Phosphate Transport in Rat Liver Mitochondria

MEMBRANE COMPONENTS LABELED BY N-ETHYLMALEIMIDE DURING INHIBITION OF TRANSPORT*

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N-ethylmaleimide (NEM) inhibits the transport of phosphate in mitochondria but is without effect on permeation of other metabolites. In spite of its specificity for inhibition of phosphate transport, NEM reacts in an unspecific manner with inner membrane proteins in general. Treatment of mitochondria with [3H]NEM just sufficient to produce inhibition of phosphate transport results in labeling of at least 10 polypeptide components of the inner membrane.

A marked increase in the specificity of reaction of NEM for components of the phosphate transport system is attained by first protecting the transport system with p-mercuribenzoate (p-MB) and then by irreversibly blocking reactive sulfhydryl groups unassociated with transport by the addition of unlabeled NEM. Subsequent addition of dithiothreitol removes p-MB and restores 65 to 75% of the original phosphate transport activity. Reinhibition of transport with [3H]NEM results in both a 6-fold decrease in the amount of [3H]NEM bound by purified inner membrane vesicles and a substantial reduction in the number of labeled polypeptide components. Five distinct labeled species are detected by this method, one of which is a 32,000 molecular weight protein containing 40% of the bound radioactivity, or approximately 160 pmol/mg of inner membrane protein.

Correlation of binding of [3H]NEM by inner membrane proteins with inhibition of phosphate transport suggests that the maximum concentration of the NEM-sensitive component of the phosphate transport system is 60 pmol/mg of mitochondrial protein. This value, when combined with the $V_{max}$ of NEM-sensitive transport of 205 nmol x min$^{-1}$ x mg$^{-1}$ at 0°C (Coty, W. A., and Pedersen, P. L. (1974) J. Biol. Chem. 249, 2593) yields an approximate minimum turnover for this process of 3500 min$^{-1}$ at 0°C. This turnover number is at least 20-fold greater than similarly calculated values for adenine nucleotide transport and succinate oxidation in rat liver mitochondria at this temperature.

Taken together these results suggest that the NEM-sensitive phosphate transport system in rat liver mitochondria has an unusually high catalytic activity compared to other mitochondrial processes, and that at least one of the five NEM-binding proteins is likely to be an essential component of this transport system.

Phosphate transport in rat liver mitochondria is catalyzed by two transport systems; one is dependent on the membrane pH gradient (1-4), whereas the second exchanges Pi for dicarboxylate ions (2, 3, 5-7). Both transport systems are sensitive to mercurial reagents, such as p-MB$^1$ and mersalyl (5, 8, 9), whereas only the pH gradient-dependent phase of transport is sensitive to NEM (10).

In addition, the NEM-sensitive phosphate transport system has an extremely high activity, with a $V_{max}$ at 0°C of 205 nmol x min$^{-1}$ x mg$^{-1}$ (11). This rapid rate is consistent with proposals that pH gradient-dependent transport of phosphate, which can be thought of as a phosphate-hydroxyl antiport activity (5), plays an essential role in such mitochondrial processes as oxidative phosphorylation (12, 13), cation transport (14, 15), and net uptake or efflux of numerous anionic metabolites (2).

These facts suggest that addition of sufficient amounts of radioactive NEM to inhibit phosphate transport might result in

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The abbreviations used are: p-MR, p-mercurirolenzoate; NEM, N-ethylmaleimide; Heps, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; EGTA, ethyleneglycol-bis($\beta$-aminoethyl ether)$N,N'$-tetraacetic acid.
in labeling of an essential transport protein. The high rate of phosphate transport makes it likely that levels of the NEM-binding component could be detected, and that quantification of the labeling could be used to estimate the concentration of this component in the inner membrane. This approach to the identification of components of the P, transport system is preferred to substrate binding because the apparent affinity of the transport system for P, is quite low (K, = 1.6 mM) (11). In addition, if sufficient specificity of labeling is attained, the labeled component(s) could be used as markers in the purification of the transport system from unlabeled mitochondria.

With these thoughts in mind, the following studies were carried out to evaluate the specificity of the reaction of NEM with mitochondrial inner membrane proteins during inhibition of phosphate transport.

**EXPERIMENTAL PROCEDURE**

### Materials

Adult, male CD albino rats were obtained from The Charles River Breeding Laboratories, Wilmington, Mass., and were fed ad libitum with a Rockland rat diet purchased from Teklad, Winfield, Ohio.

The following chemicals were purchased from the indicated sources and were used as received: p-MB and sodium dodecyl sulfate from Sigma Chemical Co.; NEM, diginiton, dithiothreitol, and Hepes from Calbiochem; Coomassie brilliant blue and ultrapure urea from Schwarz/Mann; Lubrol WX from I.C.I. Organics, Providence, R.I.; and defatted bovine albumin from Sigma Chemical Co. The radioactively labeled compounds H-45PO4, 2H3NEM, and [3-14C]acetic anhydride were obtained from New England Nuclear. The H+P, was heated in a boiling water bath for 3 hours in 0.1 N HCl prior to use; all other radiochemicals were used as supplied.

Reagents for acrylamide gel electrophoresis, acrylamide (electrophoresis grade), and N,N'-methylenebisacrylamide were obtained from Eastman Organic Chemicals. NCS, a guanidinium ammonium base in toluene was obtained from Amersham-Searle.

The following proteins were used as molecular weight standards in polyacrylamide gel electrophoresis and were obtained from the indicated sources: chymotrypsinogen A (bovine pancreas) from Worthington Biochemicals Corp.; cytochrome c (horse heart), catalase (bovine liver), and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) from Sigma Chemical Co.; bovine albumin (electrophoretically pure) and β-lactoglobulin (bovine) from Miles Laboratories.

### Methods

**Isolation of Mitochondria**—Rat liver mitochondria were isolated by differential centrifugation as described by Schnaitman and Greenwald (16). The isolation medium contained 220 mM d-mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4, and 0.5 mg/ml of defatted bovine albumin.

**Subfractionation of Mitochondria**—The inner membrane-matrix (mitoplast) fraction was obtained by limited digitonin treatment of freshly isolated mitochondria as described by Schnaitman and Greenwald (16). This preparation was fractionated further by treatment with the nonionic detergent Lubrol WX, according to the procedure of Chan et al. (17). These procedures enabled a separation of mitochondria into three fractions, corresponding to the outer membrane plus intermembrane space, the matrix, and the inner membrane. Enzyme markers for these compartments were assayed as previously described (16, 17).

**Phosphate Transport Measurements**—Determinations of initial rates of phosphate transport were made exactly as previously described (11), using a rapid sampling device and an inhibitor-stop assay technique.

**Measurement of Phosphate Transport by Passive Swelling**—Passive swelling in isosmotic solutions of ammonium sulfate was used as a rapid qualitative means of measuring phosphate transport. Mitochondria were diluted to a final concentration of 10 mg of protein/mI in solutions containing isolation medium and inhibitors of transport, where appropriate. After a suitable incubation period at 0°, a 0.1-m1 aliquot (1.0 mg of protein) was transferred to a cuvette containing 0.9 ml of the swelling medium at 25°, mixed rapidly, and the absorbance of this mixture was monitored at 750 nm. Biuret reaction (23) in the presence of 0.26% sodium cholate. Soluble protein was measured by the method of Lowry et al. (24); if sodium phosphate transport inhibitors p-MB and NEM were standardized spectrophotometrically. The molar extinction coefficient of p-MB at 290 nm at pH 7.0 is 1690 (18), whereas NEM has a molar extinction coefficient of 620 at 305 nm at neutral pH (19).

**Treatment and Purity of NEM**—In order to minimize the chemical breakdown of [3H]NEM, this compound was stored at -20° in pentane. Immediately prior to use, the pentane was removed by low temperature evaporation (care was taken to ensure that the [3H]NEM did not sublime; vacuum never exceeded 25 cm of Hg). After redissolving in aqueous solution, the [3H]NEM concentration was measured as described above, and the specified activity was determined by measurement of radioactivity and comparison with a [3H]toluene standard; this value was within 10% of the specific activity specified by the manufacturer. In addition, concentrations of [3H]NEM determined by standard spectrometric methods were in excellent agreement with the extinction coefficient method, and thin layer chromatographic analysis on Silica Gel G showed no significant impurities, using a solvent system of petroleum ether/acetone (9:1, v/v) and visualizing authentic NEM with a spray reagent containing 5% o-mercuribenzenoic acid in saturated methanolic NaOH.

**Polyacrylamide Gel Electrophoresis**—Mitochondrial membrane proteins were analyzed by electrophoresis in polyacrylamide gels using the procedure of Laemmli (25) and the guidelines for molecular weight standards-Six proteins with well-defined subunit molecular weights were used as molecular weight markers.

**Preparation of Radioactive Proteins for Molecular Weight Standards**—Six proteins with well-defined subunit molecular weights were used as markers. The following proteins were analyzed by electrophoresis in polyacrylamide gels using the procedure of Laemmli (25) and the guidelines for molecular weight standards: chymotrypsinogen A (bovine pancreas) from Worthington Biochemicals Corp.; cytochrome c (horse heart), catalase (bovine liver), and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) from Sigma Chemical Co.; bovine albumin (electrophoretically pure) and β-lactoglobulin (bovine) from Miles Laboratories.

**Determination of Radioactivity**—All determinations of radioactivity were made in a Beckman 100 series liquid scintillation spectrometer. The scintillation grade toluene was obtained from Amersham-Searle. Preparation of Radioactive Proteins for Molecular Weight Standards-Six proteins with well-defined subunit molecular weights were used as molecular weight markers (3-14C)acetic anhydride were obtained from New England Nuclear. The 8*P, was heated in a boiling water bath for 3 hours in 0.1 N HCl prior to use; all other radiochemicals were used as supplied.

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RESULTS

Specificity of NEM as an Inhibitor of Phosphate Transport — Data accumulated in a number of different laboratories have led to the now rather generally accepted view that NEM reacts specifically with an essential sulfhydryl group of the phosphate transport system catalyzing $P_i/\text{OH}^-$ exchange rather than nonspecifically altering membrane permeability. Thus, other mitochondrial transport systems which catalyze exchange of dicarboxylates for phosphate (4, 10), other dicarboxylates (25), or tricarboxylates (25), and transport of adenine nucleotides (13) are not inhibited by NEM. Results presented in Fig. 1 are consistent with this view and show that NEM also fails to inhibit permeation of substances such as CO$_2$ and acetate, which penetrate the mitochondrial inner membrane by simple diffusion (5, 26). This is demonstrated by measurements of passive swelling of mitochondria in ammonium salts of phosphate, bicarbonate, and acetate. Swelling is observed in solutions containing these three anions, but not in the presence of Cl$^-$, which does not penetrate the membrane (Fig. 1A) (5). NEM specifically inhibits swelling in ammonium phosphate, whereas swelling in ammonium acetate or bicarbonate is not affected (Fig. 1B).

Labeling of Inner Membrane Proteins with $[^{3}H]\text{NEM}$ — The specificity of inhibition of phosphate transport by NEM suggested that one or more of the protein components involved might be readily labeled with radioactive NEM. For this reason, experiments were undertaken to inhibit initial rates of transport with radioactive NEM and to assess the labeled protein components by electrophoresis in sodium dodecyl sulfate.

As shown in Fig. 2, the initial rate of phosphate transport in rat liver mitochondria is maximally inhibited by approximately 20 nmol of NEM/mg of protein. Mitochondria were treated with this amount of $[^{3}H]\text{NEM}$, and after a 3-min incubation at $0^\circ$, a 10-fold excess of dithiothreitol was added to block any further reaction. These $[^{3}H]\text{NEM}$-treated mitochondria were then fractionated to yield a purified inner membrane preparation (See "Methods") incapable of transporting phosphate. As shown in Table I this fraction contained approximately 28% of the total mitochondrial protein and bound 3.9 nmol of $[^{3}H]\text{NEM}/\text{mg of protein}$. Over 90% of the radioactivity recovered was in fractions enriched in either outer membrane plus intermembrane space or matrix activities.

Analysis of $[^{3}H]\text{NEM}$-labeled inner membrane proteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate is shown in Fig. 3. At least 10 different molecular weight classes of labeled polypeptides can be resolved. Because of the large amount of labeling attained, some of these radioactive peaks actually may represent more than one type of protein subunit.

These data show that NEM, under the conditions used, reacts unspecifically with a variety of proteins in the inner membrane. Therefore, it became necessary to find a method for increasing the specificity of $[^{3}H]\text{NEM}$ for the phosphate transport system.

Protection against NEM Inhibition of Phosphate Transport by p-MB — In the search for an agent which would protect the phosphate transport system against inhibition by NEM, the most obvious choice for initial experiments was the substrate

![Fig. 1. Effect of NEM on passive swelling of mitochondria. Swelling media contained 5.0 mM Hepes, pH 7.4, 1.0 mM EGTA, and either NH$_4$Cl (125 mM), NH$_4$ acetate (125 mM), NH$_4$HCO$_3$ (125 mM), or NH$_4$Pi (100 mM). Mitochondria were incubated at 10 mg/ml for 2 min at $0^\circ$ in isolation medium either without NEM (A) or with NEM, 500 $\mu$M, 50 nmol/mg of protein (B). Aliquots of 0.1 ml were rapidly mixed with 0.9 ml of the above swelling mixtures, and passive swelling was monitored at 750 nm as described under "Methods."](http://www.jbc.org/)

![Fig. 2. Inhibition by NEM of the exchange of $^{32}$P$_i$ with Pi-loaded mitochondria. Mitochondria were suspended in 2.0 ml of isolation medium (See "Methods") at a protein concentration of 5 mg/ml. Concentrations of NEM were as indicated on the figure. KP$_i$, pH 7.4, was added to give a final concentration of 1.0 mM. After equilibration of added $P_i$, $^{32}$P$_i$ was added to rapidly stirred aliquots, and 0.2 ml was rapidly pipetted at different time intervals ranging from 1 to 30 s into vials containing 2.0 ml of isolation medium plus 0.1 mM p-MB. After assay, the samples were diluted to 8.0 ml with isolation medium and centrifuged. Procedures for measuring accurately the elapsed time, for counting the pellet fraction, and for calculating the initial rates of $P_i$ transport are referenced under "Methods."](http://www.jbc.org/)

| Fraction                        | Distribution of Protein | Distribution of $[^{3}H]\text{NEM}$ bound |
|---------------------------------|-------------------------|------------------------------------------|
| Mitochondria                    | 100%                    | 100 nmol/mg of protein                   |
| Outer membrane plus inter-      | 31%                     | 45 nmol/mg of protein                    |
| membrane space                  | 69%                     | 55 nmol/mg of protein                    |
| Mitoplast (inner membrane       | 41%                     | 48 nmol/mg of protein                    |
| plus matrix)                    | 20%                     | 7 nmol/mg of protein                     |

Table I

Distribution of protein and radioactivity in submitochondrial fractions of $[^{3}H]\text{NEM}$-labeled mitochondria

Mitochondria were suspended to a concentration of 5 mg/ml in isolation medium and were treated at $0^\circ$ with $[^{3}H]\text{NEM}$ (15.0 nmol/mg of protein) for 3 min. Dithiothreitol (0.83 mM) then was added to block further reaction, and after an additional incubation of 5 min at $0^\circ$, the labeled mitochondria were diluted to 2 mg/ml with isolation medium and centrifuged. The mitochondrial pellet was resuspended to 100 mg/ml. Subfractionation of the $[^{3}H]\text{NEM}$-labeled mitochondria was carried out exactly as described under "Methods."
phosphate. However, in agreement with the results of Debise and Durand (27), phosphate was found to have no effect on the inhibition of transport by NEM. For this reason, an alternative method of protection using the reversible sulfhydryl group inhibitor p-MB was investigated.

The phosphate swelling experiments summarized in Fig. 4 indicate that p-MB and NEM react with the same sulfhydryl group of the phosphate transport system. Fig. 4, A and B show that whereas inhibition of swelling by p-MB is reversed by thiol reagents such as dithiothreitol, NEM irreversibly inhibits swelling. As noted in Fig. 4C, p-MB added in amounts sufficient to inhibit swelling in ammonium phosphate protects against irreversible inhibition of transport by subsequently added NEM. This protective effect was observed with NEM concentrations as high as 200 nmol/mg of protein, or 10-fold higher than necessary to completely inhibit transport in untreated mitochondria.

It is important to note that although this experiment was performed using the passive swelling technique, the results were confirmed by direct measurement of the rates of transport using the $^{32}$P$_i$ exchange assay. Using this method, p-MB was able to protect 65 to 75% of phosphate transport activity against inhibition by 125 nmol of NEM/mg of protein.

This phenomenon of p-MB protection against NEM inhibition is potentially a useful tool for increasing the specificity of $[^3H]$NEM labeling of inner membrane proteins, as shown in Fig. 5. Treatment with a large excess of NEM after protecting $P_i$ transport activity with p-MB is likely to block irreversibly most of the accessible $-SH$ groups of the inner membrane. These mitochondria can be treated with dithiothreitol to restore transport and washed to remove the excess dithiothreitol. Phosphate transport then can be re inhibited by the further addition of $[^3H]$NEM. Some of the $-SH$ groups that are labeled directly with $[^3H]$NEM may be blocked with the unlabeled NEM added after p-MB protection and, therefore, will not be labeled by the addition of $[^3H]$NEM in the final step.

![Fig. 3. Gel electrophoretic analysis of $[^3H]$NEM-labeled inner membrane proteins. Labeling of mitochondria with $[^3H]$NEM and isolation of the inner membrane fraction were carried out essentially as described in Table I and under “Methods.” This inner membrane fraction was treated with sodium dodecyl sulfate, mixed with $[^14C]$labeled standard proteins, and analyzed on sodium dodecyl sulfate polyacrylamide gels as described under “Methods.” The migration distance of the standard proteins and the dye front are indicated by arrows. The standard proteins are from left to right: bovine albumin (BSA), catalase (CAT), glyceraldehyde-3-phosphate dehydrogenase (GPD), chymotrypsinogen A (CT), $\beta$-lactoglobulin ($\beta$LG), and cytochrome c (Cyt c). Molecular weights of these proteins are summarized under “Methods.” Recovery of radioactivity placed on the gels exceeded 90%.

![Fig. 4. A, reversal of p-MB inhibited phosphate transport by dithiothreitol. Where indicated, mitochondria (10 mg/ml) in isolation medium were treated with p-MB, 125 $\mu$M, 12.6 nmol/mg of protein, and dithiothreitol, 2.5 mM. These reagents were added separately in the sequences indicated with 2-min incubations at 0° following each addition. Ammonium phosphate swelling measurements were made on aliquots of these mixtures at 750 nm as described under “Methods.” B, irreversible inhibition of phosphate transport by NEM. Conditions are the same as in A with NEM present at 1.0 mM or 100 nmol/mg of protein. C, protection by p-MB of NEM inhibition of phosphate transport. Conditions are as noted above. The order of addition of p-MB, NEM, and dithiothreitol is as noted in the figure.

![Fig. 5. The effect of p-MB protection on the specificity of labeling of the phosphate transport system with $[^3H]$NEM. This diagram compares the labeling of the mitochondrial phosphate transport system and other membrane proteins with $[^3H]$NEM using two different procedures: direct labeling (upper reaction), and labeling after p-MB protection and blocking with NEM (lower scheme). In this figure, radioactive NEM is indicated by $\bullet$ and the phosphate transport system by $\bigcirc$. If phosphate transport is inhibited by direct addition of $[^3H]$NEM, a number of membrane proteins are labeled in addition to the NEM-binding component of the phosphate transport system. The p-MB protection procedure, however, provides a possible means of reducing the extent and increasing the specificity of labeling. This procedure consists of the following four steps: (a) Protection of the phosphate transport system—SH group with p-MB. (b) Blocking of accessible membrane—SH groups with excess “cold” NEM. (c) Removal of p-MB protecting group with dithiothreitol, followed by washing of the mitochondria to remove excess reagent. (d) Reinhibition of phosphate transport with $[^3H]$NEM.
Sensitivity of Phosphate Transport to NEM after p-MB Protection and Blocking of —SH Groups—Mitochondria were treated first with sufficient p-MB to inhibit phosphate transport and then with an excess of NEM. After restoration of transport with dithiothreitol and removal of excess sulfhydryl reagent by centrifugation and resuspension, the concentration dependence of reinhibition of transport by NEM was determined. As shown in Fig. 6, the concentration needed to produce half-maximal inhibition is reduced approximately 7-fold, from 10 nmol/mg in untreated mitochondrial to 1.5 nmol/mg. Thus, this treatment has sensitized phosphate transport to inhibition by NEM, because it has reduced the levels of accessible, reactive membrane —SH groups. Mitochondria treated in this manner are referred to below as “inhibitor-sensitized.”

Labeling of Inhibitor-sensitized Mitochondria with [3H]NEM—Mitochondria were treated with [3H]NEM after inhibitor sensitization, as described above, and the inner membrane fraction then was isolated. As noted in Table II, the inner membrane fraction contained less than 0.7 nmol of

![Fig. 6](http://www.jbc.org/)

Fig. 6. Effect of p-MB protection and blocking procedure on the concentration dependence of NEM inhibition of phosphate transport. Rates of phosphate transport were measured by the §P exchange assay in the presence of varying amounts of NEM, as described under “Methods.” The concentration dependence of NEM inhibition was measured for untreated mitochondria (O—O) or for mitochondria treated by the p-MB protection and NEM blocking procedure (Δ—Δ). This treatment was carried out as follows: mitochondria (5 mg/ml) were suspended in isolation medium and centrifuged at 0° with p-MB, 12.5 nmol/mg for 2 min; then NEM, 125 nmol/mg for 5 min; and finally an excess of dithiothreitol (0.5 mM) for 5 min. The mitochondria were diluted to about 2 mg/ml with isolation medium and centrifuged. The pellet fraction was suspended in isolation medium, re-centrifuged to remove any residual dithiothreitol, and then suspended to a concentration of 100 mg/ml for transport assays.

| Table II |

Distribution of protein and radioactivity in submitochondrial fractions of mitochondria labeled with [3H]NEM after inhibitor sensitization

Mitochondria were inhibitor-sensitized with p-MB as described in Fig. 6 and then were treated with [3H]NEM (2.4 nmol/mg of protein) under the same conditions as in Table I. After dithiothreitol addition and centrifugation, these labeled mitochondria were subfractionated as described under “Methods.”

| Fraction         | Distribution of [3H]NEM bound |
|------------------|-------------------------------|
|                  | Protein | [3H]NEM bound | pmol/mg of protein |
| Mitochondria     | 100     | 100            | 1.0                |
| Outer membrane plus intermembrane space | 28     | 46             | 1.9                |
| Mitoplast (inner membrane plus matrix) | 71     | 54             | 0.7                |
| Matrix           | 50      | 41             | 0.8                |
| Inner membrane   | 21      | 13             | 0.7                |

Table III

Gel electrophoretic analysis of inner membrane proteins isolated from mitochondria labeled with [3H]NEM after inhibitor sensitization with p-MB

The protein fractions correspond to the [3H]NEM-labeled peaks in Fig. 7 resolved by gel electrophoresis in sodium dodecyl sulfate. Apparent molecular weights were determined by comparison with standard curves of mobility versus log molecular weight using [14C]-acetyl standard proteins, as described in Fig. 7. Binding of [3H]NEM was estimated by comparison of the amount of radioactivity in each peak and the specific activity of the [3H]NEM used.

| Fraction | Molecular weight | [3H]NEM bound pmol/mg of protein |
|----------|-----------------|---------------------------------|
| I        | 95,000          | 40                              |
| II       | 75,000          | 30                              |
| III      | 92,000          | 80                              |
| IV       | 47,000          | 70                              |
| V        | 32,000          | 160                             |
membrane protein comprising the mitochondrial fraction, and of inner membrane (Table III) by 0.25, the fraction of inner membrane sulfhydryl groups unrelated to transmembrane phosphate transport as determined by direct measurement of binding of NEM in these studies is correlated with inhibition of most of the membrane sulfhydryl groups. Thus, under conditions where phosphate transport is inhibited by NEM at least 10 inner membrane proteins are labeled (Fig. 3).

In order to enhance the specificity of NEM for reaction with protein components of the phosphate transport system, p-MB, a reversible inhibitor of both protein sulfhydryl groups and the phosphate transport system was employed. When this agent was added initially to mitochondria to inhibit phosphate transport, unlabeled NEM could be added to block irreversibly most of the membrane sulfhydryl groups unrelabeled transport. Addition of dithiothreitol to remove p-MB and restore transport (Fig. 4), followed by the addition of labeled NEM, resulted in a 6-fold decrease in the membrane-bound NEM observed under unprotected conditions (Tables I and II) and the labeling of only five distinct polypeptide components (Fig. 7).

From these data it is possible to estimate both the maximum concentration of the NEM-sensitive protein component of the phosphate-hydroxyl exchange diffusion carrier and the turnover number of this process. Such estimates are possible because NEM specifically inhibits phosphate transport by blocking at least one essential sulfhydryl group and because binding of NEM in these studies is correlated with inhibition of phosphate transport as determined by direct measurement of initial rates of transport (11).

The maximum concentration of the NEM-sensitive protein component calculated from the amount of NEM bound by inner membrane proteins under the p-MB protection conditions noted above is 60 pmol/mg of mitochondrial protein. The value is obtained by multiplying 160 pmol of NEM bound/mg of inner membrane (Table III) by 0.25, the fraction of inner membrane protein comprising the mitochondrial fraction, and by 1.5 to correct for the fact that inhibitor-sensitized mitochondria retained approximately 67% of their original P_1 transport activity. Sixty picomoles per milligram of mitochondria is about 25% of the concentration of respiratory chain components such as cytochrome a (17) and approximately the same level as the estimated concentration of atractyloside binding sites of the adenine nucleotide transport system (28).

If it is assumed that there is a 1:1 correspondence between the NEM-reactive protein and the active site for phosphate transport, then for a V_max of 205 nmol x min^{-1} x mg^-1 at 0^\circ (11), a turnover number of approximately 3500 min^{-1} can be calculated. Similar calculations of the turnover number for succinate oxidation and adenine nucleotide transport give values of 110 and 175 min^{-1} at 0^\circ, respectively (17, 28, 29).

The unusually high catalytic activity of the phosphate transport system at this low temperature may relate to the mechanism of transport. A "gated pore" mechanism (30) involving relatively little protein movement would require a much lower activation energy than a "mobile carrier" mechanism (31, 32). Measurements of the temperature dependence of phosphate transport are being made to test this possibility.

The five polypeptides labeled with NEM are possible components of the transport system which catalyzes P_1/OH^- exchange in rat liver mitochondria. Although it seems unlikely that all of these components are parts of the P_1/OH^- carrier, it is not unreasonable to suggest that as many as two of these polypeptides may be associated with this process. Pertinent here are the recent results of Fonyo (33) which suggest that at least two sulfhydryl groups may be associated with the transport system catalyzing P_1/OH^- exchange.

These results, therefore, provide a basis for future progress toward isolation of the phosphate transport system. The [3H]NEM-labeled proteins can be used as markers for the isolation of the same proteins from untreated mitochondrial membranes, which then could be tested for activity in a reconstitution assay for phosphate transport. Experiments currently are being carried out toward this objective, with special attention being given to the 32,000 molecular weight component which contained 40% of the total NEM bound to the inner membrane.

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