Structural Studies on Bio-active Compounds. Part 11.[1] Molecular Modelling, Crystallographic, and Biochemical Studies of the Interactions of (±)-α-Vinylserine with the Enzyme Serine Hydroxymethyltransferase

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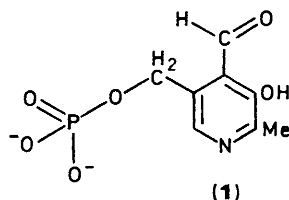
(±)-α-Vinylserine has previously been found to be a competitive inhibitor of serine hydroxymethyltransferase, an important target in anti-tumour chemotherapy. The crystal structure of (±)-α-vinylserine was determined by *X*-ray diffraction techniques, and molecular modelling was used to build the amino acid pyridoxal 5'-monophosphate Schiff's base. Molecular-mechanics calculations indicate that the reacting conformation for the cleavage of the α–β bond of the compound when bound to pyridoxal 5'-monophosphate is achievable. However, this compound, when incubated with homogeneous rabbit-liver serine hydroxymethyltransferase, was not dehydroxymethylated under any conditions, nor could any quinonoid intermediates be detected. Circular dichroism spectroscopy indicates that this molecule is not an effective enzyme inhibitor because it is not forming an imine base with the pyridoxal phosphate at the active site of the enzyme.

Serine hydroxymethyltransferase (EC 2.1.2.1), an enzyme which requires pyridoxal 5'-monophosphate (**1**), catalyses the interconversion of L-serine (**2**) and glycine (**3**) in the presence of tetrahydrofolate, generating a one-carbon unit in the form of N^5,N^{10}-methylenetetrahydrofolate.[2] This one-carbon unit is used in the biosynthesis of purines, pyrimidines, and methionine. The enzyme has been shown by many workers to be an apposite target for anticancer therapy.[3] While effective inhibitors for many other pyridoxalphosphate-requiring enzymes have been developed by a combination of serendipity and rational synthesis, few have been found for this enzyme. D-Fluoroalanine[4] (**4**) is a weak inhibitor with a K_i of 10–60 mmol dm^{-3}. L-α-Vinylserine (**5**) was rationally designed to be an enzyme-activated irreversible inhibitor of serine hydroxymethyltransferase, based on the knowledge that the enzyme is capable of dehydroxymethylating α-substituted serine analogues.[5] However, (±)-α-vinylserine was found to be a competitive inhibitor[5] with K_i 15.2 mmol dm^{-3}. The weakness of inhibition and the lack of time dependence may be attributable to one of three possible events occurring at the active site of the enzyme. (*a*) The compound may compete with L-serine for the active site but fail to bind *via* formation of an external aldimine. (*b*) The compound may bind to the active site pyridoxal phosphate without undergoing dehydroxymethylation. (*c*) The compound may bind and be dehydroxymethylated, but the product may be a Michael acceptor which reacts with other nucleophiles before it can react with the enzyme (or is unreactive). In order to distinguish between these hypotheses, the crystal structure of (±)-α-vinylserine has been determined, its mode of interaction with the enzyme has been predicted by molecular modelling, and the extent of the reaction with the enzyme has been investigated by biochemical techniques.



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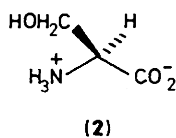

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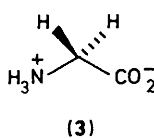

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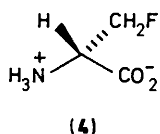

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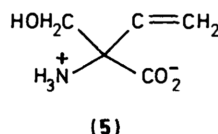

(**5**)

The likelihood of dehydroxymethylation was considered first. Dunathan[6] predicted that if an amino acid, bound to pyridoxal phosphate as an external aldimine, is to lose a group from its α-carbon, enhancement of the delocalisation energy of the system must occur. The greatest drop in energy will be achieved when the bond to be broken is in a plane perpendicular to the delocalised system, giving an overlap of the σ bond orbitals of the α–β bond and the π orbitals of the imine function.

Molecular modelling was used to construct the serine pyridoxal phosphate Schiff's base, as shown in Figure 1, starting from the crystal structure of pyridoxal 5'-phosphate as derived by Fujiwara.[7] When the serine N(6)–C(2) bond is rotated in increments of 10°, two relative energy minima appear (Figure 2). The conformation corresponding to one of these mimima (θ

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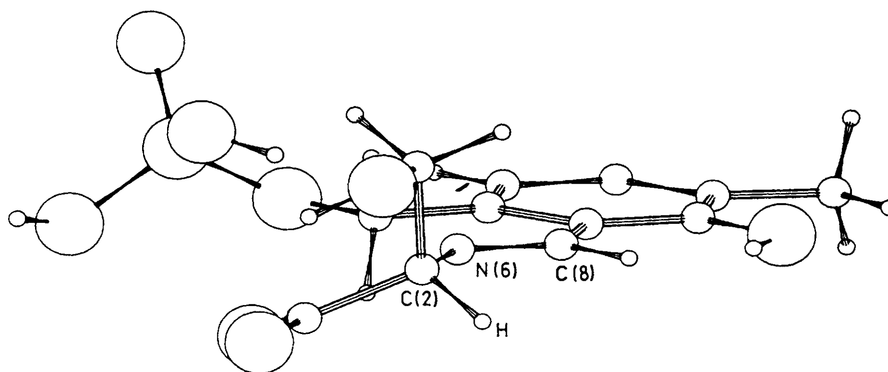

Figure 1. The modelled L-serine-pyridoxal phosphate Schiff's base.

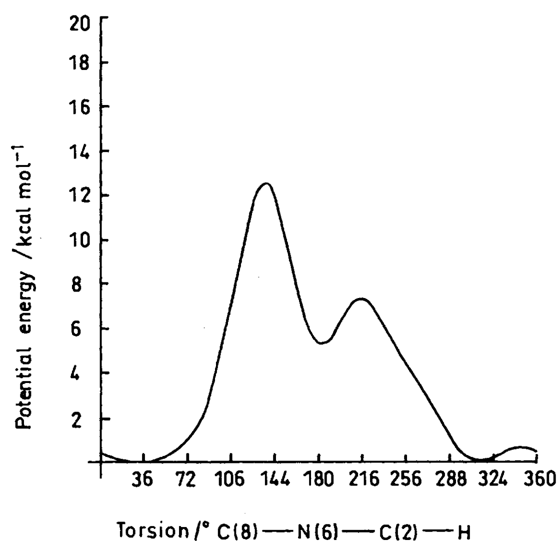

Figure 2. Effect of the rotation of the N(6)–C(2) bond of the modelled L-serine-pyridoxalphosphate Schiff's base on the potential energy of the model.

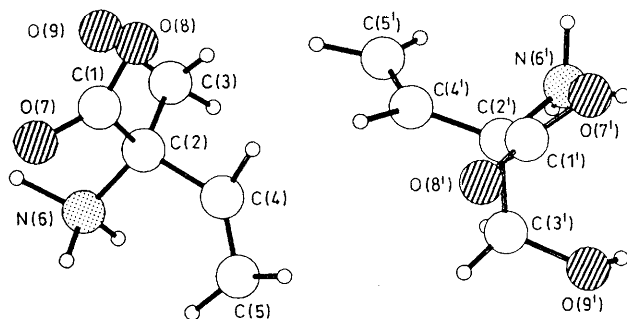

Figure 3. PLUTO drawing of the two structurally independent molecules of α-vinylserine in the asymmetric unit.

Table 1. Fractional co-ordinates with estimated standard deviations in parentheses.

Atom	x	y	z
C(1)	0.775 3(6)	0.257 2(6)	0.669 2(6)
C(2)	0.725 8(5)	0.241 1(6)	0.777 1(6)
C(3)	0.713 3(6)	0.370 8(7)	0.835 2(7)
C(4)	0.602 0(6)	0.179 2(8)	0.705 5(8)
C(5)	0.568 8(7)	0.069 5(9)	0.726 2(10)
N(6)	0.810 7(5)	0.158 6(4)	0.893 9(5)
O(7)	0.853 4(4)	0.180 5(4)	0.673 3(4)
O(8)	0.726 8(5)	0.344 9(5)	0.584 1(5)
O(9)	0.824 3(5)	0.440 0(4)	0.887 9(5)
C(1')	0.124 6(5)	0.209 1(6)	0.115 9(6)
C(2')	0.182 3(5)	0.243 8(6)	0.270 7(6)
C(3')	0.170 4(6)	0.130 6(6)	0.354 4(6)
C(4')	0.314 2(6)	0.275 3(7)	0.323 2(7)
C(5')	0.374 3(7)	0.364 9(7)	0.408 3(8)
N(6')	0.112 3(5)	0.355 7(5)	0.288 8(5)
O(7')	0.038 0(4)	0.276 4(4)	0.034 5(4)
O(8')	0.169 7(4)	0.116 8(4)	0.080 9(4)
O(9')	0.049 0(4)	0.093 6(4)	0.312 5(5)
H(1)	0.917	0.204	0.962
H(2)	0.818	0.067	0.861
H(3)	0.772	0.155	0.975
H(4)	0.663	0.371	0.904
H(5)	0.659	0.405	0.758
H(6)	0.559	0.239	0.623
H(7)	0.494	0.062	0.640
H(8)	0.638	0.028	0.833
H(9)	0.854	0.455	0.968
H(1')	0.021	0.337	0.199
H(2')	0.142	0.350	0.401
H(3')	0.117	0.444	0.247
H(4')	0.217	0.053	0.356
H(5')	0.210	0.156	0.438
H(6')	0.367	0.216	0.261
H(7')	0.469	0.371	0.443
H(8')	0.338	0.426	0.459
H(9')	0.015	0.136	0.362

320°) has the scissile bond nearly perpendicular to the π system and is very similar to the preferred reacting conformation for the cleavage of the α–β bond.[6] Other bonds were kept fixed in this study. ^{31}P N.m.r. studies have shown that no change in the environment of the phosphate group occurs when pyridoxal phosphate binds L-serine.[8] The fixed phosphate position is responsible for the apparent high-energy interaction when the torsion angle is *ca.* 130°, but this is irrelevant to the successful construction of a model with low energy as well as favourable geometry for dehydroxymethylation.

The *X*-ray diffraction crystal structure of (±)-α-vinylserine was determined by direct methods and refined by the full-matrix least-squares method. The presented data for the zwitterionic compound confirm the inferences from spectroscopic data,[5] with bond angles and lengths being essentially as predicted. The study reveals two structurally independent but nearly superimposable molecules as shown in Figure 3. Both have the carboxy group and C(2)–N(6) bond in the same plane, with one hydrogen atom of the ammonium group being in a near-eclipsed conformation that favours intramolecular hydrogen bonding (Table 5). Corresponding bond distances in the two molecules do not differ significantly ($\Delta < 1.7\sigma$) from each other. Differences from those in (±)-serine, which was refined from neutron-diffraction data by Frey *et al.*[9] starting with the co-ordinates proposed by Shoemaker et al.,[10] are slight. The

Table 2. Bond lengths/Å for non-hydrogen atoms in (±)-α-vinylserine with estimated standard deviations given in parentheses.

	Unprimed	Primed
C(1)–C(2)	1.545(8)	1.533(8)
C(1)–O(7)	1.238(7)	1.249(7)
C(1)–O(8)	1.242(7)	1.252(7)
C(2)–C(3)	1.528(9)	1.533(9)
C(2)–C(4)	1.513(9)	1.501(9)
C(2)–N(6)	1.496(8)	1.515(7)
C(3)–O(9)	1.423(8)	1.413(8)
C(4)–C(5)	1.271(11)	1.287(10)

Table 3. Bond angles/° for non-hydrogen atoms in (±)-α-vinylserine with estimated standard deviations given in parentheses.

	Unprimed	Primed
C(2)–C(1)–O(7)	117.8(5)	117.6(5)
C(2)–C(1)–O(8)	114.5(4)	117.1(5)
O(7)–C(1)–O(8)	127.7(6)	125.3(6)
C(1)–C(2)–C(3)	110.9(5)	109.7(5)
C(1)–C(2)–C(4)	108.3(4)	110.9(5)
C(1)–C(2)–N(6)	109.3(4)	107.9(5)
C(3)–C(2)–C(4)	108.9(5)	108.9(5)
C(3)–C(2)–N(6)	109.1(5)	108.0(5)
C(4)–C(2)–N(6)	110.3(5)	111.5(5)
C(2)–C(3)–O(9)	111.0(5)	113.1(5)
C(2)–C(4)–C(5)	129.0(7)	127.6(7)

Table 4. Torsion angles/° for all non-hydrogen atoms in (±)-α-vinylserine.

	Unprimed	Primed
O(7)–C(1)–C(2)–C(3)	137.7	119.15
O(7)–C(1)–C(2)–N(6)	17.4	2.2
O(8)–C(1)–C(2)–C(4)	74.4	58.6
C(1)–C(2)–C(4)–C(5)	114.1	139.8
C(3)–C(2)–C(4)–C(5)	−125.3	−99.4
O(7)–C(1)–C(2)–C(4)	−102.9	−120.2
O(8)–C(1)–C(2)–C(3)	−45.0	−61.7
O(8)–C(1)–C(2)–N(6)	−165.3	−179.1
N(6)–C(2)–C(3)–O(9)	67.0	58.9
N(6)–C(2)–C(4)–C(5)	−5.6	19.6

The estimated standard deviations range from 0.7–1.0°.

Table 5. Hydrogen bonds present in the unit cell of α-vinylserine.

Bond	Distance (Å)			Angle (°)
a–b···c	a–b	b–c	a···c	a–b–c
N(6)–H(1)···O(7′^{I})	1.27	1.54	2.806	172
N(6)–H(2)···O(8′^{II})	1.04	2.00	2.888	141
N(6)–H(3)···O(8)	1.16	1.51	2.677	177
N(6′)–H(1′)···O(7^{III})	1.13	1.96	2.888	136
N(6′)–H(2′)···O(8′)	1.10	1.84	2.910	164
N(6′)–H(3′)···O(9^{IV})	1.04	2.25	3.182	149
O(9)–H(9)···O(7^{V})	0.79	2.63	3.189	130
O(9)–H(9)···O(7′^{I})	0.79	2.77	2.935	94
O(9′)–H(9′)···O(7′^{V})	0.92	1.97	2.803	149

Symmetry code: (I) $1 + x, y, 1 + z$; (II) $1 - x, -y, 1 - z$; (III) $-1 + x, \frac{1}{2} - y, -\frac{1}{2} + z$; (IV) $1 - x, 1 - y, 1 - z$; (V) $x, \frac{1}{2} - y, \frac{1}{2} + z$.

fractional co-ordinates for (±)-α-vinylserine are given in Table 1, and the bond lengths and angles in Tables 2 and 3 respectively. Torsion angles are given in Table 4.

Similar conformational calculations were then carried out for the modelled L-α-vinylserine–pyridoxal 5′-phosphate imine (Figure 4). The C(2)–C(4) and N(6)–C(2) bonds were rotated simultaneously and the potential energy was calculated. The data are presented in the form of a contour plot in Figure 5. Relative minima of 1 kcal mol^{-1} above the global minimum are observed at conformations (300°, 330°) and (70°, 20°). The former corresponds to the required conformation for cleavage of the α–β bond.[6] This indicates that, for a modelled system, there are no conformational energy restrictions which inhibit the dehydroxymethylation of L-α-vinylserine. It is unknown whether this conformation can occur in the active site of the enzyme. Although calculations suggest that both serine and α-vinylserine would be able to be dehydroxymethylated by serine hydroxymethyltransferase the crystallographic packing of these two racemic compounds differs markedly. Four pairs of isomers of (±)-α-vinylserine are found in the unit cell, with the enantiomers bound together by hydrogen bonds of which four have a H···O distance <2.3 Å, as shown in Table 5. In the crystalline state, (±)-α-vinylserine is orientated such that the polar carboxylate, alcohol and ammonium groups point towards the edge of the unit cell. The vinyl groups are directed towards the centre of the unit cell, creating alternate polar and non-polar planes extending throughout the crystal. This packing is different to that found in (±)-serine,[9,10] where the unit cell was found to contain sheets of serine stacked parallel to the [100] face.

In order to investigate whether (±)-α-vinylserine is dehydroxymethylated by the enzyme, the compound (20 mmol dm^{-3}) was incubated with homogeneous rabbit-liver cytosolic serine hydroxymethyltransferase and tetrahydrofolate over a period of 1 h. No production of methylenetetrahydrofolate could be detected under any conditions, suggesting that no cleavage of the α–β bond was occurring with this compound. This hypothesis was then further investigated using u.v. spectroscopy. The u.v. absorption spectrum of the enzyme has been studied and the intermediates produced on dehydroxymethylation have been characterised.[11] It is known that the quinonoid intermediate produced upon α–β bond cleavage has a characteristic absorption at 500 nm;[12] this can be amplified by the addition of tetrahydrofolate, which inhibits the re-protonation of the quinonoid.[13] When (±)-α-vinylserine (20 mmol dm^{-3}) was incubated with the rabbit-liver enzyme, no change in the u.v. absorption spectrum occurred and no quinonoid intermediate was detected. The effect of incubating the enzyme with glycine (5 mmol dm^{-3}) and tetrahydrofolate (0.7 mmol dm^{-3}) is shown in Figure 6.

Thus α-vinylserine is not bio-activated. This failure may imply that the compound binds to the active site of the enzyme without cleavage of the α–β bond, or the compound may not even bind to the active site. Circular dichroism (c.d.) spectroscopy was used to investigate these alternative explanations. Although pyridoxal phosphate is achiral, it has an induced optical activity when bound in the asymmetric environment of an enzyme.[14] In pyridoxal-phosphate-dependent enzymes a strong positive Cotton effect is observed due to $\pi \rightarrow \pi^*$ transitions in the aldimine chromophore.[14] The c.d. spectra of cystosolic rabbit-liver enzyme[8] and the *Escherichia coli* enzyme[15] have this absorption band present. Conversion of the internal aldimine into the external aldimine induces a change in the 320–500 nm region of the c.d. spectrum.[15] The effect of 50 mmol dm^{-3} (±)-α-vinylserine on the c.d. spectrum is shown in Figure 7. Similar results were obtained for the buffer control. The drop in the $\theta_{max.}$ is due to a dilution effect and indicates that no new aldimine bond is being formed by this compound, *i.e.* that the compound does not bind covalently to the pyridoxal phosphate in the active site of the enzyme. This is unlike the effect of the addition of 50 mmol dm^{-3} glycine, a positive control. This amino acid is forming a new aldimine bond and therefore changes the c.d. spectrum. The finding that (±)-α-vinylserine is not a substrate due to the inability of the

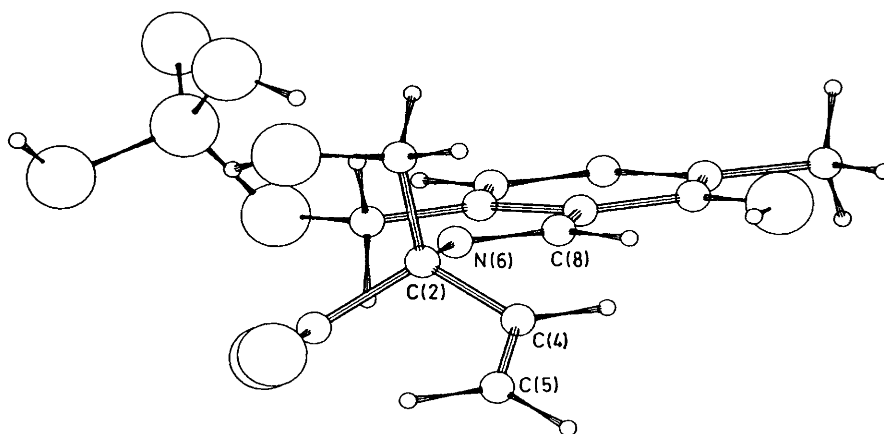

Figure 4. The modelled α-vinylserine–pyridoxal phosphate Schiff's base.

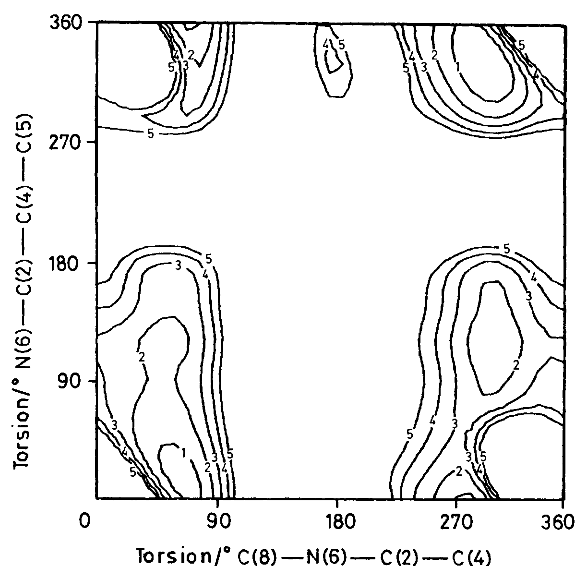

Figure 5. Effect of the simultaneous rotation of the C(2)–C(4) and N(6)–C(2) bonds of the modelled α-vinylserine–pyridoxal phosphate on the potential energy of the model (contours in kcal mol^{-1}).

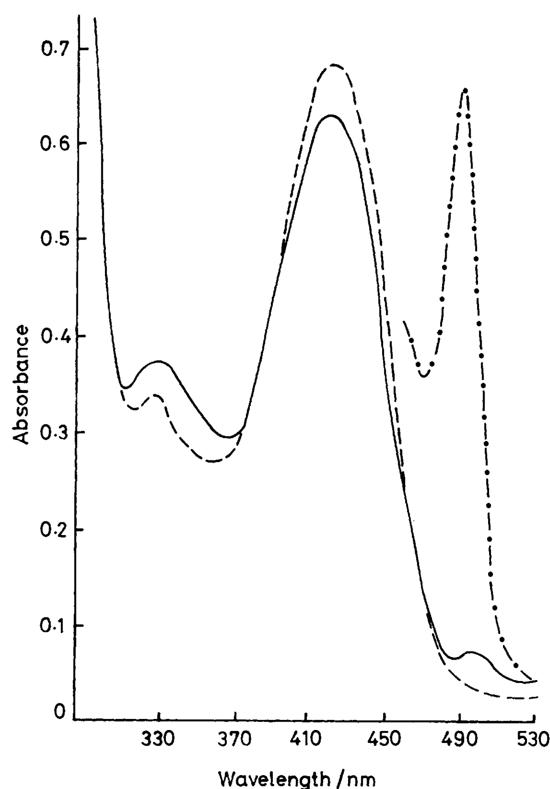

Figure 6. Effect of the addition of glycine 5 mmol dm^{-3} (– – – –) and then tetrahydrofolate (0.7 mmol dm^{-3}) (– · – · –) on the absorption of purified rabbit-liver serine hydroxymethyltransferase (——).

compound to bind covalently to the pyridoxal phosphate at the active site of the enzyme is unexpected. It is known that other α-vinylamino acids are enzyme-activated irreversible inhibitors of pyridoxal phosphate-dependent enzymes, *e.g.* α-vinyldopa inhibits dopa decarboxylase.[16] The mechanism of transimination is well known[17] and there is no apparent reason why it should not occur with α-vinylserine, especially as it is known that serine hydroxymethyltransferase can dehydroxymethylate α-substituted serines such as α-methyl-, α-ethyl-, and α-hydroxymethylserine.[18] These amino acids have α-substituents that are approximately isosteric with the vinyl group. The reasons for the lack of binding of this compound is unknown.

This work suggests that the design and synthesis of new α-substituted serine analogues as potential inhibitors of serine hydroxymethyltransferase may not yield effective inhibitors of this enzyme. It is, however, noted that α-fluoromethylserine with its small α-substituent may be an active inhibitor and routes to the synthesis of this compound are being investigated in this laboratory.

Experimental

Preparation of (±)-*α-Vinylserine* [(±)-2-*Amino*-2-*hydroxymethylbut*-3-*enoic Acid*].—(±)-α-Vinylserine was prepared as previously described.[5] The compound (100 mg) was dissolved in water (10 cm^3) and warmed gently. Hot ethanol (20 cm^3) was added and the solution was allowed to stand for 14 days at ambient temperature. The crystalline florets of needles were separated by sonicating the suspension for 2 × 5 s. The suspension was then filtered and the crystals dried *in vacuo* for 3 days. A specimen 0.7 × 0.07 × 0.055 mm was mounted about the needle axis on a glass fibre for collection of *X*-ray diffraction data.

Crystal Data.—$C_5H_9NO_3$, M = 131.1, monoclinic, a = 12.346(3), b = 10.442(4), c = 10.763(5) Å, β = 116.70(3), V = 1 239.7 Å^3 (by least squares analysis of setting angles of 25 reflections, λ = 0.710 69 Å), space group $P2_1/c$, Z = 8,

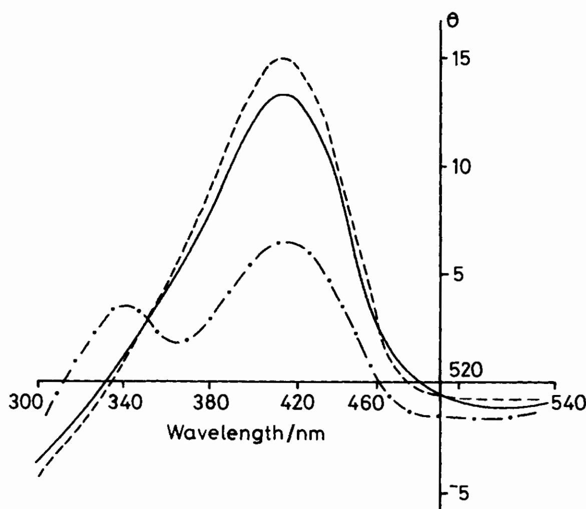

Figure 7. Effect of the addition of amino acids on the c.d. absorption spectrum of purified rabbit-liver serine hydroxymethyltransferase; (- - - -) enzyme alone; (——) effect of addition of buffer alone or α-vinylserine (50 mmol dm^{-3}); (- · - · -) effect of the addition of glycine (50 mmol dm^{-3}).

D_m = 1.41 g cm^{-3}, D_x = 1.42 g cm^{-3}, μ(Mo-K_α) = 0.75 cm^{-1}, $F(000)$ = 560.

Data Collection and Processing.—Enraf–Nonius four circle CAD-4 diffractometer, ω/2θ scans, ω scan width (1.0 + 0.35 tanθ)°, ω scan speed of 0.5–5° min^{-1} depending on intensity, graphite monochromated Mo-K_α), 4 612 reflections measured (2 < θ < 25°), 2 177 unique (merging R 0.0561). 1 037 Reflections deemed observed with $|F_o| > 3\sigma(|F_o|)$. Standard deviations were calculated on the basis of counting statistics and instrument instability. Intensity and orientation monitor reflections indicated no need to correct for time-dependent instability. Maximum (sinθ)/λ reached was 0.63 with range h, k, l, 0–12, −14 to 14, and −12 to 12 respectively. No correction was made for absorption.

Structure Analysis and Refinement.—The crystal structure was solved by direct methods using the SHELX-76 program;[19] hydrogen atoms were located in difference electron density maps. Non-hydrogen atom positions and anisotropic thermal parameters together with group isotropic temperature factors for the hydrogen atoms were refined by the full-matrix least-squares technique based on the stored scattering factors (using the same temperature factors for structurally equivalent hydrogen atoms in the two independent molecules comprising the asymmetric unit).* The weighting scheme $w = (\sigma^2(|F_o|) + 0.008|F_o|^2)^{-1}$ gave satisfactory agreement analyses. The final R and R_w values are 0.0648 and 0.0693 respectively, with a goodness-of-fit ratio of S = 1.35. Molecular drawings were obtained using the PLUTO program developed by Motherwell and Clegg.[20]

Molecular Calculations.—Molecular-mechanics conformational-energy calculations were performed using the Glaxo Group Research molecular modelling system. The structure of L-serine was taken from fragments constructed from the internal database of the system. The crystal structure of pyridoxal 5′-phosphate derived by Fujiwara[7] was obtained from the Cambridge Crystallographic Database.[21] Conformational-energy calculations were performed by the summation of the individual components for the non-bonded and torsional energies.[22]

Enzyme Purification and Activity Assays.—The cytosolic rabbit-liver serine hydroxymethyltransferase was purified as previously described.[23] The purity of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis and the enzyme found to be a single band with the same R_F value as that previously obtained.[23] The determination of enzyme activity and the detection of the dehydroxymethylation of (±)-α-vinylserine utilised a coupled assay involving N^5,N^{10}-methylenetetrahydrofolate dehydrogenase as described by Schirch *et al.*[24]

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 rabbit-liver enzyme (2 mg ml^{-1}) in 50 mmol dm^{-3} potassium phosphate pH 7.3 was recorded between 300–530 nm against buffer alone. The amino acid under test dissolved in the buffer was then added and the absorption spectrum of the enzyme was re-recorded during 1 h.

In order to amplify the production of the stabilised quinonoid intermediate, 0.7 mmol dm^{-3} tetrahydrofolate and 30 mmol dm^{-3} 2-mercaptoethanol was added and the cuvette was sealed. The absorption spectrum between 460–530 nm was re-recorded over a time period of 30 min.

C.d. spectra were recorded on a Jasco J-500C spectropolarimeter. A 1 cm pathlength cell was used with all buffers degassed prior to use. The c.d. spectrum of homogeneous cytosolic rabbit-liver enzyme (3.1 mg cm^{-3}) in 50 mmol dm^{-3} potassium phoshate pH 7.3 was recorded between 300–530 nm at a scan rate of 50 nm min^{-1}. The amino acid under test (5 mmol dm^{-3} in buffer) was added to the cell and the spectrum was re-recorded.

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* *Supplementary data* (see section 5.6.3 of Instructions for Authors, in the January issue). Thermal parameters have been deposited at the Cambridge Crystallographic Data Centre.

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