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MITOCHONDRIAL PHOSPHATE TRANSPORT AND THE *N*-ETHYLMALEIMIDE BINDING PROTEINS OF THE INNER MEMBRANE

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Summary

Mitochondria have been prepared from the flight muscles of mature blowflies (*Sarcophaga bullata*). Phosphate transport by these mitochondria, determined by rates of passive swelling in ammonium phosphate, is sensitive to inhibition by *N*-ethylmaleimide. 20 nmol of *N*-ethylmaleimide/nmol cytochrome *a* inhibit the swelling by 90%. When the mitochondria are inhibited by *N*-[^{3}H]ethylmaleimide, then solubilized in dodecyl sulfate/mercaptoethanol at 100°C and then electrophoresed on dodecyl sulfate-polyacrylamide gels, many labeled protein bands can be detected, including a large labeled peak that has the same mobility as the tracking dye, bromophenol blue. Sonic submitochondrial particles that are prepared from the *N*-[^{3}H]ethylmaleimide-labeled mitochondria, solubilized, and electrophoresed on dodecyl sulfate-polyacrylamide gels, possess only seven major labeled protein bands with no radioactive peak at the tracking dye. These labeled proteins have molecular weights of 71, 68, 64, 45, 32, 30, and approx. $10 \cdot 10^3$. The nmol *N*-[^{3}H]-ethylmaleimide bound to each of these proteins per nmol cytochrome *a* are 0.15, 0.19, 0.35, 0.45, 0.87, 0.10, and 0.17, respectively, when the mitochondria are inhibited with 21.5 mol *N*-[^{3}H]ethylmaleimide/mol cytochrome *a* at 10 μM cytochrome *a*. Coty and Pedersen ((1975) J. Biol. Chem. 250, 3515—3521) sensitized rat liver mitochondria to *N*-[^{3}H]ethylmaleimide and identified five labeled proteins. Only the labeled $32 \cdot 10^3$ dalton and the $45 \cdot 10^3$ dalton proteins are common to both systems.

Introduction

Inorganic phosphate must be transported into the mitochondrial matrix in order to participate in oxidative phosphorylation. The phosphate can be

Abbreviations: Mops, 4-morpholinopropane sulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

transported into the matrix space by at least two different modes: (a) phosphate : hydroxyl exchange on the phosphate transport system [1] and (b) phosphate : dicarboxylate or phosphate : phosphate exchange on the dicarboxylate transport system [2]. In rat liver mitochondria the phosphate transport system is the major pathway for phosphate transport [3]. This probably is also true in heart mitochondria, since here the dicarboxylate transport system has been shown to have a lower activity than in liver mitochondria [4]. Mitochondria from the flight muscle of the blowfly appear to possess only the phosphate transport system [5,6] and represent thus a unique membrane for the study of phosphate transport.

The phosphate transport system is sensitive to SH reagents such as mersalyl and *N*-ethylmaleimide [7]. The dicarboxylate transport system, while it is sensitive to *n*-butylmalonate and mersalyl, is not sensitive to *N*-ethylmaleimide [2,8—12].

Attempts have been made to isolate a phosphate transport protein by taking advantage of the SH reagent sensitivity of the transport. A chloroform-soluble protein fraction has been isolated from rat liver mitochondria [13]. The phosphate binding of this protein is partially sensitive to *N*-ethylmaleimide and other SH reagents [13]. Coty and Pedersen [14] used a *p*-hydroxymercuribenzoate sensitization method and found only five *N*-[^{3}H]ethylmaleimide-labeled protein bands on dodecyl sulfate-polyacrylamide gels of a Lubrol-insoluble inner membrane fraction from rat liver mitochondria to which sufficient *N*-[^{3}H]ethylmaleimide had been added to inhibit its phosphate transport. Banerjee and coworkers [15] isolated a protein fraction from beef heart mitochondria which upon incorporation into liposomes catalyzes phosphate : hydroxyl and phosphate : phosphate exchange that is sensitive to *N*-ethylmaleimide and other SH reagents.

It is the purpose of the present communication to demonstrate that the highly specialized inner membrane of the insect flight muscle mitochondria has only few *N*-ethylmaleimide-reactive SH groups. Of these, the $32 \cdot 10^3$ or $45 \cdot 10^3$ molecular weight protein is most likely the *N*-ethylmaleimide binding protein of the mitochondrial phosphate transport system.

Materials and Methods

Mitochondria were prepared from the flight muscles of adult (9—14 days after the pupal stage) blowflies (*Sarcophaga bullata*) without the protease nagarse in the following manner. Thoraces of 40 flies were gently squeezed in 10 ml KCl medium (100 mM KCl/10 mM potassium Mops/0.2 mM EGTA, pH 7.2, 0°C) in a Teflon pestle homogenizing vessel, 30 ml KCl medium were added and the suspension was put through two layers of cheese-cloth; the filtrate was centrifuged at 100 × *g* for 5 min; the supernatant was centrifuged at 900 × *g* for 5 min. The pellet was taken up in 100 µl Mops medium (100 mM Mops/0.2 mM EGTA, adjusted to pH 7.2 with KOH, 4°C). Cytochrome *a* was determined as described [16].

Sonic submitochondrial particles were prepared according to the following method, carried out at 0—4°C: the mitochondria from 40 thoraces were suspended in 10 mM potassium phosphate/0.3 mM EDTA (pH 7.2) and cen-

trifuged at 3000 × *g* for 5 min; resuspended in 150 mM KCl and centrifuged at 1100 × *g* for 5 min; resuspended in 2 ml of 30 mM potassium phosphate/1 mM EDTA (pH 7.2). The suspension was sonicated with a Bronwill Biosonik IV sonifier at maximum power for twice 5 s with time between sonications for cooling. The suspension was centrifuged at 3000 × *g* for 10 min and the resulting supernatant for 30 min at 100 000 × *g*. The pellet was suspended in 70—100 μl of 0.25 M sucrose and dissolved in dodecyl sulfate/mercaptoethanol at 100°C as the mitochondria.

The swelling rate of the mitochondria was recorded with an Aminco-Chance spectrophotometer. *N*-Ethylmaleimide (Sigma Chemical Co.) was dissolved in Mops medium and added during vortexing at 4°C to 30 μl mitochondrial suspension (Mops medium) of a final concentration of 11.6 μM cytochrome *a*. After 2 min at 4°C, 10 μl 32 mM dithiothreitol in Mops medium were added during vortexing. After 1 min at 4°C, the suspension was rapidly mixed into 3 ml of 100 mM ammonium phosphate/5 mM ammonium-Mops/1 mM EGTA at pH 7.2 and 22°C. The increase in %*T* was recorded at 650—450 nm.

Mitochondria for labeling and electrophoresis were prepared identically to the swelling experiments. The pentane of the *N*-[^{3}H]ethylmaleimide solution (New England Nuclear) was allowed to evaporate at 22°C and Mops medium was immediately added. The *N*-[^{3}H]ethylmaleimide concentration was determined spectrophotometrically, using an $\epsilon_{mM}^{cm^{-1}} = 0.62$ at 305 nm [17] and was found not to change during 8 h at 0°C. The specific activity of the *N*-[^{3}H]-ethylmaleimide was determined by using [^{3}H]toluene as standard and was found to be within 10% of the value specified by New England Nuclear.

The *N*-ethylmaleimide reaction was quenched with dithiothreitol and the mitochondria pelleted in an Eppendorf microcentrifuge. The supernatant was removed with an aspirator. The pellet was taken up in 20 μl of 0.25 M sucrose/dithiothreitol (5 mg dithiothreitol/ml of 0.25 M sucrose) and rapidly injected into 160 μl of 1.5% dodecyl sulfate/3% mercaptoethanol at 100°C. The mixture was kept at 100°C for 3 min and then rapidly centrifuged to collect liquid that had condensed on the wall of the test tube. The final volume was determined for later corrections. 60 μl of sample buffer (0.5 M Tris · HCl/0.16% dodecyl sulfate, pH 6.8), 5 μl mercaptoethanol (Sigma), and 5 μl 0.1% bromophenol blue were added. 25 μl of this mixture were added to a few crystals of sucrose and after they were dissolved, the whole solution was transferred to the dodecyl sulfate-polyacrylamide gel.

The dodecyl-polyacrylamide gels were prepared with minor modifications according to Laemmli [18]. The gel solution (15 ml acrylamide (22.2%)/*N*,*N*′-methylene-bis-acrylamide (0.6%); 6.7 ml 1.5 M Tris · HCl/0.16% dodecyl sulfate, pH 8.8; 4.3 ml H_2O; 0.55 ml $(NH_4)_2S_2O_8$ (1.7 mg/ml) was deaerated with an aspirator for 2.5 min and 13.5 μl *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were added. The solution was layered with water-saturated and argon-deoxygenated isobutanol, using a 1-μl disposable Drummond pipette [19]. After 4 h at 22°C only those gels with flat surfaces were used. The isobutanol and the unpolymerized gel solution were aspirated and the deaerated stacking gel solution was added (3 ml sample buffer, 7 ml water, 2.7 ml 22.2% acrylamide/0.6% *N*,*N*′-methylene-bis-acrylamide, 0.25 ml $(NH_4)_2S_2O_8$ (3.3 mg/ml), 5 μl *N*,*N*,*N*′,*N*′-tetramethylethylenediamine) and layered with iso-

butanol. Before sample application, the isobutanol was removed with an aspirator.

Electrophoresis was carried out at 22°C with a Hoefer electrophoresis cell. The running buffer was 25 mM Tris base, 190 mM glycine, 0.04% dodecyl sulfate. The upper reservoir was enlarged with 4 l running buffer by pumping buffer out of the upper reservoir into the 4-l reservoir and letting gravity equilibrate the two reservoirs during the electrophoresis run. The gels were run at 0.25 mA/gel until the sample had entered the gel. Then the constant current was raised to 1.5 mA per gel. After 7 h the bromophenol blue reached the bottom of the gel and after another 10 h, the $25 \cdot 10^3$ dalton proteins had reached the bottom. At the end of the run, the gels, after removal from the glass tubes, were either frozen in liquid nitrogen and stored in the freezer, or they were stained with Coomassie Brilliant Blue and then destained. The gels were scanned with a Gilford spectrophotometer and then sliced with a Hoefer gel slicer. The slices were identified with the stained protein bands and were digested according to Malim and Lofberg [20]. A scintillation cocktail [20] was chosen to eliminate chemiluminescence. The counting efficiency was determined by adding a fixed amount of $C_6{}^3H_5CH_3$ to vials with digested slices of low radioactivity.

The recovery of *N*-[^{3}H]ethylmaleimide activity from the labeled gels was higher than 80%. It was determined in the following manner. *N*-[^{3}H]ethylmaleimide-labeled submitochondrial particles were solubilized and dialyzed at 22°C for 4 h against 1.5% dodecyl sulfate/3% mercaptoethanol. The dialyzed sample was counted and converted to dpm. Samples of dialyzed ^{3}H-labeled submitochondrial particles were placed on gels and electrophoresed. The gels were sliced and the sum of the counts of all the slices (less background counts) were added and converted to dpm.

Standard proteins were heated in dodecyl sulfate/mercaptoethanol like the mitochondria and then dialyzed against dodecyl sulfate/mercaptoethanol before use. The proteins used were: carbonic anhydrase (Sigma C7500, $M_r = 31 \cdot 10^3$), glyceraldehyde-3-phosphate dehydrogenase (Sigma G5126, $M_r = 36 \cdot 10^3$), ovalbumin (Sigma A5503, $M_r = 45 \cdot 10^3$), L-glutamic dehydrogenase (Sigma G2626, $M_r = 53 \cdot 10^3$), and bovine serum albumin (Sigma A6003, $M_r = 67 \cdot 10^3$).

Results

Inhibition of mitochondrial swelling

The flight muscle mitochondria that are isolated in KCl medium are suspended in Mops medium to minimize swelling during storage at 4°C. The concentration of the mitochondria is adjusted so that, after the addition of *N*-ethylmaleimide, with a final volume of 30 µl, it is 11.5 µM cytochrome *a*. After the addition of dithiothreitol (see Materials and Methods) the suspension is rapidly added to the ammonium phosphate in an optical cuvette. Rapid mixing is achieved by plunging a flattened glass stirrer, onto which the suspension has been added as a drop, into the ammonium phosphate solution. The light scattering changes are recorded and the initial slope determined. Fig. 1 shows that the initial swelling rate is inhibited about 90% when the

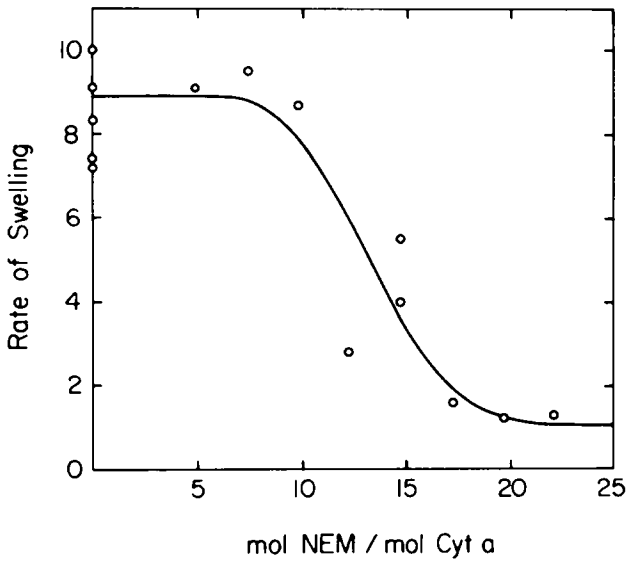

Fig. 1. Inhibition of mitochondrial swelling in ammonium phosphate by *N*-ethylmaleimide (NEM). Swelling experiments were carried out as described in Materials and Methods.

mitochondria are exposed to 20 mol *N*-ethylmaleimide/mol cytochrome *a* for 2 min at 0—4°C.

The 2 min incubation time was decided upon after an analysis of the amount of *N*-[^{3}H]ethylmaleimide bound to some of the membrane proteins. A 2 min incubation at 0—4°C yields 70—80% of the label incorporated by an 8 min incubation by the major proteins. Thus, since the labeling reaction does continue beyond 2 min, it is important to stop the reaction with dithiothreitol.

Electrophoresis of N-[^{3}H]ethylmaleimide-labeled mitochondria

Fig. 2a shows a dodecyl sulfate-polyacrylamide gel, stained with Coomassie Blue G-250, of solubilized mitochondria. Many protein bands are present. The dye front was marked in the unstained gels with a needle puncture and thus appears in the scan of the stained gel as a spike. When the destained gel (Fig. 2a) is sliced and the radioactivity determined, Fig. 2b is obtained. The majority of radioactive peaks appears in the first 70 slices. The peak at about slice 130 corresponds exactly to the position marked with a solid triangle in Fig. 2a. This is the $10 \cdot 10^3$ dalton protein of the inner membrane (see also Fig. 4b). When the gel is sliced before staining (Fig. 2c) the same radioactive pattern as in Fig. 2b is obtained, except for a large radioactive peak near the tracking dye. This radioactive peak will be discussed in more detail later.

Fig. 3a shows a gel from which the low molecular weight proteins have been run off. The remaining high molecular weight protein bands are now apart far enough to be readily separated by the gel slicer. Fig. 3b shows the radioactive bands that can be identified in the gel of the high molecular weight proteins. While most of the bands are well separated, some are still close superpositions of several labeled proteins.

Fig. 3a identifies with solid triangles the stained protein bands that contain

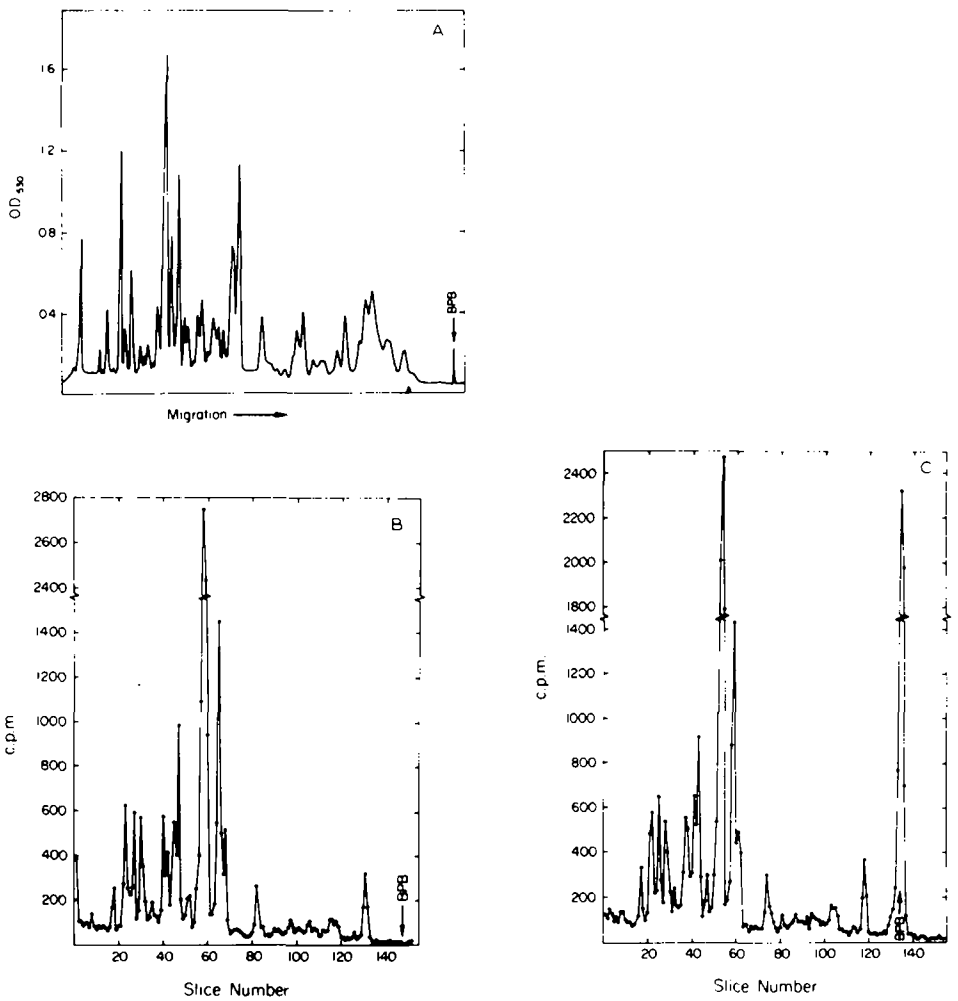

Fig. 2. (A) Stained dodecyl sulfate-polyacrylamide gel of labeled mitochondria. BPB is the tracking dye bromophenol blue. For details see Materials and Methods. (B) Radioactivity of the slices of the stained dodecyl sulfate-polyacrylamide gel of (A). (C) Radioactivity of the slices of an unstained dodecyl sulfate-polyacrylamide gel of *N*-[^{3}H]ethylmaleimide-labeled mitochondria.

the *N*-[^{3}H]ethylmaleimide and that are part of the inner membrane (see end of Results and Fig. 5b). The two (?) highest molecular weight proteins (solid triangles) can often not be separated and have been considered as one entity ($71 \cdot 10^3$ daltons).

Analysis of labeled submitochondrial particles

N-Ethylmaleimide reacts with SH groups of matrix proteins [14] and proteins of the outer membrane. It also reacts with proteins that are only loosely bound to the inner membrane and thus likely without a role in phosphate transport [21]. In order to identify those proteins that are intrinsic to the inner mitochondrial membrane and thus could be phosphate transport

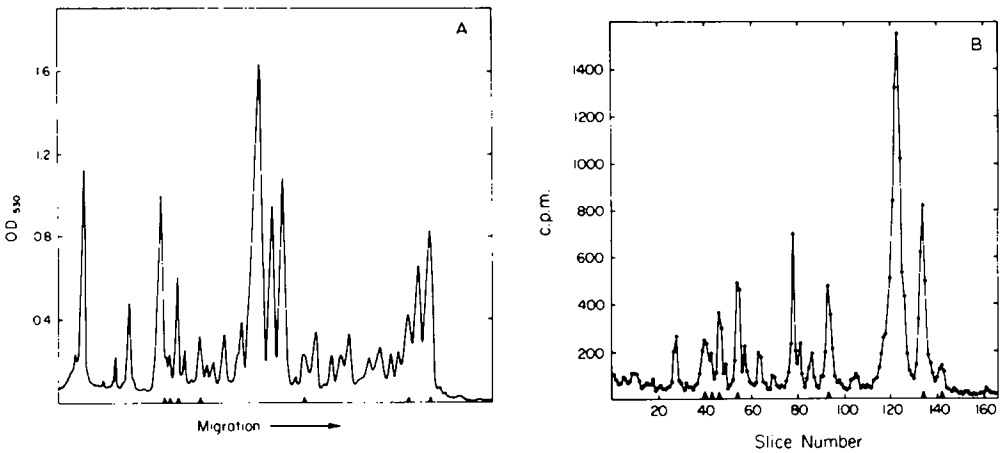

Fig. 3. (A) Stained dodecyl sulfate-polyacrylamide gel of Fig. 2A with the low molecular weight proteins run off the gel. Solid triangles are the protein peaks of the labeled proteins intrinsic to the inner membrane. (B) Radioactivity of the slices of the stained gel of (A). Solid triangles as in (A).

proteins, we prepared sonic submitochondrial particles from the *N*-[^{3}H]ethylmaleimide-labeled mitochondria. If we place as much submitochondrial cytochrome *a* on the gels as mitochondrial cytochrome *a* had been placed on the earlier gels, the same radioactive pattern should result, except for those peaks that are not intrinsic to the inner membrane.

We determined the cytochrome *a* concentration of the submitochondrial particles. The resulting gels contained fewer but more heavily stained and more highly ^{3}H-labeled protein bands. A more careful determination of the cytochrome *a* concentration of the sonic submitochondrial particles showed that about 40% of the cytochrome *a* is only very slowly reduced. If the slowly reduced portion is included in the concentration, the proteins of the two types of gels correlate quite well.

Fig. 4a is a scan of a stained SDS-polyacrylamide gel of submitochondrial particles. It is quite clear from a comparison with Fig. 2a that many proteins are missing or are significantly reduced in amount. It should be noted that generally the protein bands of the submitochondrial particles gels stained darker than those of the mitochondria, based on the amount of cytochrome *a* added, even if the slowly reduced oxidase is taken into account. This suggests that some of the oxidase becomes extremely slowly reduced.

Fig. 4b is the radioactivity of the slices of the stained submitochondrial particles gel. Many of the mitochondrial radioactive bands are missing. The radioactivity of the unstained submitochondrial particles gel (Fig. 4c) is the same except for a remaining trace of the large radioactive band near the tracking dye which is so obvious in the mitochondrial gel (Fig. 2c). The most dramatic differences between Figs. 2c and 4c are the band at the tracking dye and that at about slice 52.

When the low molecular weight proteins are run off the gel (Fig. 5a), clearly many proteins are missing with respect to the mitochondrial gel (Fig. 3a). The radioactive peaks of this gel (Fig. 5b) consist of only six or seven protein

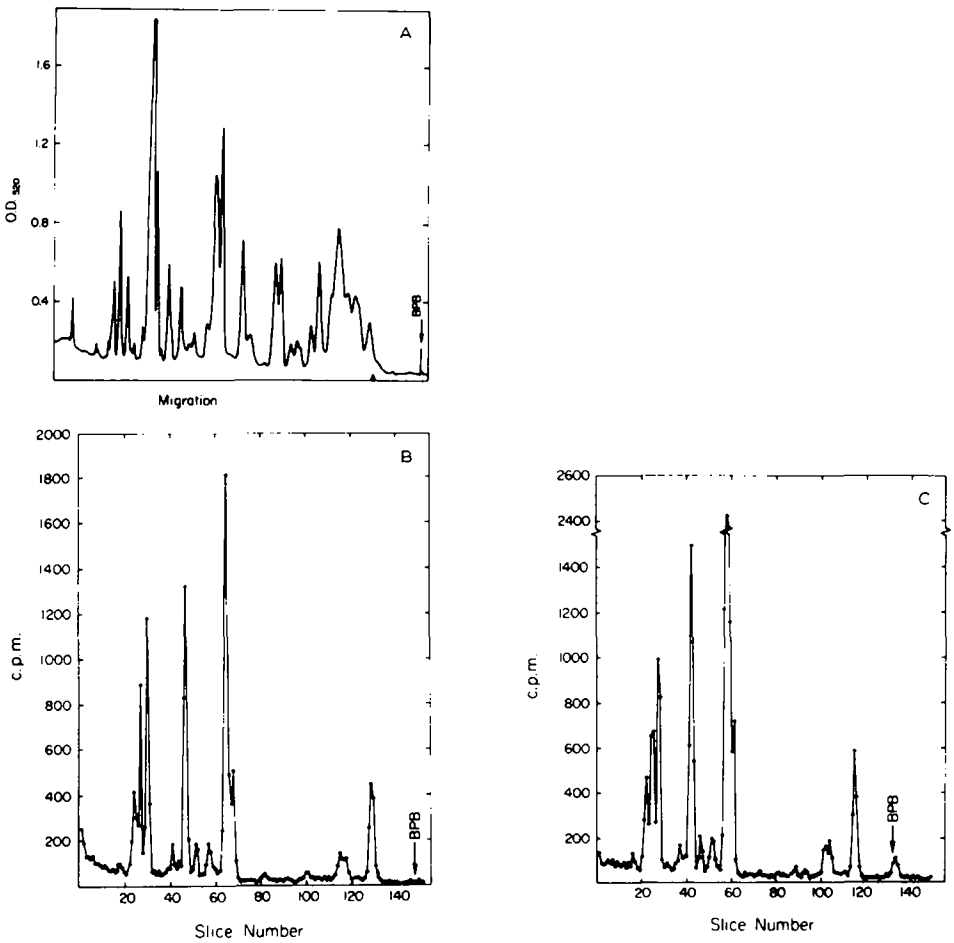

Fig. 4. (A) Stained dodecyl sulfate-polyacrylamide gel of sonic submitochondrial particles. The solid triangle identifies the position of the $10 \cdot 10^3$ dalton labeled protein of the inner membrane. (B) Radioactivity of the stained and sliced gel of (A). (C) Radioactivity of the sliced and unstained gel of (A).

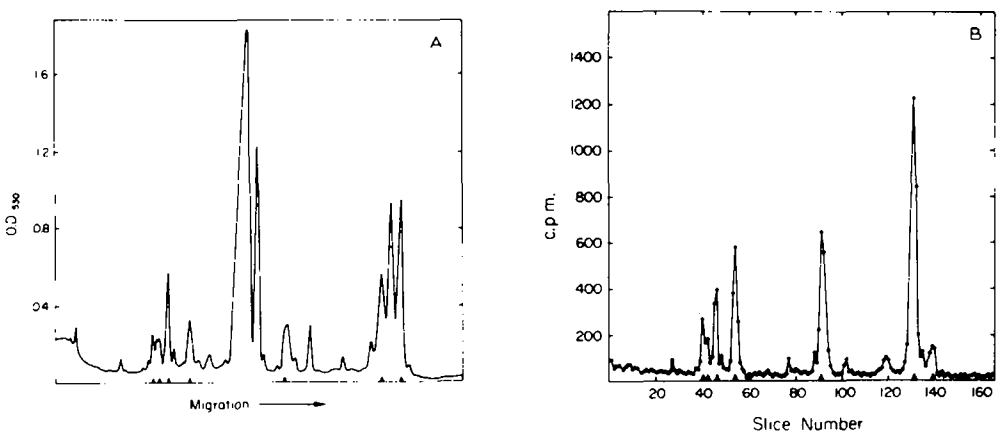

Fig. 5. (A) The stained dodecyl sulfate-polyacrylamide gel of *N*-[^{3}H]ethylmaleimide-labeled submitochondrial particles with the low molecular weight proteins run off the gel. (B) The radioactivity of the slices of the stained gel of (A).

TABLE I

N-ETHYLMALEIMIDE BINDING PROTEINS OF FLIGHT MUSCLE AND RAT LIVER MITOCHONDRIAL INNER MEMBRANES

Flight muscle *		Rat liver **	
Molecular weight	pmol *N*-[^{3}H]ethylmaleimide bound per nmol cytochrome *a*	Molecular weight	pmol *N*-[^{3}H]ethylmaleimide bound per nmol cytochrome *a*
—	—	95 000	70
—	—	76 000	50
71 000	150	—	—
68 000	190	—	—
64 000	350	—	—
—	—	52 000	140
45 000	450	47 000	120
32 000	870	32 000	270
30 000	100	—	—
10 000	170	—	—

* These values were obtained from the sonic submitochondrial particles of the flight muscle mitochondria.

** These values were calculated from the data of Chan and coworkers [22] and Coty and Pedersen [14] using the Lubrol-insoluble fraction of the inner membrane.

bands. The solid triangles identify the major labeled proteins that are present in the stained mitochondrial and submitochondrial particles gels. Many of the smaller peaks are probably remaining traces of mitochondrial proteins that are not intrinsic to the inner membrane.

Molecular weight estimations

The molecular weights of the major labeled inner membrane proteins (Fig. 5b) were determined by running standard proteins on the same gel with solubilized mitochondria. Since the radioactive peaks had been accurately identified with stained proteins of the mitochondrial gels (Fig. 3a), their position was accurately identifiable with relation to the standard proteins on the same gel. Table I shows the calculated molecular weights of the major labeled proteins with a comparison to those identified by Coty and Pedersen [14] with their sensitization method.

Discussion

This investigation presents a new membrane system for characterizing the mitochondrial phosphate carrier. The original expectation was that the high specialization [5] of the flight muscle mitochondrial membrane would minimize the number of *N*-ethylmaleimide-reactive SH groups not associated with the phosphate transport system and that the high metabolic capacity of these mitochondria would imply a very high concentration of phosphate transport systems.

Rhodin and Racker [21] have shown that SH reagent-sensitive phosphate transport can be observed in sonic submitochondrial particles from beef heart mitochondria. We prepared sonic submitochondrial particles from *N*-[^{3}H]-ethylmaleimide-labeled flight muscle mitochondria. Figs. 4b and 5b together

suggest that indeed only few *N*-ethylmaleimide-reactive SH groups exist in the inner membrane of the flight muscle mitochondria. On the other hand, comparing our present results with those of Coty and Pedersen [14] (see Table I), it becomes clear that almost all labeled proteins occur at a higher concentration per cytochrome *a* in the flight muscle submitochondrial particles than in the Lubrol-insoluble inner membrane fraction of rat liver mitochondria. There are two possible explanations for the lower labeled protein concentration in the rat liver mitochondria, besides the one that there are fewer phosphate transport systems in the rat liver mitochondria: (a) while the Lubrol-insoluble rat liver mitochondrial fraction has been characterized as containing all of the cytochrome *a* [22], no evidence exists as to how much of the *N*-[^{3}H]ethylmaleimide-labeled intrinsic membrane proteins are lost; (b) we have preliminary results that suggest that our labeled intrinsic membrane proteins saturate with *N*-[^{3}H]ethylmaleimide in a manner proportional to the amount of inhibitor added instead of following the lag in inhibition (Fig. 1). This latter result would suggest that sensitization with *p*-hydroxymercuribenzoate does eliminate phosphate carrier capacity that normally is not observed (see ref. 23) and gives a lower estimate of the amount of inhibitor associated with the phosphate transport system.

The $32 \cdot 10^3$ and $45 \cdot 10^3$ dalton proteins are the only bands that appear in the pattern seen with the Lubrol-insoluble fraction of rat liver mitochondria [14] and flight muscle submitochondrial particles (Table I). Coty and Pedersen [14] used the phosphate buffer system in their dodecyl sulfate-polyacrylamide gels while we used the Tris/glycine buffer system. The different buffers could possibly affect the mobility of an intrinsic membrane protein and one should be more generous in considering overlaps in molecular weight comparisons. The $30 \cdot 10^3$ dalton protein of the flight muscle submitochondrial particles has less inhibitor bound than the $32 \cdot 10^3$ dalton protein of the rat liver mitochondrial protein and is thus an unlikely candidate. The only other labeled protein of the flight muscle submitochondrial particles that is close in molecular weight to a labeled protein of the rat liver mitochondrial fraction is the $71 \cdot 10^3$ dalton protein. However, following the arguments of Coty and Pedersen [14], the concentration of this protein is too low, especially once our preliminary experiments suggest a higher phosphate transport activity in the flight muscle mitochondria and since it appears that the $71 \cdot 10^3$ dalton protein band is made up of at least two different proteins (Fig. 5b).

The $45 \cdot 10^3$ dalton band represents 1.5% of the total submitochondrial particle protein (determined by weighing the paper of the graph of the Coomassie Blue peak) and the $32 \cdot 10^3$ dalton band represents 4% of the total submitochondrial particle protein if one assumes that the intensity of Coomassie Brilliant Blue G-250 stain of the protein bands is proportional to the amount of protein [24]. On the other hand, since the cytochrome *a* concentration per mg of protein in the submitochondrial particle is about 0.5 nmol/mg protein, about 0.44 nmol *N*-[^{3}H]ethylmaleimide are bound to the $32 \cdot 10^3$ dalton protein and 0.22 nmol to the $45 \cdot 10^3$ dalton protein per mg of submitochondrial particle protein. This implies that the labeled $32 \cdot 10^3$ dalton protein represents 1.4% and the labeled $45 \cdot 10^3$ dalton protein represents 1.1% of the total submitochondrial particle protein. Comparing this with the earlier calcu-

lations it appears that only half of the $32 \cdot 10^3$ dalton protein subunits are labeled with *N*-[^{3}H]ethylmaleimide, while all of the $45 \cdot 10^3$ dalton proteins are labeled. It should be of interest to find out whether the $32 \cdot 10^3$ dalton protein, as a dimer, is the phosphate carrier protein with inhibition essentially complete with only one subunit inhibited by *N*-ethylmaleimide. This would be contrary to the conclusion of Fonyo [23], who presented evidence that two SH groups are involved in the phosphate transport with either one alone being sufficient for transport to occur.

It is not clear what the labeled compound is that has the same mobility as the bromophenol blue tracking dye (Fig. 2c). It could be *N*-[^{3}H]ethylmaleimide-labeled lipoic acid or glutathione. Both occur in mitochondria and are expected to react with *N*-ethylmaleimide [25,26]. It is quite unlikely that the tritium peak is due to digested labeled proteins for the following reasons: (a) the mitochondria or submitochondrial particles are injected directly into 100°C dodecyl sulfate/mercaptoethanol, (b) most labeled proteins are of molecular weight larger than $30 \cdot 10^3$ with almost no radioactivity between the tracking dye and these high molecular weight proteins, and (c) submitochondrial particles posses the same protein bands with almost no tritium label at the position of the tracking dye (Fig. 4c). This last observation in view of the results of Rhodin and Racker [21], make it also unlikely that the labeled species at the tracking dye has a role in the phosphate transport. A radioactive peak at the tracking dye was not observed by Coty and Pedersen [14], since they analyzed only the Lubrol-insoluble inner membrane fraction of the rat liver mitochondria and not the proteins of the whole mitochondria as we have done.

Experiments that are being carried out now are expected to extend the present results and to identify one of the labeled protein peaks more closely with the phosphate transport. The isolation of a reconstitutively active phosphate carrier protein should be facilitated possibly utilizing the methods developed by Banerjee and coworkers [15] for the isolation of a phosphate transport-catalyzing protein fraction from beef heart mitochondria, or those developed by Kramer and coworkers [27] for the isolation of the highly purified and undenatured mitochondrial ATP-ADP carrier protein.

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References

1 Chappell, J.B. and Crofts, A.R. (1965) Biochem. J. 95, 393—402
2 Chappell, J.B. and Haarhoff, K.N. (1967) in Biochemistry of Mitochondria (Slater, E.C., Kaniuga, Z. and Wojtczak, L., eds.), pp. 75—92, Academic Press, London
3 Coty, W.A. and Pedersen, P.L. (1974) J. Biol. Chem. 249, 2593—2598
4 Scott, K.M., Jurkowitz, M. and Brierley, G.P. (1972) Arch. Biochem. Biophys. 153, 682—694
5 van den Bergh, S.G. and Slater, E.C. (1962) Biochem. J. 82, 363—371

6 Wohlrab, H. (1976) Fed. Proc. 35, 1601
7 Meijer, A.J., Groot, G.S.P. and Tager, J.M. (1970) FEBS Lett. 8, 41—44
8 Chappell, J.B. and Crofts, A.R. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., eds.), Vol. 7, pp. 293—314, BBA Library, American Elsevier Publ. Co., New York
9 Papa, S., Lofrumento, N.E., Loglisci, M. and Quagliariello, E. (1969) Biochim. Biophys. Acta 189, 311—314
10 Robinson, B.H. and Williams, G.R. (1970) Biochim. Biophys. Acta 216, 63—70
11 Hoek, J.B., Lofrumento, N.E., Meijer, A.J. and Tager, J.M. (1971) Biochim. Biophys. Acta 226, 297—308
12 Palmieri, F., Prezioso, G., Quagliariello, E. and Klingenberg, M. (1971) Eur. J. Biochem. 22, 66—74
13 Kadenbach, B. and Hadvary, P. (1973) Eur. J. Biochem. 39, 21—26
14 Coty, W.A. and Pedersen, P.L. (1975) J. Biol. Chem. 250, 3515—3521
15 Banerjee, R.K., Shertzer, H.G., Kanner, B.I. and Racker, E. (1977) Biochem. Biophys. Res. Commun. 75, 772—778
16 Wohlrab, H. (1974) Biochemistry 13, 4014—4018
17 Riordan, J.F. and Vallee, B.L. (1967) Methods Enzymol. 11, 541—548
18 Laemmli, U.K. (1970) Nature 227, 680—685
19 Neville, D.M. (1971) J. Biol. Chem. 246, 6328—6334
20 Malim, D.T. and Lofberg, R.T. (1966) Anal. Biochem. 16, 500—509
21 Rhodin, T.R. and Racker, E. (1974) Biochem. Biophys. Res. Commun. 61, 1207—1212
22 Chan, T.L., Greenawalt, J.W. and Pedersen, P.L. (1970) J. Cell, Biol. 45, 291—305
23 Fonyo, A. (1974) Biochem. Biophys. Res. Commun. 57, 1069—1073
24 Van Kley, H. and Hale, S.M. (1977) Anal. Biochem. 81, 485—487
25 Cam, K., Partis, M.D. and Griffiths, D.E. (1977) Biochem. J. 166, 593—602
26 Gaudemer, Y. and Latruffe, N. (1975) FEBS Lett. 54, 30—34
27 Kramer, R., Aquila, H. and Klingenberg, M. (1977) Biochemistry 16, 4949—4953