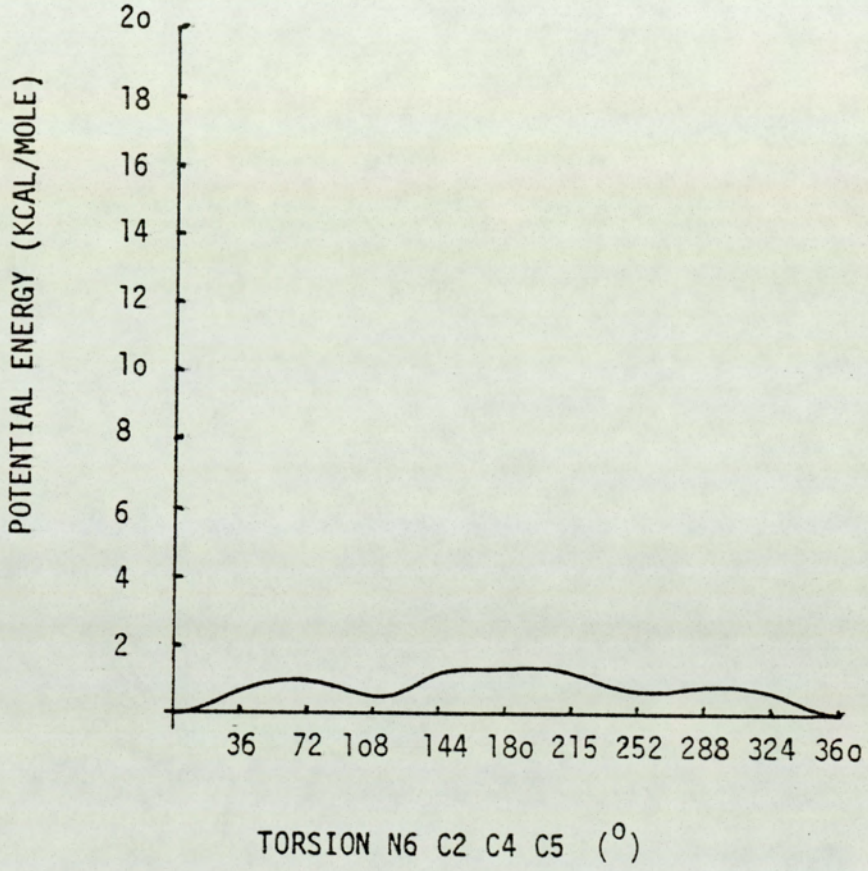


Figure 7.8. Effect of the rotation of the vinyl group of α-vinylserine on the potential energy of the molecule.

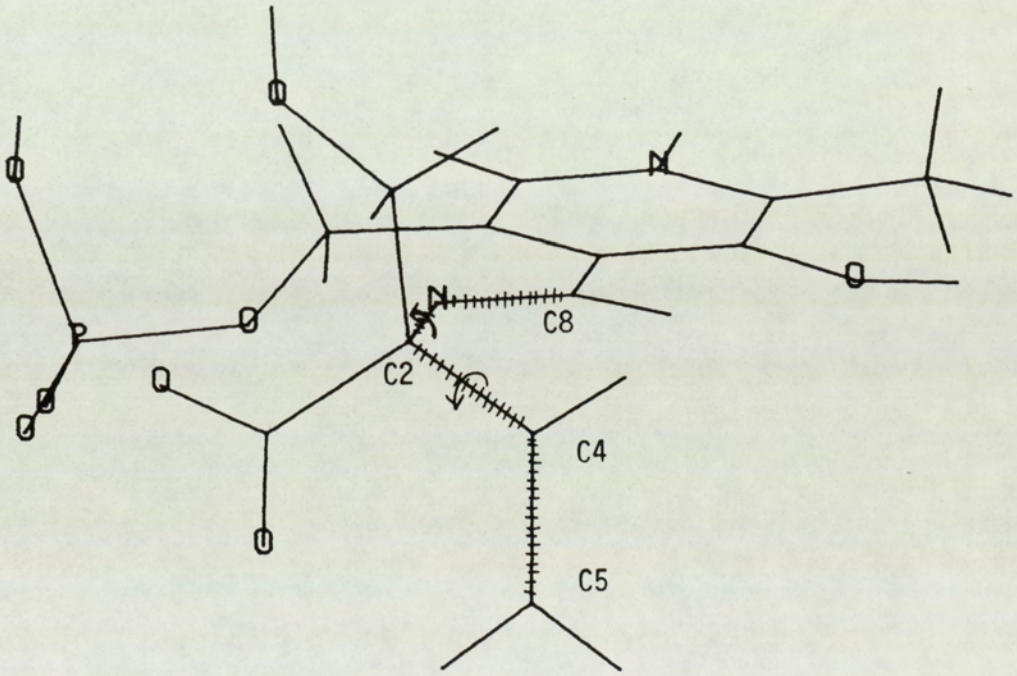


Figure 7.9. The modelled α-vinylserine-PLP Schiff's base.

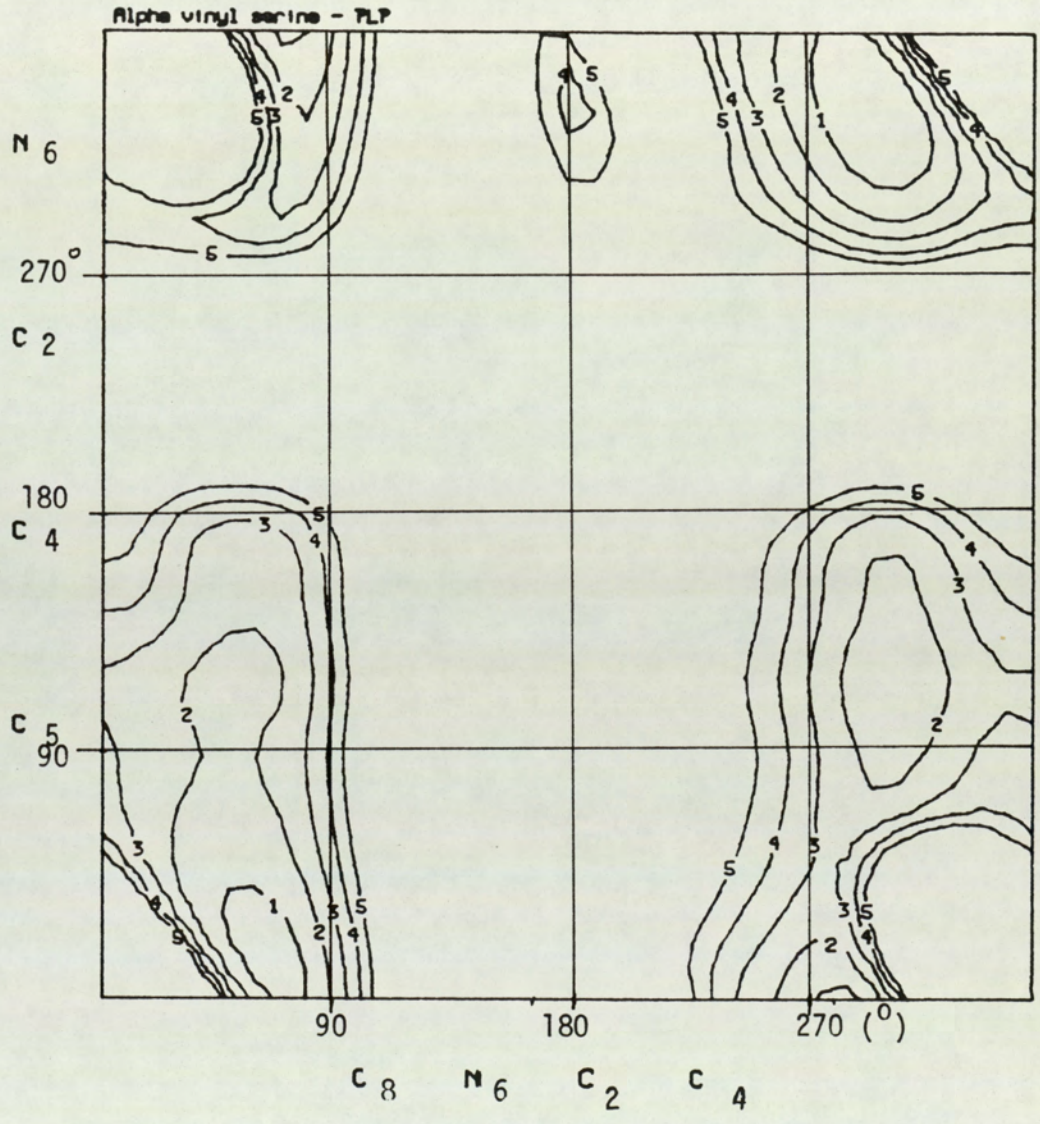


Figure 7.10. Effect of the simultaneous rotation of the C2-C4 and N6-C2 bonds on the potential energy of the modelled α-vinylserine-PLP Schiff's base.

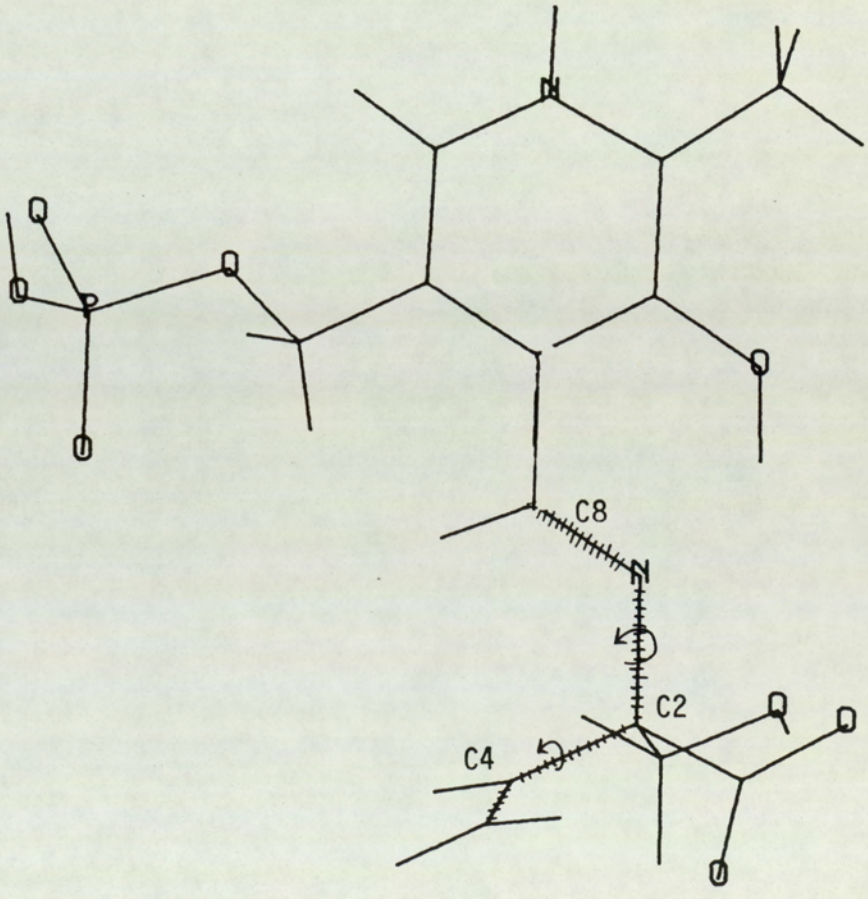


Figure 7.11. The modelled α-vinylserine-PLP Schiff's base, with hydrogen bonding present.

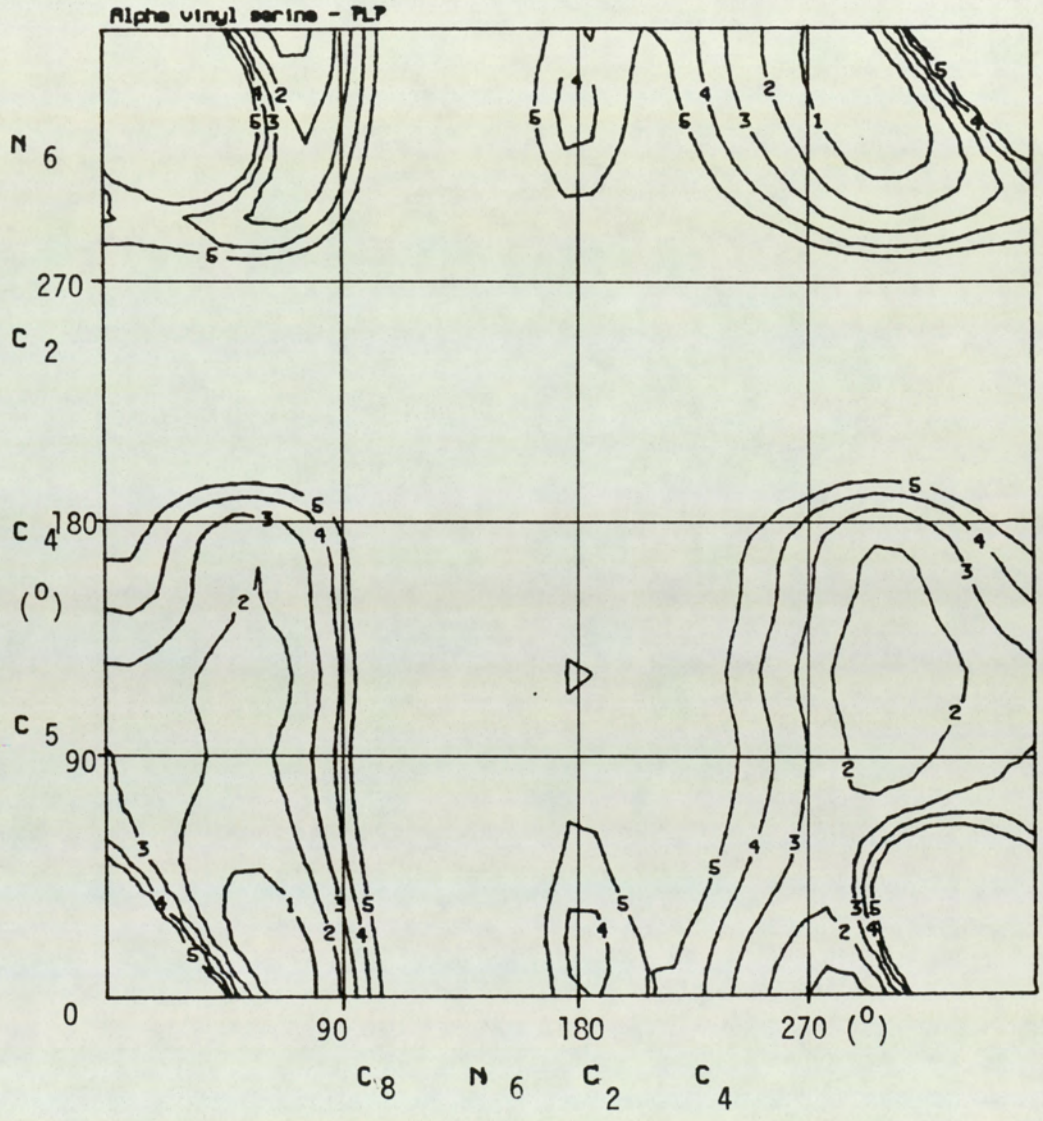


Figure 7.12. Effect of the simultaneous rotation of the C2-C4 and N6-C2 bonds on the potential energy of the modelled α-vinylserine-PLP Schiff's base, with hydrogen bonding present.

is no reason discernible from molecular modelling calculations, why a correct conformation for α-β bond cleavage should not be attained. It is unclear why the enzyme can dehydroxymethylate α-ethyl-, α-methyl- and α-hydroxymethylserine, but will not activate α-vinylserine. It is possible that this is due to steric structural requirements not being achieved in the active site of the enzyme.

Seven PLP enzymes, including SHMT, behave in a similar manner when pyridoxamine 5'-phosphate is formed. When the bound substrate-PLP Schiff's base is protonated, this occurs exclusively at the si face of the planar intermediate, yielding pyridoxamine with the enzyme labile proton in the pro(S) position[191]. This has prompted the suggestion that all the PLP enzymes have evolved from a common progenitor[191]. It may be possible to take advantage of this relationship in an attempt to model the active site of SHMT. The crystal structure of aspartate aminotransferase, a PLP enzyme, has been determined to 3.5Å resolution[182].

The decapeptide PLP binding sequence of the E. coli and the mitochondrial and cytosolic forms of rabbit liver SHMT, has been determined to be Val-Val-Thr-Thr-Thr-Thr-His-Lys(PLP)-Thr-Leu[69]. Sequence similarity is seen between this and other PLP enzymes, such as the Thr-His-Lys(PLP)- sequence in arginine and lysine decarboxylases[192]. The enzyme aspartate aminotransferase, however, shows no sequence homology with the PLP binding site of SHMT.

The structure of the enzyme is shown in plate 7.1 . The active site residues 250-260 are coloured red, with the lysine (258) residue and its bound PLP coloured green.

Plate 7.2. shows a modified active site region of aspartate aminotransferase. Residues 250-260 are shown, with residue 258, lysine (wrongly labelled as serine), with its bound PLP, coloured

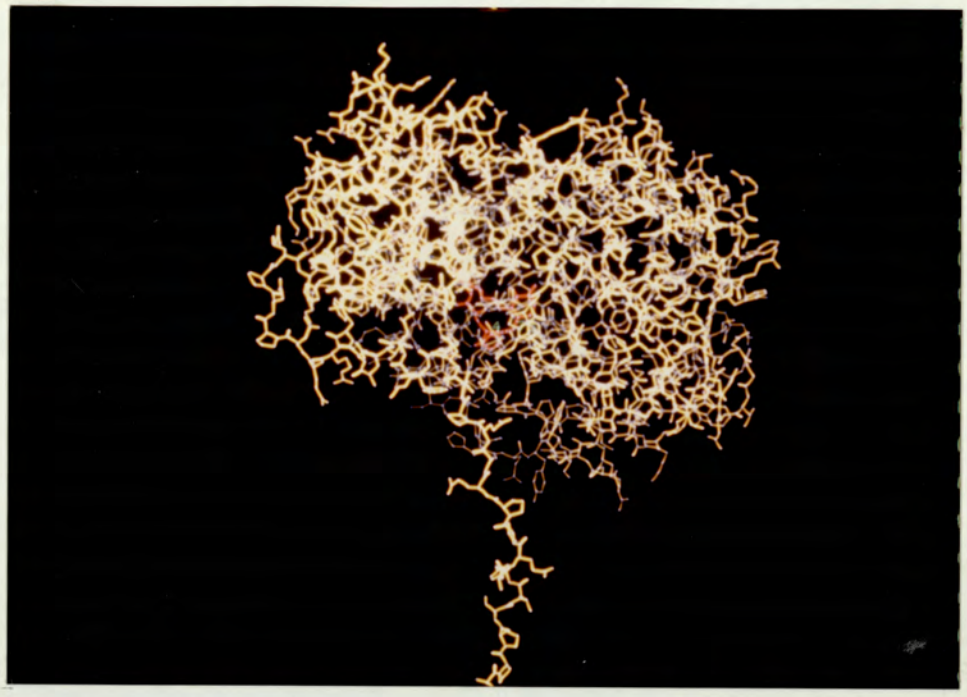


Plate 7.1. The enzyme aspartate aminotransferase.

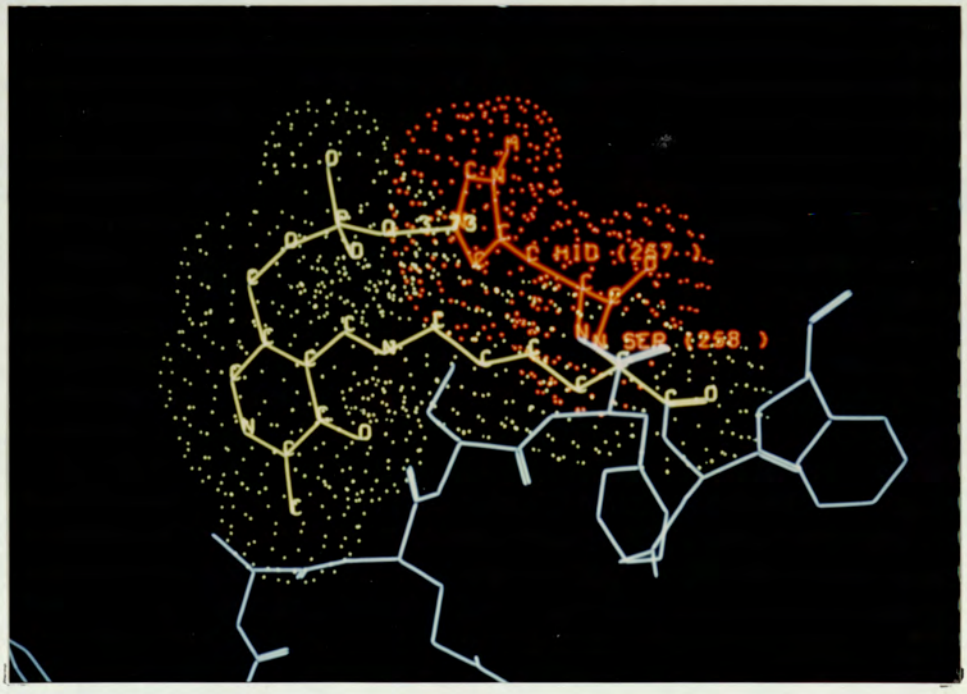


Plate 7.2. The modified active site region of aspartate aminotransferase.

green. Residue 257, serine, was changed to histidine in an attempt to model the active site of SHMT . It is however difficult to put the modelled region, once constructed, to any real use. When modelling the binding of substrate to the active site region, an infinite variation of conformations may be produced. The validity of these changes in tertiary structure is not known.

The superimposition of the binding sequence of SHMT onto the active site of aspartate aminotransferase, based on the common ancestry of the enzymes, cannot be justified without any knowledge of the tertiary structure of SHMT. Only when the crystal structure of SHMT is elucidated will it be possible to examine the structural requirements of SHMT for the binding and dehydroxymethylation of substrates.

CHAPTER 8

CONCLUSIONS

The compounds α-vinyl-, α-allyl- and α-propargylserine were designed and synthesised to be irreversible enzyme-activated inhibitors of the enzyme SHMT.

On binding to the PLP, the compounds, when dehydroxymethylated, may produce an intermediate that is a Michael acceptor activated to nucleophilic attack. Once this has occurred, the covalent linkage of the compound to the enzyme would cause the inhibition of the enzyme.

While it is known that the enzyme can tolerate β-substituted serine analogues, its ability to dehydroxymethylate α-substituted serines has never been fully explored. It is known that L-α-methyl-, L-α-ethyl- and α-hydroxymethylserine are substrates for the enzyme. The L1210 SHMT is capable of dehydroxymethylating α-hydroxymethyl-serine, albeit with a K_m of 20 mM. This suggests that the enzyme should be able to accept α-substituted serine analogues provided the α-chains are approximately isosteric to the known substrates.

The testing of the α-substituted compounds against total K562 cell SHMT showed DL-α-vinylserine to be a competitive inhibitor, with a Ki of 15.2 mM. As only the L-isomer will be a substrate for the enzyme, the Ki for L-α-vinylserine may be effectively halved. This rate of inhibition may be a reflection of the rate constant obtained for the dehydroxymethylation of α-hydroxymethylserine compared to L-serine itself. If the compound is dehydroxymethylated and inhibits the enzyme irreversibly, then, despite the low rate of inhibition, all of the SHMT may be inhibited with time.

DL-α-Allyl- and DL-α-propargylserine caused no inhibition of the enzyme from the K562 cells. This may suggest that the latter two compounds have substituents that are either too bulky for the enzyme to accept, or that the β-proton removal by the enzyme, a prerequisite for the activation of these compounds, does not occur. No dehydroxymethylation of the α-substituted compounds could be detected using the partially purified L1210 SHMT enzyme. The compounds were also not dehydroxymethylated by the homogeneous rabbit liver enzyme, nor was there any evidence for quinonoid formation, or any change in the absorbance of the enzyme. This suggests that the inhibition caused by DL-α-vinylserine is due to it blocking the active site of the enzyme, although it is not itself a substrate for dehydroxymethylation.

Molecular modelling studies show that for a modelled α-vinylserine-PLP conjugate there is no reason why the correct orientation for α-β bond cleavage should not be achieved. The requirements for bond cleavage in the active site of the enzyme are not, however, known.

Circular dichroism spectroscopy shows that the reason why the α-substituted serine analogues are not dehydroxymethylated is more fundamental. The compounds do not appear to bind to the active site PLP. Why this occurs when serine, glycine and α-hydroxymethylserine are substrates for the enzyme is unknown. There is no reason why an α-substituted amino-acid should not form a Schiff's base with PLP. The CD spectroscopy indicates that the compounds do not bind to the PLP due to some subtle structural or electronic requirement in the active site of the enzyme not being achieved.

The competitive inhibition caused by α-vinylserine appears to be due to the compound blocking L-serine reaching the active site of the enzyme.

In order to be able to further investigate the requirements of SHMT for binding substrates, the crystal structure of the enzyme will have to be determined. This, in conjunction with high field FT NMR, will enable the regions of the enzyme that have an important role in the binding of substrates to be identified.

The lack of activity of the compounds suggests that the synthesis of other α-substituted serine analogues, such as α-fluoromethylserine, would be hard to justify, especially when D-fluoroalanine (which is effectively dehydroxymethylated α-fluoromethylserine) is such a poor inhibitor. The design of THF transition state analogues as inhibitors of SHMT may be considered in the light of the binding constant for folates being two orders of magnitude below that of serine. These compounds may be able to inhibit selectively the unique transfer of the one-carbon unit from the active site nucleophile to THF.

It is unknown why the enzyme SHMT is so resistant to inhibition. Similar rational synthetic approaches and serendipity have produced effective inhibitors for many other enzymes. In order to produce an effective inhibitor of SHMT, further knowledge of the mechanism of the active site cleavage is required.

CHAPTER 9

EXPERIMENTAL

9.1. Notes on instrumentation and compound analysis.

1. All melting points are reported uncorrected.
2. Infra-red spectra were recorded on a Pye-Unicam SP200 or a Perkin Elmer 1310 spectrophotometer and, unless otherwise stated, as potassium bromide discs.
3. ^1H NMR (60 MHz) were recorded on a Varian EM360A spectrophotometer in suitable solvents using tetramethylsilane as an internal standard. The signals were assigned δ p.p.m. downfield from tetramethylsilane.
4. ^1H FT NMR (200 MHz) were conducted by Glaxo Group Research, Greenford, Middlesex, in suitable solvents using tetramethylsilane as an internal standard.
5. Mass spectra were recorded on a Micromass 128 single-focussing mass spectrometer.
6. Elemental analyses were carried out by Glaxo Group Research and the Department of Molecular Sciences, Aston University.
7. Ethanol refers to 95% ethanol and petrol refers to the petroleum fraction with the boiling range 60-80°C.
8. For this section only the abbreviation THF will be used for tetrahydrofuran.

9.2. DL-N-Chloroacetylserine.

DL-Serine (3.0 g, 0.286 mol) was dissolved in 1M aqueous sodium hydroxide (28.6 ml, 1.14 mol) and the solution was cooled to 4°C. Chloroacetyl chloride (4 g, 0.0509 mol) and 1M aqueous sodium

hydroxide (37.7 ml) were added dropwise over a period of 25 min. 1M Hydrochloric acid (28.6 ml, 1.029 mol) was added and the solution was reduced in volume under reduced pressure to give a yellow oil with sodium chloride crystals suspended in it. The oil was extracted three times with hot ethyl acetate (25 ml). The combined organic layers were evaporated under reduced pressure to give a clear viscous oil which crystallised on standing.

Recrystallisation from ethyl acetate/petrol gave the named compound as a white powder.

(2.05 g; 39.5%); mp 125-7°C, lit m.p. 122-3°C[193]; ν_{max} = 1520, 1650, 1705 cm^{-1}; δ (D_2O) 3.9(2H,d,$\underline{CH_2}OH$), 4.2(2H,s,$\underline{CH_2}Cl$), 4.4-4.8-(4H,m,3xDHO+$\underline{CH}$).

9.3. DL-N-Dichloroacetylserine.

The substitution of dichloroacetyl chloride for chloroacetyl chloride in method 9.2, resulted in the named compound. The compound was recrystallised from aqueous ethanol.

(2.4 g; 38.4%); m.p. 176-8°C, lit m.p. 172-3°C[33]; ν_{max} = 1575, 1660, 3300 cm^{-1}; δ (D_2O) 3.9(2H,d,$\underline{CH_2}OH$), 4.1-4.8(4H,m,3xDHO+$\underline{CH}$), 6.5-(1H,s,$\underline{CH}Cl_2$).

9.4. DL-N-Trichloroacetylserine.

The substitution of trichloroacetyl chloride for chloroacetyl chloride in method 9.2, resulted in the named compound. The compound was recrystallised from ethyl acetate/petrol.

(2.8 g; 39.2%); m.p. 112-4°C, lit m.p. 106-8°C[36]; ν_{max} = 1510, 1680-1725, 3000 cm^{-1}; δ (D_2O) 4.1(2H,d,$\underline{CH_2}OH$), 4.2-4.9(4H,m,3xDHO+$\underline{CH}$).

9.5. DL-O-Chloroacetylserine.

Chloroacetic anhydride (12.1 g, 0.0708 mol) was dissolved in ethyl chloroacetate (10 ml). DL-Serine (1.06 g, 0.01 mol) in perchloric acid (2.0 g, 0.01 mol) was added dropwise over a period of 1 hr, with cooling applied. The reaction mixture was heated to 55°C for 3 hr, and then allowed to stand overnight.

The brown solution was diluted with distilled water (2 ml), and triethylamine (4.5 g, 0.045 mol) was added to neutralise the salt. The suspension was poured into warm diethyl ether (200 ml), upon cooling crystallisation occurred.

(0.7 g; 41%); m.p. 120-1°C, lit m.p. 122-3°C[36]; ν_{max} = 1600, 1745, 2500-3000 cm^{-1}.

9.6. DL-O-Dichloroacetylserine hydrochloride.

DL-Serine (3 g, 0.0286 mol) was suspended in dichloroacetic acid (100 ml). The solution at 0°C was saturated with dry hydrogen chloride gas and the reaction left at room temperature for 2 days. Addition of anhydrous diethyl ether caused the precipitation on standing of the product as white crystals.

(2.4 g; 39.5%); m.p. 128-30°C, lit m.p 125-6°C[36]; ν_{max} = 1500, 1600, 1760, 2500-3000 cm^{-1}.

9.7. DL-O-Dichloroacetylserine hydrochloride via a N→O haloacyl shift.

DL-N-Dichloroacetylserine (0.5 g) was suspended in anhydrous diethyl ether (100 ml). The suspension was saturated with dry hydrogen chloride gas over a period of 6 hr, and left stirring for 72 hr. The insoluble residue of DL-O-dichloroacetylserine hydrochloride was removed by filtration. The solid was washed with diethyl ether and ethyl acetate to give a white powder; (0.12 g; 24%) as 9.6.

9.8. Chloromethyl acetate.

Acetyl chloride (25.9 g, 0.33 mol), paraformaldehyde (9 g, 0.33 mol) and a trowel point of anhydrous zinc chloride were warmed to 50°C. After a short induction period, the heat of reaction caused the solution to boil gently for 3 hr (external heat was applied for the last 40 min to maintain the boiling). The mixture was filtered through basic alumina (Brockman grade I) to give a yellow solution[149]. NMR spectroscopy revealed this to contain 92% named compound and 8% bis-chloromethyl ether. This reagent was used without further purification.

(30.3 g; 84.7%); ν_{max} = 1760 cm^{-1}; $\delta(CDCl_3)$ 2.1(3H,s,$\underline{CH_3}COO$), 5.60-(2H,s,$ClC\underline{H_2}$), 5.55(8% in total,s,$(ClC\underline{H_2})_2O$).

9.9. DL-Threonine methyl ester hydrochloride.

DL-Threonine (25 g, 0.21 mol) was suspended in methanol (freshly distilled from magnesium methoxide) (100 ml) and the suspension was boiled for 22 hr under a constant stream of dry hydrogen chloride. The suspension was evaporated under reduced pressure. Water present was removed by azeotropic distillation with toluene (3x100 ml) and absolute ethanol (2x100 ml), to give a fine white powder. Recrystallisation from acetonitrile gave the named product as fine white needles.

(31.5 g; 88.5%); m.p. 118-9°C, lit m.p. 125 dec °C[194]: ν_{max} = 1500, 1600, 1745, 2500-3000 cm^{-1}; $\delta(D_2O)$ 1.95(3H,d,$\underline{CH_3}$), 3.8-6.0(9H,m).

9.10. DL-Methyl 2-amino-3-chlorobutanoate hydrochloride.

To a suspension of threonine methyl ester hydrochloride (5.0 g, 0.29 mol) in dichloromethane (260 ml), phosphorus pentachloride (6.35 g, 0.03 mol) was added over a period of 1 hr. The solution was left

stirring for 3 hr at room temperature and then left to stand for 16 hr at 0°C. The white crystals deposited were removed by filtration and washed with diethyl ether and petrol. DL-Methyl 2-amino-3-chlorobutanoate hydrochloride was recrystallised from acetonitrile. (3.7 g; 67.6%); m.p. 169-71°C, lit m.p. 169-72°C[147]; ν_{max} = 1500, 1600, 1740, 2500-3000 cm^{-1}; δ (D_2O) 1.5(3H,d,$\underline{CH_3}$), 3.75(3H,s,$\underline{CH_3}$), 3.75(3H,s,$\underline{CH_3}$), 4.0-5.0 (m).

9.11. Methyl 2-(N-benzylideneamino)but-2-enoate.

To a well-stirred slurry of DL-methyl 2-amino-3-chlorobutanoate hydrochloride (8.3 g, 0.44 mol) in dichloromethane (80 ml) at 5°C was added freshly distilled benzaldehyde (4.5 g, 0.44 mol), triethylamine (6.15 ml, 0.044 mol) and dried magnesium sulphate (4 g). The suspension was stirred at room temperature for 21 hr, and then distilled water (100 ml) was added and the layers were separated. The aqueous portion was extracted with dichloromethane (3x50 ml) and the organic solvents were combined, washed with saturated aqueous sodium chloride and then dried with anhydrous magnesium sulphate (4 g).

Following evaporation of the solvents under reduced pressure DL-methyl 2-(N-benzylidineamino)-3-chlorobutanoate (11.1 g) was obtained as an oil. This was dissolved in dichloromethane (80 ml) and the solution was cooled to 5°C. 1,5-Diazobicyclo(5.4.0)undec-7-ene (6.5 ml) was added dropwise over a period of 5 min. After 2.5 hr, distilled water (100 ml) was added and the organic layer was dried with anhydrous magnesium sulphate (3 g). Following evaporation of the solvent under reduced pressure, the oil was filtered through Brockman grade III neutral alumina (20 g) in 10:1 hexane:diethyl ether to give a 2:1 mixture of E:Z isomers[146] of methyl 2-(N-benzylideneamino)but-2-enoate.

(oil; 8.4 g; 93.7%); ν_{max}(liquid film)=1715, 1640, 1600, 1580 cm^{-1}; $\delta(CDCl_3)$ 1.9(2H,d,$C\underline{H}_3CH$), 3.75(3H,s,$C\underline{H}_3$), 5.85(1H,q,$CH_3C\underline{H}$(E)), 6.5-(1H,q,$CH_3C\underline{H}$(Z)), 8.2(1H,s,NC$\underline{H}$(E)), 8.4(1H,s,NC$\underline{H}$(Z)), 7.2-7.9-(5H,m,$C_6\underline{H}_5$); m/z 203 (M^+) 143 (100%).

9.12. DL-α-Vinylserine (DL-2-amino-2-(hydroxymethyl)but-3-enoic acid).

This reaction was undertaken in a nitrogen atmosphere using glassware which had been previously dried by heating at 120°C for 16 hr. The THF had been distilled from calcium hydride prior to use.

Lithium hexamethyldisilazide was prepared by the addition of butyl lithium (8 ml, 1.55 M in hexane, 0.0123 mol) to hexamethyl-disilazane (2.0 g, 0.0123 mol) in THF (5 ml), at -70°C. To this solution, hexamethylphosphoramide (7 ml) was added, followed by methyl 2-(N-benzylideneamino)but-2-enoate (2.3 g, 0.0112 mol), which addition produced an intense red colour. Chloromethyl acetate (1.35 g, 0.0123 mol), in THF (10 ml), was added and the reaction was stirred for 1 hr at -70°C and then for 2 hr at room temperature.

The reaction mixture was diluted with saturated aqueous ammonium chloride and extracted three times with diethyl ether (50 ml). The combined organic fractions were washed twice with distilled water (50 ml) and once with saturated aqueous sodium chloride solution (50 ml). The organic layer was dried with magnesium sulphate (5 g) and evaporated under reduced pressure to give a yellow oil (2.8 g). 2M Hydrochloric acid (40 ml) was added and the reaction was left stirring for 2 min. The solution was washed twice with diethyl ether (50 ml) and twice with chloroform (50 ml). 6M Hydrochloric acid (20 ml) was added and the solution was boiled for 2 hr. The mixture was washed twice with dichloromethane (10 ml) and decolourised with

activated charcoal (100 mg).

The solution was evaporated to 10 ml under reduced pressure and then neutralised with aqueous ammonia. Hot ethanol (25 ml) was added and, upon cooling, fine white crystals were deposited. Recrystallisation from aqueous ethanol yielded DL-α-vinylserine.

TLC single spot RF=0.375 (silica gel, 2:1 propan-1-ol:water)-(ninhydrin); (0.17 g; 11.6%); m.p. 173-5 °C (decomp.); Found C,44.9; H,7.0; N,10.7. Calculated for $C_5H_9NO_3.1/8H_2O$ C,45.0; H,7.0; N,10.5; ν_{max} (liquid paraffin) = 1510, 1590, 1610, 1640 cm^{-1}; δ (D_2O) 3.8(1H,d,Jgem 12Hz,CHHOH), 4.2(1H,d,Jgem 12Hz,CHHOH), 4.68(DHO), 5.35(1H,d,Jtrans 18Hz, CHCHH), 5.44(1H,d,Jcis 12Hz,CHCHH), 6.02(1H,dd,Jcis 12Hz,Jtrans 18Hz,$CHCH_2$); m/z 100 (M^+-CH_2OH) 54 (100%).

9.13. DL-α-Hydroxymethylserine (DL-3-hydroxy-2-hydroxymethyl-2-amino-propanoic acid).

To 1M aqueous sodium carbonate (1.3 l), 1M aqueous copper (II) sulphate (33.0 ml) and (38%) aqueous formaldehyde (200 ml), glycine (25 g, 0.33 mol) was added. The solution was boiled for 2 hr, cooled to room temperature and then filtered. The filtrate was acidified with glacial acetic acid and concentrated under reduced pressure to a volume of about 300 ml.

Aliquots of 50 ml were adsorbed onto a column of Dowex 50-W X8 (mesh size 200-400) (2x10 cm) which had been previously washed with 2M hydrochloric acid (50 ml) and distilled water (until eluate was neutral). The column was washed with distilled water (until neutral) and then the named compound was eluted from the column with 2M aqueous ammonia (100 ml).

Evaporation of appropriate eluates yielded the named compound as

a fine white powder which was recrystallised from aqueous ethanol.

TLC single spot Rf=0.53 (silica gel, 2:1 propan-1-ol:water) (ninhydrin); (32.9 g; 73.8%); m.p. 110°C with decomposition, lit m.p. unrecorded[129]; ν_{max} = 1510, 1575, 1640, 1650, 2500-3800 cm^{-1}; $\delta(D_2O)$ 3.74(2H,d,Jgem 12Hz,2xCHHOH), 3.93(2H,d,Jgem 12Hz,2xCHHOH); m/z 104 (M^+- CH_2OH) 42 (100%).

9.14. DL-α-Benzyloxymethylserine (DL-2-amino-3-hydroxy-2-(benzyloxymethyl)propanoic acid).

DL-O-Benzylserine (5 g, 0.025 mol) was dissolved in 0.2 M aqueous sodium carbonate (500 ml) and 1M aqueous copper (II) sulphate (2.5 ml). Aqueous (38%) formaldehyde solution (15.1 ml) was added and the solution boiled for 25 min. The azure solution was cooled, filtered and concentrated under reduced pressure to a volume of 100 ml.

Aliquots of 50 ml were adsorbed onto a column of Dowex 50-W X8 (mesh size 200-400) (2x10 cm) which had been previously washed with 2M hydrochloric acid (50 ml) and distilled water (until eluate was neutral). The column was washed with distilled water (until neutral) and then the named compound was eluted from the column with 2M aqueous ammonia (100 ml). Evaporation under reduced pressure followed by cooling to 0°C caused the deposition of DL-α-benzyloxymethylserine as a fine white powder which was recrystallised from aqueous ethanol.

TLC single spot Rf=0.61 (silica gel, 2:1 propan-1-ol:water) (ninhydrin); (1.6; 27.7%); m.p. 164-7°C; Found C,58.7; H,6.7; N,6.2. Calculated for $C_{11}H_{15}NO_4$ C,58.7; H,6.7; N,6.2; ν_{max} (liquid paraffin) = 1460, 1590, 1640 cm^{-1}; δ (CF_3COOD) 3.95(1H,d,Jgem 12Hz,CHHOH),

4.10(1H,d,Jgem 12Hz,CHHOH), 4.30(1H,d,Jgem 13Hz,CHH), 4.43(1H,d,Jgem 13Hz,CHH), 4,68(2H,s,$C_6H_5CH_2O$), 7.25-7.60(5H,m,C_6H_5); m/z 194 (M^+ -CH_2OH) 91 (100%).

9.15. DL-Diethyl hydroxymethylacetamidomalonate (DL-Ethyl 3-hydroxy-2-acetamido-2-ethoxycarbonyl propanoate).

Diethyl acetamidomalonate (14.5 g, 0.067 mol) was suspended in distilled water (10 ml) containing neutralised formaldehyde (5.7 g, 0.069 mol). 4M Aqueous sodium hydroxide (0.5 ml) was added and the suspension was left to stir for 2 hr at room temperature. 2M Hydrochloric acid was used to neutralise the solution. Evaporation under reduced pressure yielded the named compound as an opalescent gel which crystallised upon standing under petrol.
(15.5 g; 93.9%); m.p. 52-55°C, lit m.p. 62-4°C[114]; ν_{max} = 1650, 1750, 2700-3100, 3100-3600 cm^{-1}; δ($CDCl_3$) 1.26(6H,t,CH_3), 2.1(3H,s,CH_3), 3.9(1H,s,OH) (disappears with D_2O), 4.2(2H,s,CH_2OH), 4.3(4H,q,CH_2), 4.3(4H,q,CH_2), 7.2(1H,s,NH).

9.16. DL-Diethyl ethylacetamidomalonate (DL-Ethyl 2-acetamido-2-ethoxycarbonylbutanoate).

To sodium metal (1.15 g, 0.05 mol) dissolved in absolute ethanol (30 ml), diethyl acetamidomalonate (10.9 g, 0.05 mol) and bromoethane (5.45 g, 0.05 mol) were added. The solution was boiled for 16 hr under reflux and after being cooled, the solvent was evaporated under reduced pressure. The white solid residue was washed with distilled water (30 ml). The named compound was recrystallised from toluene/-diethyl ether.

(8.1 g; 66.0%); m.p. 88-9°C, lit m.p. 83°C[130]; ν_{max} = 1530, 1650, 1740, 2500-3000 cm^{-1}; δ ($(CD_3)_2SO$) 0.75(6H,t,$\underline{CH_3}$), 1.2(3H,t,$\underline{CH_3}$), 2.1(5H,m,$\underline{CH}CO$, $\underline{CH_2}$), 4.1(4H,q,$\underline{CH_2}$), 7.9(1H,s,$\underline{NH}$).

9.17. DL-Diethyl (3-chlorobenzyl)acetamidomalonate (DL-Ethyl 2-acetamido-3-(3-chlorophenyl)-2-ethoxycarbonylpropanoate).

The substitution of 3-chlorobenzylchloride for bromoethane in method 9.16 resulted in the named compound. The compound was recrystallised from toluene/diethyl ether.

(6.4 g; 42%); m.p. 141-2°C, lit m.p. 141-2°C[195]; ν_{max} = 1520, 1600, 1640, 1740 cm^{-1}; δ ($CDCl_3$) 1.2(6H,t,$\underline{CH_3}$), 2.0(3H,s,$\underline{CH_3}$), 3.65(2H,s,$\underline{CH_2}$), 4.25(4H,q,$\underline{CH_2}$), 6.65(1H,s,$\underline{NH}$), 7.15(6H,m,$C_6\underline{H_5}$).

9.18. DL-Diethyl allylacetamidomalonate (DL-Ethyl 2-acetamido-2-ethyloxycarbonylpent-4-enoate).

The substitution of allyl bromide for bromoethane in method 9.16 resulted in the named compound in the form of an uncrystallisable oil.

(Oil; 10.4 g; 80.7%); lit m.p. 46°C[130]; ν_{max} = 1510, 1660, 1735 cm^{-1}; δ($(CD_3)_2SO$) 1.15(6H,t,$\underline{CH_3}$), 1.9(3H,s,$\underline{CH_3}$), 2.75(2H,d,$\underline{CH_2}$), 4.15-(4H,q,$\underline{CH_2}$), 4.9-5.9(3H,m,$\underline{CH}\underline{CH_2}$), 8.20(1H,s,$\underline{NH}$).

9.19. DL-Ethyl allylacetamidocyanoacetate (DL-ethyl 2-acetamido-2-cyanopent-4-enoate).

The substitution of allyl bromide for bromoethane, and ethyl acetamidocyanoacetate for ethyl acetamidomalonate, in method 9.16 resulted in the named compound. The compound was recrystallised from aqueous ethanol.

(4.1 g; 39.3%); m.p. 83-5°C, lit. m.p. 86-8.5°C[196]; ν_{max} = 1515, 1650, 1740 cm^{-1}; δ ($CDCl_3$) 1.25(3H,t,$\underline{CH_3}$), 2.05(3H,s,$\underline{CH_3}$), 2.75-(2H,d,$\underline{CH_2}CH$), 4.25(2H,q,$\underline{CH_2}CH_3$), 5.1-6.1(3H,m,$\underline{CH}\underline{CH_2}$), 7.5(1H,s,$\underline{NH}$); m/z 211 ($M^+$ +1) 95 (100%).

9.20. DL-Diethyl vinylacetamidomalonate (DL-ethyl 2-acetylamino-2-ethoxycarbonyl-but-3-enoate).

To a three-necked round bottom flask fitted with a septum, an input valve, and a stirrer, was added bis-acetonitrile palladium (II) dichloride (0.5 g, 0.0019 mol) in dry THF (38.5 ml) (freshly distilled from calcium hydride). The solution was cooled to -70°C and it was saturated with ethylene; after 5 min triethylamine (0.54 ml, 0.0038 mol) was added. After 15 min diethyl acetamidosodiomalonate (0.0019 mol in dry THF 10 ml) was added. The solution was warmed to -5°C and stirred for 1 hr. It was finally warmed to room temperature during 70 min.

After the palladium (0) was removed by filtration, evaporation of the solvent from the filtrate under reduced pressure gave a brown oil. Column chromatography on silica gel with ethyl acetate yielded DL-diethyl vinylacetamidomalonate.

(Oil; 0.29 g; 61.9%); ν_{max} = 1520, 1640, 1740 cm^{-1}; δ ($CDCl_3$) 1.25-(6H,t,$\underline{CH_3}$), 2.1(3H,s,$\underline{CH_3}$), 4.3(4H,q,$\underline{CH_2}$), 5.2(2H,m,$CH\underline{CH_2}$), 6.5-(1H,m,$\underline{CH}CH_2$), 7.2(1H,s,$\underline{NH}$).

9.21. DL-Ethyl ethylacetamidomalonate (2-acetamido-2-ethoxycarbonyl butanoic acid).

Diethyl ethylacetamidomalonate (2 g, 0.0081 mol) was dissolved in absolute ethanol (40 ml). Potassium hydroxide (0.457 g, 0.0081 mol) in absolute ethanol (20 ml) was added and the solution was left

to stir for 17 hr at room temperature. The solution was acidified with 6M hydrochloric acid, filtered and the solvent was evaporated under reduced pressure to give the named compound as a white powder. This material was recrystallised from ethyl acetate/water.
(1.2 g; 70.0%); m.p. 128-129° C lit m.p. unrecorded[197].
ν_{max} = 1515, 1620, 1710, 1740, 2500 cm^{-1}; δ ($(CD_3)_2SO$) 0.75(3H,t,$\underline{CH_3}$), 1.3(3H,t,$\underline{CH_3}$), 1.8-2.4(5H,m,$\underline{CH_2}$,$\underline{CH_3}CO$), 4.2(2H,q,$\underline{CH_2}$), 7.9(1H,s,$\underline{NH}$); m/z 217(M^+) 58 (100%).

9.22. DL-α-Allylserine (DL-2-amino-2-hydroxymethyl-pent-4-enoic acid.

Ethyl allylacetamidocyanoacetate (3.5 g, 0.016 mol) was suspended in dry THF (10 ml). Lithium borohydride (0.36 g, 0.016 mol) in dry THF (10 ml) was added dropwise over a period of 5 min. The solution was boiled for 180 min, then diluted with methanol (50 ml) and acidified with 6M hydrochloric acid (2 ml). Evaporation under reduced pressure followed by the repeated addition and evaporation (under reduced pressure) of methanol (50 ml) gave a yellow semi-solid oil.

6M Hydrochloric acid (20 ml) was added and the solution was boiled for 2 hr. Evaporation of water under reduced pressure followed by neutralisation of the residue with 2M aqueous ammonia gave DL-α-allylserine as a white powder. This was recrystallised from aqueous ethanol. TLC single spot Rf=0.52 (silica gel, 2:1 propan-1-ol:water) (ninhydrin); (0.46 g; 20.0%); m.p. 215-7°C decomp.; Found C,49.6; H,7.7; N, 9.7. Calculated for $C_6H_{11}NO_3$ C, 49.6; H, 7.6; N, 9.6; ν_{max} = 1510, 1530, 1610, 1640, 2500-3200, 3450 cm^{-1}; δ (D_2O) 2.45(1H,dd,Jgem 14.6Hz Jvic9.3Hz,CH$\underline{H}$CH), 2.60(1H,dd,Jgem 14.6Hz Jvic6.3Hz,C$\underline{H}$HCH), 3.71(1H,d,Jgem 11.2Hz,CH$\underline{H}$OH), 3.95(1H,d,Jgem

11.2Hz,CHHOH), 4.65(DHO), 5.27(1H,d,Jcis 12.2Hz,CHCHH), 5.28(1H,d,Jtrans 15.1Hz,CHCHH), 5.55-5.85(1H,m,CHCH$_2$); m/z 126 (M^+-CH_2OH) 114 (100%).

9.23. DL-Ethyl propargylacetamidocyanoacetate (DL-ethyl 2-acetamido-2-cyanopent-4-ynoate.

To toluene (100 ml) which had previously been dried azeotropically by boiling for 10 min in a Dean and Stark apparatus, sodium hydride (4.8 g, 50% in oil, 0.1 mol), ethyl acetamidocyanoacetate (17 g, 0.1 mol) and 70% propargyl bromide solution (in toluene) (14.8 g, 0.18 mol) were added at room temperature. The solution was boiled for 3 hr, followed by the addition of ethyl acetate (20 ml), distilled water (50 ml) and diethyl ether (50 ml). The combined organic layers were reduced in volume by evaporation and, upon standing, light brown crystals of DL-ethyl propargylacetamidocyanoacetate were deposited. The compound was recrystallised from ethanol/petrol.

(5.2 g; 25%); m.p. 87-8°C; ν_{max} = 1660, 1760, 2500-3500 cm^{-1}; $\delta(CDCl_3)$ 1.56(3H,t,CH_3CH_2), 2.15(3H,s,CH_3), 2.24(1H,t,CCH), 3.08(1H,d,Jgem 13Hz,CHHOH), 3.44(1H,d,Jgem 13Hz,CHHOH), 4.41(2H,q,CH_2CH_3), 9.06(1H,s,NH); m/z 208(M^+) 135 (100%).

9.24. DL-α-propargylserine (DL-2-amino-2-hydroxymethylpent-4-ynoic acid).

The named compound was synthesised by the method shown in section 9.22 using DL-ethyl propargylacetamidocyanoacetate as the starting material. Neutralisation with 2M aqueous ammonia yielded a tar. The tar was dissolved in distilled water (5 ml) and acidified with glacial acetic acid. The solution was adsorbed onto a column of

Dowes 50-W X8 (mesh size 200-400) (2x10 cm) which had been previously washed with 2M hydrochloric acid (50 ml) and distilled water (until eluate was neutral). The column was washed with distilled water (until neutral) and then the DL-α-propargylserine was eluted from the column with 2M aqueous ammonia (100 ml). The compound was recrystallised from aqueous ethanol.

TLC Single spot Rf=0.56 (silica gel, 2:1 propan-1-ol:water)-(ninhydrin); (0.39 g; 17%); m.p. 157-60°C; Found C, 50.0; H,6.2; N, 9.7. Calculated for $C_6H_9NO_3$ C, 50.3; H, 6.3; N, 9.8.; ν_{max} = 1590, 1620, 2500-3700 cm^{-1};$\delta(D_2O)$ 2.57(1H,dd,Ja1.9Hz,Jb2.4Hz,CH_2CCH), 2.75-(1H,dd,Jgem 17.6Hz,Ja1.9Hz,CHHCCH), 2.82(1H,dd,Jgem 17.6Hz,Jb2.4Hz,-CHHCCH), 3.80(1H,d,Jgem 11.2Hz,CHHOH), 3.95(1H,d,Jgem 11.2Hz,CHHOH), 4.7(DHO); m/z 112 (M^+ -CH_2OH 100%).

9.25. DL-Benzyl formamidocyanoacetate (DL-benzyl 2-formamido-2-cyanoethanoate).

Sodium nitrite (10.35 g, 0.15 mol) and glacial acetic acid (10 ml) were added to benzyl cyanoacetate (21.8g, 0.12 mol) dissolved in iced water (100 ml), over a period of 5 min. The solution was stirred for 1 hr at 4°C and then for 16 hr at room temperature. The precipitate was removed by filtration and dissolved in 3M hydrochloric acid (20 ml). The aqueous layer was extracted using diethyl ether (4x50 ml) and the organic solvent dried with magnesium sulphate (5 g). Evaporation under reduced pressure gave benzyl 2-cyano-2-(hydroxyimino)acetate as a crude yellow wax.

The oxime was stirred with 100% formic acid (65.25 ml) and water (7.25 ml) and warmed to 60-3 °C. Zinc powder (13.6 g, 4.8 mol) was added cautiously over a period of 1.5 hr. Following a short induction period cooling was required (to 4°C). The suspension was left to stir

for an additional 30 min at 4°C and for a further 30 min at room temperature. The precipitate was removed by filtration and was washed with hot ethyl acetate (3x100 ml). The ethyl acetate was left to stand for 16 hr and was then refiltered. Evaporation under reduced pressure gave the named compound as a white powder which was recrystallised from chloroform/cyclohexane.

(4.2 g; 16%); m.p. 108-10°C, lit m.p. 102-5°C[145]; ν_{max} = 1520, 1660, 1740, 2900-3000, 3270 cm^{-1}; δ ($CDCl_3$/$(CD_3)_2SO$) 5.2(2H,s,$C\underline{H}_2$), 5.7-(1H,s,C$\underline{H}$), 7.45(5H,s,$C_6\underline{H}_5$), 8.15(1H,s,CO$\underline{H}$), 9.1(1H,d,N$\underline{H}$); m/z 218 (M^+) 65 (100%).

APPENDIX 1

LEVELS OF SHMT IN VARIOUS TUMOUR CELLS.

Tumour type	gradient	y-intercept[1]	correlation coefficient	R^2(%)	$^{14}CH_2O$ produced in 10 minutes[2]
TLX5	0.028	0.014	0.973	94.7	0.38
L1210	0.021	-0.073	0.981	96.4	0.44
L1210 MTX	0.041	-0.120	0.990	98.0	0.29
HL60	0.051	0.097	0.993	98.5	0.61
Raji mer$^-$	0.061	-0.107	0.999	99.9	0.49
Raji mer$^+$	0.066	-0.044	0.995	99.0	0.62
PC6	0.084	0.714	0.998	99.7	1.55
K562	0.026	0.129	0.958	91.7	0.38
HT29	0.047	0.128	0.986	98.6	0.60
Colon 26	0.042	0.221	0.956	91.2	0.64
L1210 (Glaxo)	0.021	0.037	0.993	98.7	0.25
L1210 CB	0.031	0.094	0.984	96.8	0.40
L1210 NCI	0.023	0.040	0.991	98.3	0.27
Walker 256	0.087	0.224	0.990	98.0	1.09
Walker ascites	0.063	0.043	0.988	97.7	0.67
Lewis lung	0.121	0.290	0.987	97.5	1.45
Sarcoma 180	0.036	0.086	0.989	98.0	0.45

1 = nmoles $^{14}CH_2O$ mg protein^{-1}

2 = nmoles $^{14}CH_2O$ mg protein^{-1}10min^{-1}

APPENDIX 2.

THE ANTITUMOUR TESTING OF N-DICHLOROACETYLSERINE.

M5076 TUMOUR

N-Dichloroacetylserine sodium salt pentahydrate CCRG 83005.

Experiment number: 83A12

Date on: 13.01.83.

Host group: BDF Mice Sex: Female

Site: Intramuscular Tissue: Homogenate

Parameter used: Tumour volume Level: $6x10^5$

Drug administration.

Vehicle: Saline Route: Intraperitoneal

Evaluation day: 1-24

Total number of injections: 17

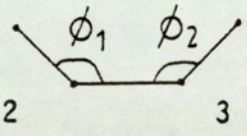
Treatment schedules: 17 daily injections

Animals weighed day 1 and 24.

Dose(g) per injection	Toxic dose survivors	Weight difference (T-C)	Control body	Evaluations Test	Control	T/C
8	05/05	+4.8	+6.5	1.93	2.89	67
4	04/05	+6.0	+6.5	2.06	2.89	71
2	05/05	+6.0	+6.5	2.78	2.89	96
CTRL	10/10	+6.5	+6.5	2.89	2.89	100

APPENDIX 3

STANDARD DEVIATION OF A TORSION ANGLE



Assume variances of atomic positions are isotropic:

$$\sigma^2(x_n)=\sigma^2(y_n)=\sigma^2(z_n)=\sigma_n^2$$

Then[198]:

$$\sigma^2(\tau)=\frac{\sigma_1^2}{r_{12}^2\sin^2\phi_1}+\frac{\sigma_2^2}{r_{23}^2}\left[\left[\frac{r_{23}-r_{12}\cos\phi_1}{r_{12}\sin\phi_1}\right]^2-2\left[\frac{r_{23}-r_{12}\cos\phi_1}{r_{12}\sin\phi_1}\right]\cot\phi_2\cos\tau\right.$$

$$\left.+\cot^2\phi_2\right]+\frac{\sigma_3^2}{r_{23}^2}\left[\cot^2\phi_1-2\left[\frac{r_{23}-r_{34}\cos\phi_2}{r_{34}\sin\phi_2}\right]\cot\phi_1\cos\tau+\left[\frac{r_{23}-r_{34}\cos\phi_2}{r_{34}\sin\phi_2}\right]^2\right]$$

$$+\frac{\sigma_4^2}{r_{34}^2\sin^2\phi_2}$$

APPENDIX 4

OBSERVED AND CALCULATED STRUCTURE FACTORS FOR α-VINYLSERINE

OBSERVED AND CALCULATED STRUCTURE FACTORS FOR VINYLSERINE

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
2	0	0	1063	-1103	6	4	0	57	-87	-8	1	1	208	206	-5	3	1	294	295	2	5	1	107	-92
3	0	0	656	-659	11	4	0	89	-93	-7	1	1	144	142	-4	3	1	600	602	4	5	1	151	151
4	0	0	327	-348	2	5	0	92	97	-6	1	1	101	168	-3	3	1	97	117	5	5	1	83	78
5	0	0	290	311	3	5	0	119	128	-5	1	1	52	63	-1	3	1	133	138	6	5	1	73	74
6	0	0	307	294	4	5	0	242	-273	-4	1	1	403	-411	0	3	1	425	-460	7	5	1	195	180
7	0	0	287	292	5	5	0	104	-89	-3	1	1	368	300	1	3	1	218	213	-9	6	1	152	-154
8	0	0	165	-164	7	5	0	144	-148	-1	1	1	116	-142	2	3	1	781	803	-8	6	1	123	-128
9	0	0	293	-293	8	5	0	87	-104	1	1	1	267	-241	3	3	1	319	328	-7	6	1	90	89
12	0	0	109	-89	9	5	0	71	36	2	1	1	357	-341	4	3	1	417	-403	-5	6	1	98	-97
1	1	0	257	274	0	6	0	170	-142	3	1	1	269	-271	5	3	1	239	-235	-3	6	1	77	79
2	1	0	584	-654	1	6	0	109	-102	4	1	1	185	195	7	3	1	151	150	0	6	1	192	211
3	1	0	752	-845	4	6	0	228	-232	5	1	1	115	123	8	3	1	87	108	1	6	1	92	-89
4	1	0	380	-361	8	6	0	145	145	6	1	1	185	-186	10	3	1	100	-101	2	6	1	155	-151
5	1	0	250	250	1	7	0	339	333	7	1	1	157	-154	11	3	1	106	-110	3	6	1	209	203
6	1	0	244	-243	2	7	0	263	274	9	1	1	64	43	-12	4	1	80	79	4	6	1	203	203
7	1	0	312	-281	3	7	0	206	208	11	1	1	97	102	-9	4	1	87	80	6	6	1	105	123
9	1	0	82	-69	4	7	0	161	-175	-9	2	1	203	204	-7	4	1	178	178	7	6	1	132	130
10	1	0	82	-87	5	7	0	220	-224	-8	2	1	165	169	-3	4	1	146	-152	-9	7	1	141	-143
13	1	0	69	-50	6	7	0	115	122	-6	2	1	382	-388	-2	4	1	148	155	-7	7	1	121	125
0	2	0	1294	-1360	7	7	0	230	233	-5	2	1	438	456	1	4	1	105	-122	-6	7	1	162	166
1	2	0	343	-358	8	7	0	182	180	-4	2	1	676	-681	0	4	1	554	-529	-4	7	1	108	99
2	2	0	462	483	10	7	0	92	-65	-3	2	1	154	168	1	4	1	554	-553	-3	7	1	179	-194
3	2	0	80	-91	0	8	0	188	189	-2	2	1	287	284	2	4	1	60	-59	0	7	1	261	275
4	2	0	80	-71	2	8	0	184	-191	-1	2	1	153	-163	3	4	1	259	281	1	7	1	156	155
5	2	0	65	75	4	8	0	136	132	1	2	1	534	-553	4	4	1	254	245	2	7	1	69	-22
7	2	0	105	-110	7	8	0	73	-54	2	2	1	310	336	5	4	1	165	-178	3	7	1	138	139
2	3	0	166	-171	2	9	0	84	-71	3	2	1	401	-407	6	4	1	225	-236	4	7	1	182	188
5	3	0	54	-59	3	9	0	159	-168	4	2	1	210	-198	7	4	1	122	-131	5	7	1	226	218
6	3	0	83	-76	4	9	0	181	171	5	2	1	401	421	9	4	1	145	155	6	7	1	130	-128
7	3	0	190	188	5	9	0	211	210	7	2	1	77	86	10	4	1	64	59	7	7	1	198	-205
9	3	0	100	-104	0	10	0	189	-208	8	2	1	106	105	-9	5	1	112	96	-6	8	1	84	98
0	4	0	573	-558	2	10	0	211	216	9	2	1	111	100	-7	5	1	194	-202	-5	8	1	161	161
1	4	0	110	-98	4	10	0	63	-55	10	2	1	68	64	-6	5	1	213	-207	-1	8	1	97	67
2	4	0	99	99	5	10	0	58	-68	-8	3	1	160	-163	-5	5	1	359	-375	0	8	1	151	171
3	4	0	235	212	1	11	0	183	-185	-7	3	1	136	-138	-4	5	1	65	-87	1	8	1	284	285
4	4	0	265	249	2	11	0	87	-72	-6	3	1	165	-145	1	5	1	164	-169	2	8	1	78	86

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
3	8	1	264	-266	6	0	2	118	124	10	2	2	85	84	5	4	2	130	-126	7	7	2	123	-132
4	8	1	216	-217	7	0	2	270	-283	11	2	2	66	76	7	4	2	120	113	9	7	2	71	86
7	8	1	69	-67	9	0	2	61	39	-11	3	2	113	-123	9	4	2	79	-82	-8	8	2	106	107
9	8	1	74	-43	-13	1	2	73	-83	-10	3	2	110	-97	11	4	2	92	-68	-6	8	2	103	-106
-9	9	1	71	49	-11	1	2	138	147	-9	3	2	212	-200	-12	5	2	148	143	-5	8	2	117	120
-4	9	1	115	-108	-10	1	2	149	151	-6	3	2	113	-122	-5	5	2	208	212	-2	8	2	83	79
-3	9	1	109	-110	-9	1	2	82	81	-5	3	2	200	-170	-4	5	2	200	202	-1	8	2	134	-126
-2	9	1	91	70	-7	1	2	251	-262	-4	3	2	467	-472	-2	5	2	103	-125	2	8	2	214	214
2	9	1	176	-173	-6	1	2	291	264	-3	3	2	239	-213	-1	5	2	55	64	4	8	2	159	-173
3	9	1	133	-130	-5	1	2	240	215	-2	3	2	333	-346	2	5	2	140	-144	6	8	2	71	70
4	9	1	131	-128	-4	1	2	94	-89	-1	3	2	394	395	4	5	2	115	-113	-7	9	2	115	99
-6	10	1	72	-74	-3	1	2	378	354	0	3	2	672	707	5	5	2	261	-274	-6	9	2	128	130
-5	10	1	127	-125	-2	1	2	506	-514	1	3	2	456	429	6	5	2	121	113	-5	9	2	82	75
-3	10	1	123	124	0	1	2	194	106	2	3	2	344	313	7	5	2	180	196	-3	9	2	135	-140
1	10	1	67	-63	1	1	2	211	-224	3	3	2	69	-55	9	5	2	131	-123	-2	9	2	118	-114
2	10	1	78	88	2	1	2	60	66	4	3	2	202	202	10	5	2	111	-101	0	9	2	120	130
3	10	1	175	165	3	1	2	75	72	5	3	2	358	359	-11	6	2	90	84	4	9	2	224	-233
-3	11	1	69	72	5	1	2	126	-114	6	3	2	95	93	-9	6	2	108	-124	5	9	2	122	-116
-1	11	1	115	118	8	1	2	293	-309	7	3	2	130	-123	-8	6	2	275	-267	7	9	2	90	75
3	11	1	70	-71	9	1	2	158	-172	8	3	2	75	99	-7	6	2	93	78	-7	10	2	84	-75
-3	12	1	102	-88	10	1	2	71	58	9	3	2	119	106	-6	6	2	109	123	-5	11	2	80	-98
-13	0	2	68	28	11	1	2	126	115	-11	4	2	86	-107	-5	6	2	164	167	-4	11	2	78	50
-12	0	2	139	130	-8	2	2	64	54	-10	4	2	90	-77	-4	6	2	259	264	-3	11	2	135	136
-11	0	2	89	78	-7	2	2	126	116	-9	4	2	243	235	-2	6	2	270	-257	-2	11	2	109	95
-10	0	2	131	-138	-6	2	2	67	72	-8	4	2	129	132	-1	6	2	179	-194	0	11	2	71	-77
-9	0	2	111	-128	-5	2	2	279	-271	-7	4	2	133	-140	0	6	2	173	176	1	11	2	130	-121
-6	0	2	164	182	-4	2	2	429	-447	-6	4	2	100	-104	2	6	2	133	-131	4	11	2	103	100
-3	0	2	203	-165	-3	2	2	523	-524	-4	4	2	114	132	4	6	2	157	164	-1	12	2	93	-78
-2	0	2	782	711	-2	2	2	741	-731	-3	4	2	597	574	6	6	2	137	-157	-13	1	3	75	52
-1	0	2	99	-87	-1	2	2	316	319	-2	4	2	640	646	7	6	2	74	-95	-9	1	3	225	-225
0	0	2	978	-902	0	2	2	286	284	-1	4	2	264	269	10	6	2	88	-52	-8	1	3	262	-256
1	0	2	344	323	1	2	2	525	-558	0	4	2	127	-113	0	7	2	74	-89	-7	1	3	126	127
2	0	2	310	-269	2	2	2	505	-504	1	4	2	308	295	2	7	2	84	-78	-6	1	3	86	16
3	0	2	307	276	4	2	2	97	106	2	4	2	461	451	3	7	2	271	-286	-5	1	3	155	-147
4	0	2	403	366	5	2	2	119	139	3	4	2	156	-165	4	7	2	112	109	-3	1	3	169	-174
5	0	2	217	224	6	2	2	70	81	4	4	2	228	-233	5	7	2	205	211	-2	1	3	525	508

OBSERVED AND CALCULATED STRUCTURE FACTORS FOR VINYLSERINE

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
0	1	3	525	513	5	3	3	214	-209	1	6	3	255	248	2	11	3	75	75	7	1	4	224	-213
1	1	3	171	186	6	3	3	65	-77	2	6	3	123	136	-12	0	4	136	-122	8	1	4	90	94
2	1	3	358	-355	-12	4	3	154	-157	5	6	3	70	-69	-11	0	4	85	-85	9	1	4	80	99
4	1	3	82	80	-10	4	3	127	127	6	6	3	77	70	-10	0	4	203	212	-11	2	4	154	-154
5	1	3	205	206	-9	4	3	122	130	-9	7	3	120	-120	-9	0	4	80	89	-10	2	4	148	-151
6	1	3	92	84	-8	4	3	311	324	-7	7	3	261	262	-8	0	4	186	166	-9	2	4	179	185
-13	2	3	69	46	-6	4	3	227	-224	-6	7	3	177	164	-7	0	4	422	401	-8	2	4	142	133
-11	2	3	78	-93	-5	4	3	371	-376	-3	7	3	153	168	-5	0	4	344	-402	-6	2	4	225	-223
-10	2	3	99	-90	-4	4	3	254	279	-2	7	3	161	154	-4	0	4	284	-285	-3	2	4	202	-190
-8	2	3	118	-105	-3	4	3	615	605	-1	7	3	114	109	-3	0	4	1410	1284	-2	2	4	152	-158
-7	2	3	164	-160	-2	4	3	167	158	1	7	3	117	-125	-2	0	4	1850	1670	-1	2	4	462	-411
-6	2	3	408	412	-1	4	3	192	196	2	7	3	258	-261	-1	0	4	449	516	0	2	4	166	-146
-5	2	3	386	345	1	4	3	273	-263	4	7	3	182	190	0	0	4	534	-509	1	2	4	105	98
-4	2	3	76	109	2	4	3	130	-128	7	7	3	91	-82	1	0	4	749	-709	2	2	4	153	146
-3	2	3	327	313	4	4	3	194	-186	-7	8	3	71	58	2	0	4	266	-240	4	2	4	128	-122
-2	2	3	497	455	5	4	3	189	-197	-6	8	3	66	56	4	0	4	353	340	5	2	4	162	-149
-1	2	3	495	-457	6	4	3	62	-73	-4	8	3	78	-75	6	0	4	335	-350	7	2	4	89	70
0	2	3	576	-558	-12	5	3	77	67	-3	8	3	153	-157	7	0	4	183	-196	9	2	4	129	119
1	2	3	124	130	-10	5	3	96	88	-2	8	3	120	-100	10	0	4	100	-105	10	2	4	85	73
2	2	3	215	205	-4	5	3	183	-165	1	8	3	72	-45	-13	1	4	163	166	-14	3	4	82	-93
3	2	3	390	382	-3	5	3	144	-132	4	8	3	68	-54	-12	1	4	95	95	-13	3	4	89	-69
4	2	3	107	110	-2	5	3	76	-91	-7	9	3	83	-70	-9	1	4	113	121	-11	3	4	139	132
6	2	3	185	-182	-1	5	3	245	-230	-6	9	3	120	-123	-8	1	4	194	197	-7	3	4	134	-131
7	2	3	306	-309	0	5	3	286	-293	-5	9	3	84	-89	-6	1	4	171	163	-6	3	4	194	-194
-12	3	3	92	-96	1	5	3	85	-90	-1	9	3	77	67	-5	1	4	618	588	-5	3	4	83	-101
-11	3	3	59	17	3	5	3	187	-184	0	9	3	125	-120	-4	1	4	409	-390	-4	3	4	194	212
-9	3	3	297	294	4	5	3	158	-155	1	9	3	77	-72	-3	1	4	641	-566	-3	3	4	60	46
-8	3	3	103	108	8	5	3	141	155	-6	10	3	65	-25	-2	1	4	470	408	-2	3	4	70	86
-7	3	3	339	-335	-11	6	3	73	64	-4	10	3	162	-163	-1	1	4	171	-173	-1	3	4	193	-191
-6	3	3	55	-53	-10	6	3	88	-86	-3	10	3	76	61	0	1	4	465	-402	1	3	4	271	-254
-5	3	3	212	214	-9	6	3	77	-61	-2	10	3	109	111	1	1	4	479	-446	3	3	4	270	263
-4	3	3	525	494	-8	6	3	151	-159	3	10	3	79	81	2	1	4	194	-172	4	3	4	86	-96
-2	3	3	573	-579	-7	6	3	171	-161	4	10	3	132	138	3	1	4	155	-140	6	3	4	200	200
1	3	3	207	164	-4	6	3	145	154	5	10	3	95	88	4	1	4	128	-128	7	3	4	138	130
2	3	3	560	546	-3	6	3	202	-203	-1	11	3	104	74	5	1	4	149	164	-11	4	4	215	234
4	3	3	195	-195	-2	6	3	207	-196	0	11	3	122	123	6	1	4	166	-156	-9	4	4	285	-293

OBSERVED AND CALCULATED STRUCTURE FACTORS FOR VINYLSERINE

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
-8	4	4	196	-196	-10	7	4	134	-132	-4	1	5	155	-149	5	4	5	65	-66	-2	9	5	86	-94
-6	4	4	197	202	-9	7	4	184	-188	-3	1	5	223	-216	7	4	5	109	95	1	9	5	188	-190
-5	4	4	382	377	-7	7	4	164	178	-2	1	5	176	-178	-9	5	5	125	-130	3	9	5	123	121
-4	4	4	312	314	-6	7	4	109	69	0	1	5	411	-362	-8	5	5	230	-240	-4	10	5	109	107
-3	4	4	295	-282	-4	7	4	162	-154	2	1	5	94	90	-7	5	5	104	-113	0	10	5	90	93
-2	4	4	655	-619	-3	7	4	143	-141	5	1	5	81	-93	-6	5	5	70	68	1	10	5	132	119
-1	4	4	139	152	-2	7	4	82	66	9	1	5	105	91	-4	5	5	172	193	-3	11	5	90	86
0	4	4	437	416	-1	7	4	260	262	-11	2	5	74	69	-3	5	5	90	99	-14	0	6	120	125
3	4	4	124	122	0	7	4	305	318	-9	2	5	103	-118	-1	5	5	107	-114	-13	0	6	121	140
8	4	4	83	-55	2	7	4	81	-65	-7	2	5	259	-260	4	5	5	67	84	-11	0	6	409	-393
9	4	4	171	-162	3	7	4	78	-49	-6	2	5	484	-491	6	5	5	71	-56	-10	0	6	168	-170
-9	5	4	106	115	5	7	4	98	105	-1	2	5	215	193	7	5	5	74	-39	-9	0	6	279	267
-7	5	4	245	235	-8	8	4	107	-103	0	2	5	198	176	-12	6	5	114	-93	-4	0	6	464	428
-6	5	4	175	174	-7	8	4	103	126	1	2	5	83	-69	-11	6	5	104	-94	-1	0	6	243	-215
-5	5	4	285	-283	-6	8	4	133	143	2	2	5	173	167	-2	6	5	93	-95	0	0	6	134	-113
-4	5	4	142	-131	-5	8	4	63	-48	3	2	5	138	140	1	6	5	152	141	1	0	6	378	382
-3	5	4	58	-72	-3	8	4	154	-149	-9	3	5	153	-157	2	6	5	198	219	2	0	6	305	305
2	5	4	204	-218	-1	8	4	96	110	-8	3	5	122	103	3	6	5	64	-51	3	0	6	245	266
3	5	4	166	-159	1	8	4	112	108	-7	3	5	159	158	5	6	5	78	89	4	0	6	108	-117
4	5	4	192	186	-7	9	4	181	-170	-6	3	5	129	108	-9	7	5	179	185	5	0	6	218	-233
6	5	4	225	-240	-5	9	4	114	104	-5	3	5	197	181	-8	7	5	105	111	6	0	6	70	-62
7	5	4	67	-79	-3	9	4	84	93	-4	3	5	151	-147	-3	7	5	61	64	-8	1	6	189	177
-12	6	4	71	30	-2	9	4	135	140	-1	3	5	235	207	-2	7	5	303	307	-7	1	6	186	168
-11	6	4	73	-79	-1	9	4	67	47	0	3	5	550	542	1	7	5	177	194	-6	1	6	172	-162
-9	6	4	108	117	0	9	4	119	-116	2	3	5	345	-340	2	7	5	187	194	-5	1	6	126	-117
-8	6	4	88	76	-7	10	4	102	-54	3	3	5	200	-201	4	7	5	157	-165	-4	1	6	334	311
-7	6	4	223	-235	-5	10	4	135	130	5	3	5	179	186	5	7	5	139	-124	-3	1	6	152	-134
-6	6	4	193	-206	-4	10	4	154	159	6	3	5	88	76	-10	8	5	78	42	-2	1	6	352	-336
-5	6	4	268	-268	-6	11	4	82	-82	9	3	5	149	-144	-2	8	5	167	167	4	1	6	187	-190
-4	6	4	209	-212	-4	11	4	90	128	-8	4	5	224	-229	-1	8	5	133	136	5	1	6	311	-318
-2	6	4	131	117	-2	11	4	139	-120	-5	4	5	121	126	1	8	5	245	-251	-12	2	6	96	-81
-1	6	4	112	-121	-1	11	4	157	-165	-2	4	5	327	-317	2	8	5	201	-202	-11	2	6	234	235
0	6	4	161	-169	2	11	4	131	139	-1	4	5	409	-377	4	8	5	66	-60	-10	2	6	91	87
1	6	4	85	86	-12	1	5	101	67	0	4	5	102	108	-6	9	5	120	-118	-9	2	6	201	-212
8	6	4	73	50	-8	1	5	66	65	1	4	5	248	249	-4	9	5	69	40	-8	2	6	147	-136
-12	7	4	73	50	-5	1	5	82	-72	4	4	5	92	-85	-3	9	5	83	-53	-7	2	6	183	-186

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
-6	2	6	202	-196	-2	5	6	216	-224	-3	1	7	230	242	2	4	7	124	-129	-5	0	8	463	435
-5	2	6	381	-341	-1	5	6	77	-86	-2	1	7	277	269	3	4	7	139	-140	-4	0	8	268	265
-4	2	6	101	-112	1	5	6	79	-89	0	1	7	207	-209	-9	5	7	67	-70	-2	0	8	276	-273
-3	2	6	67	48	3	5	6	116	-128	1	1	7	153	-138	-7	5	7	72	-75	0	0	8	138	-126
0	2	6	79	-83	4	5	6	82	-71	2	1	7	85	95	-4	5	7	272	-270	1	0	8	106	83
4	2	6	100	103	5	5	6	88	76	-9	2	7	94	71	-3	5	7	181	-180	2	0	8	145	142
5	2	6	111	126	6	5	6	117	99	-8	2	7	158	175	0	5	7	107	108	3	0	8	292	-294
-12	3	6	88	-94	-7	6	6	261	273	-7	2	7	153	163	1	5	7	82	91	4	0	8	175	-189
-11	3	6	227	-219	-6	6	6	203	194	-5	2	7	192	199	5	5	7	73	68	-6	1	8	185	-174
-7	3	6	124	-137	-4	6	6	237	-220	-4	2	7	171	160	6	5	7	103	89	-5	1	8	214	-212
-4	3	6	102	-84	-3	6	6	188	-184	-3	2	7	400	-393	-5	6	7	216	-218	-4	1	8	112	-106
-3	3	6	338	340	3	6	6	100	-90	-2	2	7	200	-190	-4	6	7	208	-209	-1	1	8	137	-136
-2	3	6	555	538	-10	7	6	99	-102	1	2	7	137	155	-3	6	7	77	74	0	1	8	172	-175
-1	3	6	178	174	-4	7	6	139	-141	3	2	7	75	57	-2	6	7	194	193	1	1	8	201	-209
0	3	6	135	-144	-3	7	6	121	-118	4	2	7	190	-190	-1	6	7	216	239	-11	2	8	119	-111
2	3	6	111	97	-2	7	6	99	92	5	2	7	136	-126	0	6	7	224	224	-7	2	8	65	-87
3	3	6	167	164	-1	7	6	104	104	6	2	7	78	66	2	6	7	134	-131	-6	2	8	169	-151
4	3	6	198	196	0	7	6	139	-155	-12	3	7	107	90	5	6	7	112	103	-3	2	8	111	-112
6	3	6	119	-130	1	7	6	75	-97	-11	3	7	205	225	-9	7	7	109	115	-2	2	8	147	-144
-9	4	6	149	154	2	7	6	63	60	-9	3	7	218	-210	-7	7	7	135	-135	0	2	8	110	96
-8	4	6	82	94	3	7	6	121	123	-7	3	7	325	320	-5	7	7	76	62	1	2	8	69	-74
-7	4	6	118	-121	-9	8	6	102	93	-6	3	7	79	103	-4	7	7	184	190	-10	3	8	86	-101
-5	4	6	337	351	-8	8	6	90	79	-5	3	7	117	-107	0	7	7	191	-198	-9	3	8	194	-195
-4	4	6	228	194	-3	8	6	77	62	-3	3	7	176	-159	-10	8	7	106	-92	-8	3	8	139	-118
-3	4	6	82	96	-1	8	6	96	86	-1	3	7	184	175	-6	8	7	115	-119	-6	3	8	122	-123
-2	4	6	73	-83	1	8	6	128	-122	0	3	7	235	235	-4	8	7	59	32	-5	3	8	136	132
0	4	6	148	151	4	8	6	96	96	2	3	7	196	-194	-8	9	7	74	-73	-4	3	8	345	354
2	4	6	93	-104	-8	9	6	94	74	-11	4	7	220	223	-1	9	7	67	-93	-3	3	8	133	-125
3	4	6	88	-64	-6	9	6	175	-161	-10	4	7	129	133	1	9	7	134	133	-2	3	8	330	-338
4	4	6	101	-117	-5	9	6	80	-86	-8	4	7	131	-139	-4	10	7	67	63	0	3	8	232	243
5	4	6	63	-69	-3	9	6	79	-14	-7	4	7	190	-190	-13	0	8	104	-111	1	3	8	201	201
-11	5	6	56	72	-4	10	6	71	-82	-6	4	7	204	203	-12	0	8	143	138	-13	4	8	70	53
-9	5	6	98	-99	-11	1	7	161	-169	-5	4	7	145	138	-11	0	8	309	317	-10	4	8	99	-80
-8	5	6	94	74	-9	1	7	194	202	-2	4	7	142	-162	-9	0	8	122	128	-9	4	8	170	-179
-4	5	6	97	106	-7	1	7	93	-87	-1	4	7	154	-160	-7	0	8	233	-251	-8	4	8	91	90
-3	5	6	173	168	-4	1	7	86	72	0	4	7	140	-132	-6	0	8	98	99	-7	4	8	455	455

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
-6	4	8	235	234	-6	9	8	122	132	-9	7	9	90	70	-13	2	10	124	97	0	2	11	124	127
-5	4	8	233	-220	-7	1	9	92	-103	-6	7	9	95	80	-10	2	10	104	-106	-11	3	11	148	-156
-4	4	8	296	-301	-6	1	9	141	-124	-5	7	9	85	104	-9	2	10	142	-144	-9	3	11	159	161
-2	4	8	237	237	-5	1	9	105	-98	0	7	9	123	129	-8	2	10	122	-138	-5	3	11	115	-128
0	4	8	79	90	-2	1	9	106	-92	-6	8	9	91	-90	-6	2	10	140	147	-10	4	11	127	-114
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2	4	8	131	-131	-8	2	9	182	-167	-4	8	9	92	87	-6	3	10	200	185	-4	4	11	107	-95
3	4	8	90	-81	-4	2	9	196	192	-2	8	9	134	-134	-5	3	10	76	54	-8	5	11	73	-77
4	4	8	81	87	-3	2	9	254	250	-1	8	9	103	-100	2	3	10	111	91	-7	5	11	76	-105
-9	5	8	187	187	4	2	9	108	103	-13	0	10	178	-189	-5	4	10	65	-43	-6	5	11	68	-72
-7	5	8	97	-123	-3	3	9	257	248	-12	0	10	130	-136	-4	4	10	81	60	-5	5	11	100	-103
-6	5	8	101	98	-2	3	9	137	123	-10	0	10	148	161	-1	4	10	84	78	-3	5	11	100	104
-4	5	8	337	-332	-1	3	9	185	-199	-8	0	10	111	137	0	4	10	84	-107	-7	6	11	75	-98
-2	5	8	243	249	0	3	9	230	-231	-5	0	10	135	146	1	4	10	143	-154	-6	6	11	104	-112
0	5	8	160	-164	4	3	9	74	82	-3	0	10	105	-122	-3	5	10	78	-84	-5	6	11	122	122
4	5	8	89	-87	-11	4	9	140	-142	-1	0	10	162	171	-2	5	10	126	-114	-4	6	11	200	198
-7	6	8	319	-320	-7	4	9	187	186	0	0	10	119	128	-5	6	10	100	-77	-9	0	12	182	-178
-6	6	8	115	-110	-6	4	9	72	61	1	0	10	128	-116	-4	6	10	141	-129	-7	0	12	174	180
-4	6	8	208	191	-4	4	9	268	-263	-13	1	10	76	-80	-5	7	10	87	-100	-6	0	12	105	137
-1	6	8	137	119	-3	4	9	155	-143	-11	1	10	189	187	-2	7	10	87	75	-5	0	12	119	-114
3	6	8	73	78	-1	4	9	96	114	-10	1	10	78	83	-11	1	11	98	141	-9	1	12	107	-75
-8	7	8	150	155	-11	5	9	96	-44	-8	1	10	76	-70	-5	1	11	179	178	-6	1	12	84	81
-6	7	8	201	-199	-10	5	9	110	-90	-4	1	10	110	114	-3	1	11	90	-82	-4	1	12	79	-74
-4	7	8	180	189	-7	5	9	112	115	-3	1	10	212	-217	-2	1	11	148	-168	-2	1	12	90	-81
-3	7	8	194	196	-3	5	9	148	-170	-2	1	10	257	-255	-7	2	11	139	138	-6	2	12	113	-116
0	7	8	83	28	-1	5	9	100	97	0	1	10	126	130	-5	2	11	166	-156	-6	3	12	124	128
-2	8	8	92	77	-5	6	9	99	72	2	1	10	166	-163	-4	2	11	86	-75	-4	3	12	106	-90
-7	9	8	128	113	-2	6	9	81	87															

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