Human Hepatic Methionine Biosynthesis

PURIFICATION AND CHARACTERIZATION OF BETAINE: HOMOCYSTEINE S-METHYLTRANSFERASE*

(Received for publication, November 9, 1981)

William E. Skiba‡§, Mark P. Taylor‡, Marilyn S. Wells¶¶, John H. Mangum**, and William M. Awad, Jr.‡***

From the Departments of ‡Medicine and ¶¶Biochemistry, University of Miami School of Medicine, Miami, Florida 33101, the ¶¶Department of Medicine, Veterans Administration Hospital, Miami, Florida 33125, and the **Department of Chemistry, Brigham Young University, Provo, Utah 84602

Human hepatic betaine:homocysteine S-methyltransferase has been purified to apparent homogeneity after a 250-fold separation. The isolation required the presence of homocysteine and a product of the reaction, dimethylglycine, in order to stabilize the protein. An apparent molecular weight of 270,000 was determined by calibrated gel filtration. A single peptide chain of Mr = 45,000 was found by calibrated sodium dodecyl sulfate-acrylamide gel electrophoresis, suggesting that the native protein is a hexamer of identical subunits. The enzyme is stable at pH values greater than 5.5. No effect of EDTA was observed on the activity of the enzyme. In the absence of thiol reagents, the hexameric protein appeared to polymerize to integral aggregates. Isovalerate and 3,3-dimethylbutyrate, analogs of dimethylglycine and betaine, respectively, are good inhibitors of the enzyme. The inhibitions are competitive with respect to betaine, indicating that a positive charge is not required for binding at the betaine/dimethylglycine site. These findings are similar to those reported for acetylcholine esterase where the neutral analogs of choline show good binding to that enzyme. The binding site for betaine/dimethylglycine may exist in two states, one permitting the binding of a positively charged group and the other a neutral group.

Mammalian methionine biosynthesis is catalyzed by two enzymes, N\textsuperscript{2}-methyltetrahydrofolate:homocysteine transmethylase (commonly known as methionine synthase) and betaine:homocysteine S-methyltransferase. The former is distributed throughout most tissues, whereas the latter, except for an occasional finding of minor activity in the kidney, is confined to the liver (1). Betaine:homocysteine S-methyltransferase can utilize also certain thetins instead of betaine, the methyl donor. This enzyme has been purified from rat liver by use of a 1-ml column of Dowex AG 1-X4 (hydroxyl form, Bio-Rad) which retained radioactive impurities. Preparation of homocysteine thiolacetic acid (SH) was treated according to the technique of Mudd et al. (2) to provide sufficient material for definitive studies on a methyl transfer mechanism. 2) Being confined ordinarily to the liver, the protein may be released into blood following hepatocellular injury and serve as a specific marker for liver disease; 3) Since methionine biosynthesis is important in tumor metabolism, it would be interesting to note the distribution of this enzyme in various forms of human malignancy. 4) The development of a specific inhibitor with high affinity could permit future animal studies on the physiological importance of this enzyme in one-carbon metabolism.

Although the general methyl donor in biological reactions is S-adenosylmethionine, chemically the reaction of betaine with homocysteine must be very similar to those methyltransferases which are S-adenosylmethionine-dependent. Both betaine and S-adenosylmethionine are high energy oumon methyl donors. In fact, the ability to utilize thetins indicates that the betaine-dependent enzyme can also be sulfonium-dependent. Thus, studies on betaine transmethylase may serve as a model for various S-adenosylmethionine-dependent reactions. The simplicity of the substrates and products of the betaine-dependent mechanism should facilitate such a study.

EXPERIMENTAL PROCEDURES

Betaine, dimethylglycine, choline chloride, \textit{L}-ethionine, glycine, and \textit{DL}-homocysteine thiolactone-HCl were obtained from Sigma. \textit{L}-Methionine was obtained from Nutritional Biochemical Corp. Isovaleric acid, 3,3-dimethylbutyric acid, and EDTA were obtained from Aldrich. New England Nuclear was the source of \textsuperscript{14}C betaine labeled in the methyl groups (1.49 mCi/mmol); further purification of this material was achieved by passage through a Dowex AG 1-X4 column (hydroxyl form, Bio-Rad) which retained radioactive impurities. Prestained microgramar carboxymethyl- and DEAES-celluloses (Whatman CM- and DE52) were obtained from H. Reeve Angel and Co. Sepharose 6B, Sephacryl S-300, and Sephadex were obtained from Pharmacia Fine Chemicals. All other chemicals were of the highest purity available.

Measurement of Enzyme Activity-Assay were performed according to a modification of the technique of Finkelstein and Mudd (6). To 10 ml of 50 mM [\textsuperscript{14}C]betaine (specific activity, 0.149 mCi/mmol) were added 20 ml of 0.1 M homocysteine, 20 \mu l of 1 M potassium phosphate (pH 7.4), 10–30 \mu l of enzyme solution, and water to give a final volume of 200 ml. Before each experiment, homocysteine thiolactone was treated according to the technique of Mudd et al. (7) to generate fresh homocysteine. Samples were incubated at 37°C for 1 h or more. The reaction was linear for at least 24 h if less than 10% of the betaine was utilized. Reactions were stopped by rapid freezing; the products, methionine and dimethylglycine, were separated from betaine by use of a 1-ml column of Dowex AG 1-X4 (hydroxyl form). After application of the sample, the column was washed with 4 ml of water to remove betaine. Thereafter, methionine and dimethylglycine were eluted with 4 ml of 1.5 M HCl into the scintillation vials to which were added 10 ml of Aquasol (New England Nuclear). All activity measurements are expressed as counts/min/10 \mu l of enzyme sample.
for a 1-h incubation. It should be noted that, of the counts measured, methionine contributes one-third and dimethylglycine, two-thirds.

**Purification of Enzyme**—Livers were obtained from cadavers determined by established procedures to have cerebral death (secondary to a variety of causes) with, however, sustained cardiopulmonary function; these bodies were to serve primarily as donors for renal transplantation. Permission was granted by next of kin for removal of the liver for investigational purposes. Following surgical removal of the kidneys and also some abdominal lymph nodes for tissue-typing purposes, aseptic hepatic extrication was performed under continuous perfusion. Immediately thereafter, the liver was sliced and layered over powdered dry ice for storage at 20°C. In any purification run, tissue from a single subject was utilized and procedures were conducted in the usual manner.

Nine grams of frozen human liver were rinsed and thawed in 180 ml of 25 mM potassium phosphate buffer (pH 7.4), 1 mM dimethylglycine, 1 mM homocysteine. After dicing to about 2-g cubes, the liver was homogenized in a blender for about 5 min at 4°C. The homogenate was centrifuged three times at 27,000 x g for 30 min; pellets and floating lipid layers were discarded after each run. The cloudy aqueous supernatant fraction was placed in a 75°C water bath; the temperature in the contained solution was allowed to rise to 70°C and maintained there for 5 min with gentle stirring throughout the heating procedure. Immediately thereafter, the solution was placed in an ice bath where it was left for 1 h. At this point, the bulk of methionine was removed by centrifugation for 20 min at 27,000 x g. As an alternative to the heating procedure, the supernatant solution obtained after initial sedimentation at 27,000 x g was placed in an ultracentrifuge and sedimented at 105,700 x g for 90 min. By either procedure, a clear supernatant fraction was obtained which was applied directly to a DEAE-cellulose column (6 x 13 cm) equilibrated in 25 mM potassium phosphate (pH 7.4), 1 mM dimethylglycine, 1 mM homocysteine. After a period of wash with this solution, stepwise elution was applied with 0.3 and 3.0 mM KCl in the same buffer. Breakthrough fractions containing the major portion of the enzyme activity were pooled and diluted to 5 times the initial volume with 1 mM dimethylglycine, 1 mM homocysteine. Following a period of wash with 700 ml of this solution, the enzyme was eluted with a step addition of 0.3 mM KCl in the same buffer. Fractions with enzyme activity were pooled and concentrated by ultrafiltration. The concentrated protein was applied to a Sepharose 6B column (2.6 x 90 cm) and eluted with 5 mM potassium phosphate, 1 mM dimethylglycine, 1 mM homocysteine (pH 7.4). Fractions with enzyme activity were pooled and concentrated by ultrafiltration through a PM-10 membrane (Amicon Corp.). The enzyme solution was equilibrated with 5 mM potassium phosphate, 1 mM dimethylglycine, 1 mM homocysteine (pH 6.5) by passage through a Sephadex G-25 column. The eluted enzyme fractions were pooled and applied to a CM-cellulose column (1.5 x 5 cm) equilibrated in the same buffer. The enzyme did not bind to this resin; other proteins that were retained were eluted with a step application of 0.3 mM KCl in the same buffer. The initial fractions containing the enzyme were combined, concentrated by ultrafiltration, and stored at 20°C in a solution of 50% glycerol. Aliquots of this material were utilized for further purification by equilibration with 5 mM potassium citrate, 1 mM dimethylglycine, 1 mM homocysteine (pH 5.7) and subsequently passed through a CM-cellulose column (1.5 x 3.2 cm) equilibrated with the same buffer. After washing with this solution, the column was eluted with a linear gradient formed with 100 ml each of the pH 5.7 buffer and the same buffer containing 1 mM KCl.

**Chromatography**—The homogeneity of the methyltransferase was analyzed by 5% acrylamide gel electrophoresis at pH 9.5 in a Canalco model 12 apparatus. The buffer composition was that described by the "Disc Electrophoresis" bulletin of the Canal Industrial Corp., New York; stacking gels were used. The amount of protein applied was 50 pg/gel; these were run in the presence and absence of 30 mM DL-homocysteine. Gel scanning was performed at 700 nm with a Gilford 240 spectrophotometer possessing a scanning attachment. Protein (5 pg) in 1% sodium dodecyl sulfate, 1% mercaptoethanol was applied also to 5% acrylamide gels. Protein standards used for calibration were as follows: l-lactate dehydrogenase (peptide molecular weights of 92,000, 68,000, and 37,000, respectively). In all cases, electrophoresis was done at room temperature at 8 mA/tube for 3 h. Standard and sodium dodecyl sulfate gels were stained by the Coomassie blue method (2-250) and (R-250), respectively.

**Stability of Transmethylase**—The stability of the enzyme at various pH values was determined by incubating the enzyme in 10 mM buffers of the following compositions: potassium citrate for pH values of 3.5, 4.5, and 5.5; potassium phosphate for pH values of 6.5 and 7.5; Tris-HCl for pH 8.5; and glycine for pH values of 9.5 and 10.5. After incubation for 4 h at 37°C, 20-pl aliquots were assayed in the usual manner except that the potassium phosphate buffer concentration was 100 mM.

**Other Studies**—The apparent molecular weight of the methyltransferase was determined by descending gel filtration through Sephacryl S-300 calibrated by the following protein standards (with their indicated molecular weights): urease (480,000), apoferritin (450,000), β-glucuronidase (280,000), catalase (240,000), aldolase (158,000), bovine serum albumin (68,000), and ovalbumin (43,000). The K_m values were determined at concentrations of betaine ranging from 25 μM-2.5 mM and of homocysteine from 50 μM-100 mM. The assumption was made that only the L-isomer of the homocysteine reacted. The data were interpreted according to the method of Lineweaver and Burk (8). The enzyme concentration used was 1.8 μg/ml. Otherwise, the usual assay technique was applied.

Amino acid composition of the methyltransferase was obtained by methansulfonic acid hydrolysis (4 N, 100°C for 20 h). The effect of possible inhibitors on enzyme activity was examined by including these compounds at concentrations from 0.5-50 mM. Assays were conducted in the homol manner; the enzyme concentration was 1.8 μg/ml. The inhibition constants by 3,3'-dimethylbutyrat and isovalerate were determined by the plot according to Dixon (9).

**RESULTS**

Fig. 1 depicts the elution pattern through DEAE-cellulose of the supernatant material containing the betaine-dependent methyltransferase activity after the heating and centrifugation procedures to remove particulate material. As indicated, the major component with activity does not bind to DEAE-cellulose in the presence of 25 mM potassium phosphate. However, 20-30% of the activity is retained and is eluted only after application of 0.3 mM KCl. When passed separately through the same column under the same buffer conditions, there was no change in the elution pattern of each of the two components, indicating that the separated transmethylases did not reflect column overloading or rapid generation of one component from the other. These components were not products of the heating step since the same elution pattern of two activities (in the same proportion) was noted if the alternative procedure of ultracentrifugation was substituted for heating to 70°C. Since in any purification procedure tissue was derived from one subject only, these results do not represent genetic variation between individuals. In samples studied from several individuals, electrophoresis was done at room temperature at 8 mA/tube for 3 h. Standard and sodium dodecyl sulfate gels were stained by the Coomassie blue method (2-250) and (R-250), respectively.
subjects, two components were observed in each case with elution patterns as noted above. Further purification steps were directed toward isolating the component with the major activity. It was found that the enzyme was moderately labile as demonstrated by the substantial loss of activity with further steps of purification and by the generation of products of decreasing molecular weight containing activity during gel filtration steps. Neither thiol-containing agents, such as mercaptoethanol, nor a series of divalent cations appeared to stabilize the enzyme. The empirical decision was made to include one substrate (1 mM homocysteine) and one product (1 mM dimethylglycine) in all steps during the purification. This led to a remarkable stabilization, preventing both the loss of activity and the generation of lower molecular weight active products. Difficulties were encountered during the purification steps after gel filtration through Sepharose 6B. Chromatography through CM-cellulose through either pH 6.5 or 5.7 alone did not yield a homogeneous preparation. At pH 6.5, the protein did not bind to CM-cellulose although material absorbing at 410 nm was retained. If chromatography through CM-cellulose at pH 5.7 was attempted directly after gel filtration through Sepharose 6B, the component with absorption at 410 nm appeared to denature and distribute throughout the fractions containing the methyltransferase. This led to the successful procedure of utilizing the pH 6.5 run before the pH 5.7 run. As indicated in Fig. 2, a major component with transmethylase activity is obtained; if rechromatographed under the same conditions, a homogeneous preparation was seen. A 250-fold purification was required to achieve homogeneity with a yield of approximately 15%. The protein was labile to lyophilization but could be stored in 50% (v/v) glycerol solutions at -20 °C in the native state for many months. Spectral analysis indicated no evidence of chromophores other than those expected or aromatic residues in the protein in the ultraviolet range.

Fig. 3 demonstrates the analytical acrylamide gel electrophoretic patterns of the purified protein. As indicated, a single wide band is found on the conventional gel if homocysteine is present. In the absence of homocysteine, a series of discrete bands is generated which migrate more slowly than the major band. Scanning spectrophotometry of the gel permitted a measurement of the concentration of the proteins that were present in the four densest bands. The proportion of protein present in these bands in order of decreasing migration was calculated to be 67:28:4:1. The fifth and smallest band was too scant to be quantitated accurately. The relative migration of the peaks of absorption of these bands was plotted against assumed integral aggregation numbers. As Fig. 4 indicates, these points lie on a straight line. Acrylamide gel electrophoresis analysis in sodium dodecyl sulfate of the purified betaine:homocysteine methyltransferase indicated the presence of a single band (Fig. 3) with an apparent molecular weight of 45,000 as determined with calibrated gels. Gel filtration analysis through a calibrated Sephacryl S-300 column provided an apparent molecular weight of 270,000 for the methyltransferase. Amino acid analysis of the protein is indicated in Table I.

The pH range of stability for the enzyme was fairly broad with activity starting to fall only at values below 7.5 and especially below 5.5. The inclusion of homocysteine and di-
methylglycine in buffers at pH values greater than 5.5 stabilized the protein. Chromatography of the protein in CM-cellulose at pH 5 instead of 5.7 yielded a protein which appeared homogeneous but was unstable despite the presence of homocysteine and dimethylglycine. No effect of the enzyme's activity was observed in the presence of 10 mM EDTA at pH 7.4. The $K_m$ values for homocysteine and betaine were 0.12 and 0.10 mM, respectively. The effect of various possible inhibitors was tested. Neither L-methionine nor L-ethionine inhibited significantly. Of the precursors and products of betaine metabolism, only dimethylglycine appeared to be a potent inhibitor. The inhibitions by 3,3-dimethylbutyrate and isovalerate, which are, respectively, analogs of betaine and dimethylglycine with nitrogens replaced by carbon atoms, were competitive against betaine (Figs. 5 and 6). $K_i$ values of 0.45 and 0.30 mM, respectively, were found.

**DISCUSSION**

In 1939, DuVigneaud and co-workers (10) demonstrated that betaine could support growth of rats when homocysteine was provided as the only sulfur source. In 1955, the first report of the partial purification of the enzyme from rat and pig liver appeared (3). These early studies indicated that the enzyme consisted of a heat-labile apoprotein and a heat-stable cofactor. Both components were reported to be required for activity. In 1957, the homocysteine transmethylase from horse liver was found to be almost homogeneous after 100-fold purification (5). A molecular weight between 150,000 and 250,000 was noted with aggregation occurring in the absence of mercaptans. Betaine could serve also as the methyl donor. Ion exchange chromatography studies indicated the presence of several different components possessing activity without the appearance of major contaminants; this heterogeneity was thought to represent formation of mixed disulfides with low molecular weight thiol-containing compounds (11). Aggregates of this protein were seen in electron microscopic studies (12). Analyses in sodium dodecyl sulfate-containing compounds with a native protein of 180,000 daltons revealed subunits of 50,000 daltons (13). In 1960, Ericson (1, 14) reported upon the purification and characterization of betaine:homocysteine transmethylase from pig liver (2). Two different procedures with 75- or 150-fold purification yielded essentially a homogeneous enzyme preparation by sedimentation and chromatographic analysis. The molecular weight of the protein was found to be 270,000. In contrast to earlier studies, no cofactor requirements were found and, in particular, neither folate, vitamin B$_6$, nor pyridoxine was found to be associated with the enzyme. As in the present case, the pig liver enzyme showed significant heat stability, a property which facilitated its purification. The specificity of the methyl donor was examined, and it was found that betaine could be substituted by its methyl or ethyl ester and to a lesser degree by 2-acetonitrile-trimethylammonium chloride, carboxymethyltrimethylammonium chloride, $a$-carboxyethyltrimethylammonium chloride, or by carboxymethylthiethylammonium chloride. Dimethylglycine was found to be a strong inhibitor. An analysis of the distribution of the methyltransferase indicated that the activ-
ity was confined mainly to the liver; in almost all species, the activity in other organs was absent. Minimal activity in the spleen and pancreas of the monkey and moderate activity in the kidneys of guinea pigs were noted. Although Ericson (1) did not find enzyme activity in any of the plants or microorganisms that were tested, later reports have indicated the presence of betaine:homocysteine methyltransferase in *Pseudomonas denitrificans* (15) and *Aspergillus nidulans* (16).

In recent years, studies on betaine:homocysteine transmethylase have been conducted primarily by one laboratory (17–21). A study of the regulation of homocysteine methyltransferase in rat tissue revealed that the betaine- and folate-dependent enzymes responded differently to dietary changes. The betaine-dependent transmethylase increased with protein and methionine intake, whereas the folate-dependent enzyme increased under conditions when there was suggested a need for methionine biosynthesis. The conclusion was that methionine synthase contributes significantly to the regulation of methionine metabolism in mammals with the high protein diet repressing the synthesis of this enzyme in liver. Betaine:homocysteine methyltransferase activity was very high in the liver, whereas the activities in the kidney, pancreas, spleen, adrenal gland, testes, and adipose tissue were less than 1% of the hepatic content. The betaine-dependent enzyme showed greater responses to hormones with substantial increments in activity following hydrocortisone administration and decreased activity following treatment with thyroxine, whereas minimal changes were noted with the folate-dependent activity in liver. Modest inhibition of betaine:homocysteine methyltransferase was noted by S-adenosylhomocysteine (being competitive with respect to homocysteine). Since S-adenosylhomocysteine activates cystathionine synthase, the distribution of homocysteine between betaine-dependent methyltransferase and cystathionine synthase may be very significantly regulated by the level of S-adenosylhomocysteine. Studies on methionine metabolism in rat hepatomas indicated that betaine-dependent transmethylation was present in all liver tumors studied although the values did not correspond in a linear fashion to the growth rates of the different tumors. The levels of enzyme activity in hepatic tumors do not respond to the dietary manipulations that cause changes in the enzyme activity of normal liver.

Our interest in utilizing the derivatives of dimethylglycine and betaine where carbon atoms substitute for nitrogen stemmed from the observations that acetylcholine esterase bound quite well charged acetylcholine analogs with the same kind of atom replacements (22–25). The structural relationship of choline to betaine is obvious. Because scission of the methyl ammonium bond was unique to the methyltransferase, the expectation was that the requirement of the positive charge for binding would be more stringent in the present case. It could be that both the single carboxymethyl and the three methyl groups contribute much more to the binding of betaine than does the positively charged nitronium atom. However, it is difficult to understand this explanation. For instance, the binding sites of trypsin and chymotrypsin have very low affinities for neutral and positively charged residues, respectively. An alternative basis for the inhibition by 3,5-dimethylbutyrate could be that the binding site for betaine/dimethylglycine exists in two states, controlled perhaps by the movement of an anionic group. When such a group moves toward the substrate site, only betaine and dimethylglycine will bind, whereas when the group moves away, the binding of analogs occurs.

Despite the earlier metabolic studies (9, 17, 20), the physiological role of the betaine-dependent methyltransferase is unclear. Why is the content of the protein uniformly so high in all livers studied and why is the enzyme confined primarily to the liver? It is difficult to see how a protein in such high concentrations could have a minor role in hepatic one-carbon metabolism. In contrast, the widely distributed methionine synthase is at much lower concentrations in various tissues including the liver (26, 27). The role of the folate- and B12-dependent pathway has been elucidated in part by disease states with specific enzyme deficiencies (28, 29). The absence of any report of derangements in the betaine-dependent pathway has limited the perspective of that enzyme's role. The development of compounds with the ability to specifically inhibit this enzyme may provide in future physiological studies an insight into this problem.

Acknowledgments—The cooperation of the Renal Transplantation Service of the Department of Surgery of the University of Miami School of Medicine is appreciated. We express our thanks for the continued support that George and Madeleine Joannou have extended to the efforts of this laboratory.

REFERENCES

1. Ericson, L.-E. (1960) *Acta Chem. Scand.* **14**, 2102–2122
2. Fromm, H. J., and Nordlie, R. C. (1959) *Arch. Biochim. Biophys.* **81**, 328–335
3. Ericson, L.-E., Williams, J. N., and Elvehjem, C. A. (1955) *J. Biol. Chem.* **212**, 537–544
4. Ericson, L.-E. (1960) *Acta Chem. Scand.* **14**, 2113–2126
5. Durell, J., Anderson, D. G., and Cantoni, G. L. (1957) *Biochim. Biophys. Acta* **26**, 270–282
6. Finkelstein, J. D., and Mudd, S. H. (1967) *J. Biol. Chem.* **242**, 873–880
7. Mudd, S. H., Finkelstein, J. D., Irreverre, F., and Laster, L. (1965) *J. Biol. Chem.* **240**, 4382–4392
8. Lineweaver, H., and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 685–686
9. Dixon, M. (1950) *Biochem. J.* **55**, 170–171
10. DuVigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M. (1939) *J. Biol. Chem.* **131**, 57–76
11. Klee, W. A. (1960) *Biochim. Biophys. Acta* **45**, 537–544
12. Klee, W. A., and Cantoni, G. L. (1960) *Biochim. Biophys. Acta* **45**, 545–553
13. Klee, W. A. (1962) *Biochim. Biophys. Acta* **59**, 562–568
14. Ericson, L.-E. (1960) *Acta Chem. Scand.* **14**, 2127–2134
15. White, R. F., Kaplan, L., and Birnbaum, J. (1973) *J. Bacteriol.* **113**, 218–223
16. Balinska, M., and Pazewski, A. (1979) *Biochem. Biophys. Res. Commun.* **91**, 1095–1100
17. Finkelstein, J. D., Kyle, W. E., and Harris, B. J. (1971) *Arch. Biochem. Biophys.* **146**, 84–92
18. Finkelstein, J. D., Harris, B. J., and Kyle, W. E. (1972) *Arch. Biochem. Biophys.* **153**, 320–324
19. Finkelstein, J. D., Kyle, W. E., and Harris, B. J. (1974) *Arch. Biochem. Biophys.* **165**, 774–779
20. Finkelstein, J. D., Cello, J. P., and Kyle, W. E. (1974) *Biochim. Biophys. Res. Commun.* **61**, 525–531
21. Grossman, M. R., Finkelstein, J. D., Kyle, W. E., and Morris, H. F. (1974) *Cancer Res.* **34**, 794–800
22. Adams, D. (1949) *Biochim. Biophys. Acta* **3**, 1–14
23. Adams, D. H., and Whitaker, V. P. (1949) *Biochim. Biophys. Acta* **3**, 358–366
24. Mounter, C. A., and Cheatham, R. M. (1963) *Enzymologia* **25**, 215–224
25. Hassan, F. B., Cohen, S. G., and Cohen, J. B. (1980) *J. Biol. Chem.* **255**, 3898–3904
26. Taylor, R. T., and Weissbach, H. (1973) in *The Enzymes* (Boyer, P. D., ed) 3rd Ed, Vol IX, pp. 121–165, Academic Press, New York
27. Mangum, J. H., and North, J. A. (1971) *Biochemistry* **10**, 3765–3769
28. Rosenberg, L. E. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., eds) 4th Ed, pp. 411–429, McGraw-Hill, New York
29. Rowe, P. B. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., eds) 4th Ed, pp. 430–457, McGraw-Hill, New York