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We have satisfactorily purified the two crucial enzymes of dihydropteroate synthetase and dihydrofolate synthetase several fold from different strains of meningococci and gonococci (different levels of sulfonamide resistance). However, for some unknown reasons that we are currently exploring intensively, these two enzymes showed extreme instability toward the usual ion-exchange purifications. The allosterism as observed with the enzyme of dihydropteroate synthetase of gonococcal extract may have contributed to such a failure, or is the structure of such enzymes so fragile that they are easily disrupted even by such mild chemical treatment?

However, by using the partially purified enzyme of dihydropteroate synthetase extracts we have examined the efficacy of a substantial number of sulfur derivatives, new and old and in vitro systems. We feel we can safely conclude that based on such structure-activity relationship studies that the modifications of sulfur containing molecules as inhibitors hold exciting chemotherapeutic promise.

In order to prove the allosteric roles of dihydropteroate synthetase and dihydrofolate synthetase in the phenomenon of synergism, or have the structure-activity relationship being more accurately interpreted, highly purified (homogeneous) preparations must be obtained. So far, we have developed an affinity column chromatographic technique for the enzyme of dihydropteroate synthetase. The preparation of the column has been successful. We are now in a unique position to explore such a purification in great detail.

We have demonstrated the activity of dihydrofolate synthetase in meningococcal and gonococcal extracts and feel that this hitherto ignored intermediate enzyme may be crucial to an understanding of the effect of drugs on folic acid biosynthesis. However, more experimental data must be obtained about this enzyme before an affinity chromatography technique can be developed.

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SUMMARY

The purpose of this work is to provide a rational chemotherapeutic approach to diseases of bacterial origin. It focuses on gonorrhoea and meningitis but has wider applicability. The methodology involves molecular considerations and therefore requires highly purified enzymes that are responsible for folic acid biosynthesis, and the synthesis and biological evaluations in cell-free systems as well as in vitro of new and complex sulfur containing drugs.

We have satisfactorily purified the two crucial enzymes of dihydropteroate synthetase and dihydrofolate synthetase several fold from different strains of meningococci and gonococci (different levels of sulfonamide resistance).

However, by using the partially purified enzyme extracts of dihydropteroate synthetase we have examined the efficacy of a substantial number of sulfur derivatives, new and old and in vitro systems. We feel we can safely conclude that based on such structure-activity relationship studies that the modifications of sulfur containing molecules as inhibitors hold exciting chemotherapeutic promise.

In order to further prove the allosteric role of dihydropteroate synthetase in the phenomenon of synergism and have the structure-activity relationship being more accurately interpreted, highly purified (homogeneous) preparations must be obtained. Therefore, we are developing an affinity column chromatography technique in hopes of purifying the enzyme in a single step to homogeneous state. The affinity resin has been prepared by a linkage of a sulfonamide to Sepharose. However a successful elution of the active fractions of the enzyme from the column depends on the presence of pure DHPP (i.e. 2-amino-4-hydroxy-6-hydroxymethyl-pteridine pyrophosphate, enzyme substrate). Our published procedure of preparing DHPP was found most recently to contain small amounts of impurities which in turn cause difficulties in purifying dihydropteroate synthetase by affinity column chromatography. In order to detect the impurities and further purify the prepared DHPP, paper chromatography, thin layer PEI-cellulose chromatography and its electrophoreses have been employed. Furthermore, PEI column chromatography was found to be the most satisfactory method of purifying the substrate of DHPP.

Satisfactory preliminary purification of the enzyme dihydropteroate synthetase by our newly developed affinity column chromatography has been achieved. The methods are still undergoing changes in order that the technique can be further perfected.

A highly purified enzyme of dihydropteroate synthetase will provide not only more meaningful kinetic data but also insight into the mechanism of action of the enzyme. The conclusion based on such experimental data has an important bearing upon the mode of action of the antibacterial sulfonamides and upon the design of alternative chemotherapeutic inhibitors of dihydropteroate synthetase that is usually present in most of the microorganisms.

Dihydrofolate synthetase studies in crude gonococcal extracts has been initiated. The enzyme showed sigmoidal response to the addition of Mg^{++} and we are currently investigating further along these lines and hoping this will lead to some important bearings on the behavior of this enzyme.

- (A) New method of synthesis and purification of 2-amino-4-hydroxy-methyl-pteridine pyrophosphate by PEI cellulose column.

- (B) Preparation of affinity column chromatography for enzyme dihydropteroate synthetase.

(A) New method of synthesis and purification of 2-amino-4-hydroxy-methyl-pteridine pyrophosphate by PEI cellulose column.

1. Preparation of 2-amino-4-hydroxy-6-hydroxymethyl pterine.

Hydrazine hydrate (2.3 ml, 90%) was added to an aqueous solution (20 ml) of crude dihydroxyacetone (3.5 g, 0.04 moles) and the mixture was kept at room temperature for 30 minutes before being added to a suspension of 2,4,5-triamino-6-hydroxy pyrimidine sulfate (5.1g, 0.02 moles) in water (60 ml) containing sodium acetate · 3H₂O (5 g) and boric acid (2.4 g). The resulting mixture was heated under nitrogen on the water bath for two hours and the yellow product (2 g) was therefore collected, washed with cold water, absolute ethanol, ethanol-ether (50:50) and finally with ether. The small amount of powder was dissolved in a minimum amount of sodium hydroxide solution (about 1M). The solution was applied as a small spot on chromatographic paper (3 MM), water was used as solvent. Only one spot was detected under ultraviolet light, its R_f value is 0.57 which is closely correspondent to the value reported in the literature (R_f = 0.52).

2. Preparation of 2-amino-4-hydroxy-6-hydroxymethyl pteridine pyrophosphate (2).

Pyrophosphoric acid was crushed and dried under vacuum for 4 days and 50 g was melted slowly in a glass-stoppered flask at 60-65 C. 2-Amino-4-hydroxy-6-hydroxymethyl-pteridine (600 mg, 0.0016 moles) was added slowly and the flask was protected from light by aluminum foil. The mixture was stirred and heated at 60-65 C for two hours. After addition of distilled water (150 ml), the contents was transferred to a beaker and an aqueous charcoal (G-60) suspension (6 g in 40 ml of distilled water) was added. The mixture was stirred for 30 minutes and was filtered through sintered glass funnel (M). The charcoal pad was washed with distilled water (500 ml) to remove excess pyrophosphoric acid. The pteridine adsorbed on the charcoal pad was eluted by suspending the pad in cold ammonium hydroxide (3 M, 20 ml) and the mixture was stirred for 10 minutes. The resulting suspension was filtered through the sintered glass funnel. This elution process was repeated five times with the same amount of cold ammonium hydroxide. Each filtrate was detected for impurities by paper chromatography, using water as the solvent. The solutions

were discarded if more than one spot was detected. The desired filtrates were then combined and lyophilized under reduced pressure. The greenish yellow amorphous powder was obtained. Yield was about 40%.

The impurities were detected by the following three methods:

- a. Paper chromatography. Small amounts of substance was dissolved in distilled water and the solution was applied on the chromatographic paper (3MM) as a tiny spot. The solvent system was the distilled water. One diffused fluorescent spot was detected under U.V. light. Its R_f value is 0.98.
- b. PEI-cellulose thin-layer chromatography. PEI sheet from J.T. Baker Chemical Co. was washed in order that the impurities were removed (3). The sheet was placed, layer side up, for 1 minute in the flat dish containing NaCl (2.5 M) (800-1000 ml.). Immersion was started from one end of the sheet and continued in a slow and steady motion. After excess solution had been allowed to drain and the uncoated side had been thoroughly dried with a towel, the layer was dried in the air for several hours. It was then soaked for five minutes in the distilled water (800-1000 ml) and again dried. Subsequently, the layer was washed by ascending irrigation with distilled water and was again dried.

Small amount of substance was dissolved in sodium chloride solution (2 M) and it was applied as a spot on the washed dry sheet. The solvent system was sodium chloride (2 M). Three fluorescent spots were observed under U.V. light, the R_f values are 0.19, 0.36, and 0.63.

- c. Paper electrophoresis. The electrode chambers of the Arthur H. Thomas model 20 electrophoresis cabinet were filled to the liquid level line with phosphate buffer (0.05 M, pH 5.3).

The filter paper, Whatman No. 1 strips (1 x 6 inches) were immersed in the phosphate buffer solution (0.05 M, pH 5.3) in a large beaker. The strips were placed between sheets of blotting filter paper and blotted lightly to remove excess liquid. The strips were lifted carefully over a carrier. The

carrier was then placed in the cabinet so that ends of the strips were immersed in the buffer solution. The voltage was set at 90 volts and the chamber was allowed to equilibrate for two hours.

The small amount of substance was dissolved in phosphate buffer (0.05 M, pH 5.3) and the solution was applied as a band across the wet paper strips. The current was set at 1 milliampere per one strip. The electrophoresis was allowed to run for two hours at room temperature.

After electrophoresis, the paper was removed and placed under U.V. light. Three fluorescent bands were found to move towards the anode with the distances of 3.2, 4.0 and 5.0, which were found comparable with the spots observed in Experiment 2.

4. Separation of the mixture by PEI cellulose column.

PEI cellulose, weak anion exchanger (0.5 g), was added into 30 ml of LiCl (0.5 M). After the solution was stirred for 15 minutes at room temperature, the suspended fine particles were removed by decantation. The resulting suspension was poured into a column (0.7 x 15 cm). The column was allowed to pack by gravity and then washed with 5 ml of LiCl (0.5 M). Sample (1 mg), dissolved in a minimum volume of distilled water, was applied into the column. The column was eluted by increasing concentrations of LiCl salt (1.0 M, 3 ml to 1.5 M, 3 ml were used as eluting solvents). Eluants with the flow rate of 1 ml per 15 minutes were collected. Fractions were pooled and identified by paper electrophoretic method.

(B) Preparation of affinity column chromatography for enzyme dihydropteroate synthetase.

Synthesis of 4-(4-aminobenzenesulfonamido)benzene sulfonamidoglycine.

The resins for the affinity column were prepared by the linkage of 4-(4-aminobenzenesulfonamido)benzene sulfonamidoglycine to Sepharose 4 B with a standard procedure (4). Sepharose-4B was activated with cyanogen bromide and coupled to the spacer-arm di(3-aminopropyl) amine (5). The sulfonamide of 4-(4-aminobenzenesulfonamido)-benzenesulfonamidoglycine was condensed with the Sepharose conjugate using 1-cyclohexyl-3-(3-morpholinoethyl)carbonyldiimide metho-p-toluenesulfonate in 50% aqueous dimethyl-formamide.

The sulfonamide used in the above coupling reaction was prepared by a series of reactions shown below:

1. Preparation of 4-acetamidobenzenesulfonamidoglycine.

To a solution of glycine (15 g, 0.2 moles) in aqueous sodium hydroxide (2M, 250 ml) was added 4-acetamidobenzenesulfonyl chloride (47 g, 0.2 moles). After stirring for two hours, the solution (pH 10.8) was filtered and acidified to pH 2. The white precipitate was collected and recrystallized from 60% aqueous ethanol to give 4-acetamidobenzenesulfonamidoglycine (45.5 g, 83%) as platelets, m.p. 237-238C (dec.) Literature m.p. 237.5-238.5 C.

2. Preparation of 4-aminobenzenesulfonamidoglycine.

4-Acetamidobenzenesulfonamidoglycine (45.5 g, 0.17 moles) in round bottom flask (500 ml) was heated under reflux in dilute hydrochloric acid (80 ml) for 45 minutes. The solution was evaporated until the final volume was about 50 ml, then it was kept overnight in the refrigerator. The white chunky crystal of the hydrochloride was obtained (28 g, 63%) m.p. 207-212 C (Lit. m.p. 211-213 C).

3. Preparation of 4-(4-acetamidobenzenesulfonamido)benzenesulfonamidoglycine.

4-Aminobenzenesulfonamideoglycine (28 g, 0.1 moles), 4-acetamidobenzenesulfonyl chloride (21.3 g, 0.1 moles), sodium hydroxide (13.4 g, 0.34 moles) and water (80 ml) were shaken together for three hours.

The resulting filtered yellow solution (pH 6) was acidified to pH 2. The white precipitate (22 g, 50%) was obtained. Recrystallization from 95% ethanol gave glycine derivative as tiny white crystals, m.p. 248-250 C (Lit. m.p. 247 C).

4. Preparation of 4-(4-aminobenzensulfonamido)benzene-sulfonamidoglycine.

4-(4-Acetamidobenzenesulfonamido)benzene-sulfonamidoglycine. (10 g, 0.02 moles) was heated under reflux for 45 minutes in dilute hydrochloric acid (30 ml). The white precipitate of the hydrochloride was obtained when the solution was cooled. The precipitate was then dissolved in the equimolar amount of solid sodium bicarbonate. The white needles were separated out on cooling and recrystallized from hot water. However, the melting point is probably caused by the impurity which is the hydrochloride form of the product. The crystal was again dissolved in hot water and the solution was adjusted to pH 12. After acidification to pH 3.0, the white precipitate was obtained and then recrystallized from hot water. The crystal has melting point (185-187 C) which is in agreement with the literature value (Found: C=44.14%, H=3.73%, N=10.85%, S=16.58%. Calculated: $C_{14}H_{15}N_3O_6S$, C=43.64%, H=3.82%, N=10.91%, S=16.62%).

The applications of our prepared affinity column on the enzyme dihydropteroate synthetase purification under different conditions (i.e. ionic strength and pHs) have been tried. The results in general are satisfactory. However, we are still attempting to improve the yield and purity of the enzyme in order that it can be studied in a more meaningful manner.

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