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Mitochondrial Adenosine Triphosphatase. Location of Sulfhydryl Groups and Disulfide Bonds in the Soluble Enzyme from Beef Heart†

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ABSTRACT: The soluble beef heart mitochondrial ATPase (F_1) contains eight sulfhydryl groups and two disulfide bonds. *N*-Ethylmaleimide has been used to radioactively label the sulfhydryl groups before and after cleavage of the disulfide bonds by dithiothreitol. After subjecting the labeled protein to polyacrylamide gel electrophoresis in sodium dodecyl sulfate and measuring radioactivity in each of the separated subunits, the location of all the sulfhydryl groups and the disulfide bonds may be specified. The conclusions are supported by direct examination of depolymerized, unreduced, enzyme by polyacrylamide gel electrophoresis. The results also indicate that current ideas regarding the overall subunit structure of this enzyme may be incorrect, and this is discussed in light of new data presented here.

The oligomycin-insensitive ATPase from beef heart mitochondria is a soluble enzyme (called "F_1") which is believed to carry the catalytic site at which ATP is hydrolyzed or synthesized in mitochondria (Pullman *et al.*, 1960; Penefsky *et al.*, 1960; Racker, 1970). The structure of the enzyme is complicated, and has recently been reviewed (Senior, 1973a). This large spherical enzyme complex, of molecular weight around 360,000, contains five different types of tightly bound subunits and a sixth, more loosely bound subunit called the "specific inhibitor protein." The overall subunit structure in the molecule is not known. One suggestion (Senior and Brooks, 1971) has been made, based on the relative staining intensity of the different subunits after separation by polyacrylamide gel electrophoresis and on their molecular sizes.

The native, unreduced enzyme is known to contain eight sulfhydryl groups and two disulfide bonds (Senior, 1973b). In this paper I have described experiments using radioactive *N*-ethylmaleimide, which labels the sulfhydryl groups. The locations of the sulfhydryl groups on the various subunits, and the probable positions of the disulfide bonds have been specified. Further, the data suggest that our previous proposal for the overall subunit structure may be incorrect, and other arrangements are discussed.

Materials and Methods

Preparation of ATPase. The enzyme was prepared from beef heart mitochondria as described previously (Senior and Brooks, 1970) with a slight modification at step 4 (Brooks and Senior, 1972). The eluate at step 4 was directly taken through step 6 (heat step). The specific activity of the enzyme, assayed as described (Senior and Brooks, 1970), was 112 μmol of ATP hydrolyzed per min per mg.

N-Ethylmaleimide. Nonradioactive material was purchased from Eastman Kodak, Rochester, N.Y. *N*-Ethyl[1-^{14}C]maleimide and *N*-ethyl[2-^{3}H]maleimide were purchased from New England Nuclear. Solutions (20–30 mM)

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of all were made in 0.1 M potassium phosphate buffer (pH 7.0) and were stored briefly at 4° when necessary. The final concentration of MalNEt[1] in solution was calculated using a molar extinction coefficient of 620 at 305 nm (Riordan and Vallee, 1967). The final specific radioactivity of MalNEt used was 2.19×10^5 dpm/nmol of ^3H or 1.6×10^3 dpm/nmol ^{14}C.

Measurement of Radioactivity in Polyacrylamide Gel Slices. Gels were sliced using a Canalco gel slicing implement ("egg-slicer" type) strung with 8-lb-test monofilament fishing line. Slices (1.5 mm thick) were placed singly or in pairs in scintillation vials, and 3 ml of 3% (w/v) sodium dodecyl sulfate in water was added. The vials (tightly capped) were shaken gently for 15 hr at 50° in a shaking incubator (10% gels) or for 2 days at 50° (12% gels). After the vials were cooled to 25°, 10 ml of scintillation fluor (containing 500 ml of toluene, 500 ml of Triton X-114, and 4 g of 2,5-diphenyloxazole per liter) was added. After shaking, the vials were counted in a Nuclear-Chicago Isocap 300 liquid scintillation counter, and counts were converted to disintegrations per minute using the external standard channels ratio. ^{14}C was counted at 80% efficiency and ^3H at 26% efficiency. This technique gave very reproducible results and was not susceptible to artifacts (*e.g.*, chemiluminescence, loss of $^{14}CO_2$) which have been noted in other procedures (Grower and Bransome, 1970). Furthermore it is the least expensive procedure currently available. Recovery of radioactivity applied to the gel was 95–100%. The method was applied to gels which were sliced directly after electrophoresis, or to gels which were fixed in 12.5% trichloroacetic acid before slicing, or to gels which were fixed, stained with Coomassie Blue, and destained, before slicing. It worked with equal facility in each of these cases. Color quenching (by Coomassie Blue) did not occur (as tested by internal standardization). Thus it was often convenient to stain and destain the gels and count only the slices containing visible protein bands.

Measurement of Radioactivity in Labeled Protein Samples. Small amounts of protein (up to 200 μg) were counted in 11 ml of scintillation fluid (containing 750 ml of toluene, 250 ml of Triton X-114, 100 ml of water, and 4.4 g of 2,5-diphenyloxazole in 1100 ml).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate Buffers. Gels that were 10% in polyacrylamide were prepared as previously described (Brooks and Senior, 1971). Gels that were 12% in polyacrylamide were prepared in the same way, simply by using 1.2 times the quantity of acrylamide and *N,N'*-methylenebisacrylamide. All gels used in this work were 5 cm long, 5 mm diameter, and were run at 8 mA/tube; 10% gels were run for 2 hr at room temperature; 12% gels were run for 18 hr at room temperature. Gels were fixed with shaking in 12.5% trichloroacetic acid for 1 hr (10% gels) or 4 hr (12% gels), stained with Coomassie Blue in 10% trichloroacetic acid overnight, and destained for 2–4 days in 10% trichloroacetic acid (with shaking). Densitometric traces were made in a Gilford spectrophotometer set at 590 nm.

Purification of F_1 Subunits. Individual subunits of F_1 were separated and purified by the methods described by Brooks and Senior (1972) with the following modifications. The ion-exchange chromatography step which used Whatman CM-52 was carried out at pH 7.8, giving an increase in yield of subunit 3 in the second peak. The ion-exchange chromatography on DEAE-Sephadex A-50 was then done as described, but using Whatman DE52 instead of the Sephadex. This did not increase yields, but was easier to handle.

Routine Procedures and Chemicals. Triton X-114 was obtained from Sigma. Sodium dodecyl sulfate (95%) was obtained from Mallinckrodt and used directly. All other chemicals were the best available commercial grades. Protein was estimated by Miller's method (Miller, 1959), calibrated with dry F_1 as standard (see Senior, 1973b).

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; MalNEt, *N*-ethylmaleimide.

Table I: Labeling F_1 with Radioactive *N*-Ethylmaleimide.

Treatment of F_1	mol of MalNEt Bound/mol of F_1[c]
SDS (1%)[a] and DTT (0.05 M)	12.4 (12.0–13.2; 7)
SDS (1%)[b]	8.2 (7.35–9.5; 10)

[a] The enzyme suspension in ammonium sulfate was centrifuged (104,000*g* × 10 min) and the enzyme was redissolved in buffer containing sodium dodecyl sulfate (1% w/v), Tris-SO_4 (40 mM), EDTA (1 mM), and dithiothreitol (50 mM) (pH 8.0); final protein concentration, 9 mg/ml. After 3 hr at 25°, the enzyme was passed through a Sephadex G-25 column (60 cm long × 0.9 cm diameter) equilibrated with buffer containing sodium dodecyl sulfate (1%, w/v), sodium phosphate (10 mM), and dithiothreitol (0.1 mM), pH 7.0. To a sample of the eluted protein a small amount of radioactive *N*-ethylmaleimide solution was added such that the final concentration of MalNEt was 1.4–1.5 mM. After 20 min at 25°, 10 volumes of 95% ethanol was added. The protein was precipitated by centrifugation (104,000*g* × 10 min), and washed once with the same volume of 95% ethanol. After recentrifugation the pellet was dried in a stream of N_2 and dissolved in a small volume of 3% (w/v) sodium dodecyl sulfate–0.2 M sucrose. Protein and radioactive MalNEt concentrations were assayed as described under Methods. Finally, in preparation for running samples on polyacrylamide gels, 2-mercaptoethanol was added to give a final concentration of 1% (v/v). [b] The enzyme was dissolved directly in buffer containing sodium dodecyl sulfate (1%, w/v) and sodium phosphate (10 mM), pH 7.0. Conditions for reaction with MalNEt, protein, and bound MalNEt assay, and preparation for polyacrylamide gel electrophoresis were as above. [c] Results are expressed as mean values, with range found and number of experiments done in parentheses. A molecular weight for F_1 of 360,000 was assumed.

Results

Labeling of Sulfhydryl Groups in F_1 with Radioactive MalNEt. The technique and results are described in Table I. Twelve -SH groups were found in the fully reduced enzyme. This confirms previous work in which 3,3'-dithiobis-6-nitrobenzoic acid (Senior, 1973b) and iodoacetic acid (Knowles and Penefsky, 1972) were used to estimate the -SH groups. Eight -SH groups were found here in the unreduced enzyme, also confirming previous work (Senior, 1973b). Under the conditions used here, the MalNEt is expected to react only with cysteine residues (Riordan and Vallee, 1967) and the agreement between this work and previous work showed that this was the case.

Distribution of Radioactivity in 10% Gels. Figure 1 shows the profile of radioactivity obtained after running la-

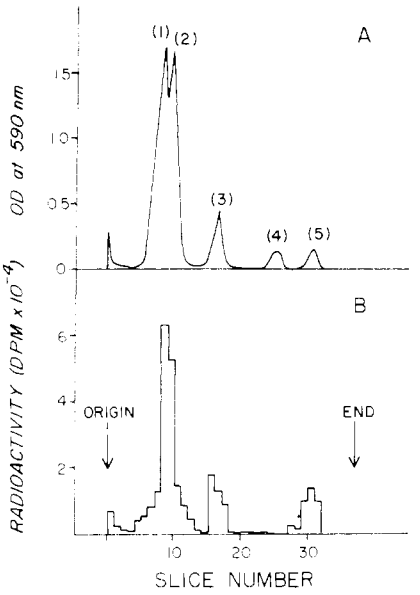

FIGURE 1: Labeling of F_1 subunits by radioactive *N*-ethylmaleimide. Samples of F_1, after reaction with radioactive MalNEt as described in Table I, were run on SDS gels which were 10% in acrylamide. Fixing, staining, densitometric tracing of stained gels, slicing, and assay of radioactivity in slices are all described under Materials and Methods. (A) Densitometric trace of a typical gel, subunits are numbered according to a previous convention (Senior, 1973a; Brooks and Senior, 1971). (B) Radioactivity measurements, the whole gel was sliced in this experiment. In this experiment 10 μl containing 33 μg of protein was applied. This sample had all 12 -SH groups labeled.

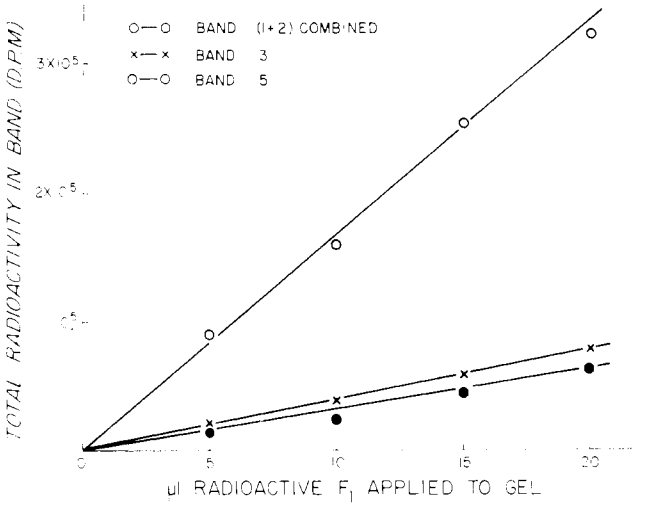

FIGURE 2: Calculation of ratios of sulfhydryl groups in different subunits of F_1. F_1, which had previously been labeled with radioactive MalNEt as described in Table I, was examined by polyacrylamide gel electrophoresis in SDS gels which were 10% in acrylamide. From each labeled F_1 sample (protein concentration 3-6 mg/ml) portions of 5, 10, 15, and 20 μl were taken and applied to gels. After electrophoresis and slicing, the radioactivity in each subunit was calculated. This graph shows the values calculated from a typical experiment which used the sample shown in Figure 1. The protein concentration of the applied samples was 3.3 μg/μl. From the slopes of the lines, the ratios of sulfhydryl groups in the various subunits can be calculated.

beled samples in sodium dodecyl sulfate polyacrylamide gel electrophoresis. The figure shows the profile obtained with a sample in which all 12 -SH groups were labeled with [^{3}H] MalNEt, but very similar profiles were also seen with unreduced samples where 8 -SH groups were labeled (see below). Subunits 1 and 2 run close together and the distribution of radioactivity between them cannot be determined in this system. It is clear, though, that radioactivity is present in bands (1 + 2), 3, and 5. Subunit 4 was not labeled. No nonstaining radioactive bands were seen. In these experiments the recovery of radioactivity in the protein bands was 70–100% of the amount applied. Some radioactivity usually remained at the origin, accounting for the other 0–30%. From each labeled sample of F_1, 5-, 10-, 15-, and 20-μl portions were run on gels and a composite graph of total radioactivity in each band could be calculated, as in Figure 2.

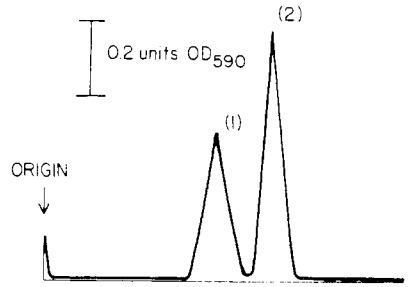

FIGURE 3: Separation of subunits 1 and 2 on polyacrylamide gels. This is a densitometric trace of an SDS gel of F_1. The gel was 12% in acrylamide (see Materials and Methods for details of the technique). This sample was treated as described in Table I, 5 μl containing 20 μg was applied. Subunits 3, 4, and 5 have run off the end of the gel.

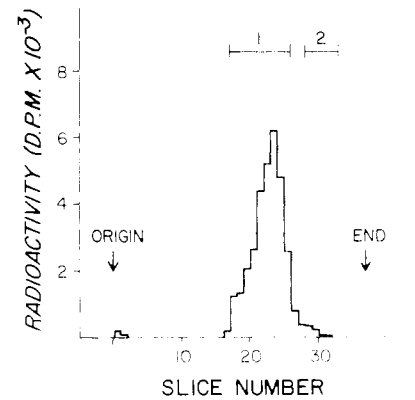

FIGURE 4: Distribution of radioactivity in subunits 1 and 2. Subunits 1 and 2 were separated as described in Figure 3 and radioactivity in gel slices was estimated as described under Materials and Methods. The whole gel was sliced in this experiment. The bars show the positions of stained bands corresponding to subunits 1 and 2.

Distribution of Radioactivity in 12% Gels. In order to separate subunits 1 and 2, 12% gels were used. Figure 3 shows that a clear separation of bands 1 and 2 was achieved under the conditions used and this technique will probably be very useful therefore in future studies on F_1. Figure 4 shows the distribution of radioactivity when MalNEt-labeled samples were run in this system. No labeling of subunit 2 occurred, only subunit 1 was labeled. This was the case with both reduced samples (all 12 -SH labeled) or unreduced samples (8 -SH groups labeled).

Polyacrylamide Gel Electrophoresis of Unreduced Unlabeled F_1. In all previous work, when F_1 was examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, β-mercaptoethanol was present during depolymerization to cleave disulfide bonds. In Figure 5 is shown a typical trace of the result when β-mercaptoethanol was not included. A new band, running between subunits 2 and 3, with a molecular weight of about 41,000 was consistently seen. At

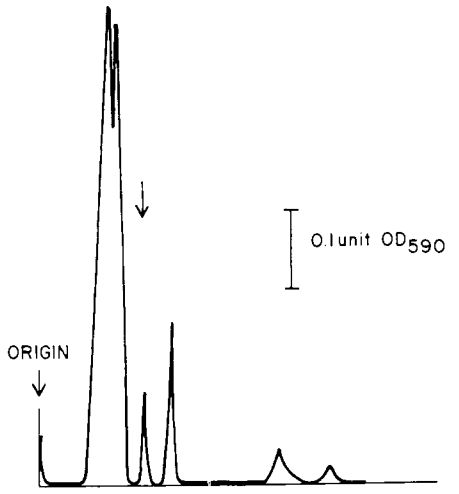

FIGURE 5: Polyacrylamide gel electrophoresis of unreduced F_1. F_1 was dissolved in 1% SDS–20 mM sodium phosphate buffer (pH 7.1) at 4 mg/ml, dialyzed against 100 volumes of 0.1% SDS–20 mM sodium phosphate (pH 7.1) overnight, and finally adjusted to 2 mg/ml and 0.2 M in sucrose by addition of dialysis buffer and 2 M sucrose. Samples were run in 10% gels. This is a densitometric trace of a typical experiment in which 30 μg were applied. The same results were obtained when F_1 was initially dissolved in 0.1% SDS–20 mM sodium phosphate and then dialyzed as above against 0.1% SDS–sodium phosphate.

Table II: Ratios of Sulfhydryl Groups in Subunits of MalNEt-labeled F_1.

	Expt	Ratio of Sulfhydryl Groups in Subunits (1 + 2) : (3) : (4) : (5)[a]
Group I		
F_1 treated with 50 mM	1	4.20 1 0 0.80
dithiothreitol before	2	3.90 1 0 0.78
reaction with MalNEt	3	4.10 1 0 0.92
(12–SH groups	4	3.95 1 0 0.91
labeled)	5	4.16 1 0 0.77
	6	3.85 1 0 0.72
	7	4.42 1 0 0.82
	Av	4.08 1 0 0.82
Group II		
F_1 reacted with	1	6.00 1 0 0.66
MalNEt directly	2	5.81 1 0 0.7
(8–SH groups	3	5.75 1 0 0.42
labeled)	4	5.93 1 0 1.0
	5	6.36 1 0 1.0
	6	5.95 1 0 0.96
	7	5.91 1 0 0.84
	Av	5.96 1 0 0.80

[a] Total radioactivity in bands on gels corresponding to subunits 3, 4, 5 and combined subunits 1 and 2 (which move as one band on 10% gels) was measured. With subunit 3 arbitrarily set at one, the ratio of radioactivity in the bands was calculated. The reaction of radioactive MalNEt with F_1 is described in Table I.

the same time subunits 3 and 5 were reduced in peak height. No species larger than subunit 1 was seen.

Cysteine Content of Purified F_1 Subunits. Each of the F_1 subunits was obtained in pure form (see Materials and Methods) and was reacted with [^{3}H]MalNEt as described in Table III. The cysteine content of each subunit was calculated as the amount of radioactive label bound and is shown in Table III.

Discussion

Location of Sulfhydryl Groups and Disulfide Bonds in F_1. In the fully reduced enzyme there are 12 sulfhydryl groups. From the ratios shown in Table II, and from the data shown in Figures 2 and 4, it may be deduced that eight sulfhydryl groups are in the subunit 1 band, that two sulfhydryl groups are in each of subunit 3 and 5 bands, and that subunits 2 and 4 contain no sulfhydryl groups. It is worth noting that this distribution of –SH groups can be made unambiguously at this stage, without separation and characterization of individual subunits, because the techniques used give complete recovery of counts from the gels. Knowles and Penefsky (1972) have previously suggested that subunit 1 contains three sulfhydryl groups and subunit 2 contains one sulfhydryl group. The data presented here (above and in Table III) are not in accord with these values.

When radioactive MalNEt was allowed to react directly with depolymerized F_1, without prior cleavage of the disulfide bonds, the distribution of radioactivity was different (Table II). There are eight sulfhydryl groups in the whole unreduced molecule, and it may be deduced from Table II and Figure 4 that the distribution of these groups is six sulfhydryl groups in subunit 1, none in subunit two, one in subunit 3, none in subunit four, and one in subunit five. The data suggest therefore that disulfide bond formation involves one –SH from subunit 3, one from subunit 5, and two from subunit 1. Since no species of molecular weight higher than subunit 1 was noted in unreduced samples (Figure 5), and a species of molecular weight intermediate between subunits 2 and 3 did appear, and bands corresponding to subunits 3 and 5 were still apparent in unreduced samples (albeit less intense), the best estimate seems to be that one disulfide bond occurs between one molecule of subunit 3 and one molecule of subunit 5, and that one disulfide bond is an intrachain bond in subunit 1. I am aware that such estimate of disulfide bond position might well be open to question if disulfide-bond interchange occurred during depolymerization in SDS. The results of the radioactive-labeling experiments and the gel electrophoresis were consistent, however, and the fact that only one new species appeared in gels of unreduced F_1, with no smearing evident, seems to show that no significant "scrambling" of disulfide bonds occurred. Further, these remarks apply only to the enzyme preparation used here. Interchange of disulfide bonds might occur in native F_1 during preparation (*e.g.*, at the stage of sonication at high pH). Examination of F_1 made by different procedures may throw light on this point.

Implications for the Overall Subunit Structure of F_1. A previous proposal was made that beef heart and rat liver F_1 contain three chains of subunit 1, three chains of subunit 2, and one chain of each of subunits 3, 4, and 5 (Senior and Brooks, 1971). The evidence available has been reviewed (Senior, 1973a) and was thought to be generally supportive of the proposal, but not conclusive. Now the evidence presented here indicates that this proposal is incorrect, for the distribution of sulfhydryl groups and disulfide bonds and the cysteine content of subunits (Table III) seems incompatible with the proposed overall subunit structure.

Subunits 2 and 4 were found not to react with MalNEt (Figures 1 and 4 and Table III) suggesting they contain no cysteine, thus the technique used here cannot be employed to calculate the number of these subunits in the F_1 mole-

Table III: Labeling of Purified F_1 Subunits with Radioactive *N*-Ethylmaleimide.[a]

Subunit	mol of MalNEt Bound/mol of Protein
$F_1 - 1$	4.08, 3.96
$F_1 - 2$	0.17, 0.20
$F_1 - 3$	0.91; 0.88; 0.81, 0.78
$F_1 - 4$	0.04; 0.1
$F_1 - 5$	0.81; 0.78

[a] Lyophilized subunits were dissolved in 1% SDS-40 mM Tris-SO_4-1 mM EDTA-50 mM DTT (pH 8.0) at 5-8 mg/ml. Further treatment was as described in Table I, superscript *a*. Molecular weight values for subunits used in calculation were those suggested as "best values" currently available (Senior 1973a, p 255).

cule. Combining the data presented in Tables I, II, and III, the results obtained using this technique of labeling with radioactive MalNEt indicate that there are two molecules of subunit 1, two molecules of subunit 3, and two molecules of subunit 5 per molecule of F_1. Thus this technique gives results which are in conflict with previous suggestions. Present work in this laboratory is aimed at development of other techniques which will enable the stoichiometry of subunits 2 and 4 to be investigated, and which might allow application of the general principle of this method to examination of other membrane-bound enzyme complexes, such as electron-transfer complexes.

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Modification of the Cysteine Residue of Streptococcal Dihydrofolate Reductase†

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ABSTRACT: Modification of the single cysteine residue of streptococcal dihydrofolate reductase with 5,5′-dithiobis(2-nitrobenzoic acid) results in virtually complete inactivation of the enzyme. Reduction of the enzyme-*S*-nitrobenzoate mixed disulfide with dithiothreitol indicates that 1.0 cysteine has been modified and approximately 70% of the original enzyme activity restored. Circular dichroic and fluorescence studies suggest that a localized conformational change involving aromatic residues, possibly tryptophan, has occurred following modification. In addition, nearly stoichiometric amounts of *p*-hydroxymercuribenzoate lead to complete inactivation of the reductase. It is suggested that the *S*-nitrobenzoate moiety bound to the cysteine residue of the enzyme may perturb one or more aromatic side chains and lead to a distortion of the hydrophobic substrate binding site.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduced triphosphopyridine nucleotide-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, the coenzyme involved in many one-carbon transfer reactions. This enzyme appears to be the primary target for the action of 4-amino analogs of folate, such as aminopterin (4-amino-4-deoxyfolate) and amethopterin (4-amino-10-methyl-4-deoxyfolate). The latter compound has been widely used in the chemotherapeutic treatment of certain leukemias, lymphomas, and other clinical disorders (Huennekens, 1968; Blakley, 1969).

Although a considerable amount of work has been done on the design and synthesis of folate antagonists (reviewed by Blakley, 1969), little is known about the mechanism of

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