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Dihydrofolate Reductase of *Streptococcus faecium*

II. PURIFICATION AND SOME PROPERTIES OF TWO DIHYDROFOLATE REDUCTASES FROM THE AMETHOPTERIN-RESISTANT MUTANT, *STREPTOCOCCUS FAECIUM* VAR. *DURANS* STRAIN A*

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SUMMARY

From a single amethopterin-resistant organism, *Streptococcus faecium* var. *durans* strain A, two different dihydrofolate reductases have been obtained as essentially homogeneous proteins in good yield. One of the reductases has a similar substrate specificity and turnover number (about 8000 moles per min per mole of enzyme) to the single reductase found in the amethopterin-sensitive strain of *S. faecium* var. *durans*, ATCC 8043, and has therefore been designated "wild type." The other enzyme, which is distinguished by its ability to catalyze the reduction of folate, in addition to dihydrofolate, and by its lower turnover number (about 900 with dihydrofolate), has been designated "mutant type." Since the wild type and mutant type reductases have sedimentation constants ($s_{20,buffer}$) of 2.58 S and 2.04 S, respectively, they are probably significantly different in molecular weight. Each exhibits a single pH optimum at pH 5.8 and is inactivated by urea. Neither is affected by methylmercuric salts but the wild type reductase is inactivated by phenylmercuric acetate and *p*-mercuribenzoate. Monovalent cations increase the activity of the mutant type reductase but decrease that of the wild type reductase. It is suggested that the amethopterin resistance *in vivo* of strain A depends at least partly on the folate reductase activity of the mutant type reductase.

Dihydrofolate reductase, the enzyme specifically inhibited by the 4-amino-4-deoxy analogues of folic acid, has been obtained in substantially pure form from a number of normal tissues (2, 3) and from cultured mammalian cell lines selected for resistance to amethopterin (4-amino-4-deoxy-10-methylfolic acid) (4, 5). Amethopterin resistance is frequently associated with the presence in the mutant line of dihydrofolate reductase with unchanged properties but at higher levels than in the sensitive parent strain. This has been reported for resistant mutants of bacteria (6, 7), of cultured mammalian cells (8, 9), and of transplantable tumors (10–14). However, there are a number of cell lines in which amethopterin resistance is apparently associated with biosynthesis of an altered dihydrofolate reductase (1, 6, 7). Whether resistance *in vivo* to the 4-amino-4-deoxy analogues of folic acid is partly caused by synthesis of dihydrofolate reductase with changed structure has yet to be determined. In order to obtain data relating to such a possible mechanism of amethopterin resistance, we have continued our studies of the dihydrofolate reductase of strain A of *Streptococcus faecium* var. *durans*, the organism formerly referred to as *Streptococcus faecalis* R SF/A (15). Previously, we reported evidence that more than one species of dihydrofolate reductase is present in this organism (1). This report describes the separation of two dihydrofolate reductases from this source, their purification to substantially homogeneous proteins, and a comparison of some of their properties.

EXPERIMENTAL PROCEDURE

Materials—Deoxyribonuclease, chromatographically pure folic acid, and *p*-mercuribenzoate were obtained from Sigma, phenylmethanesulfonylfluoride from Calbiochem, and NADPH and NADP from P-L Biochemicals. Pterin (2-amino-4-hydroxypteridine) 6-carboxylic acid, 6-formylpterin, and 6-methylpterin, obtained as gifts from Cyanamid International, (Pearl River, New York), by courtesy of Dr. A. Heltai, were chromatographically pure. Folyldiglutamic acid, 3′,5′-dichlorofolic acid, 10-methyl folic acid, and 9-methyl folic acid, also obtained as gifts from the same source, contained appreciable contaminants when examined by paper chromatography with, as solvent, 100 mM potassium phosphate buffer, pH 7.2, equilibrated with excess *iso*-amylalcohol; they were therefore partially purified by the butanol extraction procedure described by Blakley (16) for the purification of folic acid. The purified compounds and pteroic acid, also from Cyanamid International, contained only minute amounts of contaminants on examination by the same chromatographic system. The method of Blakley (17) was used to prepare 10-formylfolate.

Dihydrofolate was prepared by the method of Blakley (18);

* For the previous paper in this series, see Reference 1.
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dihydrofolyldiglutamic acid, 10-methyldihydrofolic acid, 9-methyldihydrofolic acid, 3′,5′-dichlorodihydrofolic acid, and 10-formyldihydrofolic acid were prepared by reduction of the parent compounds according to the method of Friedkin, Crawford, and Misra[1]; dihydropteroic acid and dihydropterin 6-carboxylic acid were prepared by catalytic hydrogenation (16). Catalytic hydrogenations were continued until 1 molecular proportion of hydrogen had been taken up. (±), L-Tetrahydrofolate was prepared as previously described (16). Aminopterin (4-amino-4-deoxyfolic acid) from International Chemical and Nuclear Corporation (Yorktown, California) contained appreciable amounts of contaminants on chromatographic examination and was therefore twice purified by the method of Loo (19).

Methylmercuric bromide, methylmercuric iodide, and phenylmercuric acetate were obtained from K and K Laboratories. Iodine solutions were standardized by the method of Cunningham and Nuencke (20).

Solutions of protamine sulfate (Mann), were prepared by stirring 20 g of the solid with 1 liter of water, adjusting to pH 5.6 with 5 N NaOH, and stirring for 5 hours at room temperature. After removal of insoluble material by centrifugation, the concentration of soluble protein was 13.2 mg per ml. Cellulose tubing (Visking Corporation, Chicago, Illinois) was soaked in 50 mM EDTA for 16 hours before use.

Bead form Sephadex G-75 (Pharmacia) was equilibrated by stirring with buffer for 48 hours prior to being poured to form a column. DEAE-Sephadex (A-50, medium; Pharmacia) was allowed to swell in water for 48 hours, during which period fines were removed; it was then cycled through 0.5 N HCl and 0.5 N NaOH with copious washing in water, and equilibrated with buffer before being poured to form a column. Hydroxyapatite (Bio-Rad) was stirred with 0.75 g of Whatman standard cellulose powder per 1 g of hydroxyapatite (dry weight) in buffer before being used to form columns. The function of the cellulose was to give good flow rates in columns with sufficient capacity and resolving power.

Methods—Spectrophotometric assay of dihydrofolate reductase was carried out at 37° with a Cary model 14 recording spectrophotometer. The standard reaction mixture contained 33 μM dihydrofolate, 50 μM NADPH, 5 mM mercaptoethanol, and 50 mM potassium phosphate buffer at pH 6.9 in a total volume of 3 ml. After recording the rate of absorbance change in the absence of dihydrofolate, the reaction was initiated by the addition of dihydrofolate and mercaptoethanol. A value of 12,300 (21) was used for the molar extinction change at 340 mμ in the reaction. Folate reductase activity was measured by a similar assay, except that in the standard reaction mixture 50 mM sodium citrate buffer, pH 6.1, replaced phosphate buffer and 33 μM folate replaced dihydrofolate. The molar extinction coefficient used for this reaction was 18,400 (1). One unit of enzyme activity is defined as that amount catalyzing the reduction of 1 μmole of dihydrofolate, or of folate, to tetrahydrofolate per min in the standard assay system at 37°.

In the determination of the equilibrium constant for the dihydrofolate reductase reaction both reaction and reference cells initially contained, in 3.0 ml, 50 mM Tris-chloride buffer (pH 8.2) (or 50 mM potassium phosphate buffer, pH 6.7), 20 mM 2-mercaptoethanol, 13 mM ascorbate, 1.2 to 2.38 mM (±), L-tetrahydrofolate, and enzyme (0.6 mμmole of mutant type reductase or 56 μμmoles of wild type reductase) at 37°. After recording the steady base line absorbance at 340 mμ, the volume in each cell was made to 3.0 ml by the addition of water to the reference cell and an equal volume of NADP solution to the reaction cell to give a final concentration of NADP in the range 0.27 to 6.7 mM. The absorbance change was followed to equilibrium, which was attained within 10 min, before measurement of the pH of each solution. The equilibrium concentrations of dihydrofolate and NADPH were determined by the use of the molar extinction change for the reaction (21) and the equilibrium constant for the reaction was calculated on the basis that only half of the chemically prepared (±), L-tetrahydrofolate is the reactive diastereoisomer. Preliminary experiments showed that the absorbance at 340 mμ due to the above concentrations of 2-mercaptoethanol and ascorbate was only 0.03, but that these concentrations afforded reasonable stability to tetrahydrofolate during the period required for each experiment at about pH 8. However, tetrahydrofolate was noticeably less stable at pH 6.7. Moreover, the lower the pH, the less favorable is the equilibrium for measurement of the reverse reaction and consequently the results of experiments at pH 6.7 were less satisfactory than those of experiments at about pH 8.

Protein concentrations greater than 1 mg per ml were determined by the biuret method of Gornall, Bardawill, and David (22) with bovine serum albumin as the standard. At lower concentrations protein was determined by the biuret method of Ellman (23). Since with albumin as standard the two biuret methods gave different values for protein concentration in enzyme preparations, the method of Ellman was used with a standard consisting of a 30-fold purified preparation of dihydrofolate reductase, concentration of which had been determined by the method of Gornall *et al.* (22) with albumin as standard. The protein concentration of fractions eluted from columns was estimated by absorbance measurements at 280 mμ.

Disc electrophoresis on polyacrylamide gel was carried out by a modification of the method of Davis (24) with a 10% resolving gel, a 6% spacer gel, and polymerization by ammonium persulfate. The resolving gel was 5 cm long and 5 mm in diameter, and samples varied from 10 to 110 μg of protein. After polymerization, excess persulfate was removed by electrophoresis in the Tris-glycine buffer (25 mM with respect to Tris and pH 8.5) before the sample was applied by layering it over the spacer gel as a solution containing 300 mg of sucrose per ml. Following electrophoresis, gels were treated in one of three ways, as follows. (*a*) Amido schwarz was used to stain for protein (24). After photography, the relative densities of protein bands were compared by a Spinco Analytrol Densitometer. (*b*) Gels were cut at right angles to the long axis at 1-mm intervals, and each of the discs produced was divided into two; one half was stained by Amido schwarz to detect protein-containing sections and the other half was extracted with 10 mM potassium phosphate buffer, pH 7.2, containing 0.5 mM EDTA, and the extract was tested for dihydrofolate reductase activity in the standard assay. (*c*) Gels were stained for dihydrofolate reductase activity by incubation at 37° in a modification of the histological stain described by Gunlack, Neal, and Williams (25). The reaction mixture contained 1 mM NADPH, 1 mM dihydrofolate, 50 mM phosphate buffer (pH 6.9), and 0.4 mg of thiazolyl blue per ml of solution. Preliminary spectrophotometric experiments showed that the formation of a blue insoluble reduced derivative of this tetra-

[1] M. Friedkin, E. J. Crawford, and D. Misera, *Fed. Proc.*, **21**, 176 (1962).

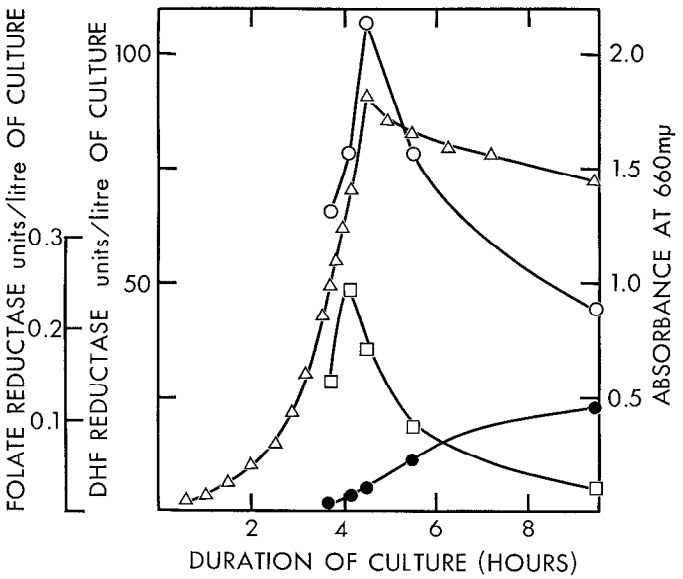

FIG. 1. Growth of SF/A and synthesis of enzyme as a function of time. Harvested cells were lysed at 37° by treatment for 60 min with 10 mg of lysozyme per g of bacterial paste suspended in 4 ml of buffer solution (10 mM potassium phosphate, pH 7.2, containing, in each liter, 5 mg of deoxyribonuclease and 2 mg of phenylmethanesulfonylfluoride). Enzyme activities are expressed per volume of culture for the dihydrofolate reductase of cell extracts (○——○), the folate reductase of cell extracts (□——□), and the extracellular dihydrofolate reductase in the medium (●——●). The specific activities of the enzyme extracted from cells paralleled the total activities. The absorbance at 660 mμ is indicated on a linear scale (△——△). In this and other figures, *DHF* is dihydrofolate.

zolium dye is dependent on the formation of tetrahydrofolate provided that mercaptoethanol is omitted from the reaction mixture.

The sedimentation[2] of the two purified dihydrofolate reductase preparations was carried out in the Beckman model E analytical ultracentrifuge at 50,740 rpm in a 12-mm cell with a double sector centerpiece. Schlieren optics were used and the temperature was controlled near 1°. Samples were in 10 mM potassium phosphate buffer, pH 7.2, containing 0.5 mM EDTA and the same buffer was used in the reference sector of the cell. Sedimentation coefficients were corrected to water at 20° as outlined by Schachman (26).

Bacterial Cultures—S. faecium var. *durans* strain A (SF/A)[3] was grown as described by Hillcoat and Blakley (1) with the following modifications. The stock culture was twice recloned and a single colony was used to inoculate 8 liters of sterile medium which was incubated at 37° until it reached a dense turbidity (about 20 hours), and was then inoculated into 50 liters of unsterilized medium. Growth at 37° was measured by determining the absorbance of samples at 660 mμ. When the culture reached an absorbance of 1.5 (late exponential growth phase), it was rapidly cooled to 12° and harvested on a Sharples centrifuge. The wet weight of bacteria obtained was 130 to 150 g.

[2] Kindly measured by Dr. J. R. Dunstone of the Department of Physical Biochemistry, Australian National University, Canberra, Australia.

[3] An amethopterin-resistant strain selected as a multistep mutant and kindly supplied by Dr. D. J. Hutchison of the Sloan-Kettering Institute.

RESULTS

Biosynthesis of Dihydrofolate Reductase during Growth of Cultures

During the phases of exponential and linear growth of cultures of SF/A, dihydrofolate reductase activity of extracts of the cells increased in parallel with growth as shown in Fig. 1. However, during the stationary phase, both the dihydrofolate reductase content of cells and, to a lesser extent, the turbidity of the culture decreased. The specific activity of the reductase paralleled the rise and fall of total activity. Folate reductase activity began to decrease even earlier, during the course of the exponential growth phase, and again the specific activity followed total activity. At least part of the fall in cellular dihydrofolate reductase activity could be accounted for by the appearance of reductase activity in the medium. The remainder of the fall in activity would be readily explained if the reductase were denatured under extracellular conditions at 37°, as is suggested by the results of cell lysis at this temperature (see below). The finding of extracellular dihydrofolate reductase, together with the decrease in turbidity of the culture during stationary phase, suggested that cells may lyse during the latter phase of growth, with the additional possibility that prior to lysis the cell wall may become permeable to dihydrofolate reductase.

When an examination was made of the growth characteristics and content of dihydrofolate reductase of the amethopterin-sensitive *S. faecium* var. *durans* (formerly referred to as *Streptococcus faecalis* R, ATCC 8043 (27)) no decrease of dihydrofolate reductase activity or of the absorbance of the culture was observed during stationary phase. The total dihydrofolate reductase activities of extracts of cells from exponential phase cultures of SF/A were consistently 12-fold greater than those of *S. faecium* var. *durans* if cells were harvested at comparable stages of growth.

Lysis of SF/A Cells

The capacity of cells of SF/A to undergo autolysis was confirmed by stirring harvested cells in the buffer solution described in the legend to Fig. 2, which illustrates the time course for the release of cell protein and reductase activities at 3°. The sediment which resulted from stirring cells for 20 hours at 3° in buffer consisted mostly of cell debris, an observation indicative that the procedure resulted in cell lysis. The autolysis was not affected by prior freezing of the cells to −20° or even to −195°, but the rate of autolysis was more rapid at 37° than at 3°. The greatest recovery of reductase of high specific activity was obtained by stirring the cells in buffer for 20 hours at 3°; this was therefore adopted as the standard procedure for the extraction of enzyme.

To examine the possibility that the cultures of SF/A might have been infected by a lysogenic phage, cell extracts were tested for their ability to cause plaque formation on lawn cultures of SF/A and of *S. faecium* var. *durans*. No plaques were obtained but after prolonged incubation there was a general lysing of the lawn cultures which was not related to the sites of application of the cell extracts. This result may be considered consistent with the effects of a cell wall lytic enzyme released by the cells, rather than with those of a lysogenic phage.

Purification of Dihydrofolate Reductase

The procedures adopted have been developed from those previously published (1). The cells from 58 liters of culture of SF/A were harvested as described in "Methods" and were extracted without washing in order to prevent loss of enzyme by autolysis.

Step 1: Extraction—Cells were allowed to autolyze for 20 hours at 3° as described in the previous section. They were then centrifuged at 35,000 × *g* for 2 hours. This and all subsequent steps were carried out at 3°. The supernatant solution was kept, the gelatinous insoluble contents of lysed cells, which formed a loosely packed upper layer in the sedimented material, were tipped off, and any unlyzed cells remaining as a tightly packed bottom layer were resuspended and autolyzed for a further period of 12 hours. The suspension was centrifuged, and the supernatant was combined with that from the previous extraction.

Step 2: Protamine Sulfate Treatment—The solution from Step 1 was adjusted to pH 5.6 with 1 *N* acetic acid and 0.175 volume of protamine sulfate solution was added. The mixture was stirred 90 min before centrifugation at 20,000 × *g* for 60 min. The precipitate was discarded.

Step 3: Ammonium Sulfate Precipitation—For complete precipitation of dihydrofolate reductase by ammonium sulfate it was found necessary to have a relatively high enzyme concentration in the solution from the previous step. A dihydrofolate reductase activity of 10 units per ml was satisfactory. To the solution obtained from the previous step EDTA was added to a final concentration of 0.5 mM; then solid ammonium sulfate was slowly added to bring the final concentration of ammonium sulfate in the solution to 3.5 M. After stirring for 16 hours, the mixture was centrifuged at 18,000 × *g* for 90 min and the precipitate was suspended in a minimal volume (about 50 ml) of 50 mM potassium phosphate buffer, pH 7.2, containing 0.5 mM EDTA. This buffer was used throughout Steps 3 to 6. The suspension was dialyzed, with rocking, against 2.5 liters of this buffer containing sucrose (150 g per liter) for 18 hours, by which time the precipitate had completely dissolved. The addition of sucrose prevented an increase of volume within the sac, during dialysis.

Step 4: First Sephadex G-75 Column—The solution from Step 3 was applied to a column of Sephadex G-75, 125 × 4 cm, eluted by buffer, and collected in fractions of 20 ml. The enzymically active fractions from the column were pooled. The column was washed with buffer before re-use.

Step 5: Ammonium Sulfate Precipitation—The enzyme in the pooled fractions was precipitated with ammonium sulfate, suspended in buffer, and dialyzed as before.

Step 6: Second Sephadex G-75 Column—The solution from Step 5 was applied to a column of Sephadex G-75 (125 × 4 cm) and eluted by buffer. The enzymic activity elution profiles shown in Fig. 3 suggested the presence of two types of dihydrofolate reductase. The elution of dihydrofolate reductase activity followed an unsymmetrical curve with a shoulder on the trailing edge of the main peak. Folate reductase activity was coincident with the main peak but the shoulder appeared to represent a type of dihydrofolate reductase which was devoid of detectable folate reductase activity and which was more slowly eluted from Sephadex G-75. The poor resolution of these two

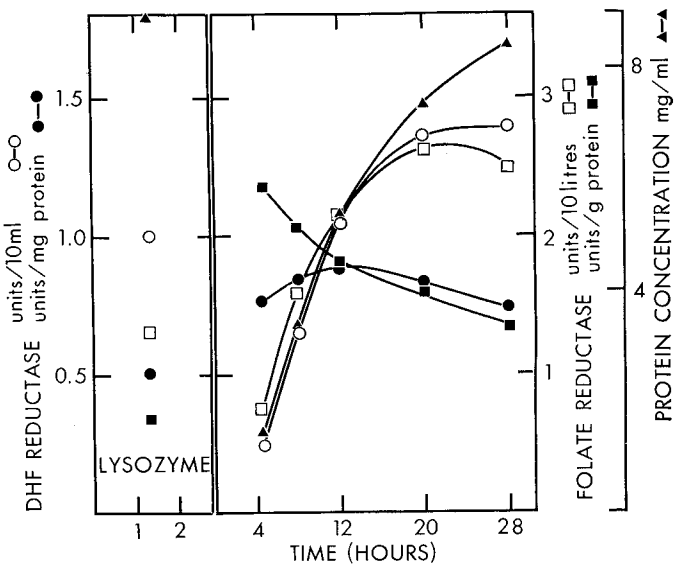

FIG. 2. Time course of cell lysis and extraction of dihydrofolate reductase from cells of SF/A. *Left panel;* results for cells stirred at 37° for 80 min with lysozyme, as described for Fig. 1. *Right panel;* results for cells stirred at 3° in the buffer solution described for Fig. 1, but without the addition of lysozyme.

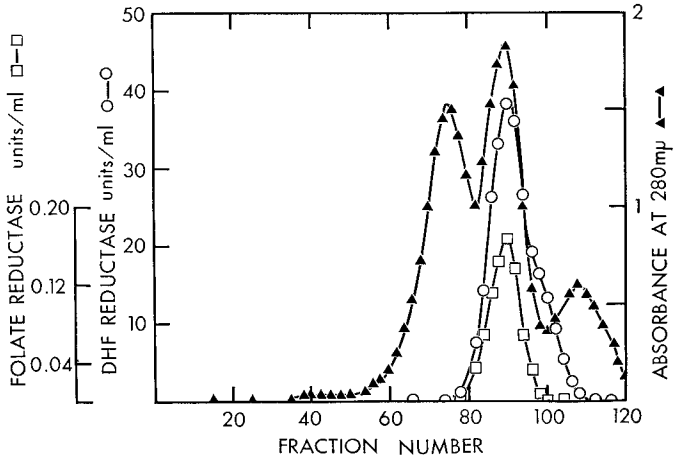

FIG. 3. Purification of dihydrofolate reductase by gel filtration on Sephadex G-75 (Step 6). Fractions of 10 ml were collected during elution from the column which measured 125 × 4 cm.

types of reductase by gel filtration suggested that they were not greatly dissimilar in molecular size.

Step 7: DEAE-Sephadex Ion Exchange Chromatography—The enzymically active fractions from Step 6 were pooled and adsorbed to a column of DEAE-Sephadex which measured 22 × 4 cm and which had been equilibrated with 50 mM potassium phosphate buffer, pH 7.2. Proteins were eluted from the column by potassium phosphate buffer, pH 7.2, the concentration of which was increased in a gradient linear from 50 to 300 mM. As shown in Fig. 4, two distinct peaks of dihydrofolate reductase activity were eluted from the column. The dihydrofolate reductase eluted first (designated 7m in Table I) also catalyzed the reduction of folate since the elution profile for folate reductase activity closely paralleled that for dihydrofolate reductase activity. The dihydrofolate reductase eluted second (designated 7w in Table I) did not catalyze the reduction of folate.

Step 8: Hydroxyapatite Chromatography—The enzymically active fractions for each of the two peaks eluted from DEAE-Sephadex were pooled and the two resulting enzyme preparations were each dialyzed against distilled water for 20 hours, in which period the ionic strength of each was reduced to less than that of 10 mM potassium phosphate, pH 7.2. The enzyme from Step 7m, with folate reductase activity, was adsorbed to a hydroxyapatite column, 20 × 4 cm, which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.2. A trace of inactive protein was eluted by 10 mM potassium phosphate, pH 7.2; then fractions with dihydrofolate reductase and folate reductase activities were eluted by 20 mM potassium phosphate, pH 7.2. The enzyme from Step 7w, without folate reductase activity, was adsorbed to a hydroxyapatite column, 6 × 4 cm, which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.2. Inactive protein was eluted by 10 and 20 mM potassium phosphate, pH 7.2; fractions which had dihydrofolate reductase activity but no folate reductase activity were eluted by 40 mM potassium phosphate, pH 7.2. Fractions of 10 ml were collected during elution from each hydroxyapatite column. The preparations resulting from Step 8 are designated 8m and 8w, respectively, in Table I. It may be seen that during purification by hydroxyapatite chromatography the two dihydrofolate reductase fractions retained their specificities with respect to folate reductase activity and that they were further distinguished by the ionic strength of buffer required to elute each from the columns.

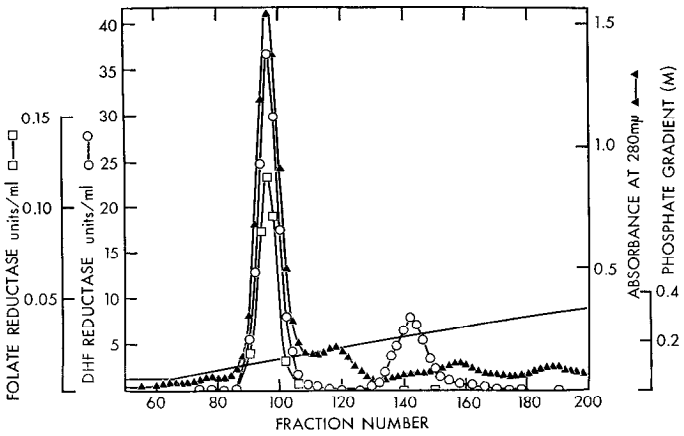

FIG. 4. Separation and purification of two types of dihydrofolate reductase by ion exchange chromatography on DEAE-Sephadex. Fractions of 10 ml were collected. Other details are described in the text.

Table I shows that the steps giving the greatest purification were the inital fractionation on Sephadex gel and the separation on DEAE-Sephadex. Gel filtration of the preparation through Sephadex a second time enabled greater utilization of the fractionation capacity of Sephadex than could be achieved during the first gel filtration stage. For convenience, the dihydrofolate reductase preparation which does not catalyze the reduction of folate (Fractions 7w and 8w) is designated "wild type" and the other dihydrofolate reductase (Fractions 7m and 8m) is designated "mutant type." Reasons for these designations are discussed below. The specific activity of the wild type dihydrofolate reductase was increased to 520 times the specific activity of the crude extract and the purification of the mutant type reductase resulted in enzyme whose dihydrofolate reductase activity was 82 times that of the crude extract. The over-all recovery of dihydrofolate reductase activity in the two preparations was 35% and the recovery of folate reductase activity was 37%, all of which was associated with the mutant type reductase. If it is assumed that the recoveries of the two types of dihydrofolate reductase are equal during purification from the crude extract then it is possible to calculate the proportion of the dihydrofolate reductase activity of the crude extract which was attributable to each of the two types of enzyme. On this basis, 69% of the dihydrofolate reductase activity of the crude extract would have been due to mutant type reductase. The wild type reductase has therefore been purified to a specific activity 1670 times that of this enzyme in the crude extract and the mutant type reductase to a specific activity 120-fold that of this species in the crude extract.

Properties of Two Dihydrofolate Reductases

Polyacrylamide Gel Electrophoresis—Fig. 5 shows the results of electrophoresis, on polyacrylamide gel at pH 8.5, of the two

TABLE I

Purification of dihydrofolate reductase and folate reductase from Streptococcus faecium *var.* durans *strain A*

Stage of fractionation	Volume	Protein concentration	Dihydrofolate reductase			Folate reductase		
			Total enzyme activity	Recovery	Specific activity	Total enzyme activity	Recovery	Specific activity
	ml	*mg/ml*	*units*	%	*units/mg protein*	*units*	%	*units/mg protein*
1	590	20	6700	(100)	0.55	14.4	(100)	0.0012
2	635	11.1	6580	98	0.93	13.0	90	0.0018
3	92	61	6440	96	1.13	12.1	84	0.0022
4	325	2.1	5000	75	7.2	11.9	83	0.0170
5	11.5	44	5750	86	11.2	11.4	79	0.0223
6	265	1.01	4600	69	16.5	14.4	100	0.0513
7m[a]	260	0.24	2340	35	37.6	9.12	63	0.148
7w[a]	290	0.019	1040	16	184	0	0	0
8m[a]	480	0.073	1650	25	45	5.55	39	0.160
8w[a]	226	0.015	640	10	285[b]	0	0	0

[a] At Step 7 dihydrofolate reductase activity was separated into two fractions designated m (mutant type) and w (wild type), each of which was purified separately through step 8.

[b] Specific activity in five peak fractions of protein concentration 0.05 mg per ml.

purified preparations of dihydrofolate reductase from SF/A. The wild type reductase migrated to the anode as a single discrete zone of protein with which was associated all of the dihydrofolate reductase activity detectable in the gels at the completion of electrophoresis. Under the same conditions the mutant type reductase migrated more slowly to the anode as a single complex in which four zones of protein could always be distinguished, unless the gel was overloaded. Part of this complexity is seen in the shoulders of the main peak and the presence of the secondary peak in Fig. 5. The preparation of mutant type reductase also contained a trace of contaminating protein which migrated to the anode more rapidly than the mutant type reductase. The dihydrofolate reductase activity of the preparation of mutant type reductase was entirely associated with the whole of the single complex zone of protein. The reason for the complexity of the zone of mutant type reductase is not apparent. It can be seen from Fig. 5 that the ratio of dihydrofolate reductase activity to protein content is approximately constant over the whole of the complex. The appearances of gels stained directly for dihydrofolate reductase activity were similar to those stained for protein.

Velocity Sedimentation—Photographs of schlieren patterns obtained during the sedimentation of the two dihydrofolate reductase preparations (Fig. 6) show single, apparently symmetrical peaks, although the earliest patterns suggest that each preparation may contain trace amounts of components sedimenting faster than the main peak. The spreading of the boundary that was evident during the course of the experiments was probably due to diffusion of these proteins of relatively low molecular

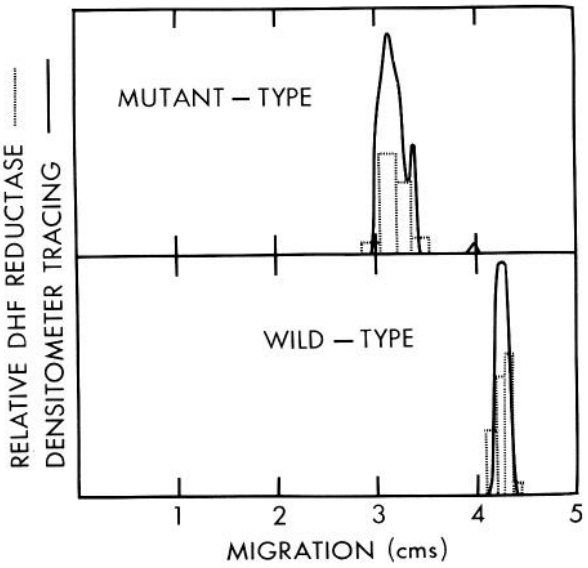

FIG. 5. Polyacrylamide gel electrophoresis of the preparations of mutant type and wild type reductase. The figure shows densitometer tracings of photographic negatives of gels stained for protein and the relative dihydrofolate reductase activity extracted from half-slices of comparable gels. The amplitude of the densitometer trace is expressed in arbitrary units since it is dependent on the settings of the scanner and the conditions used for the development of the negative. The enzyme activity extracted is also given in arbitrary units since it depended on the conditions of electrophoresis and extraction. A total of about 20 to 30% of the activity applied to the gels was present in the extracts. The activity values were matched with the densitometer trace by reference to the amount of protein staining in the other half of each slice.

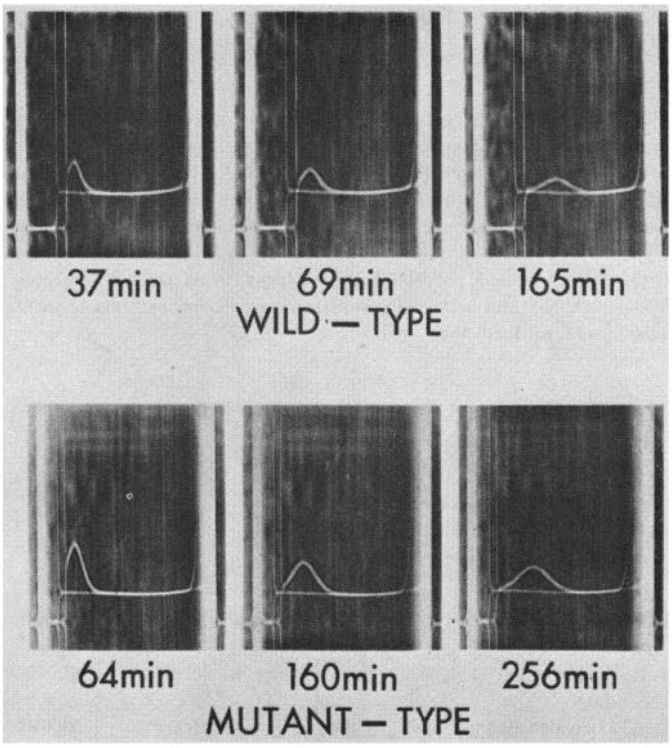

FIG. 6. Velocity sedimentation. Photographs are shown of the schlieren patterns which were obtained at the times indicated during the course of ultracentrifugation. The protein concentrations were 2.4 mg per ml and 3.3 mg per ml, and the phase plate angles were 55° and 60° for the wild type and mutant type preparations, respectively.

weight. Since the protein concentration used for each experiment was limited to the amount of enzyme which could be purified from 58 liters of SF/A culture it was not possible to determine the dependence of the sedimentation coefficient on concentration. The sedimentation coefficients determined were as follows: for the wild type reductase, $s_{20,buffer}$ = 2.58 S; for the mutant type reductase, $s_{20,buffer}$ = 2.04 S. Approximate estimates of the minimum molecular weight of each dihydrofolate reductase, obtained from their sedimentation coefficients by comparison with that of albumin (26, 28), were 28,000 for the wild type reductase and 20,000 for the mutant type reductase.

By the use of the approximate values estimated for the molecular weights and the specific activities of the two dihydrofolate reductases, and on the assumption that each is not contaminated by other proteins, the approximate turnover numbers for the two enzymes can be calculated. At 37° and at pH 5.8, the optimum pH for both dihydrofolate reductases (see below), the approximate turnover numbers for dihydrofolate are 8000 and 900 moles per min per mole of enzyme for the wild type and mutant type reductases, respectively. The approximate turnover number for folate reduction catalyzed by the mutant type reductase is 7.5 moles per min per mole of enzyme at 37° and pH 4.7.

Spectra—Each dihydrofolate reductase exhibited a single absorbance maximum at 278 mμ and a distinct shoulder at 290 mμ, but no absorbance spectrum in the visible region. The presence of significant amounts of tryptophan, indicated by the shoulder at 290 mμ, may account for the unusually high absorb-

TABLE II

Specificity of dihydrofolate reductases for dihydropteridine and pteridine derivatives as substrates

Dihydro derivatives of pteridine, pteroate, and folate compounds were tested as substrates for the purified reductases in an assay system as described in "Methods," except that 100 μM NADPH was used, the pH was 6.6, and the substrate under test was used at a concentration of 50 μM. Reaction mixtures contained 0.64 $\mu\mu$mole of wild type reductase or 3.1 $\mu\mu$moles of mutant type reductase. Unreduced derivatives were tested similarly, but at pH 6.1 and with 36 $\mu\mu$moles of wild type reductase or 600 $\mu\mu$moles of mutant type reductase.

Dihydropteridine derivative	Wild type reductase	Mutant type reductase	Pteridine derivative	Mutant type reductase
	$\Delta A_{340}/min/$ mμmole reductase			$\Delta A_{340}/min/$ mμmole reductase
Dihydrofolate	31	3.1	Folate	0.051
Dihydrofolyldiglutamate	20	1.62	Folyldiglutamate	0.054
10-Methyldihydrofolate	5.0	1.34	10-Methylfolate	0.030
9-Methyldihydrofolate	0	0	9-Methylfolate	0.004
3′,5′-Dichlorodihydrofolate	5.3	1.44	3′,5′-Dichlorofolate	0.040
10-Formyldihydrofolate	0.78	0.31	10-Formylfolate	0.20
Dihydropteroate	2.3	1.50	Pteroate	0.11
Dihydropterin 6-carboxylate	0	0	Pterin 6-carboxylate	0
			6-Formylpterin	0
6-Methyldihydropterin	0	0	6-Methylpterin	0

ance of each reductase at 280 mμ. In each case a solution of protein of concentration 1 mg per ml had an absorbance of 2.0 at 280 mμ.

Substrate Specificity—The ability of the wild type and mutant type reductases to catalyze the reduction of a number of pteridine, pteroate, and folate derivatives was tested. The wild type reductase did not catalyze the reduction of any of the unreduced derivatives tested. Results are compared in Table II in terms of the absorbance change per min per mμmole of the respective dihydrofolate reductases, because the molar extinction changes for the reduction of many of those compounds to the corresponding tetrahydro derivatives have not been established. In the case of most substrates tested there was little difference between the rates observed at 50 and 100 μM substrate concentrations, an observation indicating that these concentrations were near saturating. Only in the case of 9-methylfolate was the rate of reduction much faster (double) at a substrate concentration of 100 μM than at 50 μM. At either concentration the rate of reduction of 9-methylfolate was very slow compared to the reduction of folate. The reduction of 10-formylfolate catalyzed by mutant type reductase was much more rapid than that of folate, although 10-formyldihydrofolate was reduced slowly by either reductase, compared to the reduction of dihydrofolate. Acidification of the reaction mixture to pH 1 after reduction of 10-formylfolate or 10-formyldihydrofolate resulted in alteration of the product of the enzymic reaction to a compound whose absorbance spectrum had a peak at 350 mμ, consistent with the assumption that the product of the reduction of 10-formylfolate or of 10-formyldihydrofolate is 10-formyltetrahydrofolate. It should be noted that pteroate and dihydropteroate were appropriate substrates but simple pterins did not serve as substrates for either reductase. An attempt was made to determine whether these pterins were able to bind to either dihydrofolate reductase by testing them as inhibitors. Neither pterin-6-carboxaldehyde, nor pterin-6-carboxylate, nor 6-methylpterin, at concentrations up to 100 μM, appreciably affected the rate of dihydrofolate reductase at pH values from pH 6.1 to 7.0. These negative results indicate that these simple pterins do not bind to either reductase.

pH Optima—As shown in Fig. 7 there was a single optimum

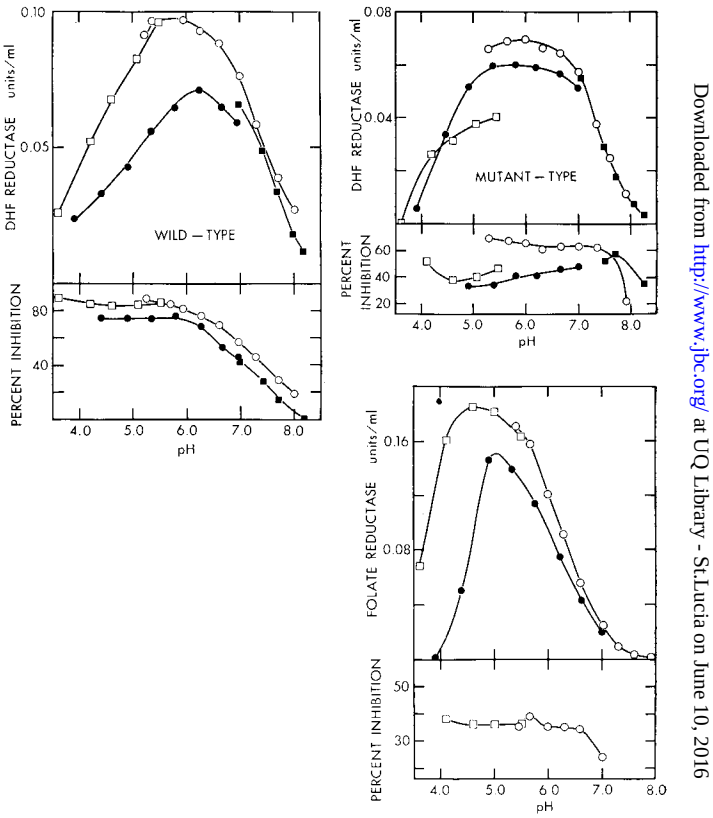

FIG. 7. Relationship between pH and reductase activity, and between pH and aminopterin inhibition, of the two types of dihydrofolate reductase. Assays at 37° were performed with the use of a 0 to 0.1 absorbance slide wire in the spectrophotometer. Reaction mixtures of 3.0 ml contained 50 μM NADPH, 5 mM mercaptoethanol, 33 μM dihydrofolate (or 33 μM folate), and the following buffers at a concentration of 50 mM: □, acetate; ●, citrate; ○, potassium phosphate; ■, Tris-chloride. For determination of the effect of pH on activity identical mixtures were placed in the reference and reaction cells and the reaction was initiated by 0.5 $\mu\mu$mole of wild type reductase (*upper left panel*), 3.5 $\mu\mu$moles of mutant type reductase (*upper right panel*), or 0.7 mμmole of mutant type reductase (*lower panel*). Following the reactions the pH of both reference cell and reaction cell was measured. To examine aminopterin inhibition of dihydrofolate reduction as a function of pH, each reaction mixture contained 5 $\mu\mu$moles of aminopterin and 3 $\mu\mu$moles of wild type reductase (*upper left*) and 7 $\mu\mu$moles of mutant type reductase (*upper right*). When inhibition of folate reduction was estimated (*lower*), the reaction mixtures contained 0.5 mμmole of aminopterin and 1.4 mμmoles of mutant type reductase.

pH value for the wild type and mutant type dihydrofolate reductases prepared from SF/A at pH 5.8. The optimum pH for the reduction of folate by mutant type reductase was pH 4.7 in acetate buffer. In citrate buffer the last mentioned pH optimum appeared to be higher (pH 5.1) but the lower stability of substrates in citrate buffer than in acetate buffer, at low pH values, may explain the latter finding (21).

The relative aminopterin inhibition of the enzymic activities, shown in Fig. 7, was approximately constant between pH 4.5 and 5.5 in each case, but at higher pH values the inhibition decreased. At pH values above 8 the dissociation constants for the complexes of each reductase with aminopterin would therefore be expected to be much greater than those at pH 5.5.

Effects of Ions, Urea, and Sulfhydryl-reacting Agents on Activities of Dihydrofolate Reductases—The effects of ions on the catalytic activities of the reductases were examined at pH 5.5 in imidazole-acetate buffer, 50 μM with respect to imidazole. The activity of the mutant type reductase was increased up to 2-fold by monovalent cations, the greatest activation due to Na^+ being observed with 0.2 mM NaCl. The effect was also observed with K^+, Li^+, Ru^+, Cs^+, and NH_4^+. In contrast, the activity of the wild type reductase was decreased 1.6-fold by concentrations of monovalent cations of 0.1 mM and above.

The effects of urea and of guanidine hydrochloride on the catalytic activities of the two purified reductases were examined both by preincubation with the enzyme (both in the presence and absence of mercaptoethanol and of either substrate, followed by dilution of the mixture into the standard assay reaction mixture) and by addition of the agent to the completed reaction mixture after first recording the initial reaction velocity. In every case there resulted a decrease in catalytic activity, and the decrease was greater the higher the final concentration of the agent in the assay reaction mixture.

The effects of phenylmercuric acetate (100 μM), methylmercuric iodide (100 μM), methylmercuric bromide (50 μM), and *p*-mercuribenzoate (500 μM) on the activities of the two reductases were examined by preincubation for 10 min at 37° with 1.5 μμmoles of wild type reductase or 20 μμmoles of mutant type reductase, in the presence of 200 μM NADPH. These agents were tested both in 50 mM potassium phosphate, pH 6.6, and in 50 mM Tris-chloride, pH 7.0, both with and without 1 mM mercaptoethanol. Following preincubation, the activities were assayed by completion of the standard reaction mixture. The mutant type reductase activity was not affected by any of the tested mercurial reagents under any test condition. In contrast, the wild type reductase activity was decreased by *p*-mercuribenzoate or phenylmercuric acetate but not by methylmercuric bromide or methylmercuric iodide. The observed losses of activity were not prevented by the presence of 1 mM mercaptoethanol.

The activities of the two reductases were not affected by iodine, tested at a concentration of 1.6×10^{-6} meq per ml in a procedure similar to that used for the mercurial reagents. However, at 1.6×10^{-5} meq per ml, iodine caused a loss of activity of each reductase, and this loss could be prevented by the presence of mercaptoethanol during the preincubation, presumably because of its direct reaction with the iodine.

Neither reductase was affected by preincubation for 45 min at 23° with *N*-ethylmaleimide in 10^6-fold excess over the concentration of enzyme, either in the presence or in the absence of 5 mM mercaptoethanol.

Since phenylmethanesulfonyl fluoride was added during extraction of the dihydrofolate reductases from cells of SF/A, for the purpose of inhibiting any proteolytic enzymes in the cell extract, it too was tested for any effect on the activity of the two reductases, by preincubation of the reagent (up to 115 μM concentration) at 37° for 30 min. The activity of the reductases was not affected.

Determination of Equilibrium Constant for Reaction Catalyzed by Pure Dihydrofolate Reductase

The procedure used for the determination of the equilibrium constant for Reaction 1 is described in "Methods."

$$\text{Dihydrofolate} + \text{NADPH} + H^+ \rightleftharpoons \text{tetrahydrofolate} + \text{NADP}^+ \quad (1)$$

The mean value for 12 determinations at pH 8 was 8.4×10^{10} with a standard deviation of 1.6×10^{10}. Similar results were obtained with wild type and mutant type reductases. The mean value obtained in determinations attempted at pH 6.7 was 2.1×10^{10} but, for reasons mentioned in "Methods," the determinations at this pH were less accurate.

The value obtained at pH 8 agrees reasonably well with the value of 5.6×10^{11} at pH 8.5 obtained by Mathews and Huennekens (29). Previous failure to detect the reverse reaction in this laboratory (30) was presumably due to the fact that the expected absorbance change calculated from the above equilibrium constant would have been about 0.006 absorbance unit under the experimental conditions used. The low value of this predicted absorbance change is largely due to the fact that the reaction was carried out at pH 6.7 or lower, with consequent movement of the equilibrium in the direction of the forward reaction. The failure of Mathews and Sutherland (31), with dihydrofolate reductase from *Escherichia coli* and T6 bacteriophage, to detect the reverse reaction is not as readily explained on such grounds since the absorbance change predicted in their experiments would be about 0.15.

DISCUSSION

It is apparent from the data presented that the mutations giving rise to the amethopterin resistance of SF/A have resulted in the synthesis by the mutant of two distinct proteins, each having dihydrofolate reductase activity and distinguished from each other by physical and enzymic properties. The "wild type" reductase has been given that designation because, in respect to substrate specificity and turnover number, it is similar to the single dihydrofolate reductase which can be found in extracts of the amethopterin-sensitive strain of *S. faecium* var. *durans*, ATCC 8043 (1, 6). The "mutant type" reductase has been given that designation because, in the above respects, it differs from the single reductase of the amethopterin-sensitive strain and its biosynthesis in appreciable amounts appears to be determined by one or more of the mutations giving rise to strain SF/A.

The physical data obtained provide strong evidence that each purified preparation of dihydrofolate reductase is essentially homogeneous. This has been further checked by titration of enzyme of known activity against aminopterin and by the use of the approximate molecular weights and specific activities of the two purified preparations. The results of such titrations[4] are consistent with the view that the preparations are essen-

[4] P. F. Nixon, R. L. Blakley, and J. F. Morrison, unpublished data.

tially pure, or alternatively that the molecular weight estimations are in error to such an extent as to compensate exactly for the extent to which the preparations are impure. On the ground that the two purified preparations are essentially homogeneous it follows that the observed enzymic activities and characteristics of each are the properties, in each case, of a single protein.

The characteristics of the two dihydrofolate reductases from SF/A serve to distinguish them from the reductases purified from other sources, as well as from each other. The pH activity curves, with a single optimum pH, are qualitatively similar to those of the dihydrofolate reductases prepared from other microorganisms (6, 31, 32–34) and from calf thymus (3) and sheep liver (35). They are also similar to the pH activity curve for the reductase from chicken liver (36, 37), after that reductase has been activated by urea or mercurial reagents, but they are dissimilar from the pH activity curve of the native dihydrofolate reductase prepared from chicken liver. The effects of urea, guanidine hydrochloride, iodine, and mercurial reagents also serve to distinguish the dihydrofolate reductases of SF/A from those of chicken liver, Ehrlich ascites cells (38), and the mouse leukemia L1210 cells (5). It may be significant that the only mercurial reagents to affect the wild type reductase were those having aromatic ring structures. Since their effects were not reversed by mercaptoethanol and were not duplicated by other sulfhydryl-reacting reagents it is probable that their effects were the result of reaction with some groups of the reductase other than sulfhydryl groups.

Relation of Mutant Reductase to Amethopterin Resistance—The relevance of the presence of two dihydrofolate reductases in SF/A to the amethopterin resistance *in vivo* of this strain may be assessed in the light of the results described. The total dihydrofolate reductase activity of extracts of SF/A is consistently 12-fold higher than that of extracts of the amethopterin-sensitive strain, ATCC 8043. When the lower turnover number of the mutant type reductase is taken into account the total molar concentration of the combined dihydrofolate reductases in crude extracts of SF/A can be calculated to be 87-fold increased over that of the sensitive strain. This increase is due to two factors: first, a 3- to 4-fold increase of the concentration of wild type reductase and, second, the appearance of mutant type reductase. However, these increases are relatively insignificant compared to the increase of resistance to amethopterin exhibited by SF/A over that of the sensitive, wild type organism. Whereas the molar amount of reductase is 87-fold greater in strain A, the resistance is 400 to 300,000 times greater. It is unlikely, therefore, that the high resistance of the mutant is attributable simply to the larger amount of reductase with which the drug within the mutant cells must combine before thymidylate synthesis and other reactions involving tetrahydrofolate derivatives are arrested.

Since neither the sensitive nor the mutant organism can synthesize folate derivatives *de novo*, and since no reduced folate is normally supplied in the culture medium of either organism, tetrahydrofolate and its derivatives must all be derived from folate under these conditions. The sensitive strain must therefore possess some enzyme capable of reducing folate, even though the capacity of cell extracts to perform this reduction is not detectable, and it is reasonable to assume that this postulated slow reduction of folate in the sensitive strain is inhibited by amethopterin. As the concentration of amethopterin is increased in the growth medium for the sensitive strain, the reduction of folate may therefore be the step in folate metabolism that is first critically inhibited. In the case of SF/A, on the other hand, the folate reductase activity of cell extracts is readily detected, being at least 22-fold greater than the minimum sensitivity of the assay, so that it is likely that folate reduction will proceed at an adequate rate at higher concentrations of amethopterin than that inhibiting growth of the sensitive strain. Hence it is possible that the increased resistance of SF/A to amethopterin represents the increase in capacity for folate reduction. If this is correct, the increased resistance to SF/A is a direct result of the presence of the mutant dihydrofolate reductase not found in the sensitive strain. In this connection it is of interest to compare the turnover number of the mutant enzyme with that of dihydrofolate reductase from other sources. Data on the chicken liver enzyme (2) permit calculated turnover numbers for folate of about 87 at pH 4 and about 11 at pH 6. The enzyme purified from sarcoma 180 (4) is reported to have turnover numbers for folate of 5.2 at pH 6.3. For the mutant type reductase from SF/A the turnover numbers for folate were 7.5 at pH 4.7 and 1 at pH 7.

Differences in the extent of inhibition of the purified reductases by the 4-amino analogues of folic acid will be discussed in relation to differences in resistance to amethopterin in the next paper of this series.[4]

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Dihydrofolate Reductase of *Streptococcus faecium* : II. PURIFICATION AND SOME PROPERTIES OF TWO DIHYDROFOLATE REDUCTASES FROM THE AMETHOPTERIN-RESISTANT MUTANT, STREPTOCOCCUS FAECIUM VAR. DURANS STRAIN A

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