Eosin-5-maleimide is impermeable to the inner mitochondrial membrane, exhibiting essentially no reactivity with matrix glutathione or with β-hydroxybutyrate, but is located on the matrix surface of the inner membrane.

In intact mitochondria, eosin-5-maleimide is unreactive with the ADP/ATP antiporter even under conditions which promote maximal labeling by N-[^3H]ethylmaleimide (i.e., ADP present). However, eosin-5-maleimide readily labels the ADP/ATP antiporter in "inverted" inner membrane vesicles even in the presence of N-ethylmaleimide. Labeling is prevented if the vesicles are prepared from mitochondria pretreated with carboxyatractyloside.

In contrast to the ADP/ATP antiporter, essential sulfhydryl groups of the Pi/H⁺ symporter are accessible to eosin-5-maleimide in intact mitochondria with optimal inhibition of phosphate transport being observed at 25 °C. Eosin-5-maleimide also prevents labeling of the P/H⁺ symporter by N-[^3H]ethylmaleimide.

These results show that essential sulfhydryl groups of the ADP/ATP antiporter and the P/H⁺ symporter differ in reactivity and locations in functionally intact mitochondria. With respect to eosin-5-maleimide, sulfhydryl groups of the ADP/ATP carrier occur in two distinct classes, both of which are inaccessible in intact mitochondria. Only one class, depending on conditions, can be exposed in submitochondrial particles. In contrast, sulfhydryl group(s) of the P/H⁺ symporter behave as a single reactive class which is readily accessible in mitochondria at 25 °C.

The ADP/ATP antiporter and P/H⁺ symporter systems are essential components of the mitochondrial phosphorylating assembly. They facilitate the transport of ADP and inorganic phosphate into mitochondria and thus enable ADP to become phosphorylated (1–3). These two transport systems are clearly the most studied of the mitochondrial carriers. Both have been extensively characterized (1–7) and successfully isolated in a reconstitutively active state (8–12).

Although the ADP/ATP antiporter and P/H⁺ symporter are different proteins (9, 11, 13), they possess several similar properties. Both are hydrophobic, integral (8, 11) membrane proteins consisting of subunits with similar molecular weights. The subunit monomer of the ADP/ATP antiporter is close to 30,000 (9, 14), with two monomers in dimeric form constituting the functional carrier unit (15). The subunit of the P/H⁺ symporter has a molecular weight somewhat higher, 35,000–35,000 (11–13, 16, 17), and a dimeric functional unit has been suggested (13). Both carrier systems can be solubilized by the non-ionic detergent Triton X-100, and in both cases, the major step of purification is adsorption chromatography on hydroxylapatite (8, 9, 11).

An important feature of the ADP/ATP antiporter and P/H⁺ symporter is that both are sulphydryl-containing proteins with one or more of these sulphydryl groups being required for function. The P/H⁺ symporter was one of the first carriers proteins found to be highly sensitive to chemical modification of sulfhydryl groups (18, 19). Phosphate transport can be inhibited by a number of sulphydryl group reagents, e.g., mercurials, disulfides, and maleimides (4). Their inhibitory effect is relatively specific since sulfhydryl groups of the P/H⁺ symporter represent perhaps the most reactive class of sulfhydryl groups in intact mitochondria (4, 20). Although the total number of sulphydryl groups associated with the P/H⁺ symporter is still unknown, two types have been proposed (21–23). It is suggested that these reactive sulphydryl groups may be localized near the outer surface of the inner membrane in intact mitochondria (21–23).

The ADP/ATP antiporter was originally thought to be insensitive to sulphydryl-reactive reagents. Sensitivity, however, can be demonstrated if mitochondria are preincubated with a low concentration of either ADP or ATP (24–26). Under these conditions, the sulphydryl groups are unmasked and become reactive toward N-ethylmaleimide. As a result, the function of the carrier is inhibited (24–26). The location of these sulphydryl groups is unclear, but they do not seem to change orientation from one side of the membrane to the other (27).

In the characterization of protein sulphydryl groups, maleimides have proven to be especially useful. Because the reaction of maleimides with sulphydryl groups of proteins is not readily reversed, proteins containing sulphydryl groups can be radioactively labeled and monitored by gel electrophoresis. Unfortunately, the most commonly used radioactive maleimide, N-ethylmaleimide, is highly permeable (4). Maleimide analogs of low permeability, like carboxylated maleimides (22) or N-acetyl-4-sulfamoylphenylmaleimide (28), are either not available in radioactive form, or they are not completely impermeable. However, another maleimide analog, eosin-5-maleimide, is fluorescent, relatively large in size, and bears several charged groups. Hence, a limited permeability might be expected. Significantly, this compound has been...
reported recently to react readily with the ADP/ATP antiporter in submitochondrial particles with the protein-bound eosin-5-maleimide being detected easily by fluorography (29).

With the above thoughts in mind, it was of interest to establish whether eosin-5-maleimide was, in fact, impermeable to the mitochondrial inner membrane and, if so, whether the use of this probe could provide insight into the relative topographies of the P/\text{H}^+ symporter and ADP/ATP antiporter in intact mitochondria.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following chemicals were purchased from the indicated sources: NAD*, NADPH, ADP, d-\((+)-\)mannitol, d-\(\beta\)-hydroxybutyric acid, N-ethylmaleimide, mersalyl, DTNB, p-chloromercuribenzoic acid, N-ethylmaleimide, acrylamide, and molecular weight standards for SDS gel electrophoresis from Bio-Rad; N,N,N,N-tetramethylethylenediamine from Eastman; 2-methyl-2H-tetrazole from Schwarz/Mann; eosin-5-maleimide from Molecular Probes; 2,3-diphenyloxazole from Mallinkrodt; X-OMAT R films from Kodak; N-[3H]ethylmaleimide from New England Nuclear.

**Methods**

**Rat Liver Mitochondria**—Rat liver mitochondria were prepared according to the procedure of Bustamante et al. (30) in H medium containing 220 mM d-\(\beta\)-mannitol, 70 mM sucrose, 0.5 mg of bovine albumin/mL, 2.0 mM HEPES, pH 7.4 (KOH). Mitochondria prepared in this manner routinely exhibited acceptor ratios (6.5–7.5) with \(\beta\)-hydroxybutyrate as substrate. For direct use in inhibitor studies, freshly isolated mitochondria were suspended in H medium without bovine serum albumin.

**Mitoplasts and Membrane Vesicles**—Rat liver mitoplasts were prepared according to the procedure of Schnaitman and Greenawalt (31). Rat liver inner membrane vesicles were prepared according to the procedure of Wehrle et al. (32).

**Bovine Heart Mitochondria**—Bovine heart mitochondria were prepared according to the procedure of Smith (33) in STE medium containing 250 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4 (HCl). Such mitochondria routinely exhibited acceptor control ratios of 4.2–5.3 with \(\beta\)-hydroxybutyrate as substrate.

**Submitochondrial Particles**—Bovine heart submitochondrial particles were prepared according to the procedure of Hansen and Smith (34).

Glutathione was measured according to Tietze (35). The assay contained: 0.1 M NaPi, 5 mM EDTA, 0.6 mM DTNB, 10 \(\mu\)g of glutathione reductase/mL, 0.2 M NADPH, and an aliquot of mitochondrial extract. The change in absorbance at 412 nm was measured for 4 min after the reaction was initiated by NADPH and the \(\Delta A\) control (without extract) was subtracted. The \(\Delta A\) versus glutathione concentration plot was linear up to 0.4 nmol of glutathione.

\(\beta\)-Hydroxybutyrate Dehydrogenase Activity—This activity was assayed polarographically in 1 ml of H medium (without bovine albumin), supplemented with 10 mM \(\beta\)-hydroxybutyrate, 2.5 mM KPi, 1 \(\mu\)M FCCP, 1 mg of mitochondrial or submitochondrial particle protein, and, in the case of submitochondrial particles, 75 mM NAD*.

Incubations with Eosin-5-maleimide—Incubations of mitochondria, submitochondrial particles, or mitoplasts with eosin-5-maleimide were performed routinely at 0°C in H medium without bovine albumin (rat liver) or in STE medium (bovine heart) at a protein concentration of 2 mg protein/mL with 150 \(\mu\)M eosin-5-maleimide for 20 min in the dark. (Eosin-5-maleimide was dissolved in dimethylsulfoxide to give a 10–15 mM stock solution.) The reaction was terminated by addition of sufficient 2 M dithiothreitol to give a final concentration of 50 mM. After an additional 10 min the labeled membranes (3–4 mg) were washed twice with 10–12 ml of H or STE medium.

1 The abbreviations used are: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

**Results**

**Eosin-5-maleimide Is Impermeable to the Mitochondrial Inner Membrane**—To determine whether or not eosin-5-maleimide penetrates the inner mitochondrial membrane, two different methods were used, both of which were employed previously to measure the permeability of sulfhydryl reagents (28, 32). The first method determines the ability of the compound to react with mitochondrial glutathione which is localized exclusively in the mitochondrial matrix (28, 40), whereas the second method is based on the sensitivity of \(\beta\)-hydroxybutyrate dehydrogenase to sulfhydryl-reactive reagents (32). Since the dehydrogenase is localized on the matrix side of the inner membrane, only permeable sulfhydryl reagents are capable of inhibiting the activity in intact mitochondria.

Data presented in Table I and Fig. 1 clearly show that, unlike N-ethylmaleimide, which is readily permeable to the inner mitochondrial membrane, the fluorescent analog, eosin-5-maleimide, is highly impermeable. Thus, a 10–20-min incubation of freshly isolated rat liver mitochondria with 300 \(\mu\)M N-ethylmaleimide at 0°C reduces the content of glutathione completely, whereas the same concentration of eosin-5-maleimide is without effect, i.e., the amount of glutathione recovered was identical with the untreated control mitochondria (Table I).

As shown in Fig. 1B, eosin-5-maleimide is as effective an inhibitor of \(\beta\)-hydroxybutyrate dehydrogenase as N-ethylmaleimide. Incubation of inner membrane vesicles which are more than 90% inverted (32) with 10 \(\mu\)M eosin-5-maleimide or N-ethylmaleimide for 10 min at 25°C results in 95% inhibition of enzyme activity. In freshly isolated mitochondria
Table I
Eosin-5-maleimide does not affect the glutathione content of mitochondria

Rat liver mitochondria were suspended in H medium without bovine albumin at a protein concentration of 10 mg/ml. To each sample consisting of a final volume of 0.35 ml, the N-ethylmaleimide or eosin-5-maleimide at concentrations indicated below were added. Samples were left on ice for 10 (Experiment I) or 20 min (Experiment II). Samples were then diluted with 13 ml of H medium and sedimented (10 min, 19,000 x g), washed once more with 13 ml of H medium, and again sedimented (10 min, 19,000 x g). Each pellet was suspended in 0.2 ml of H medium and 1 ml of 5% trichloroacetic acid, 0.01 N HCl. After 5 min at 0 °C, precipitated proteins were sedimented (20 min, 19,000 x g). Supernatants were extracted 5 times with 1 ml of H2O-saturated diethyl ether. The remaining ether was evaporated under nitrogen. Aliquots (25-40 μl) were assayed for glutathione.

| Inhibitor          | Concentration (μM) | Glutathione content (nmol/mg protein) | % of control |
|--------------------|--------------------|--------------------------------------|--------------|
| Control            |                    |                                      | 100          |
| Eosin-5-maleimide   | 200                | 3.17                                 | 98           |
| Eosin-5-maleimide   | 300                | 3.25                                 | 102          |
| N-Ethylmaleimide    | 300                | 0.03                                 | 1            |
| Control            |                    |                                      | 100          |
| Eosin-5-maleimide   | 200                | 2.92                                 | 100          |
| Eosin-5-maleimide   | 300                | 3.06                                 | 106          |
| N-Ethylmaleimide    | 300                | 0.07                                 | 2            |

Fig. 1. Impermeability of intact mitochondria to eosin-5-maleimide. Mitochondria (A) or inner membrane vesicles (B) isolated from rat liver were incubated for 1-2 min with stirring at 25 °C in 1 ml of H medium (without bovine albumin), containing 2.5 mM KPi, 1 μM FCCP, and 1 mg/ml of protein. Ten min after the addition of indicated concentrations of N-ethylmaleimide or eosin-5-maleimide, the activity of β-hydroxybutyrate dehydrogenase was determined as β-hydroxybutyrate oxidase activity by measuring the respiration initiated by the addition of 10 mM β-hydroxybutyrate. Control values represented as 100% in the figure were 24 and 35 ng atoms of oxygen/min/mg of protein for mitochondria and submitochondrial particles, respectively.

(Fig. 1A), 100 μM eosin-5-maleimide is without effect, and 150 μM of this reagent decreases the enzyme activity by only 7%. The same concentration of N-ethylmaleimide inhibits β-hydroxybutyrate dehydrogenase activity by 85%.

It seems clear from these studies that eosin-5-maleimide does not penetrate the mitochondrial inner membrane at 0 °C or 25 °C, and can, therefore, be used as a highly specific probe for sulfhydryl groups which lie on the cytoplasmic surface of the mitochondrial inner membrane.

Eosin-5-maleimide Preferentially Labels Sulfhydryl Groups of the ADP/ATP Antiporter in Submitochondrial Particles Rather Than in Intact Mitochondria—Eosin-5-maleimide was recently shown to react selectively with sulfhydryl groups of the ADP/ATP antiporter (29). Although labeling was performed in bovine heart submitochondrial particles, it was suggested that eosin-5-maleimide penetrates the inner mitochondrial membrane, reacting with the cytosolic side of the carrier (29). Since the data summarized above seem to exclude this possibility, experiments were carried out to establish on which side of the membrane the eosin-5-maleimide-reactive sulfhydryl group is located. For this purpose, mitochondria and submitochondrial particles were prepared from bovine heart and rat liver. In all cases membranes were incubated with 150 μM eosin-5-maleimide at 0 °C for 20 min. After quenching the reaction with excess dithiothreitol (50 mM), samples were analyzed by SDS-PAGE. The distribution of protein-bound eosin-5-maleimide was visualized by fluorography (29).

As shown in Fig. 2, the amount of the bound label and its distribution among individual mitochondrial proteins differs greatly in mitochondria and submitochondrial particles. Both types of submitochondrial particle preparations (Fig. 2, B and D), in contrast to the mitochondria from which they were derived (Fig. 2, C, 2F), are highly labeled by eosin-5-maleimide. The majority of the label is associated with a 30-kDa protein in bovine heart and a 29.3-kDa protein in rat liver; for simplicity this band will be referred to below as having a molecular mass of 30 kDa. Significantly, no labeling of the...
30-kDa band is observed in freshly isolated rat liver mitochondria (Fig. 2F) nor rat liver mitoplasts (Fig. 2E), which are “outer membrane-free” mitochondria (32). Increasing the temperature to 25 °C also failed to induce labeling of the 30-kDa band, whereas bands with molecular weights corresponding to the Pi/H⁺ carrier were labeled (see Fig. 7).

In freshly isolated bovine heart mitochondria, only very weak labeling of the 30-kDa band is observed (Fig. 2C). The respiratory control ratio of bovine heart mitochondria is always somewhat lower than that of rat liver mitochondria (4.2–5.3 versus 6.7–7.5 with β-hydroxybutyrate as substrate). Also, about 10% of the total β-hydroxybutyrate dehydrogenase activity is inhibited by 10 μM eosin-5-maleimide in the bovine heart mitochondrial preparation, the remaining 90% being insensitive to 150 μM eosin-5-maleimide. Thus, the low but visible labeling of the 30-kDa protein in bovine heart mitochondria is most likely related to the presence of a certain percentage of damaged mitochondria, rather than to a difference between bovine heart and rat liver.

When the time of the labeling is increased from 5 to 30 min and the concentration of eosin-5-maleimide increased from 50 to 200 μM, the same basic pattern of the labeling is obtained for both rat liver and bovine heart preparations. The intensity of all labeled bands is decreased when lower concentrations and shorter incubation times are used (not shown).

To ascertain whether the 30-kDa band labeled by eosin-5-maleimide in submitochondrial particles is, in fact, the ADP/ATP antiporter, the eosin-5-maleimide-labeled submitochondrial particles were used to isolate the ADP/ATP carrier. Isolation according to Klingenberg et al. (14) was carried up to the Ultrogel step. The resultant preparation contains more than 75% of the total protein as a 30-kDa band with the major contaminant being a protein of 34–35 kDa. As shown in Fig. 2A, the isolated transporter is highly labeled by eosin-5-maleimide with all of the label being associated with the 30-kDa band.

Results presented in Fig. 2 demonstrate that the ADP/ATP antiporter is intensively labeled in both bovine heart and rat liver submitochondrial particles, while the corresponding mitochondria are labeled very little (bovine heart) or not at all (rat liver). Prior incubation with ADP or ATP had no effect on these labeling patterns. Because submitochondrial particles prepared by the methods described here (see “Methods”) have a predominantly “inverted” inner membrane orientation relative to intact mitochondria (32), these results indicate that the eosin-5-maleimide-reactive sulfhydryl groups of the ADP/ATP antiporter are expressed exclusively on the matrix side of the inner mitochondrial membrane.

Submitochondrial Particles Prepared from Carboxyatractyloside-Treated Mitochondria Are Not Readily Labeled with Eosin-5-maleimide—When submitochondrial particles are prepared from mitochondria which have been pretreated with carboxyatractyloside, there is very little labeling by eosin-5-maleimide (Fig. 3, C and F) relative to control submitochondrial particles (Fig. 3, A and D) or to submitochondrial particles to which carboxyatractyloside is added directly (Fig. 3, B and E). Thus, carboxyatractyloside, when added to mitochondria, “freezes” the carrier in a form in which eosin-5-maleimide-reactive sulfhydryl groups remain inaccessible even after preparation of submitochondrial particles. It is known that carboxyatractyloside is a nonpenetrant inhibitor of the ADP/ATP antiporter and stabilizes the so-called “C-state” of the carrier, i.e., that state of the carrier in which the nucleotide-binding site faces the matrix space of the inner membrane (41, 42). Thus, data presented here suggest that sulfhydryl groups reactive with eosin-5-maleimide at the matrix surface of the inner membrane may be expressed only after the transporter has undergone a transition from the C-state to the so-called “M-state” (41, 42), a state in which the nucleotide-binding site faces the matrix space.

Eosin-5-maleimide and N-Ethylmaleimide Appear to Label Different Sulfhydryl Groups of the ADP/ATP Antipporter—Previous studies have shown that when the freely permeable sulfhydryl agent N-ethylmaleimide is added to bovine heart mitochondria, it is able to react with sulfhydryl groups of the ADP/ATP antipporter (24–26). Although reactivity is slow, it can be markedly stimulated by adding ADP to the mitochondria. Pretreatment of the mitochondria with carboxyatractyloside prevents labeling by N-ethylmaleimide. Results presented in Fig. 4 confirm these observations in rat liver mitochondria by showing that N-[3H]ethylmaleimide moderately labels the 30-kDa protein in the absence of ADP (Fig. 4A), more so in the presence of ADP (Fig. 4B), and not at all when carboxyatractyloside is present (Fig. 4, C and D).

Significantly, sulfhydryl groups of the ADP/ATP antipporter labeled by N-ethylmaleimide appear to differ from those labeled by eosin-5-maleimide. Thus, data presented in Fig. 5 show that, in the presence of ADP, neither bovine heart nor rat liver mitochondria are labeled with eosin-5-maleimide (Fig. 5, C and G). Moreover, as shown in Fig. 6, pretreatment of either bovine heart or rat liver submitochondrial particles with N-ethylmaleimide (Fig. 6, B and E) or N-ethylmaleimide + ADP (Fig. 6, C and F) fails to alter labeling of the ADP/ATP antipporter by eosin-5-maleimide.

These results indicate that, in intact mitochondria, the C-state of the ADP/ATP antipporter contains two classes of sulfhydryl groups, one of which has only limited accessibility to N-ethylmaleimide and both of which are inaccessible to

![Fig. 3. Diminished capacity of submitochondrial particles prepared from carboxyatractyloside-treated mitochondria to react with eosin-5-maleimide. Control submitochondrial particles were labeled with eosin-5-maleimide exactly as described in the legend to Fig. 2A, bovine heart; D, rat liver. Carboxyatractyloside (100 μM) was added directly to submitochondrial particles 2 min before addition of eosin-5-maleimide. B, bovine heart; E, rat liver. Carboxyatractyloside-treated mitochondria to which carboxyatractyloside is added directly (Fig. 3, A and D).](image-url)
**FIG. 4.** *N*-[^3]H]Ethylmaleimide labels the ADP/ATP antiporter in intact mitochondria. Control rat liver mitochondria (1 mg of protein/ml) were incubated for 2 min at 25 °C in H medium without bovine albumin, 5 mM succinate, and 5 μg of oligomycin/ml. A, N-ethylmaleimide (100 μM) was then added, and followed after 5 min by *N*-[^3]H]ethylmaleimide. The reaction was terminated after 6 min with 50 mM dithiothreitol. Aliquots were then taken for SDS-PAGE as described under “Methods.” B, conditions were identical with A with labeling in the presence of 100 μM ADP added together with *N*-[^3]H]ethylmaleimide. C, identical conditions with A, but with labeling in the presence of 100 μM carboxyatractyloside added 1 min before *N*-[^3]H]ethylmaleimide. D, identical conditions with A but with both ADP and carboxyatractyloside present (added as in B and C). Radioactivity in the gel was detected by fluorography.

**FIG. 5.** Inability of ADP to induce reactivity of eosin-5-maleimide with the ADP/ATP antiporter in intact mitochondria. Control mitochondria: B, bovine heart; F, rat liver. Submitochondrial particles: A, bovine heart; D, rat liver. Preparation of submitochondrial particles (i.e., inversion of the inner membrane) is able to “unmask” the eosin-5-maleimide-reactive groups in the absence of ADP. However, ADP must be present to fully unmask the N-ethylmaleimide-reactive groups.

**FIG. 6.** Inability of *N*-ethylmaleimide and ADP to alter labeling of the ADP/ATP antiporter in submitochondrial particles by eosin-5-maleimide. Control submitochondrial particles were labeled by eosin-5-maleimide as in Fig. 2 (A, bovine heart; D, rat liver). Labeling was also performed after 5 min of prior incubation with 1.5 mM *N*-ethylmaleimide (B, bovine heart; E, rat liver) or with 1.5 mM *N*-ethylmaleimide and 100 μM ADP (C, bovine heart; F, rat liver). Fluorographs were obtained from 100-μg protein samples (bovine heart) or 150-μg proteins samples (rat liver) after SDS-PAGE.

Eosin-5-maleimide. Preparation of submitochondrial particles (i.e., inversion of the inner membrane) is able to “unmask” the eosin-5-maleimide-reactive groups in the absence of ADP. However, ADP must be present to fully unmask the N-ethylmaleimide-reactive groups.

**Eosin-5-maleimide Inhibits the P_/H^+ Symporter in Intact Mitochondria**—In functionally intact mitochondria, the ADP/ATP antiporter must work in synchrony with the P_/H^+ symporter in order to supply the proton ATPase with ADP and P_ for ATP synthesis. It was, therefore, of interest to examine the reactivity of the P_/H^+ symporter with eosin-5-maleimide to establish whether the sulfhydryl group(s) of this transporter exhibit a membrane orientation similar to or different from that of the ADP/ATP antiporter. Results presented in Fig. 7A show that, at 0 °C, eosin-5-maleimide has only a slight inhibitory effect on phosphate transport in mitochondria as measured by the classical ammonium phosphate swelling technique (43). This finding is in contrast to that observed with the well-established phosphate transport inhibitor N-ethylmaleimide, which inhibits phosphate transport nearly completely (Fig. 7A). At 25 °C, however, it can be seen in Fig. 7B that eosin-5-maleimide becomes almost as effective as N-ethylmaleimide in inhibiting phosphate transport. Significantly, dithiothreitol prevents inhibition by both reagents, indicating that sulfhydryl groups are involved (data not shown). Moreover, the inner mitochondrial membrane remains impermeable to eosin-5-maleimide at 25 °C (Fig. 1). It seems clear, therefore, that essential sulfhydryl groups of the P_/H^+ symporter reactive with eosin-5-maleimide are oriented near the cytoplasmic surface of the inner membrane in intact mitochondria.

**Eosin-5-maleimide Prevents Labeling of the P_/H^+ Symporter by N-[^3]H]Ethylmaleimide in Intact Mitochondria**—Work carried out in this laboratory (44) first showed that the P_/H^+ symporter could be labeled rather selectively by N-[^3]H] ethylmaleimide in rat liver mitochondria by (a) first protect-
Fig. 7. Inhibition of phosphate-induced swelling of mitochondria by N-ethylmaleimide and eosin-5-maleimide. Rat liver mitochondria were incubated in H medium without bovine albumin at a protein concentration of 5 mg/ml for 2 min with indicated concentration of N-ethylmaleimide or eosin-5-maleimide, either at 0 °C (A) or at 25 °C (B). Aliquots (1 mg) were then injected into 3 ml of 120 mM NH₄F, 0.5 mM EDTA, 0.04 mM rotenone, pH 7.2, and swelling was measured at 540 nm. The change in absorbance after 30 s compared with that of control mitochondria was taken as 100%.

Fig. 8. Comparison of the effects of eosin-5-maleimide, N-ethylmaleimide and mersalyl on the labeling of Pᵢ/H⁺ symporter by N-[³H]ethylmaleimide. Rat liver mitochondria (5 mg of protein/ml) were incubated in H medium without bovine albumin at 0 °C for 1 min with 10 nmol of mersalyl/mg of protein and then for 5 min with 100 nmol of N-ethylmaleimide/mg of protein. After addition of dithiothreitol (500 nmol/mg of protein), the mitochondria were washed twice with 25 ml of H medium without bovine serum albumin. Labeling with N-[³H]ethylmaleimide (20 nmol/mg of protein) for 2 min was then performed with or without prior incubation with various sulphydryl reagents as indicated: A, control; B and C, 4 and 8 nmol of mersalyl/mg of protein, 1 min, 0 °C; D and E, 12 and 16 nmol of N-ethylmaleimide/mg of protein, 2 min, 0 °C; F and G, 20 nmol of eosin-5-maleimide/mg of protein, 45 min at 0 °C and 5 min at 25 °C. Following SDS-PAGE, radioactivity in the gels was detected by fluorography. Arrows 1 and 2 refer to bands (32 kDa and 33.5 kDa) which are labeled with N-ethylmaleimide and thought to correspond to the Pᵢ/H⁺ carrier. The arrow 3 refers to the 30-kDa position of the ADP/ATP carrier (see Fig. 4).

DISCUSSION

Results of experiments described here provide new information about the fluorescent probe eosin-5-maleimide, showing clearly that this agent is impermeable to the mitochondrial inner membrane (Table I and Fig. 1). Thus, eosin-5-maleimide was found to be unreactive with both matrix glutathione and β-hydroxybutyrate dehydrogenase, an enzyme localized on the matrix surface of the inner membrane. These findings should prove valuable in extending the use of this agent to other membrane proteins where knowledge of the topography of the protein is a prerequisite to understanding the mechanism.

Significantly, in the present study the use of eosin-5-maleimide together with the permeable sulphydryl reagent, N-ethylmaleimide, provides new insight into the relative locations of sulphydryl groups of the Pᵢ/H⁺ and ADP/ATP carriers of both rat liver and bovine heart mitochondria. These results and our interpretations of them can best be depicted as shown in Fig. 10. To the left in the figure, the Pᵢ/H⁺ carrier is shown localized in intact mitochondria, such that its essential sulphydryl group(s) face the cytosolic surface of the inner membrane. Only one class of sulphydryl groups is shown. This is consistent with the findings that both eosin-5-maleimide and N-ethylmaleimide inhibit phosphate transport in mitochondria at 25 °C (Fig. 7), and that eosin-5-maleimide and mersalyl protect the Pᵢ/H⁺ carrier from labeling by N-[³H]ethylmaleimide (Fig. 8). It is consistent also with the earlier findings from this laboratory showing that the impermeant phosphate transport inhibitor, p-chloromercuribenzoate (46), protects...
were incubated with eosin-5-maleimide described in Fig.
freshly isolated rat liver mitochondria. Rat liver mitochondria
the conditions selected for study. In any event, eosin-5-maleimide at 25
25°C for 10 min (C) or at 0°C for 30 min (B). For comparison, rat
submitochondrial particles, labeled for 30 min at 0°C (A) to reveal the 30-kDa ADP/ATP antiporter band, are shown. Arrows 1 and 2 indicate the position of bands with molecular masses of 34.7 kDa and 33.5 kDa that become labeled by eosin-5-maleimide in mitochondria at higher temperature.

In contrast to the Pi/H+ carrier, the ADP/ATP carrier is depicted in Fig. 10 as having two distinct classes of sulfhydryl groups. Cys residues 56 and 129 lie near the matrix surface whereas residues 159 and 256 are buried within the membrane. NEM, N-ethylmaleimide; EMA, eosin-5-maleimide.

With regard to the studies reported here, it should be pointed out that the amino acid sequence of the ADP/ATP antiporter is now known (47) and that cysteine residues are located at positions 56, 129, 159, and 256 in the monomeric 30-kDa protein. Two models for the folding of the carrier polypeptide in the mitochondrial inner membrane have been proposed, one by Bogner et al. (48), on the basis of the positions of lysine residues, and one by Saraste and Walker (49), on the basis of a Hydroplot computer program. The latter computer-derived model places two out of the four cysteine residues (cysteines 159 and 256) in a hydrophilic space opposite to that occupied by cysteine 56. Clearly, the observations in this study and other studies (24–26) cannot be easily accommodated by the Saraste and Walker model. Thus, the latter model predicts that, even in the absence of ADP, the carrier should be labeled readily by sulfhydryl reagents from both sides of the inner mitochondrial membrane. In contrast, the model proposed by Bogner et al. (48) can readily accommodate the observations described in this study because two cysteine residues (residues 159 and 256) reside within the inner membrane and two others (residues 56 and 129) lie near the matrix surface. The “folding” model of Bogner et al. (48) is redrawn in Fig. 10 (inset) to
emphasize the predicted locations of the four cysteine residues.

Finally, it should be noted that Muller et al. (60) have recently employed eosin-5-maleimide to study the rotational diffusion of the ADP/ATP translocator in bovine heart mitochondria. In sharp contrast to the results presented here, they conclude that eosin-5-maleimide readily labels the ADP/ATP carrier in mitochondria. However, the mitochondria used in their studies were stored frozen for an undefined time period, which is well-known to impair mitochondrial intactness. Significantly, Muller et al. provide no information about the intactness of their preparation in the way of acceptor control ratios. In the studies reported here we have always worked with freshly isolated mitochondria exhibiting high acceptor control ratios (see "Methods"). Moreover, our results are consistent with the observations from several laboratories which show that hydrophobic sulfhydryl reactive reagents are impermeable to the mitochondrial inner membrane whereas water-soluble reagents like N-ethylmaleimide are readily permeable (28, 32, 40, 49).

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