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# Improved methods for the purification of enzymes of the folate pathway in *Escherichia coli*

## I. Chromatographic methods

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ABSTRACT

A sequence of chromatographic procedures is described for the isolation of three consecutive enzymes of the folate pathway in *Escherichia coli*: hydroxymethyldihydropteridine pyrophosphokinase (E.C. 2.7.6.3) (I), 7,8-dihydropteroate synthase (E.C. 2.5.1.15) (II) and 7,8-dihydrofolate reductase, (E.C. 1.5.1.3) (III). Starting with the crude extract, ion-exchange chromatography on a DEAE-Sepharose CL-6B column with a salt gradient completely separated I, II and III. I and II were further purified by hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B, followed by size-exclusion chromatography on Ultrogel AcA 54. For III only size-exclusion chromatography was used. The overall enrichment factors, on the basis of protein, were 13 700-fold for I, 280-fold for II and 500-fold for III. Bacterial batches of more than 500 g were handled.

## INTRODUCTION

For special assignments in enzymology, the availability of purified enzymes, acting sequentially in a biochemical pathway, is sometimes necessary. We were interested in the properties of the enzymes of the folate pathway of *E. coli*, *i.e.*, hydroxymethyldihydropteridine pyrophosphokinase (E.C. 2.7.6.3) (I), 7,8-dihydropteroate synthase (E.C. 2.5.1.15) (II) and 7,8-dihydrofolate reductase (E.C. 1.5.1.3) (III), which catalyse the reactions shown in Fig. 1. For this a purification procedure combining different efficient chromatographic methods was selected, starting from whole cells of *Escherichia coli*.

Classical strategies in protein purification recommend adsorptive media with high load capacities at the beginning, followed by volume- or mass-limited techniques toward the end of the procedure. Under some circumstances, this strategy must be abandoned, *e.g.*, if unsuccessful separation efficiencies compel changes in this sequence. This seemed to be the case in the purification method for enzymes I and II, published by Richey and Brown [1]. According to their paper, size-exclusion chromatography (SEC) rather than ion-exchange chromatography (IEC) is performed first. However, in such a procedure there is a bottle-neck in the economical accumulation of sufficient amounts of enzyme.

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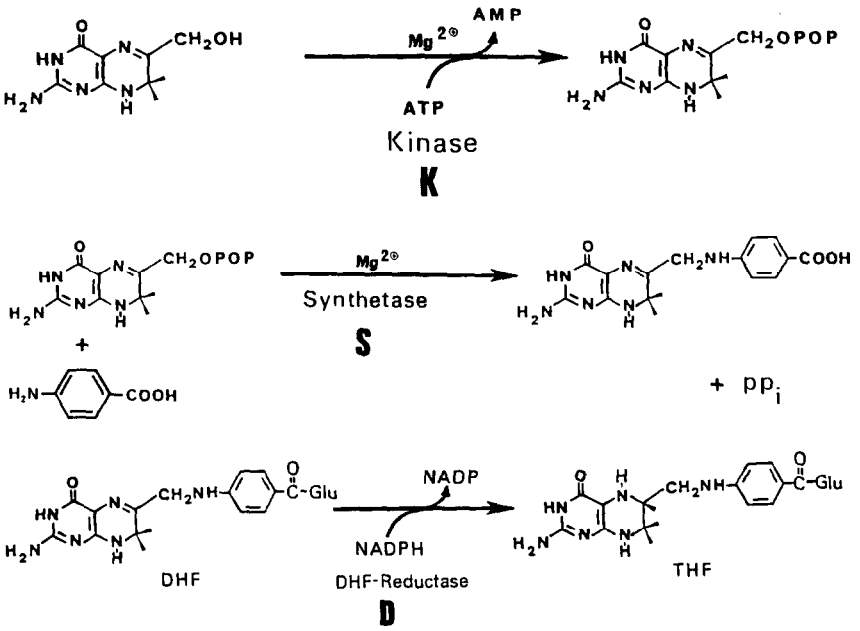


Fig. 1. Enzyme reactions.

In our procedure, the classical strategies mentioned above are used and III is additionally obtained.

## EXPERIMENTAL

### *Materials*

The column materials DEAE-Sepharose CL-6B and Phenyl-Sepharose CL-4B, were purchased from Pharmacia (Freiburg, F.R.G.) and Ultrogel AcA-54 from LKB (Bromma, Sweden). The glass columns (50 × 10 cm I.D., 30 × 5 cm I.D. and 90 × 5 cm I.D.) were obtained from Kranich (Göttingen, F.R.G.). The preparation of substrates for the tests of I and II and the sources of chemical supplies were described in ref. 2 and those for III in ref. 3. The cell mass was fermented by E. Merck (Darmstadt, F.R.G.) using *E. coli* ATCC, strain 11.7775, from the American Type Culture Collection (Rockville, MD, U.S.A.). Thin-layer chromatographic (TLC) plates, trishydroxymethyl aminomethane (Tris) and all other chemicals were purchased from E. Merck. Equipment for ultrafiltration with membrane type PM 10 was from Amicon, [Witten (Ruhr), F.R.G.].

### *Methods*

All enzyme purifiation steps were carried out at 4°C, unless stated otherwise. The salt gradients were monitored by conductivity measurements. The enzyme activities of I and II were determined by a TLC method [2], following the increase in products fluorimetrically at an excitation wavelength of 365 nm. The activity of III was

determined photometrically according to ref. 3 by following the decrease in absorbance of the substrates NADPH and dihydrofolic acid at 340 nm. Two buffer concentrations were used (pH 7.7): buffer A, 0.1 *M* Tris–HCl and buffer B, 0.01 *M* Tris–HCl.

The protein content of fraction I was determined by the method of Lowry *et al.* [4], while the one in the chromatographic fractions was estimated by the method of Warburg and Christians [5]. If storage was necessary after separation, the pooled fractions were concentrated, dialyzed against buffer B by ultrafiltration, and lyophilized.

*Crude extract*

Usually, 550 g of frozen cells were thawed and suspended in 370 ml of buffer A, resulting in a total volume of 855 ml. For easier handling, this cell suspension was divided into two parts. Each part was subjected to twenty ultrasonication intervals of 2 min each (Branson B12 sonifier, output control: 10; Heinemann, Schwäbisch Gmünd, F.R.G.). The temperature was held between 3 and 10°C. The DNA of the broken cells was digested by treatment with DNase I (27 mg of DNase I per 20 ml of buffer A) for 30 min at room temperature with stirring. Cell debris was separated by ultracentrifugation (2 h at 145000 *g*; MSE Superspeed 65 centrifuge, Colora, Lorch/Württ., F.R.G.). The supernatant of *ca.* 640 ml was subjected to fractional precipitation by addition of solid ammonium sulphate, 20–70% saturation at 4°C. A pH of 7.7 was maintained by dropwise addition of 1.0 *M* NaOH. Both precipitations were accomplished by stirring the suspension for *ca.* 1 h. The precipitated proteins were collected by centrifugation in a Beckman (Munich, F.R.G.) J2-21 centrifuge (30 min at 22000 *g*). In the 20% fraction the three enzymes remained in the supernatant whereas in the 70% step they precipitated together with the bulk of the protein. The pellet was suspended in *ca.* 210 ml of buffer A and dialysed once against 2 l of buffer A followed by 5-l batches of buffer B until the electric conductivity after the last buffer change corresponded to that of buffer B. Insoluble material was removed by centrifugation. With the aid of 4 *M* NaCl in buffer B, the clear dark yellow supernatant was adapted to the equilibration buffer of the ion-exchange column (55 m*M* NaCl in buffer B) (pH and conductivity control). If necessary, the protein concentration of this solution was adjusted to *ca.* 30 mg/ml with equilibration buffer (fraction I).

*Ion-exchange chromatography*

A DEAE-Sepharose CL-6B column (47 × 10 cm I.D.) was prepared and equilibrated with 55 m*M* NaCl in buffer B. The column was run with a flow-rate of *ca.* 3 ml/min. After fraction I (28.6 g of protein in 905 ml of equilibration buffer, 31.6 mg/ml) had been applied to the column, unbound material was washed out with starting buffer until the absorbance of the eluent matched the baseline. The column was developed with the aid of an increasing salt gradient (8.5 l of 55 m*M* NaCl in buffer B against 8.5 l of 360 m*M* NaCl in buffer B) and finally flushed with 0.8 *M* NaCl in buffer B. Fractions of 26.5 ml were collected. The activities of enzymes I, II and III, were located according to refs. 2 and 3 and pooled in fractions K, S and D, respectively (Fig. 2). The fractions K and S were further purified by HIC, whereas fraction D was submitted to SEC.

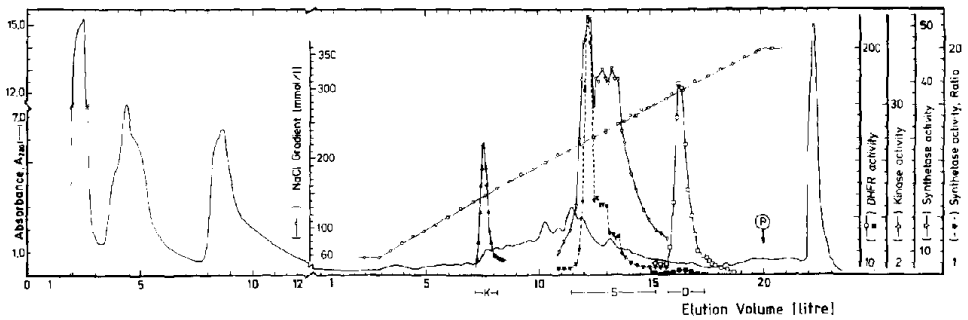


Fig. 2. Ion-exchange chromatography of fraction I on DEAE-Sepharose CL-6B. Activity of enzyme I (△) expressed in arbitrary area units. Activity of enzyme II expressed in arbitrary area units (▽) and the product/substrate ratio (▼). (□) Activity of enzyme III. Control: + 1 m*M* methotrexate (■), expressed as $-\Delta A_{340} \times 10^{-3}$/min corresponding to $81.3 \cdot 10^{-6}$ units/ml [6].

*Hydrophobic-interaction chromatography (HIC)*

Fractions K and S from the ion-exchange chromatography were separately loaded onto a Phenyl-Sepharose Cl-4B column (27 × 5 cm I.D.). To attain adequate binding and separation of the desired enzymes the total loading of protein should be lower than 10 mg/ml column material (a load of *ca.* 3 g of protein and a concentration of 4 mg/ml in each instance were successful). The flow-rate was 2 ml/min. Fractions of 27 ml were collected during the gradient. Enzyme activities were located using a previously described method [2].

For purification of enzyme I, the column was equilibrated with 3 *M* NaCl in buffer B and fraction K from IEC was adjusted to this salt concentration. After the protein solution (2.9 g of protein in 750 ml) had been applied, the column was washed with equilibration buffer. Normally, after a 9–10-l wash all of the unattached protein was eluted and the decreasing salt gradient was started (2.5 l of 3 *M* NaCl in buffer B down to 2.84 l of buffer B). The activity of I was pooled (fraction K) and finally purified by SEC (Fig. 5).

For purification of enzyme II, the column was equilibrated with 2 *M* NaCl in buffer B. Fraction S from IEC was adjusted to this salt concentration and loaded onto the column (2.6 g of protein in 612 ml). Unbound material was washed out with the equilibration buffer. Normally, after a 9–10-l wash the absorbance matched the baseline and a decreasing salt gradient in combination with an increasing alcohol gradient was started (2.5 l of 2 *M* NaCl in buffer B against 2.74 l of an aqueous solution of 5% ethanol). The activity of enzyme II was located in fraction S, which was finally purified by SEC (Fig. 6).

*Size-exclusion chromatography*

Fractions K and S from HIC and fraction D from IEC were applied separately onto an Ultrogel AcA-54 column (84 × 5 cm I.D.), equilibrated with 80 m*M* NaCl in buffer B. The flow-rate was *ca.* 1 ml/min and fractions of 20 ml were collected.

The samples applied were as follows: enzyme I, fraction K from HIC (88.5 mg in 24.7 ml); enzyme II, fraction S from HIC (248 mg in 38 ml) and enzyme III, fraction D from IEC (654 mg in 28 ml). Enzyme activities were located as indicated above.

## RESULTS AND DISCUSSION

Our aim was the development of an efficient preparation procedure to obtain the enzymes I, II and III of the folate pathway in *E. coli* in both sufficient quantity and purity.

Fig. 2 shows that a DEAE-Sepharose CL-6B column clearly separates these three enzymes under the conditions described under Experimental. The enzymes I and III were eluted as single sharp peaks centred at NaCl concentrations of 145 and 285 m*M*, respectively. Because of the favourable elution pattern of the proteins in this range of the gradient, the purification effects calculated on the basis of protein content were 25- and 40-fold for I and III, respectively.

However, the elution pattern of enzyme II is complicated and shows at least two peaks of activity, as determined by the enzymatic product, covering a range from about 210 to 270 m*M* NaCl near the bulk of the protein. Thus the purification effect of this step is only *ca.* 5-fold. We observed this phenomenon always when the separation techniques had been based on differences in charge and adsorption behaviour, no matter whether a crude sample preparation [7] or a highly purified enzyme had been used [8]. Therefore, we assume dynamic changes in the conformation(s) of enzyme II during the separation process comparable to the behaviour in binding of this enzyme to an affinity matrix [9]. This hypothesis is supported by kinetic studies of Ferone and Webb [10].

If the enzyme activity of II is determined on the basis of the product/substrate ratio ($H_2$-pteroate/$H_2$-pt$CH_2$OPOP)[a], the main activity centres in the first peak of fraction S correspond to 225 m*M* NaCl. In this peak an overproportional consumption of the pyrophosphate substrate is observed, whereas a corresponding formation of the product $H_2$-pteroate is missing. In accordance with the observations of Ferone and Webb [10], this behaviour may be explained by a product inhibition of the $H_2$-pteroate which is synthesized to a sufficient level in these enzymatically most active fractions. Additionally, there seems to be a pyrophosphorylase activity in these fractions, probably caused by a contaminating enzyme [1] and/or by the enzyme II itself, despite its inhibition by its product $H_2$-pteroate. This effect was also noticed when more purified samples of II were used [11]. Therefore, we speculate on a pyrophosphorylase activity associated with a distinctive conformation of enzyme II. More experiments have to be carried out to clarify this phenomenon of pyrophosphorylase activity.

Figs. 3 and 4 show the HIC of the enzymes I and II. There are significant differences in the hydrophobic properties of these enzymes. I is less hydrophobic than II and therefore different NaCl concentrations are recommended for binding each enzyme on the Phenyl-Sepharose column. Furthermore, special conditions for the elutions are necessary.

As shown in Fig. 3, enzyme I is eluted from the column in the range 1.6–1.3 *M* NaCl. The absorbance pattern at 280 nm indicates an efficient separation effect. Finally, this enzyme was highly purified by SEC (Fig. 5) with an overall purification effect of *ca.* 13 700-fold as calculated on the basis of its protein balance.

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[a] $H_2$-pt$CH_2$OPOP = Hydroxymethyldihydropteridine pyrophosphate, see also Fig. 1.

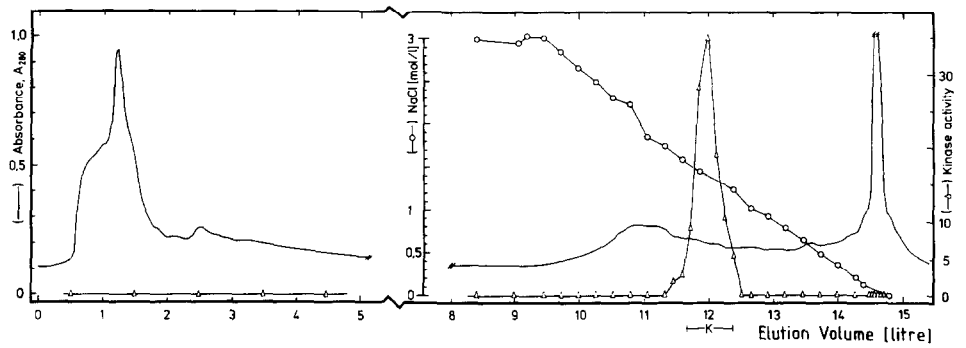


Fig. 3. Hydrophobic-interaction chromatography of fraction K from the IEC column on Phenyl-Sepharose CL-4B. Activity of enzyme I (△) expressed in arbitrary area units.

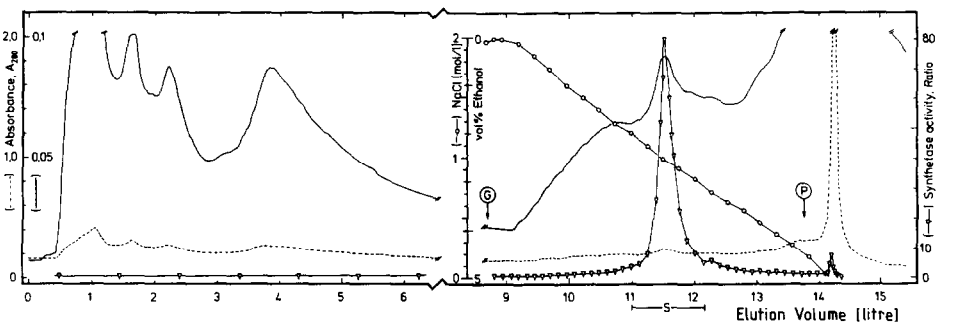


Fig. 4. Hydrophobic-interaction chromatography of fraction S from the IEC column on Phenyl-Sepharose CL-4B. Activity of enzyme II (▽) expressed as the product/substrate ratio.

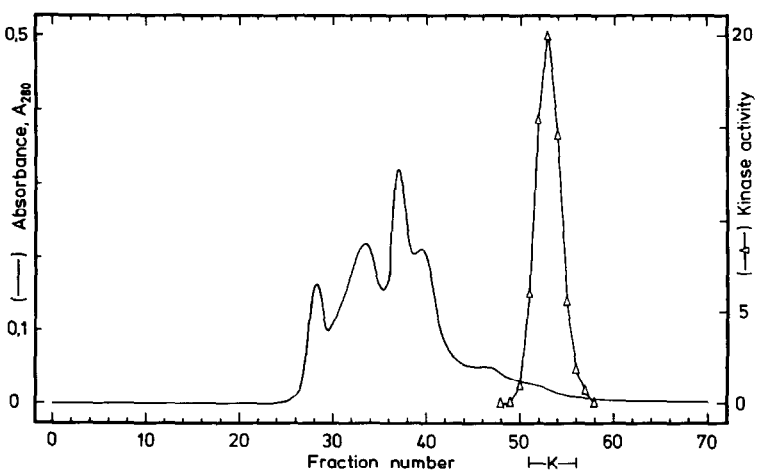


Fig. 5. Size-exclusion chromatography of fraction K from HIC on Ultrogel AcA-54. Activity of enzyme I (△) expressed in arbitrary area units.

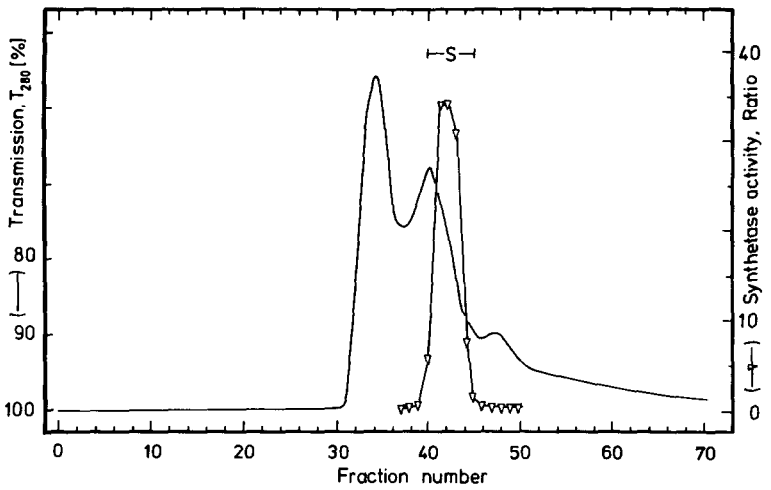


Fig. 6. Size-exclusion chromatography of fraction S from HIC on Ultrogel AcA-54. Activity of enzyme II (▽) expressed in arbitrary area units.

Fig. 4 shows the binding and elution conditions of enzyme II on the same column material, but equilibrated with a low concentration of NaCl. Because enzyme II seems to expose a hydrophobic domain, a decreasing salt gradient was run simultaneously against an increasing gradient of ethanol. Enzyme II is detached in a peak corresponding to 1 *M* NaCl and 2.5% ethanol together with a distinct peak of protein. Finally, after SEC (Fig. 6), the total purification effect was *ca.* 280-fold. This material is a suitable sample for application of an electrophoretic purification process such as preparative isoelectric focusing [8].

Fraction D from the IEC experiment was purified only by SEC (Fig. 7), yielding

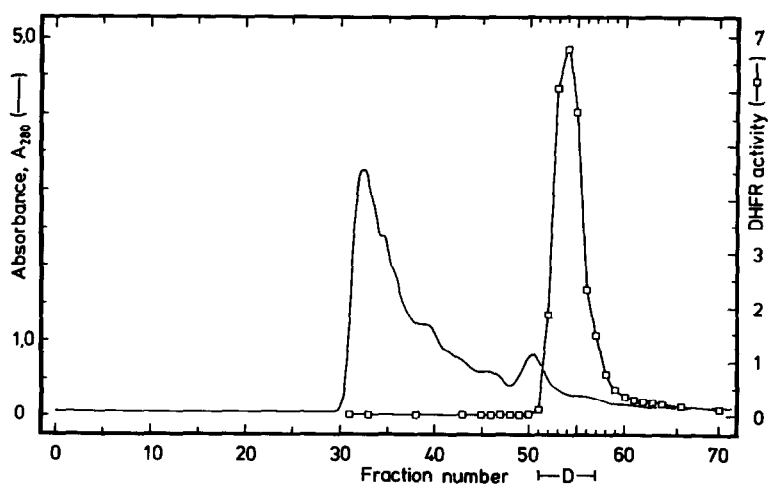


Fig. 7. Size-exclusion chromatography of fraction D from IEC on Ultrogel AcA-54. Activity of enzyme III (□) expressed as described for Fig. 1.

a purification effect of *ca.* 500-fold by these two steps. In this enzyme preparation an unspecific background consumption of the substrate NADPH of only 1% was observed, as determined by the inhibitory effect of methotrexate (data not shown) which was tolerable for our purposes.

In conclusion, ion-exchange chromatography is useful for a high loading capacity at the beginning and further is not restricted by the sample volume by special procedures. As the salt concentration of the samples obtained increases during this separation procedure, HIC should be the next step. For this task only a further increase in the salt concentration is necessary, *ie.*, by addition of solid NaCl. There is no need to reduce the sample volume. Finally, SEC separates proteins of different size and simultaneously eliminates high salt concentrations. These methods of purification were repeated several times and showed high reproducibility. Before the samples are ready for storage they have to be dialysed, concentrated by ultrafiltration and lyophilized. In our hands, bacterial masses of more than 500 g have been processed, but scaling up is easily possible.

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