Mitochondrial Neutral Amino Acid Transport: Evidence for a Carrier Mediated Mechanism†

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ABSTRACT: Swelling of rat liver mitochondria induced by various neutral amino acids indicates that the L isomers of serine, alanine, methionine, valine, threonine, and leucine enter the mitochondrial matrix by a stereospecific process. Chemical modification of mitochondria with diazobenzenesulfonate or *p*-mercuribenzoate inhibited the rapid uptake of these compounds, as well as that of L-proline and glycine, to various extents. Diazobenzenesulfonate did not inhibit the transport of compounds that enter the mitochondrial matrix by simple diffusion, i.e., thiocyanate, nitrate, formate, bicarbonate, and acetate. Inhibition by *p*-mercuribenzoate was reversed by treatment with dithiothreitol. Inclusion of various neutral amino acids in the *p*-mercuribenzoate preincubation mixture substantially prevented inactivation of transport. Glycine, D- or L-serine, L-threonine, L-methionine, L-alanine, and L-valine all individually protected against the inactivation of glycine, L-serine, L-threonine, L-methionine, L-alanine, L-valine, L-proline, and β-alanine transport, while L-proline, β-alanine, L-leucine, L-isoleucine, and L-histidine did not protect. L-Arginine potentiated *p*-mercuribenzoate inactivation of neutral amino acid transport. These findings are consistent with the presence of an inner membrane neutral amino acid carrier system having broad substrate specificity, although the presence of multiple carriers cannot be conclusively eliminated.

The intramitochondrial content of free amino acids in rat liver has been reported (Matsuzawa, 1974) and significant metabolism involving neutral amino acids occurs in the mitochondrial matrix. Alanine aminotransferase provides for the conversion of alanine to pyruvate (De Rosa and Swick, 1975), while the terminal oxidation of glycine is catalyzed by the glycine cleavage system (Motokawa and Kikuchi, 1971). In the urea cycle, ornithine is converted to citrulline in a reaction that may be regulated by L-valine, L-leucine, or L-isoleucine (Matsuzawa, 1974). L-Leucine has also been reported to activate glutamate dehydrogenase and may therefore significantly influence the overall nitrogen metabolism of the liver cell (McGivan et al., 1973). Serine transhydroxymethylase isoenzymes in the matrix and the cytosol have been proposed to function in the transfer of one-carbon units between the two compartments (Cybulski and Fisher, 1976).

Although the mitochondrial inner membrane has been shown to be permeable to a variety of neutral amino acids (Garfinkel, 1963; Buchanan et al., 1969; Jones and Jones, 1970; Gamble and Lehninger, 1973; Halling et al., 1973; King and Diwan, 1973; Meyer, 1977), their mechanism of entry has been the center of some controversy. Halling et al. (1973) reported that a number of neutral L-amino acids and DL mixtures of the amino acids enter rat heart and liver mitochondria at similar rates, and that amino acids such as DL-norvaline and β-alanine, which are apparently metabolically unimportant in these mitochondria, are rapidly transported. These authors hypothesized that neutral amino acids traverse the inner mitochondrial membrane, by a nonstereospecific passive diffusion mechanism, as a ring structure formed by hydrogen bonding between the carboxylate and amino groups. While liposomes have been shown to be permeable to neutral amino acids, the rate of transport being related to the hydrophobicity of the molecule (Klein et al., 1971; Wilson and Wheeler, 1973), the transport rate is far lower in synthetic membranes than in mitochondria (Halling et al., 1973). This discrepancy might be explained either by a difference in lipid content of the two types of membranes, or by the presence of neutral amino acid carrier systems in mitochondria. Buchanan et al. (1969) have suggested that a carrier protein participates in L-leucine uptake by rat liver mitochondria and demonstrated that the process was partially inhibited by *N*-ethylmaleimide and various amino acids. A functional sulfhydryl group has also been implicated in L-proline transport (Meyer, 1977). Gamble and Lehninger (1973) found that citrulline is rapidly transported by liver, but not by heart mitochondria, suggesting the lack of a functional carrier system in heart. We have recently demonstrated that the rates of mitochondrial swelling induced by the D and L isomers of serine are substantially different (Cybulski and Fisher, 1976), indicating a facilitated transport of serine into rat liver mitochondria.

Our interest in defining further the postulated one-carbon shuttle system involving serine transhydroxymethylase isoenzymes (Cybulski and Fisher, 1976) led us to a more detailed investigation of the mechanism of neutral amino acid transport which is reported in this paper.

Materials and Methods

Rat liver mitochondria were prepared from Sprague-Dawley male rats, which had been starved for 16 h, according to a modification of the method of Schnaitman and Greenawalt (1968), using 0.25 M sucrose as the isolation medium. Protein determinations were performed by the biuret method (Jacobs et al., 1956). Diazobenzenesulfonate was synthesized and stored at −10 °C in 20 mM potassium phosphate buffer, pH 7.5, as described by Dilley et al. (1970). Stock *p*-mercuribenzoate solutions contained 10 mM glycylglycine, pH 7.5, and were quantitated spectrophotometrically using an ϵ_{232} = 16 900 cm^{-1} M^{-1} (Boyer, 1954). Amino acids, *N*-ethylmaleimide, *p*-mercuribenzoate, and CCCP[1] were obtained from Sigma Chemical Co.

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[1] Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMB, *p*-mercuribenzoate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

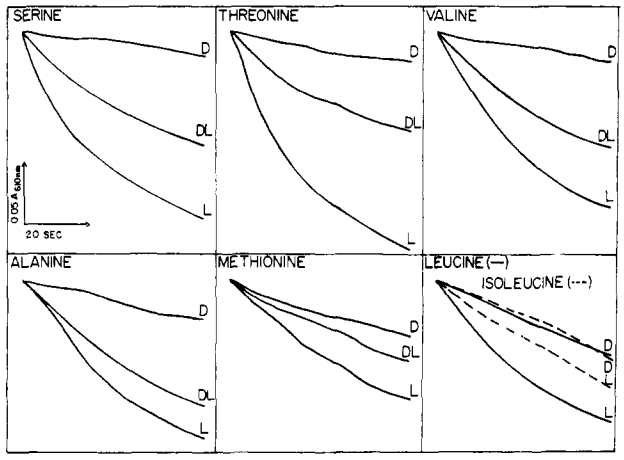

FIGURE 1: Transport of neutral amino acids. Mitochondrial swelling was induced by isoosmolar solutions of D, L, or DL mixtures of various neutral amino acids as described under Materials and Methods.

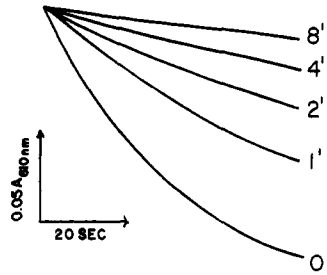

FIGURE 2: Effect of diazobenzenesulfonate on L-serine induced mitochondrial swelling. Mitochondria (20 mg of protein) were preincubated with 6 mM diazobenzenesulfonate. After indicated times, aliquots containing 1 mg of protein were combined with osmotic swelling mixtures containing 200 mM L-serine as described under Materials and Methods.

Mitochondrial transport studies using osmotic swelling techniques were performed by the general method of Chappell and Haarhoff (1967). Mitochondria (1 mg of protein) were suspended in 1 mL of a solution containing 5 mM Tris-HCl, 0.3 mM EDTA, 1.4 μg of rotenone, 1 μg of antimycin A, and either 200 mM amino acid or 100 mM of the ammonium salts of phosphate, thiocyanate, bicarbonate, formate, acetate, or nitrate at pH 7.4, and the decrease in apparent absorbance at 610 nm was monitored at room temperature.

Diazobenzenesulfonate and *p*-mercuribenzoate pretreatments of mitochondria were carried out by suspending mitochondria at 0 °C (20 and 10 mg, respectively) in a 1-mL solution containing 0.25 M sucrose and the appropriate concentration of diazobenzenesulfonate or *p*-mercuribenzoate adjusted to pH 7.5. *p*-Mercuribenzoate pretreatment in the presence of amino acids was performed similarly, with the final suspension containing 0.2 M sucrose, 60 mM amino acid at pH 7.5 and 150 μM *p*-mercuribenzoate. At the specified time, an aliquot containing 1 mg of mitochondrial protein was transferred to the transport mixtures described above. The percentage of transport inhibition was calculated from the difference in the extent of the apparent absorbance decrease during the first minute between a suspension of control mitochondria preincubated in the absence of inhibitor and those preincubated with inhibitor in the presence or absence of indicated amino acid.

Results

Transport of D and L Neutral Amino Acids. Halling et al. (1973) reported that the rates of mitochondrial swelling induced by the L isomers of alanine and valine are essentially the same as those induced by the DL mixtures of these compounds, indicating a lack of transport stereospecificity. The experiment presented in Figure 1, in which mitochondria were placed in isoosmotic solutions of various D or L neutral amino acids, shows that this is not the case. Rather, the L isomer promoted a faster rate of mitochondrial swelling than did the D isomer, although the difference between the rates varied depending upon the amino acid. For serine, alanine, valine, and threonine, the isomeric swelling rate difference was very significant, whereas for leucine and methionine it was not as large, but still substantial. In the case of isoleucine, the difference was small and, although suggestive of a stereospecific process, does not constitute conclusive evidence. As can be seen, 1:1 mixtures of the D- and L-amino acids generally gave swelling rates intermediate between the pure isomers. The mitochondrial swelling caused by an isoosmotic solution of DL-leucine could not be measured due to the insolubility of the isomeric mixture.

Diazobenzenesulfonate Inhibition of Neutral Amino Acid Transport. A criterion for the existence of a membrane transport carrier protein is inhibition of transport by protein modification reagents. Diazobenzenesulfonate may be particularly useful in elucidating carrier mediated transport since it reacts with a variety of amino acid side chains including those of tryptophan, arginine, histidine, tyrosine, lysine, and cysteine (Howard and Wild, 1957). Mitochondrial phosphate ion uptake, which occurs by a carrier mediated process, has been previously demonstrated to be inhibited by diazobenzenesulfonate (Dawson, 1974).

Pretreatment of mitochondria with 6 mM diazobenzenesulfonate was found to yield a time-dependent inhibition of L-serine induced mitochondrial swelling, with maximum inhibition being reached after 8 min (Figure 2). Under identical preincubation conditions, the uptake of a variety of other neutral L-amino acids was also inhibited, although considerable variability in the extent of maximum inhibition was observed. The transport of glycine and the L isomers of alanine, serine, threonine, proline, cysteine, valine, methionine, leucine, and isoleucine was inhibited by approximately 85, 90, 85, 85, 85, 75, 70, 60, 45, and 10%, respectively. Thus, with the exception of isoleucine, mitochondrial swelling induced by these amino acids is substantially inhibited. In order to assess whether the apparent inhibition of amino acid transport by diazobenzenesulfonate resulted from a specific modification of a carrier protein(s) or from a nonspecific modification of the osmotic characteristics of the membrane, the swelling of diazobenzenesulfonate treated mitochondria in isoosmolar ammonium thiocyanate, known to be transported by passive diffusion (Mitchell and Moyle, 1969), was studied. Thiocyanate enters mitochondria by an electrogenic process. Therefore, to induce large amplitude swelling the membrane must be made permeable to protons or to cations, which in turn reduces the membrane electrochemical potential (Chappell et al., 1972). As can be seen in Figure 3, ammonium thiocyanate did not cause swelling in untreated mitochondria until an oxidative phosphorylation uncoupler, CCCP, was added. However, with increasing time of preincubation with diazobenzenesulfonate, the mitochondrial swelling in the presence of ammonium thiocyanate increased in the absence of uncoupler. Clearly, diazobenzenesulfonate treatment does not alter mitochondrial swelling characteristics, but it does apparently increase the proton permeability of the membrane. Such a result is consistent with a report by Hanstein and Hatefi (1974) which demonstrated that certain diazo compounds uncouple mito-

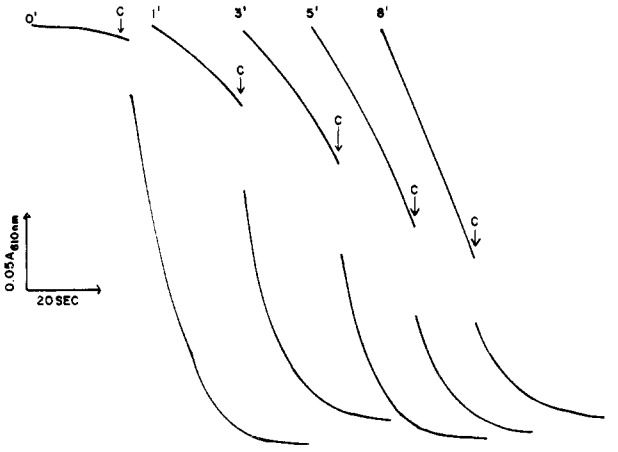

FIGURE 3: Effect of diazobenzenesulfonate on ammonium thiocyanate induced mitochondrial swelling. Mitochondria were preincubated with diazobenzenesulfonate for indicated times as described in Figure 2, and swelling was measured in 100 mM ammonium thiocyanate. Addition of 1.5 nmol of CCCP is indicated by C.

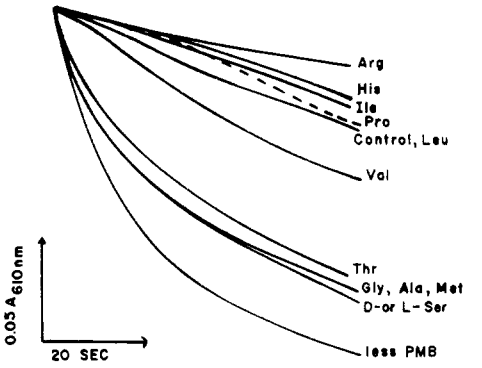

FIGURE 5: Effect of various amino acids on *p*-mercuribenzoate inactivation of glycine induced mitochondrial swelling. Mitochondria were preincubated in the presence of 150 μM *p*-mercuribenzoate and various amino acids (60 mM) as described under Materials and Methods. After 10 min, aliquots containing 1 mg of protein were removed and combined with a swelling mixture containing 200 mM glycine. All amino acids present during the preincubation were L isomers unless otherwise stated. The control preincubation contained no added amino acid.

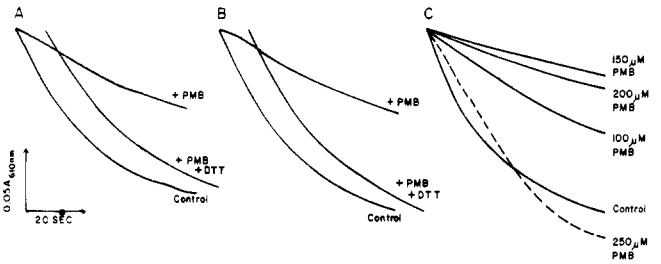

FIGURE 4: Effect of *p*-mercuribenzoate on mitochondrial swelling induced by L-serine or glycine. (Experiment A) L-Serine induced swelling of mitochondria preincubated either with 150 μM *p*-mercuribenzoate (PMB), or with 150 μM *p*-mercuribenzoate followed by a 2-min incubation with 1.5 mM dithiothreitol (+PMB + DTT). (Experiment B) Glycine induced swelling of mitochondria treated as described under experiment A. (Experiment C) Glycine induced swelling of mitochondria pretreated with various concentrations of *p*-mercuribenzoate. All *p*-mercuribenzoate preincubations were performed for 10 min as described under Materials and Methods. Control preincubations contained no *p*-mercuribenzoate or dithiothreitol.

chondria by covalent bond formation with membrane proteins. Mitochondrial swelling caused by the ammonium salts of acetate, formate, bicarbonate, and nitrate, all of which are believed to permeate the inner membrane by simple diffusion (Chappell and Haarhoff, 1967; Chappell et al., 1972; Elder and Lehninger, 1973), was also unaffected by diazobenzenesulfonate. In order to determine whether the amino acid transport inhibition properties of diazobenzenesulfonate resulted from its uncoupling property, control experiments measuring the rates of uptake of amino acids in the presence of CCCP were performed. No inhibition of swelling was observed in any of these experiments.

p-Mercuribenzoate Inhibition of Amino Acid Transport. Mercurial inhibition of neutral amino acid transport in human skin fibroblasts (Booth and Nadler, 1975) and rabbit intestinal cells (Schaeffer et al., 1973) suggests the participation of sulfhydryl groups in transport. Mitochondrial transport of leucine (Buchanan et al., 1969), proline (Meyer, 1977), and glutamate (Meijer et al., 1972) has also been reported to be inhibited by sulfhydryl reagents. The effect of preincubating mitochondria with 150 μM *p*-mercuribenzoate on L-serine or glycine transport is shown in Figure 4. The degree of *p*-mercuribenzoate inactivation varied with different mitochondrial preparations, and complete inhibition of swelling could not be obtained with higher concentrations of *p*-mercuribenzoate or longer preincubation times. At higher *p*-mercuribenzoate concentrations inhibition of swelling was reversed (Figure 4), indicating that the membrane was becoming passively permeable to amino acids. Similar results were obtained by Scott et al. (1970) in studies monitoring potassium chloride uptake by bovine heart mitochondria. The uptake of the L isomers of threonine, valine, methionine, proline, and alanine was also inhibited by 150 μM *p*-mercuribenzoate, demonstrating 70, 55, 40, 35, and 30% inhibition, respectively, as compared with 65% inhibition of glycine and L-serine transport. Addition of dithiothreitol (1.5 mM) to *p*-mercuribenzoate treated mitochondria, followed by an additional 2-min preincubation, completely restored mitochondrial swelling induced by these amino acids. L-Leucine and L-isoleucine uptake appeared to be inhibited to a small degree by *p*-mercuribenzoate, although the greatest differences between the control and inhibited rates approximate the error limits of the experiment. *N*-Ethylmaleimide up to 1.5 mM, which completely inhibits the phosphate (Johnson and Chappell, 1973) and glutamate (Meijer et al., 1972) carriers of rat liver mitochondria, had no effect on glycine or L-serine transport.

Protection against p-Mercuribenzoate Inactivation of Transport. The stereospecific nature of neutral amino acid transport coupled with its inhibition by either *p*-mercuribenzoate or diazobenzenesulfonate provides substantial evidence in support of carrier mediated transport of these substances in rat liver mitochondria. In an attempt to define whether several of the neutral amino acids are transported by a single carrier or by a multiplicity of carriers, the ability of individual amino acids to protect against the *p*-mercuribenzoate inhibition of swelling induced by other amino acids was investigated. As shown in Figure 5, D-serine, L-serine, L-alanine, glycine, L-threonine, or L-methionine in the preincubation medium at 60 mM provided substantial protection against *p*-mercuribenzoate inhibition of swelling induced in isoosmolar glycine. L-Valine gave intermediate protection, while L-leucine, L-isoleucine, L-histidine, and L-proline provided no protection. L-Arginine routinely potentiated *p*-mercuribenzoate inactivation. Figure 6 shows the effects of various amino acids on the *p*-mercuribenzoate inactivation of L-serine, L-methionine, L-threonine, L-valine, and L-alanine transport. In general, the results were analogous to those obtained for glycine-induced swelling. Similar protection profiles were also observed in

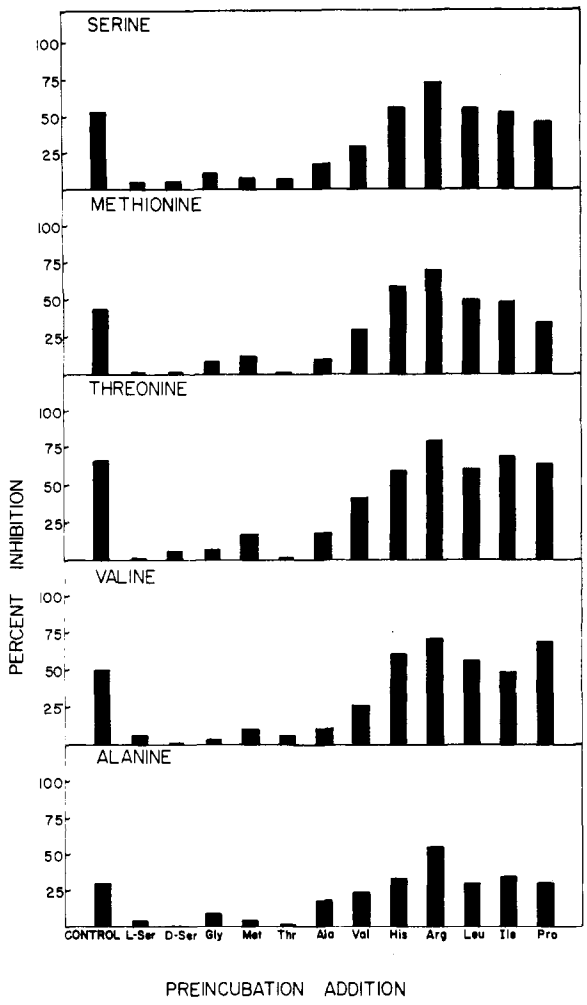

FIGURE 6: Protective effect of various amino acids on *p*-mercuribenzoate inactivation of neutral amino acid transport. Mitochondria (20 mg of protein) were preincubated with 150 μM *p*-mercuribenzoate in the absence (control) or presence of various amino acids (60 mM) as described under Materials and Methods. After 10 min of preincubation, aliquots containing 1 mg of protein were combined with osmotic swelling mixtures containing either 200 mM L-serine, L-methionine, L-threonine, L-valine, or L-alanine. Percentages of inhibition were calculated from the change in absorbance at 610 nm during the first minute of mitochondrial swelling.

studies examining the effect of these amino acids on the *p*-mercuribenzoate inactivation of proline and β-alanine transport. However, 60 mM L-proline or β-alanine protected neither their own transport nor that of the other neutral amino acids tested. Other analogues of neutral amino acids including sarcosine (*N*-methylglycine), dimethylglycine, and pyruvate failed to protect glycine or L-serine transport from *p*-mercuribenzoate inactivation.

Discussion

The stereospecific nature of serine, alanine, valine, threonine, methionine, and leucine transport suggests that the L isomers of these compounds enter the mitochondrial matrix by facilitated diffusion. Isoleucine uptake may also be stereospecific, but the difference in swelling rates induced by the two isomers of this compound was not large enough to verify this possibility. The generally low rate of D isomer uptake may be explained either by a slower transport of these compounds by a carrier, or by a passive diffusion process as observed in synthetic membranes (Klein et al., 1971; Wilson and Wheeler, 1973). Inhibition of the transport of glycine, L-serine, L-valine, L-alanine, L-methionine, L-proline, L-threonine, and L-leucine by diazobenzenesulfonate and *p*-mercuribenzoate is also consistent with the operation of a carrier. The lack of inhibition of L-isoleucine transport suggests that this compound enters mitochondria either by a carrier not affected by diazobenzenesulfonate or *p*-mercuribenzoate, or by a passive diffusion mechanism. The transport of the various amino acids was found to be inhibited to different extents by both diazobenzenesulfonate and *p*-mercuribenzoate. This variability may indicate that multiple neutral amino acid carriers, which vary in their sensitivity to the inhibitors, are present in mitochondria, or that the modification of a single carrier affects either the transport or binding of the amino acids by a single carrier in subtly different ways. Since neither *p*-mercuribenzoate (Gaudemer and Latruffe, 1975) nor diazobenzenesulfonate (Tinberg et al., 1974; Cybulski and Fisher, 1976) at the concentrations used in these experiments substantially penetrates the mitochondrial inner membrane, the site on the carrier which reacts with these compounds must be available to the outer surface of the inner membrane. This conclusion is further supported by the ability of dithiothreitol, a nonpermeant thiol (Rhodin and Racker, 1974), to reverse rapidly the inactivation of amino acid transport by *p*-mercuribenzoate. Since *p*-mercuribenzoate can inhibit the transport of a variety of neutral amino acids, at least one reactive sulfhydryl group may be present at or near the carrier(s) substrate binding site. The capability of glycine, L-serine, L-threonine, L-alanine, L-methionine, and L-valine to protect the transport of one another from *p*-mercuribenzoate inactivation indicates that a single carrier may function in the transport of these compounds. However, these properties might also be explained by the existence of several carrier proteins with different transport specificities, but with similar substrate binding characteristics. If, indeed, there is a single carrier functioning in the transport of these neutral amino acids, some of its properties can be deduced. The fact that glycine, L-serine, D-serine, L-threonine, L-alanine, L-methionine, and L-valine protect against the *p*-mercuribenzoate inactivation of L-proline and β-alanine-induced mitochondrial swelling implies that L-proline and β-alanine may also be transported by this carrier. However, the inability of these latter two compounds to protect the transport of any of the neutral amino acids, including themselves, demonstrates that a primary α-amino group is necessary for the protection of the essential sulfhydryl group. Consistent with this notion is the lack of protection observed with pyruvate. Sarcosine and dimethylglycine were unable to prevent *p*-mercuribenzoate inhibition of amino acid transport, further indicating that an unsubstituted α-amino group is necessary for the binding to the carrier. The lack of significant effect of L-leucine, L-isoleucine, L-lysine, and L-histidine on *p*-mercuribenzoate inhibition of neutral amino acid transport indicates that, even though these molecules possess a free α amino group, no binding between them and the neutral amino acid carrier occurs because of side chain restrictions. The potentiating effect of L-arginine on *p*-mercuribenzoate inactivation suggests that this compound may bind to the carrier, perhaps at a site other than that binding the neutral amino acids. Finally, the protection afforded by D-serine against sulfhydryl group inactivation shows that, while the transport of neutral amino acids is specific for L isomers, the formation of carrier–neutral amino acid complexes appears to be nonstereospecific.

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3′-Phosphatase Activity in T4 Polynucleotide Kinase†

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ABSTRACT: The purification of T4 polynucleotide kinase results in the copurification of an activity which will specifically remove the 3′-terminal phosphate from a variety of deoxyribonucleotides and ribonucleotides in the absence of ATP. This phosphatase activity requires magnesium, has a pH optimum of 6.0, and is more active with deoxyribonucleotides than ribonucleotides. T4 polynucleotide kinase and the 3′-phosphatase activity copurify by gradient elution column chromatography on DEAE-cellulose, phosphocellulose, and hydroxylapatite. The two activities are included in and comigrate on Sephadex G-200. Polyacrylamide gel electrophoresis at pH 9.2 results in comigration of the two activities together with the major protein band. The two activities respond in parallel to heat inactivation at 35 °C and ATP, a substrate for the kinase only, protects both activities from heat inactivation. It is therefore suggested that the two activities are functions of the same protein molecule.

Polynucleotide kinase from bacteriophage T_4 infected *Escherichia coli* (EC 2.7.1.78) catalyzes the transfer of the γ phosphate of ATP[1] to the 5′-hydroxyl termini of nucleic acids (Richardson, 1972). Although the biological function of this enzyme has not been established (Chan and Ebisuzaki, 1970), it is widely used for the preparation of ^{32}P-labeled nucleic acids for sequence determination (Szekely, 1972; Maxam and Gilbert, 1977) and for the synthesis of substrates for DNA and RNA ligase (Khorana et al., 1972; Walker et al., 1975). During attempts to use polynucleotide kinase to phosphorylate oligoribonucleotides with a 3′-terminal phosphate (Uhlenbeck and Cameron, 1977), it was discovered that highly purified preparations of the enzyme removed the 3′-phosphate from an oligomer during the time that the 5′-phosphate was added. This phosphatase activity did not require ATP and would specifically remove the 3′-phosphate from a variety of oligoribonucleotides. In an effort to remove this undesirable phosphatase activity, a purification procedure for polynucleotide kinase was followed with assays of both activities. Data were obtained

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; poly(U), poly(uridylic acid); PEI, polyethylenimine; ATP, adenosine triphosphate; pTp, 2′-deoxythymidine 3′,5′-bisphosphate; Tp, 2′-deoxythymidine 3′-monophosphate.