Dihydropteroate Synthase: Purification by Affinity Chromatography and Mechanism of Action [1]

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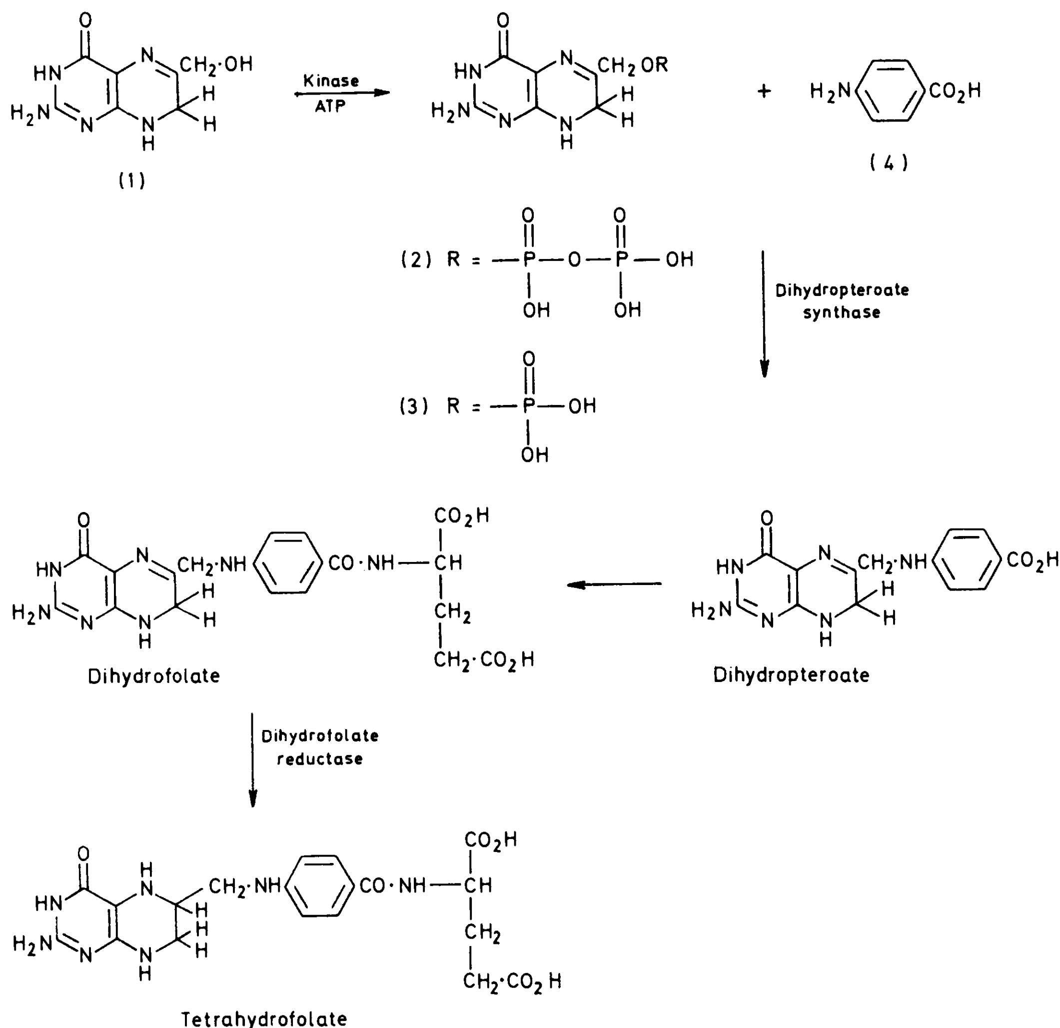
The purification of dihydropteroate synthase by affinity chromatography on a sulphonamide bound to agarose is described. The discovery that binding to the affinity column occurs only in the presence of the co-substrate, the pteridine diphosphate (2), or the corresponding monophosphate (3), is discussed in relation to an ordered kinetic mechanism of action of the enzyme.

THE enzymes involved in the biosynthesis of tetrahydrofolate by bacteria and other parasites (Scheme) have proved to be of especial significance in chemotherapy. Thus the powerful antibacterial agent Septrin consists of two components, trimethoprim (5) and a sulphonamide (sulphamethoxazole), which are specific inhibitors of two of these enzymes, dihydrofolate reductase and dihydropteroate synthase, respectively. The design of second generation inhibitors of this type will be facilitated by a detailed understanding of the structure and properties of the enzyme in question. Such studies are well under way with dihydrofolate reductase. Purification of the enzyme has been achieved in several laboratories,[2] the



SCHEME

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primary structure of the enzyme from *Escherichia coli* has been established,[3] and crystallographic studies to elucidate the conformation of the enzyme are being carried out.[4] It seemed appropriate that a similar study of dihydropteroate synthase should be undertaken. Since purification of the enzyme by classical techniques has been achieved only with difficulty,[5] we have investigated the purification of the enzyme by the biospecific technique of affinity chromatography.[6] We have also carried out some studies of the mechanism of action of the enzyme.

Affinity Chromatography.—Dihydropteroate synthase (E.C. 2.5.1.15) catalyses the reaction between 2-amino-7,8-dihydro-6-hydroxymethylpteridin-4-one diphosphate (2) and *p*-aminobenzoic acid (4).[5] The sulphonamide (6) [7] is a potent competitive inhibitor of dihydropteroate synthase (K_i 8 × 10^{-7} mol l^{-1}), and possesses a suitable functional group for attachment to the solid support without impairing the specific inhibitor–enzyme interaction.

Agarose in the form of the beaded derivative Sepharose-4B was activated with bromine cyanide [8] and coupled to the spacer arm bis-(3-aminopropyl)amine.[9] The sulphonamide (6) was condensed with the Sepharose conjugate by using 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate in aqueous dimethylformamide. The product gel contained 1.7 ± 0.8 μmol of inhibitor per ml of settled gel as shown by sulphur analysis.

Despite the potency of the sulphonamide (6) as a competitive inhibitor, crude enzyme from *Escherichia coli* preparations in either 0.2M-Tris–HCl buffer, pH 8.5, or 0.1M-potassium phosphate buffer, pH 8.0, did not bind to a 60 × 8 mm column of the sulphonamide-Sepharose (Figure 1). Both protein and enzyme activity were eluted essentially together, although the enzyme was slightly retarded by the column. However, when either buffer contained additionally 18μM-2-amino-7,8-dihydro-6-hydroxymethylpteridin-4-one diphosphate (2) and 5mM-dithiothreitol to inhibit oxidation of the dihydropteridine diphosphate, essentially all the enzyme activity was retained on the column while the bulk of the protein was eluted. Immediately upon removal of the dihydropteridine diphosphate (2) and the dithiothreitol from the buffer, enzyme activity emerged from the column (Figure 2). The obligatory presence of the dihydropteridine diphosphate (2) in the enzyme solution applied to the column and the elution effected by its removal are good evidence for the biospecificity of the process. The eluted enzyme represents a purification of 180-fold in one step.

An improved purification has been achieved by using the same inhibitor and spacer arm attached to Sepharose through an *s*-triazine derivative [10] rather than by activation of the Sepharose with bromine cyanide. The required sulphonamide–Sepharose derivative was prepared in this case by (*a*) the reaction of 2,4-dichloro-6-methoxy-*s*-triazine (7) with Sepharose under mildly basic conditions, (*b*) condensation of the resulting triazinylagarose (8) with bis-(3-aminopropyl)amine in

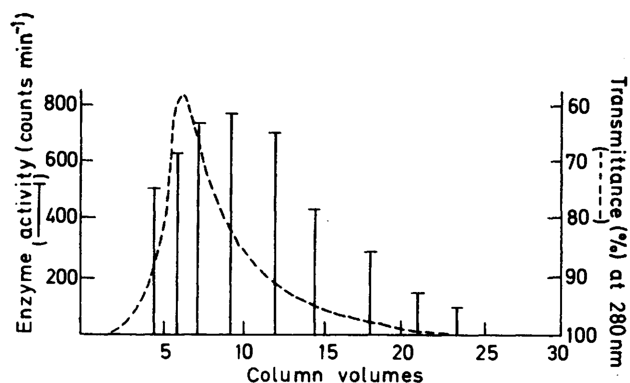

FIGURE 1

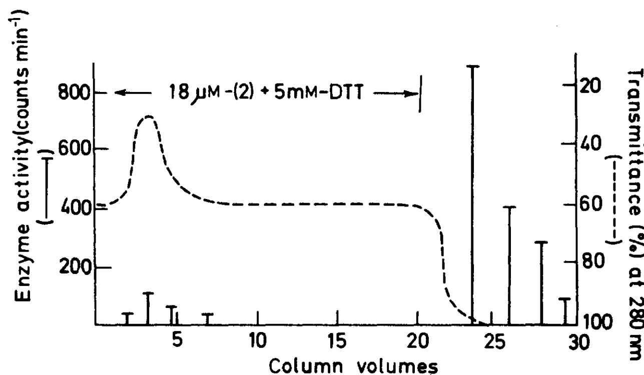

FIGURE 2

aqueous solution at pH 8.5, and (*c*) reaction of the 4-aminoalkylamino-6-methoxy-*s*-triazin-2-ylagarose (9) with the sulphonamide (6) by using a water-soluble carbodi-imide as described above. The product gel thus obtained contained 4—5 μmol of inhibitor per ml of settled gel. This material proved very effective in an affinity column for purification of dihydropteroate synthase, a purification of 730-fold being obtained under conditions identical with those described above.

That a phosphate ester is important for inducing a binding site on the enzyme for the sulphonamide, and presumably also for *p*-aminobenzoic acid since sulphonamides are competitive inhibitors, was shown by the fact that only the dihydropteridine diphosphate (2) and the corresponding monophosphate (3) were able to promote

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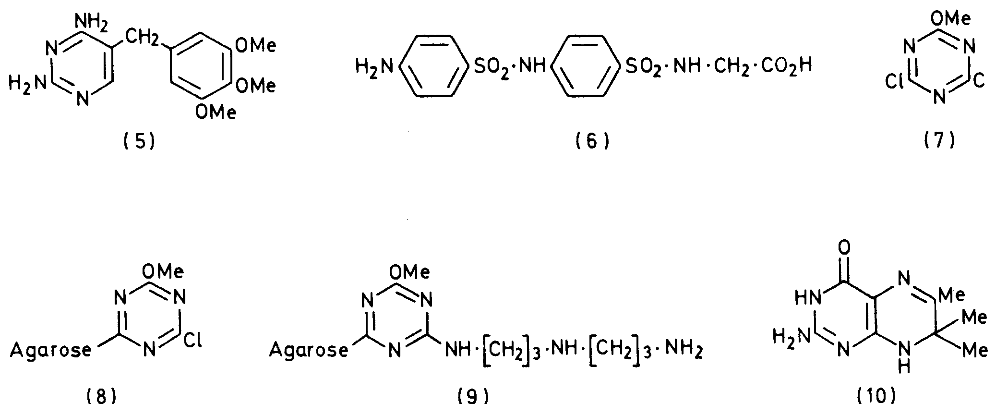
successful affinity chromatography of the enzyme as described above. Other compounds tested did not effect binding of the enzyme to the column (Table).

We also wished to establish whether or not a column prepared with *p*-aminobenzoic acid, one of the substrates of the enzyme, as ligand could function successfully in affinity chromatography. A column of *p*-aminobenzoic acid–Sepharose, prepared as described from Sepharose activated with bromine cyanide, did not bind dihydropteroate synthase under conditions identical with those which were successful with the sulphonamide–Sepharose column. This may have been due to the low degree of substitution (0.1 μmol ml^{-1}) of ligand to Sepharose achieved in this case, but more probably reflects the fact that amides derived from *p*-aminobenzoic acid have little antibacterial activity.[11]



Induction of enzyme binding

Compound	Binding of enzyme	Concentration in buffer (M)
(2)	Yes	1.8×10^{-5}
(3)	Yes	5.9×10^{-4}
Pyrophosphoric acid (PP_i)	No	1.0×10^{-3}
(1) + PP_i	No	(1) 1.2×10^{-3}; PP_i 2.4×10^{-3}
(5)	No	8.0×10^{-5}
(10)	No	4.0×10^{-5}

Although all the foregoing evidence is consistent with a biospecific process, it was important to demonstrate that the spacer arm and Sepharose itself do not contribute any non-specific binding of the enzyme. Surprisingly, a blank column composed of bis-(3-aminopropyl) amine only linked to Sepharose activated by bromine cyanide retained completely all protein and all enzyme activity. However, this column did not retain enzyme activity when the buffer contained dihydropteridine diphosphate (2) and dithiothreitol.

Mechanism of Action.—It appears that the co-substrate dihydropteridine diphosphate (2) induces a binding site on the enzyme for the second substrate, *p*-aminobenzoic acid. This implies that an ordered binding sequence is required by dihydropteroate synthase in which the dihydropteridine diphosphate (2) binds to the enzyme before *p*-aminobenzoic acid or sulphonamide. This sequence is summarised in the Cleland [12] diagram (Figure 3). Recent detailed studies of the kinetics of the enzymic reaction [13] support our mechanistic interpretation.

Similar results have been reported for both kinetic [14] and affinity chromatography [15] studies on lactate dehydrogenase although in this case the existence of an ordered mechanism was known *before* the affinity chromatography experiments were carried out. Our results therefore show the potential of affinity chromatography in the elucidation of aspects of the mechanism of enzyme action.

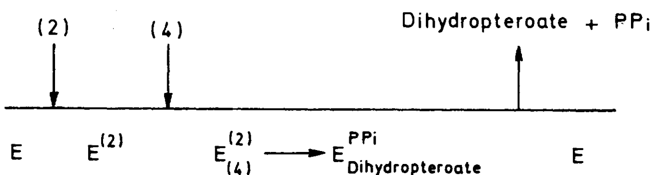


FIGURE 3 Cleland digram

Finally, an ordered mechanism for dihydropteroate synthase has an important bearing upon the mode of action of the antibacterial sulphonamides and the implications of this are under investigation.

EXPERIMENTAL

Substrates and Inhibitors.—The following compounds were prepared by literature methods: 2-amino-6-hydroxymethylpteridin-4-one by synthesis of D- or L-neopterin,[16] treatment with periodate,[17] and reduction of the 6-formylpteridine produced with sodium borohydride; [18] the corresponding pteridine monophosphate and diphosphate by treatment of the pteridine with polyphosphoric acid and separation of the mixed phosphates by ion-exchange chromatography.[18] The pteridines were converted into the corresponding 7,8-dihydro-derivatives by reduction with sodium

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[13] R. Ferone and S. R. Webb in ' Chemistry and Biology of Pteridines,' ed. W. Pfleiderer, de Gruyter, Berlin, 1975, p. 61.
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[15] P. O'Carra and S. Barry, *F.E.B.S. Letters*, 1972, **21**, 281.
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[17] M. Viscontini and J. Bieri, *Helv. Chim. Acta*, 1971, **54**, 2291.
[18] M. Viscontini and Y. Furuta, *Helv. Chim. Acta*, 1973, **56**, 1710.

dithionite to give 2-amino-7,8-dihydro-6-hydroxymethylpteridine-4-one [19] (1) and the corresponding monophosphate [18] (3) and diphosphate [18] (2).

4-(4-Aminophenylsulphonylamino)phenylsulphonylaminoglycine (6) was obtained by acidic hydrolysis [7] of the corresponding acetamido-derivative, itself prepared by the reaction [20] of 4-acetamidobenzenesulphonyl chloride with 4-aminophenylsulphonylaminoglycine. 2-Amino-7,8-dihydro-6,7,7-trimethylpteridine-4-one (10) was obtained by a modification of a published method.[21] Trimethoprim (5) was a gift from Burroughs Wellcome Co.

Materials for Affinity Chromatography.—(*a*) Agarose in the form of Sepharose-4B was activated with bromine cyanide and coupled to bis-(3-aminopropyl)amine by the published methods.[8,9] The resulting 3-(3-aminopropyl)aminopropylaminoagarose (12 ml; settled volume) was washed on a Buchner funnel with 50% aqueous dimethylformamide and sucked dry. The filter cake was resuspended in 50% aqueous dimethylformamide (20 ml) and the sulphonamide (6) (180 mg) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide methotoluene-*p*-sulphonate (700 mg) were added. The suspension was shaken gently at room temperature for 22 h and then washed with 50% aqueous dimethylformamide (200 ml), methanol (200 ml), and water (200 ml).

A sample (1 ml) was washed with acetone, dried, and weighed. Sulphur analysis indicated the presence of 1.7 $\pm$ 0.8 μmol of sulphonamide per ml of settled gel.

(*b*) With T. LANG. 4-*Chloro*-6-*methoxy*-s-*triazin*-2-*ylagarose* (8). Dioxan-washed agarose (10 g) was added to a solution of 2,4-dichloro-6-methoxy-*s*-triazine [22] (0.45 g, 0.002 5 mol) in dioxan (20 ml), and the suspension was stirred for 15 min at room temperature. Water (20 ml) was added followed by 2M-sodium carbonate (2 ml). The temperature was increased to 25—30 °C and M-sodium hydroxide (4 ml) was added dropwise over 10 min. After stirring for a further 20 min, the mixture was acidified to pH 6 with M-hydrochloric acid. The triazinylagarose was collected, and washed with dioxan (100 ml), 0.1M-sodium chloride (500 ml), and water (250 ml) (Found: N, 1.9; Cl, 2.0%. Calc. N: Cl 1.18 : 1).

4-[3-(3-*Aminopropyl*)*aminopropylamino*]-6-*methoxy*-s-*triazin*-2-*ylagarose* (9). The 4-chlorotriazinylagarose (8) (*ca.* 9 g) was stirred in a solution of bis-(3-aminopropyl)amine (1.31 g, 0.01 mol) and concentrated hydrochloric acid in water (20 ml) (pH 8.5) for 24 h at room temperature. The triazinylagarose was collected, washed with water (250 ml), a mixture of 0.01M-hydrochloric acid and 0.1M-sodium chloride (50 ml), water (50 ml), 0.01M-sodium hydroxide and 0.1M-sodium chloride (50 ml), 0.1M-sodium chloride (400 ml), and water (250 ml) (Found: N, 2.9; Cl, 0.0%).

Reaction of the triazinylagarose (9) *with the sulphonamide* (6). The sulphonamide (6) hydrochloride (100 mg), the triazinylagarose (9) (5.5 ml settled volume), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate (200 mg) were shaken gently for 48 h at room temperature in 50% aqueous dimethylformamide (20 ml). The suspension was filtered and the product gel was washed with water (200 ml), 50% aqueous dimethylformamide (200 ml), methanol (200 ml), and water (100 ml). Sulphur analysis on a dried sample showed the presence of 4.5 $\pm$ 0.8 μmol of sulphonamide per ml of settled gel. Before use in affinity chromatography this material was washed with dilute sodium hydroxide to generate the free base and then with water.

(*c*) *Coupling of* p-*aminobenzoic acid to Sepharose*. Agarose was activated with bromine cyanide and coupled to bis-(3-aminopropyl)amine as in (a). The resulting 3-(3-aminopropyl)aminopropylaminoagarose (6 ml; settled volume), *p*-aminobenzoic acid (130 mg), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate (450 mg) in 30% aqueous dioxan (30 ml) were shaken gently for 24 h. The product gel was washed as in (a). The degree of substitution (estimated by azo-dye formation) was 0.12 μmol of ligand per ml of settled gel.

Enzyme Assays.—The crude protein preparations were (*a*) a 0—50% ammonium sulphate fraction from *E. coli*, (*b*) an acetone powder prepared from *E. coli*, and (*c*) a freeze-dried preparation from *E. coli* from which 6-hydroxymethyldihydropteridine pyrophosphate kinase had been removed by gel filtration.

Protein was determined by Lowry's method.[23] Enzyme activity was assayed by using [^{14}C]-*p*-aminobenzoic acid.[5] Scintillation counting was carried out by using Bray's solution.[24] Enzyme-inhibitor dissociation constants (K_i) were determined according to Dixon's method.[25]

Affinity Chromatography.—A typical experiment was as follows. A column of sulphonamide-Sepharose (60 × 8 mm) was equilibrated with Tris–HCl buffer (0.2M; pH 8.5) containing dihydropteridine diphosphate (18μM), dithiothreitol (DTT) (5mM), and magnesium chloride (5mM). To the column was applied crude enzyme [preparation (*a*)] in this buffer (10 mg in 1.6 ml). This buffer was used to wash the column until sixteen 2 ml fractions had been collected. Washing was then continued with Tris–HCl buffer (0.2M; pH 8.5) without any additives. Samples (0.1 ml) of the various fractions were assayed for dihydropteroate synthase activity (1 h at 30 °C). Activity was found chiefly in fractions 20—22.

A column which had been in constant use for 4½ months performed essentially as described above.

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