Folate Reductase and Specific Dihydrofolate Reductase of the Amethopterin-Sensitive *Streptococcus faecium* var. *durans**

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ABSTRACT: The dihydrofolate reductase activity of the folate-dependent, amethopterin-sensitive strain of *Streptococcus faecium* var. *durans* (SF/O) has been separated into two components that differ in several properties. The major component is a highly active *specific dihydrofolate reductase;* it has been purified 400-fold with an over-all recovery of 33%. The minor component, which has a 9-fold lower turnover number in dihydrofolate reduction, catalyzes the reduction of folate also. Designated, therefore, *folate reductase*, it has been purified 40-fold with 25% yield. Structurally these enzymes differ.

The more basic protein, *specific dihydrofolate reductase*, migrates during molecular sieve chromatography as if its molecular weight were 3000 less than that of *folate reductase*. The estimated molecular weight (19,000) of *folate reductase* resembles that of animal reductases which catalyze the reduction of folate and dihydrofolate. Properties of *folate reductase* identify it as the enzyme which is the prevailing form of dihydrofolate reductase in the amethopterin-resistant mutant strain, *S. faecium* var. *durans*/A_k. Positive induction by exogenous folate of only the *specific dihydrofolate reductase* has been observed.

The bacterium *Streptococcus faecium* var. *durans* (Coultas *et al.*, 1966) requires folic acid for growth in serine-, purine-, or thymine-free medium (Johnson and Hutchison, 1964). To date, an enzyme capable of catalyzing the reduction of folic acid has not been found in any amethopterin-sensitive strain of this bacterium. However, dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NAD(P)-oxidoreductase, EC 1.5.1.3) which catalyzes the reaction

$$\text{dihydrofolate} + \text{NADPH} + \text{H}^+ \rightleftarrows \text{tetrahydrofolate} + \text{NADP}^+$$

has been isolated from several strains of this *Streptococcus* (Blakley and McDougall, 1961; Soini and Nurmikko, 1963; Albrecht *et al.*, 1966a).

Evidence suggests that in animal systems the enzyme which mediates the reduction of dihydrofolate also catalyzes the reduction of folate (Zakrzewski and Nichol, 1960; Mathews and Huennekens, 1963; Morales and Greenberg, 1964; Bertino *et al.*, 1965; Kaufman and Gardner, 1966; Zakrzewski *et al.*, 1966; Perkins *et al.*, 1967). The reduction of folate by the partially purified dihydrofolate reductase of *Lactobacillus leichmannii* implies that in *L. leichmannii*, as in animal systems, a single enzyme catalyzes the reduction of both substrates (Kessel and Roberts, 1965). Only dihydrofolate is effectively reduced by dihydrofolate reductase preparations from the amethopterin-sensitive *Streptococcus faecalis* R, ATCC 8043 (Blakley and McDougall, 1961); amethopterin-sensitive and -resistant *Diplococcus pneumoniae* (Sirotnak *et al.*, 1964a,b); *Escherichia coli* (Mathews and Sutherland, 1965); *Proteus vulgaris* and *Staphylococcus aureus* (Burchall and Hitchings, 1965); and *E. coli* infected with bacteriophage T_6 (Mathews and Sutherland, 1965).

By contrast, in SF/O, the amethopterin-sensitive strain of *S. faecium* var. *durans* used in this laboratory (Coultas *et al.*, 1966), there is apparently a tetrahydrofolate-forming system consisting of more than one enzyme (Albrecht and Hutchison, 1968). We found that one species of dihydrofolate reductase, which accounts for 90% of the total dihydrofolate reductase activity of the culture, is specific for dihydrofolate; folate increases the differential rate of synthesis of this species. Another enzyme, having a 9-fold lower turnover number in dihydrofolate reduction, catalyzes the reduction of folate also and differs from the specific dihydrofolate reductase in gel filtration behavior and electrophoretic mobility. Positive induction of this enzyme by folate has not been observed. This report deals with the partial purification and several properties of these two dihydrofolate reductases of SF/O.

Materials and Methods

Folic acid and amethopterin were supplied by American Cyanamid Co.; dihydrofolic acid was prepared, and amethopterin was purified as described previously (Albrecht *et al.*, 1966a). Working solutions of dihydrofolic acid were made daily (25°) in 0.25 M potassium phosphate buffer (pH 6.5), 17 mM in 2,3-dimercaptopropanol. 2,3-Dimercaptopropanol and Bio-Gel P-60 (Bio-Rad Laboratories) were purchased from Calbiochem; starch hydrolyzed (Connaught Medical

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Research Laboratories), ammonium sulfamate, and *N*-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Fisher Scientific Co.; 3-(4,5-dimethylthiazolyl-1-2)-2,5-diphenyltetrazolium bromide and sodium nitrite were products of Nutritional Biochemicals Corp. and Mallinckrodt Chemical Works, respectively. Mann Research Laboratories, Inc., was the source of the nonenzymic protein molecular weight markers. The commercial sources of other biochemical reagents have been cited (Albrecht *et al.*, 1966a,b).

Standard buffer was a solution of 10 mM potassium phosphate (pH 7.4), 1 mM in EDTA.

The basal culture medium, a purine- and pyrimidine-free modification of the medium of Flynn *et al.* (1951), was prepared without glucose and with supplements of 10 μg of folic acid and 100 mg of sodium acetate per l. Sterile glucose (final concentration, 2%) was added aseptically to the medium after the glucose-free medium had been autoclaved (121°, 15 psi) in 30-l. lots for 60 min or in 6–8-l. lots for 30 min.

For enzyme purification, cells of an exponential culture of SF/O in the basal medium were harvested with the Sharples centrifuge, washed and suspended in standard buffer (Albrecht *et al.*, 1966b), and disrupted with ultrasonic vibration (Albrecht *et al.*, 1968). The supernatant solution after centrifugation (27,000*g*) for 30 min was the cell extract (Albrecht *et al.*, 1968).

For the assay of dihydrofolate reductase activity, the previously described method (Albrecht *et al.*, 1966a) was modified. To follow the purification, 0.2 μmole of NADPH was added to a preincubated (3 min, 30°) mixture (2.8 ml) of a test portion of the enzyme fraction, 150 μmoles of potassium phosphate (pH 6.5), 10 μmoles of 2,3-dimercaptopropanol, 0.1 μmole of dihydrofolate, and 300 μmoles of KCl. To measure the activity of extracts obtained in the studies of controlled enzyme synthesis, dihydrofolate (0.1 μmole), as a solution in 0.3 ml of 0.25 M potassium phosphate (pH 6.5), 17 mM 2,3-dimercaptopropanol was added to a preincubated (3 min, 30°) mixture (2.7 ml) of extract, 0.2 μmole of NADPH, 300 μmoles of KCl, 5 μmoles of 2,3-dimercaptopropanol, and 75 μmoles of potassium phosphate (pH 6.5). The reaction was followed at 340 mμ for 3 min in the thermostatically controlled chamber (30°) of the Zeiss spectrophotometer, Model PMQ II. The decrease in absorbance of complete reaction mixtures was corrected for the absorbance decrease of enzyme-free mixtures and, when necessary, for the absorbance change of dihydrofolate-free mixtures. KCl was included in the standard dihydrofolate reductase assay system because, in effect, it aided the detection of the minor component. In the dihydrofolate reduction catalyzed by the extract, KCl had no apparent effect. However, it stimulated (50–100%) the relatively low dihydrofolate reductase activity of the early gel filtration fractions and inhibited the major component only 15%. One unit of enzyme activity was defined as the amount required for the reduction of 1 mμmole of dihydrofolate/min. Other details of these assay procedures and the measure of dihydrofolate reductase activity in the presence of amethopterin were as explained previously (Albrecht *et al.*, 1966a).

Folate reductase activity was determined by a system based upon the method of Raunio and Hakala (1967). Enzyme preparations were incubated (37°) for 30 (or 60) min in 1 ml of 0.1 M citrate buffer (pH 6.0) with 10 μmoles of sodium bicarbonate, 0.2 μmole of folic acid, and 0.4 μmole of NADPH. Subsequent HCl addition (0.2 ml, 5 N) stopped the enzymatic reaction and catalyzed the cleavage of the enzymatic reaction product to a diazotizable amine (Bratton and Marshall, 1939). After 30 min at 25°, 0.2 ml of acetone was added to each acidified reaction mixture and then, at 3 min-intervals, 0.1 ml of each of the following reagents in aqueous solution: 0.5% sodium nitrite, 2.5% ammonium sulfamate, and 0.5% *N*-(1-naphthyl)ethylenediamine dihydrochloride. After 15 min at 25°, the assay mixtures were centrifuged to remove any precipitate that formed on acidification and the absorbance at 560 mμ was read. A reference mixture, to which folic acid was added after acidification, was incubated for each enzyme preparation. On the assumption that the diazotizable amine was *p*-aminobenzoyl-L-glutamic acid, derived quantitatively from a reduced-folate compound, the molar extinction coefficient 55,000 M^{-1} cm^{-1} at 560 mμ (Raunio and Hakala, 1967) was used in calculating the enzyme activity. One unit of folate reductase was defined as that amount required to reduce 1 mμmole of folate/min under the conditions employed.

Protein was determined according to the method of Lowry *et al.* (1951).

The experimental method for *gel filtration* was cited earlier (Albrecht *et al.*, 1966a). To reduce the volume of effluent fractions, combined fractions (30-ml portions) were dispensed into sacs of dialysis tubing which were subsequently covered with crystalline sucrose in 200-ml graduated cylinders. Individual fractions (3.5–6.5 ml) were treated similarly in 30-ml beakers. After 16–18 hr at 4°, the sacs were removed from the sucrose, rinsed with cold standard buffer, and placed in beakers of cold buffer for 2–5 min.[1]

Zone electrophoresis (applied voltage, 170) with starch as a supporting medium and a bridge solution of 0.3 M borate (pH 8.5) was carried out at 4° for 6 hr according to the method of Smithies (1955, 1959). Starch gels were prepared in 25 mM boric acid, 45 mM Tris–1 mM EDTA (pH 8.6) (Boyer and Fainer, 1963).[2] After electrophoresis, the gel was sliced lengthwise. To visualize the enzyme loci, the inner cut surface of one part of the gel was covered with 10 ml of a buffered (50 mM Tris, pH 7.4) mixture of NADPH (0.2 mM), dihydrofolic acid (0.1 mM), and 3-(4,5-dimethylthiazolyl-1–2)-2,5-diphenyltetrazolium bromide (0.033%); the other part of the gel was treated similarly with a mixture from which dihydrofolic acid had been omitted. After 15-min incubation of the gel slices at 38° and subsequently 15 min at 25°, the complete and control reaction mixtures were removed by siphoning. Each slice was covered with 50 ml of dilute acetic acid (glacial acetic acid–water (1:11)). After 2–3 min, the

[1] Use of sucrose was suggested by Dr. Morris Friedkin.
[2] Recommended by Dr. Frank M. Huennekens.

acid was removed. The gel surface was covered with cellophane and photographed. To determine the number of proteins in the preparations, gels were stained with Amido Black (Smithies, 1955).

Results

The dihydrofolate reductase activity of the cell extract was essentially proportional to the amount of extract used for test within the limits of the assay. Endogenous diazotizable material complicated the quantitation of the folate reductase activity of the extract, and apparently other unidentified substances adversely affected folate reduction by the extract. Therefore, the folate reductase value of the extract was based upon tests with small amounts of the extract. The following purification procedure eliminated these conflicting factors (Table I).

Fractional Ammonium Sulfate Precipitation. Nucleic acids were removed from the extract as precipitates with protamine sulfate according to the previously described method (Albrecht *et al.*, 1966b). The resultant supernatant solution was treated with freshly ground ammonium sulfate according to the following modification of the cited procedures (Albrecht *et al.*, 1966a,b). Initially to each 20-ml portion of solution, 4.32 g of ammonium sulfate was added. Centrifugation sedimented the resultant precipitate as a pellet; another addition of the salt (5.76 g) to the separated supernatant solution effected the second ammonium sulfate precipitation, which also was recovered as a pellet. Each pellet was dissolved in standard buffer and dialyzed before enzyme assay. The solubilized second ammonium sulfate precipitate, the Am S fraction, contained 89% of the total dihydrofolate reductase activity of the extract. On the assumption that the folate reductase activity of the extract involved minimal interference by endogenous materials, a recovery of 88% of the folate reductase activity was calculated for the Am S fraction.

Gel Filtration. Slow migration of the dihydrofolate reductase activity of the Am S fraction through a column of Sephadex G-100 facilitated the separation of dihydrofolate reductase activity from the bulk of other protein (Albrecht *et al.*, 1966a). Moreover, this step resolved the dihydrofolate reductase activity into a minor and a major elution peak (Figure 1). The minor peak component (effluent fractions 65–77) which accounted for only 6.5% of the dihydrofolate reductase activity of the Am S fraction also catalyzed

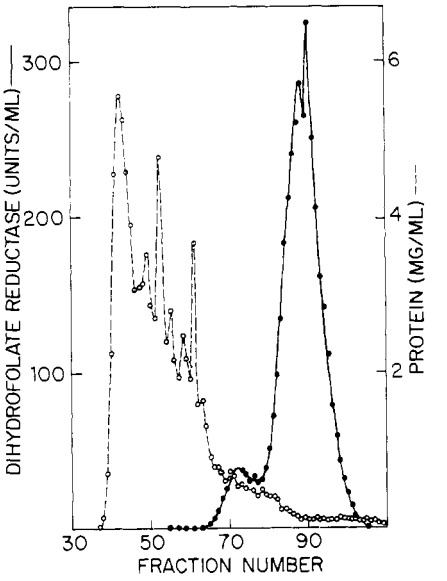

FIGURE 1: Filtration behavior of the total protein (60.8 mg) and dihydrofolate reductase activity (29,680 units) of the ammonium sulfate fraction through Sephadex G-100 (40–120 μ; 5 × 47 cm column) at 4°. The eluent was standard buffer; effluent fractions, 7.4 ml.

TABLE I: Purification of Folate Reductase and Specific Dihydrofolate Reductase of *S. faecium* var. *durans* (SF/O).

Purification Step	Total Protein[a] (mg)	Dihydrofolate Reductase Act. Sp Act. (units/mg)	Dihydrofolate Reductase Act. Total Act.[a] (units)	Folate Reductase Act. Sp Act. (units/mg)	Folate Reductase Act. Total Act.[a] (units)
Cell extract	1,614	25	40,560	0.027	44.4
Ammonium sulfate fractionation	836	61	35,948	0.050	39.0
First dextran filtration					
Fractions 65–77[b]	117	20	2,342	0.280	33.0
Fractions 81–97[b]	24	1,377	33,445	0.074	1.8
Second dextran filtration					
Fractions 64–80[b]	41	36	1,453	0.41	16.8
Polyacrylamide filtration					
Filtration A					
Fractions 45–49[b]	10.79	85	919	1.07	11.6
Filtration B					
Fraction 61	0.51	11,024	5,672	*c*	
Fraction 62	0.47	10,264	4,778	*c*	
Fraction 63	0.37	10,400	3,882	*c*	

[a] Recovery of total protein and enzyme activities was computed on the basis of extract (60 ml) of 14 l. of culture. [b] Fractions were concentrated as described in Methods. [c] Negligible activity.

the reduction of folic acid. The pattern (Figure 2) resulting from the second G-100 filtration of the minor peak fractions illustrates the parallel elution of folate and dihydrofolate reductase activities. Because of its affinity for folate and dihydrofolate, the minor component was called folate reductase. Essentially inactive in reducing folate, the major peak fractions (Figure 1, 80–105) contained 86% of the dihydrofolate reductase activity of the Am S fraction.

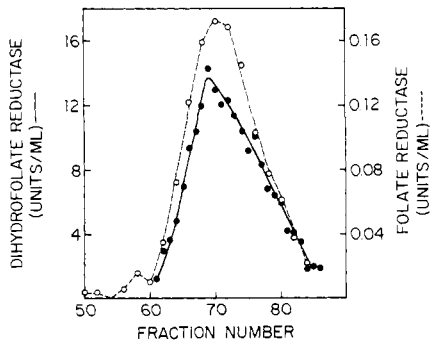

FIGURE 2: Folate reductase activity of the minor component of the total dihydrofolate reductase activity. Effluent fractions 65–77 of the first G-100 filtration (Figure 1) were combined and reduced in volume. A portion (1350 dihydrofolate reductase units) of the resultant concentrate was chromatographed on Sephadex G-100 (5 × 47 cm column). Details as in Figure 1.

Polyacrylamide gel filtration (Figure 3) of the folate reductase and of the specific dihydrofolate reductase emphasized their structural dissimilarities. The filtration behavior of the reductases and other proteins of known molecular weight on polyacrylamide gel and Sephadex G-100 suggested that the molecular weight (Whitaker, 1963) of the folate reductase is 19,000; 15,700 was estimated for the specific dihydrofolate reductase.

The summary of the purification (Table I) presents the marked activity differences between the most highly purified fractions of folate reductase (45–49; Figure 3A) and the specific dihydrofolate reductase (61–63; Figure 3B); 40-fold purification with 25% recovery was achieved for the folate reductase and 400-fold purification with 33% recovery for the specific dihydrofolate reductase.

Comparative Turnover Numbers. Figure 4 illustrates the essentially stoichiometric inhibition (Werkheiser, 1961; Schrecker and Huennekens, 1964; Albrecht *et al.*, 1964a) of the folate reductase and the specific dihydrofolate reductase by the chemotherapeutic folate analog, amethopterin. As a catalyst of folate and of dihydrofolate reduction (Figure 4A,B), the folate reductase was similarly inhibited by amethopterin but required 13 times more amethopterin than the specific dihydrofolate reductase (Figure 4C) for comparable inhibition of unit activity. Calculations based on the graphic analyses of the inhibitor–enzyme titration studies (Figure 4) partially explained the difference in inhibition by amethopterin. The essentially stoichiometric binding of inhibitor and enzyme facilitated quantitation of the reductase of the enzyme preparations: an expression, describing the reductase concentration in terms of drug binding equivalents (amethopterin binding sites), was obtained by extrapolating the linear portion of each titration plot to the abscissa, *i.e.*, to zero activity. Based on the total protein of the individual preparations, a tenfold higher binding capacity (Table II) was calculated for the specific dihydrofolate reductase preparation. This difference, partly attributable to the greater purity of the specific dihydrofolate reductase, led to the determination of a ninefold difference in the rate of dihydrofolate reduction per drug binding equivalent of the folate reductase and the specific dihydrofolate reductase (turnover number, Table II).

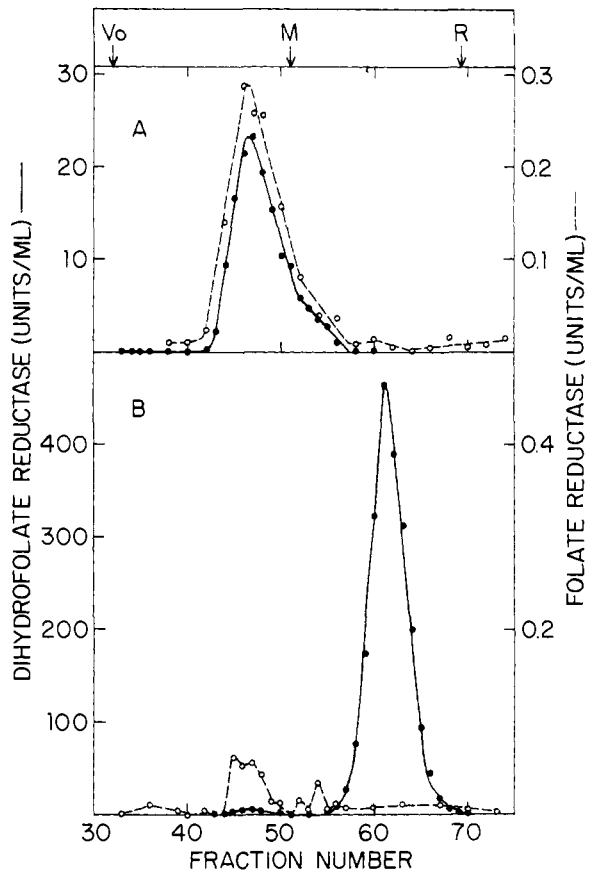

FIGURE 3: Polyacrylamide gel (Bio-Gel P60) chromatography (2.5 × 90 cm column, 4°) of the folate reductase (A) and the specific dihydrofolate reductase (B) of SF/O. In experiment A, a portion (522 dihydrofolate reductase units) of the concentrate of combined fractions 64–80 of the second G-100 filtration (Figure 2) was applied to the gel column; in expt B, a portion (9575 dihydrofolate reductase units) of the concentrate of combined fractions 81–97 of the first G-100 filtration (Figure 1) was applied to the column. Standard buffer was the eluent; effluent fractions, 3.55 ml. The exclusion front, V_0, and the peak elution of myoglobin (M, molecular weight 17,800) and ribonuclease (R, molecular weight 14,000) are indicated.

Electrophoresis of the Dihydrofolate Reductases. The electrophoretic patterns of the folate and the specific dihydrofolate reductases differed (Figure 5). Visualiza-

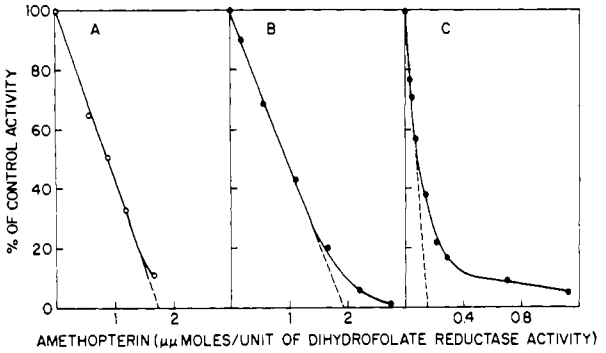

FIGURE 4: The inhibition by amethopterin of the folate reductase activity of the folate reductase (A) and of the dihydrofolate reductase activity of the folate reductase (B) and of the specific dihydrofolate reductase (C). Enzyme activity is expressed as per cent of control (activity determined in the absence of amethopterin taken as 100%). Amethopterin concentration is described as the ratio of the amount added per unit of dihydrofolate reductase activity of the preparation in the reaction. In expt A, the 40-fold-purified folate reductase (78 μg of protein, 6.8 dihydrofolate reductase units) was preincubated (10 min, 25°) in the folate reduction system minus folate in the absence (control) or presence of increasing amounts of amethopterin before the addition of folic acid and incubation at 37°. In expt B, the folate reductase (62 μg of protein, 5.5 dihydrofolate reductase units) and in expt C, the 400-fold-purified specific dihydrofolate reductase (0.6 μg of protein, 7.1 enzyme units) were preincubated (5 min, 30°) with components of the dihydrofolate reduction system except dihydrofolic acid in the absence or presence of increasing amounts of amethopterin before the addition of dihydrofolic acid. Subsequently, standard assay procedures were followed.

tion of active enzyme depended upon the formation of an insoluble colored formazan resulting from the interaction of 3-(4,5-dimethylthiazolyl-1–2)-2,5-diphenyltetrazolium bromide and the enzymatic reaction product, tetrahydrofolate (Gunlack *et al.*, 1966). A single band of activity at 4.3 cm from the origin typified the preparation of the specific dihydrofolate reductase (2 μg of protein). Two bands of dihydrofolate reductase activity comprised the electrophoretic pattern of the folic reductase preparation. Detectable bands of dihydrofolate reductase in the folate reductase preparation depended upon the electrophoresis of 75 μg of total protein in which four other protein bands were demonstrated.

Controlled Synthesis of Dihydrofolate Reductases. A study of the effect of folate supplementation of medium on the synthesis of the dihydrofolate reductases is summarized in Table III. Folate, indeed, increased the total, easily measurable dihydrofolate reductase activity in the cell extracts. Because of the interferring substances, folate reductase activity was not compared until after partial purification of the cell extracts of cultures grown in the differently supplemented media. Purification steps included the protamine sulfate treatment of extracts, ammonium sulfate fractionation, and polyacrylamide gel filtration described above. A modification of the procedure involved longer centrifugation during salt fractionation. Extracts of cells grown in purine- and thymine-free medium (Table III, culture 1) consistently contained protein which was insoluble in ammonium sulfate solution at 0.3 saturation. It was subsequently sedimented by 15-min centrifugation at 12,000*g* and yielded an opalescent but clear supernatant solution for further fractionation. Extracts of cultures provided with exogenous thymine and purines (Table III, cultures 2 and 3) contained protein which, although insoluble in ammonium sulfate solution (0.3 saturation), was not sedimented by the usual conditions. Additional centrifugation at 27,000*g* for 1 hr removed as a pellet the material "salted out" by ammonium sulfate (0.3 saturation). Usually 3–5% of the total dihydrofolate reductase activity of the extract was lost during this fractionation step which routinely preceded the preparation of the individual Am S fractions. Thus, the small amount of folate reductase activity precipitated at 0.3 saturation was included in the summation of the folate reductase activity of each culture (Table III). Folate supplementation did not affect the total amount of folate reductase activity synthesized by SF/O.

The elution profiles (Figure 6) of the polyacrylamide chromatography of the respective Am S fractions illustrate the resolution of the folate and the specific dihydrofolate reductases synthesized during growth in the different media. Figure 6 also shows that an ex-

TABLE II: Relative Dihydrofolate Reductase Activity of Folate Reductase and Specific Dihydrofolate Reductase of *S. faecium* var. *durans* (SF/O).

Enzyme	Dihydrofolate Reductase Act.[a] (units/mg) A	Amethopterin Binding Capacity[b] μμmoles/unit B	Amethopterin Binding Capacity[b] mμmoles/mg of Protein C	Turnover No.[c]
Folate reductase	136	1.90	0.17	800
Specific dihydrofolate reductase	11,024	0.15	1.50	7350

[a] Dihydrofolate reduced (millimicromoles per minute per milligram of protein). [b] Obtained from data plotted in Figure 4 by extrapolating linear portions of plots to the abscissas (B) and further calculation to express value on the basis of total protein (C). [c] Activity per drug binding equivalent: A/C, *i.e.*, moles of substrate converted per minute per mole of drug bound.

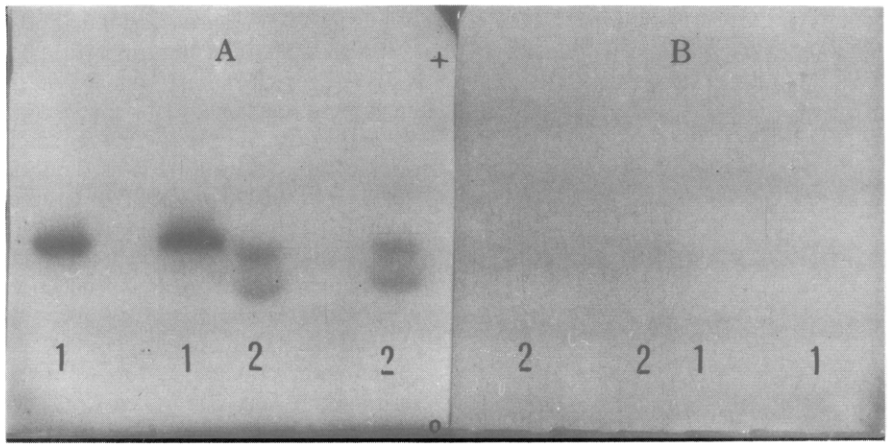

FIGURE 5: Electrophoretic patterns of preparations of (1) the specific dihydrofolate reductase (6.3 units), and (2) the folate reductase (12.1 dihydrofolate reductase units) resulting from polyacrylamide gel filtration. Experimental procedure is described in the text. A represents the portion of the starch gel which was incubated with the dihydrofolate reduction system; B, the portion which was incubated with the dihydrofolate-free solution; o, the origin, and +, the anodal region.

TABLE III: Effect of Exogenous Folic Acid on the Synthesis of Total Dihydrofolate Reductase Activity by Cultures of *S. faecium* var. *durans* (SF/O).[a]

		Dihydrofolate Reductase Activity		
Culture No.	Medium Supplements	Total[b] (units)	Associated with Folate Reductase (units)	Folate Reductase Act.[c] (units)
1	FA	3100	319	4.0
2	P + T	1543	334	3.7
3	P + T + FA	2400	284	3.9

[a] Separate cultures of SF/O were harvested during the exponential phase of growth in medium (described in the text) which had been supplemented with folic acid (FA, 10 mμg/ml) and/or purines (P, adenine, guanine, and xanthine, each at 10 μg/ml) and thymine (T, 2 μg/ml). Enzyme activity values were calculated on the basis of each 100 mg of total protein of the respective cell extracts. [b] Total activity of extract. [c] Total activity of all fractions.

tremely small amount of folate reductase activity migrated with the larger protein molecules eluted earlier than the minor peak of dihydrofolate reductase activity, the bulk of the folate reductase activity. Further, these plots emphasize the induction effect of exogenous folate on the specific dihydrofolate reductase. An indirect effect of purine and thymine supplementation on the migration or filtration properties of the folate reductase activity is also evident (Figure 6). Folate reductase activity synthesized in purine- and thymine-free medium was sharply and maximally eluted in eight effluent fractions. When derived from cultures in purine- and thymine-supplemented media, folate reductase activity was apparently retarded in its migration and, in several experiments, consistently produced a broader elution spectrum frequently characterized by two peaks. The presence of folate during growth with purine and thymine supplementation also affected the ratio of these peaks of folate reductase activity.

Discussion

A folate reductase with extremely low activity and a specific dihydrofolate reductase are constitutive enzymes of the amethopterin-sensitive *S. faecium* var. *durans* (SF/O). Evidently the folate reductase is a dihydrofolate reductase, structurally different from the specific dihydrofolate reductase detected earlier with facility in SF/O (Albrecht *et al.*, 1966a). Perhaps structural dissimilarity is responsible for the affinity of the folate reductase for folate as substrate and its decreased rate in catalyzing the reduction of dihydrofolate.

The appearance of two distinct bands on the electrophoretogram of the folate reductase preparation suggests that two species of folate reductase with identical filtration properties but different electrophoretic mobilities are synthesized by SF/O. Formation of one species as a product of the purification procedure is an alternative consideration. Studies by Mell *et al.* (1968) of the multiple forms of chicken liver dihydro-

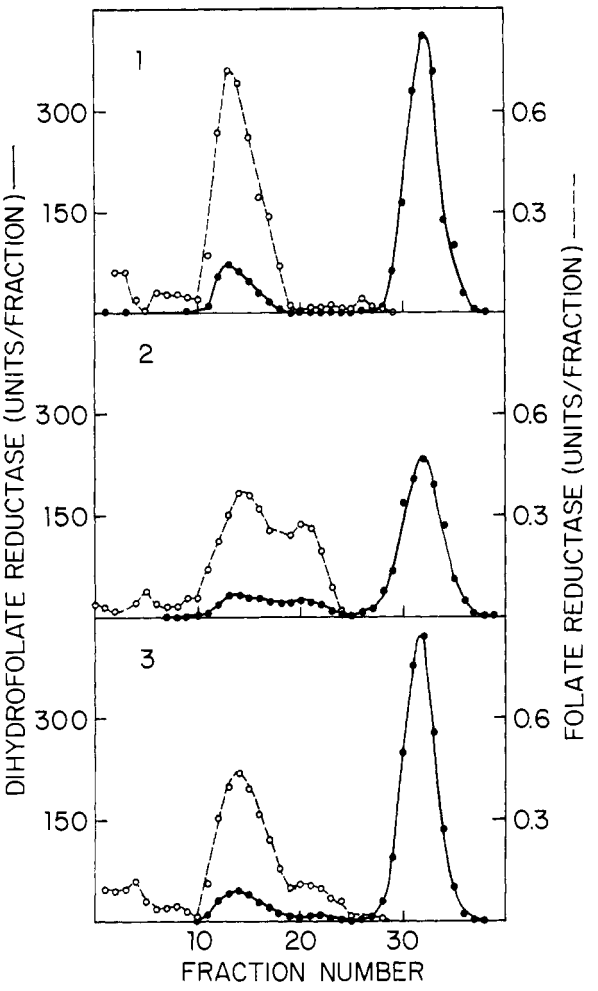

FIGURE 6: Polyacrylamide gel chromatography of the dihydrofolate reductase synthesized by differently supplemented cultures of SF/O. Ammonium sulfate fractions were derived (according to the procedure explained in the text) from cultures 1, 2, and 3 (described in Table III), and applied to the gel column (2.5 × 88 cm). Enzyme activity values of the effluent fractions (3.55 ml, standard buffer) were computed after assay on the basis of each 100 mg of total protein in the respective cell extracts. These data are plotted above for the fractions eluted after the peak elution of total protein (peak taken as zero).

folate reductase emphasize the possibility of interconversion of forms. Equally probable is a trace amount of the specific dihydrofolate reductase in the folate reductase preparation which, as shown, contained several proteins. As a trace contaminant, the specific dihydrofolate reductase, retarded in its movement on the gel by the general composition of the folate reductase preparation, could have produced the second band. Electrophoretic patterns of supplementary analyses suggested that the mobility of the specific dihydrofolate reductase could be altered by the composition of the analytical sample. It is of note that the specific dihydrofolate is so highly active that an amount of the purified enzyme undetectable as protein is readily visualized by its enzymatic activity. Although the second band remains unidentified, several electrophoretic analyses led to the tenet that the band nearer the origin represented a natural folate reductase.

Present data point out similarities between the folate reductase of SF/O and the major dihydrofolate reductase of the amethopterin-resistant mutant strain, *S. faecium* var. *durans*/A_k. Earlier studies with preparations of dihydrofolate reductase purified 80-fold from SF/O and 40-fold from SF/A_k revealed several differentiating properties between those SF/O and SF/A_k enzyme preparations (Albrecht *et al.*, 1966a). Only the SF/A_k enzyme catalyzed the reduction of folate. The apparent amethopterin resistance of the recently purified SF/O folate reductase in reducing dihydrofolate, a reflection of its low turnover, resembles the resistance of the SF/A_k enzyme. The amethopterin binding value of the purified SF/A_k enzyme was 1.53 $\mu\mu$moles/unit of activity; an eightfold lower turnover in dihydrofolate reduction was calculated. Similarity in gel filtration behavior suggests that the molecular weight of the SF/O folate reductase and the SF/A_k reductase (A. M. Albrecht, 1968, unpublished data) is 19,000. These values resemble those estimated for several animal dihydrofolate reductases that also catalyze the reduction of folate. Presumably, the folate reductase is the prevailing form of dihydrofolate reductase of SF/A_k in which the level of the specific dihydrofolate reductase is low (A. M. Albrecht, 1968, unpublished data).

Synthesis by these streptococci of more than one protein with dihydrofolate reductase activity is not extraordinary. Multiple forms of dihydrofolate reductase have been observed previously within a bacterial species (Sirotnak and Hutchison, 1966; Hillcoat and Blakley, 1966) and in *Escherichia coli* before and after infection with various T bacteriophages (Mathews, 1967). Recently, two different dihydrofolate reductases have been obtained from the amethopterin-resistant mutant strain A of *S. faecium* var. *durans* (Nixon and Blakley, 1968). Kenkare and Braganca's (1963) purification of two sheep liver enzymes, active in the separable reductions of folate to dihydrofolate and dihydrofolate to tetrahydrofolate, is unique. In consideration of such resolution, one may question the basis of the reduction of folate and dihydrofolate by the 40-fold purified SF/O folate reductase. Electrophoretic patterns of the *Streptococcus* enzymes do not exclude the possibility that the SF/O folate reductase is a preparation of a *specific* folate reductase contaminated with the *specific* dihydrofolate reductase. Electrophoretic patterns, however, substantiate in the folate reductase preparation the presence of another dihydrofolate reductase distinct from the *specific* dihydrofolate reductase.

Substrate affinity of a reductase in SF/O for folate perhaps manifests a biochemical design and physiological economy within this bacterium. Even a low level of folate reductase activity could fulfill a short-lived physiological need for folate reduction during growth. By contrast, the need for dihydrofolate reduction is maintained. Oxidation of tetrahydrofolate to dihydrofolate during thymidylate biosynthesis (Friedkin, 1963) calls for the regeneration of tetrahydrofolate from dihydrofolate. Undoubtedly the specific dihydrofolate reductase of SF/O mediates this reductive step during

growth. Enzyme data obtained with extracts of folate-supplemented and folate-free cultures substantiate such intracellular activities.

When spared of folate-dependent metabolism by exogenous purines and thymine, SF/O synthesized low levels of folate and dihydrofolate reductase. Folate supplements increased the differential rate of synthesis of just the specific dihydrofolate reductase both in the presence and absence of the purines and thymine. Presumably, dihydrofolate, once it is formed and then maintained metabolically at a certain level, effects the intracellular induction. With thymine supplementation a decreased differential rate of synthesis of the specific dihydrofolate reductase may be physiologically economical. Apparently, the ratio of purines to thymine in these studies offset any possible repression of the synthesis of the specific dihydrofolate reductase by exogenous thymine by effecting exhaustion of the thymine supplement and the subsequent synthesis *de novo* of thymine.

Identification of the immediate product of folate reduction with highly purified folate reductase may clarify the *in vivo* role of these reductases. Probably each has a primary purpose: initial reduction of folate by the folate reductase; homeostasis of the dihydrofolate–tetrahydrofolate concentration ratio by the specific dihydrofolate reductase.

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