Differential Effects of Mercurial Reagents on Membrane Thiols and on the Permeability of the Heart Mitochondrion*

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ABSTRACT: The extent of reaction of mercurial reagents with the mitochondrial membrane has been found to depend on the polarity of the reagent, the anionic composition of the suspending medium, the pH, and to some degree, on the metabolic status of the mitochondrion. At low levels of mercurial binding (*ca.* 10 mμmoles/mg), the entrance of inorganic phosphate into the matrix of the mitochondrion is inhibited. At slightly higher levels (10–20 mμmoles/mg), the energy-linked accumulation of monovalent cations and acetate is stimulated. When conditions permit binding of 15–30 mμmoles/mg of mercurial to the membrane, a passive permeability to Cl^- is produced, which is reflected in large amplitude swelling of mitochondria suspended in KCl media. A portion of the bound mercurial can be removed by the nonpenetrating thiolcysteine, and by dithioerythritol. Mitochondria, which have been swollen in KCl by addition of mercurials, can be contracted in an energy-linked reaction which is accompanied by ion extrusion if (a) cysteine is added to remove a portion of the bound mercurial, and (b) a source of metabolic energy is provided. It is proposed that under certain conditions, mercurials modify only the exterior thiol components of the coupling membrane; whereas, under more favorable conditions for reactivity, the thiols of interior components can also be modified.

We have recently reported that a number of organic mercurials, such as *p*-hydroxymercuribenzoate, *p*-chloromercuriphenylsulfonate, and mersalyl enhance the energy-linked uptake of K^+ and other monovalent cations by isolated heart mitochondria (Brierley *et al.*, 1968a). These experiments were carried out in a sucrose medium containing the monovalent cation and acetate as the permeant anion. In an extension of these studies to the effects of mercurials under other conditions, it became apparent that both the extent of reaction of certain of the mercurials with the membrane and the resulting effects of mitochondrial permeability and ion uptake were strongly dependent on the anionic composition of the suspending medium (Knight *et al.*, 1968). In media containing acetate as the predominant anion, CMS,[1] CMB, and mersalyl are approximately equivalent in effectiveness and activate only the energy-linked uptake of cations and the permeant anion. In solutions containing substantial amounts of Cl^-, however, it was noted that CMB reacted with more membrane thiols than did CMS and induced a passive permeability to K^+ and Cl^- which was not seen with the more polar CMS (Knight *et al.*, 1968). Addition of cysteine and a respiratory substrate to the osmotically swollen mitochondria which result from this treatment causes an energy-linked extrusion of ions and contraction to commence. We have suggested that these results depend on the ability of CMB in a Cl^- medium to penetrate the inner membrane of the mitochondrion and to react with interior thiol groups which are not available to mercurial reagents under other conditions. It was further suggested that the nonpermeant thiol reagent, cysteine, reversed the reaction of the mercurial only with membrane thiols which were exposed on the exterior of the inner membrane. The present communication presents the experiments upon which these suggestions were based and develops these concepts in greater detail.

Methods

Beef heart mitochondria were prepared by Nagarse treatment in the presence of EGTA (Hatefi *et al.*, 1961; Settlemire *et al.*, 1968). Submitochondrial particles were prepared as described by Jacobus and Brierley (1969).

Swelling and contraction of mitochondria were monitored by absorbance at 546 mμ using a Plexiglass cuvet equipped with a magnetic stirrer and mounted on an Eppendorf photometer. The pH and oxygen uptake were recorded simultaneously with a combination pH electrode and a Clark electrode, respectively. Water, K^+, and $^{36}Cl^-$ content of isolated particles were estimated as described by Hunter and Brierley (1969).

The binding of CMS and CMB was determined by incubating the mitochondria in various media in the presence of ^{203}Hg-labeled CMS or CMB (The Radiochemical Centre, Amersham). The mitochondria were removed by rapid filtration through Millipore filters (0.45 μ) and the bound mercurial was calculated from the decrease in radioactivity in the filtrate. Control experiments have established that the mercurials

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[1] The abbreviations used are: CMB, *p*-hydroxymercuribenzoate; CMS, *p*-Cl-mercuriphenylsulfonate; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CCP, carbonylcyanide-*m*-chlorophenylhydrazone.

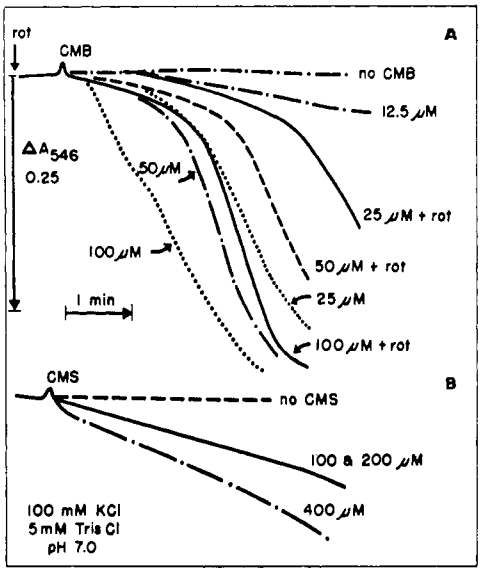

FIGURE 1: The effect of CMB and CMS on the swelling of heart mitochondria suspended in 0.1 M KCl at pH 7.0. Heart mitochondria (5 mg of protein) were added to 8 ml of a medium of KCl (100 mM) containing Tris-Cl (5 mM, pH 7.0) in a Plexiglass cuvet equipped with a magnetic stirrer, a Clark oxygen electrode, and a combination pH electrode. Absorbance at 546 mμ was recorded with an Eppendorf photometer. The effect of the addition of the indicated amount of CMB or CMS in the presence and the absence of rotenone (4 μg/mg) is shown.

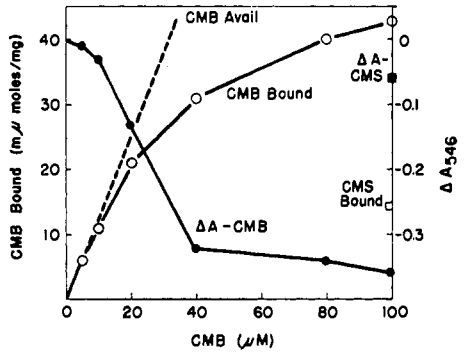

FIGURE 2: Relation of CMB-induced swelling in isotonic KCl at pH 7 to the binding of CMB to the mitochondrion. The reactions were carried out using 2.5 mg of mitochondrial protein treated with rotenone in 3-ml cuvets in the Eppendorf photometer. The decrease in absorbance at 546 mμ was recorded after 4 min in the presence of the indicated concentration of ^{203}Hg-labeled CMB or CMS. At this point, a sample was filtered rapidly through a Millipore filter and the uptake of CMB by the mitochondrion was calculated from the decrease in radioactivity in the resulting filtrate. The total amount of CMB available (mμmoles/mg) is shown by the dashed trace.

do not interact with the filters. The composition of the suspending medium and other experimental details are given with each of the individual experiments reported.

In a number of experiments, the binding of CMB has also been evaluated using ^{14}C-labeled CMB (Tracerlab, Waltham, Mass.) with results identical with those with the ^{203}Hg-labeled reagent. This supports our contentions that reaction of the organic mercurial rather than one of the various ionic forms of inorganic mercury is involved in these studies. In addition, $HgCl_2$ releases large amounts of H^+ when it interacts with submitochondrial particles (Jacobus and Brierley, 1969), and it has been established that neither CMB nor CMS releases H^+ under conditions of extensive mercurial uptake by these particles (W. E. Jacobus, unpublished).

Results

Swelling of Isolated Heart Mitochondria Induced by CMB in a Medium of 0.1 M KCl. Isolated heart mitochondria do not swell or take up ions when suspended in a lightly buffered medium of 0.1 M KCl at pH 7.0. The studies shown in Figure 1A establish, however, that low concentrations of CMB induce a rapid swelling of the mitochondria in this medium. The presence of endogenous respiration usually decreases the time of onset, but does not affect the extent of swelling. It should be noted that the mercurial strongly inhibits endogenous respiration under these conditions so that a source of energy is available only in the initial phases of the reaction regardless of the presence of rotenone. In the absence of endogenous respiration (rotenone present), a lag of 2–3 min occurs before the rapid phase of the swelling begins and this lag is a function of the concentration of CMB (Figure 1A). Even rather high concentrations of CMS (400 μM) fail to induce a comparable precipitous swelling under these conditions (Figure 1B). Phenylmercuric acetate duplicates the effects of CMB in these experiments; whereas, mersalyl closely resembles CMS in its action at comparable concentrations.

Analysis of centrifuged pellets of mitochondria which have been swollen in the presence of CMB reveals large increases in total water, mannitol-impermeable water, K^+, and Cl^- which are compatible with an osmotic swelling (Hunter, 1969). In addition, electron micrographs have established that the swelling is accompanied by extensive expansion of the matrix (Hunter *et al.*, 1969). Swelling under these conditions, therefore, appears to be the result of the uptake of water, K^+, and Cl^- into the matrix compartment of the mitochondrion. Since mitochondria appear to be impermeable to both K^+ and Cl^- in the absence of energy at neutral pH, the extensive uptake of K^+ and Cl^-, which results from CMB treatment, appears to reflect an alteration in membrane permeability. This altered permeability is a function of the amount of CMB which has reacted with the membrane (Figure 2). Concentrations of CMB up to about 10 μM are bound almost quantitatively to the mitochondrion but do not induce swelling. In the concentration range from 10 to 40 μM, an increase in bound mercurial is related to increased passive osmotic swelling in the KCl suspending medium. Above 40 μM CMB, the binding of mercurial continues to increase slightly but there is little further effect on the extent of swelling. The ineffectiveness of CMS in activating swelling under these conditions appears to be related to the inability of this reagent to react with the membrane to the same extent as CMB (Figure 2). When the amount of swelling in KCl is related to the amount of mercurial bound, rather than the concentration added, it is apparent that CMS is as effective as CMB per mole bound (Figure 2).

Energy-Linked Contraction of Mercurial-Treated Swollen Mitochondria. Mitochondria which have been induced to take up K^+, Cl^-, and water in the absence of energy by treatment with CMB can be contracted in an energy-linked

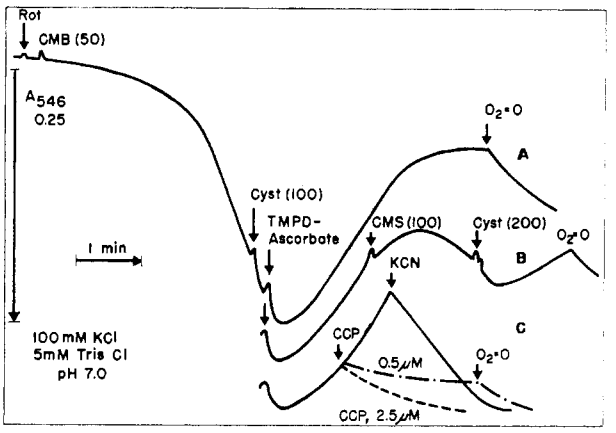

FIGURE 3: Energy-linked contraction of heart mitochondria swollen by the addition of CMB in a medium of 0.1 M KCl. The conditions were identical with those described in Figure 1. Values in parentheses are the concentrations of CMB, CMS, and neutralized cysteine (μM) added at the indicated points. The concentration of other reagents was as follows: TMPD (0.1 mM); Tris-ascorbate (3 mM), rotenone (4 μg/mg), and CCP (0.5 and 2.5 μM as shown).

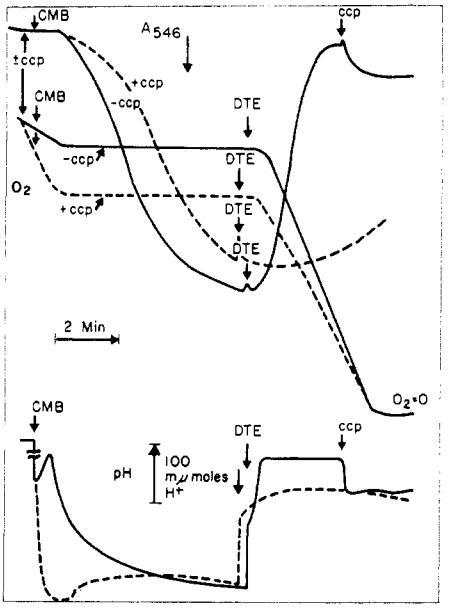

FIGURE 5: Swelling and contraction, oxygen uptake, and pH changes of mitochondria treated with CMB while respiring with succinate in a medium of 0.1 M KCl. The conditions were identical with those of Figure 4 except that K^+-succinate (2.5 mM) was added in place of the TMPD–ascorbate.

reaction, if cysteine or dithioerythritol is also added to the suspension (Figure 3). In the absence of cysteine, no contraction results from the addition of an oxidizable substrate and in the absence of a source of energy, cysteine produces no contraction (Knight *et al.*, 1968). Optimal contraction is obtained when a twofold molar excess of cysteine over CMB is added. In the reaction shown, TMPD–ascorbate respiration was used as the source of energy and the resulting contraction was abolished by cyanide and by uncouplers such as CCP. At anaerobiosis, the contraction ceased and passive swelling resulted. The concentration of CCP necessary to reverse the contraction as effectively as cyanide or anaerobiosis was about fivefold in excess of the concentration necessary to elicit maximal respiration in the absence of mercurial (0.5 μM at pH 7). Cycles of energy-linked contraction and passive swelling

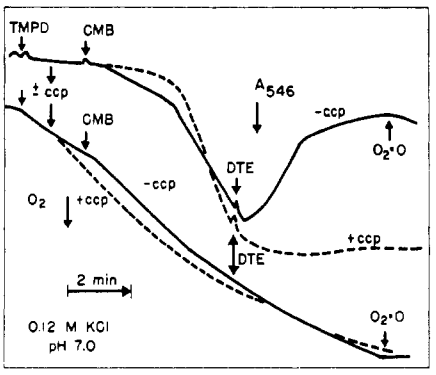

FIGURE 4: Swelling and contraction of mitochondria treated with CMB while respiring with TMPD–ascorbate in a medium of 0.1 M KCl. The conditions were identical with those of Figure 3 except that CMB (50 μM) was added after the TMPD–ascorbate. The dashed traces show the swelling and oxygen uptake in the presence of CCP (6×10^{-7} M), the solid traces there sponse in the absence of uncoupler. Where indicated dithioerythritol (100 μM) was added to remove a portion of the mercurial.

could be established by the addition of either excess mercurial (CMS and CMB are equally effective at this stage of the reaction) or excess cysteine (*cf.* Figures 3, trace B). Electron microscope studies have established that the matrix of the swollen mitochondria contracts almost completely under these conditions so that the bulk of the particles resemble unswollen mitochondria in appearance (Hunter *et al.*, 1969). K^+ and Cl^- are extruded during the contraction and the mannitol-impermeable water volume is decreased.

The effect of CMB on swelling and contraction in 0.1 M KCl in the presence of respiration with TMPD–ascorbate is shown in Figure 4. Respiration with this substrate is not inhibited by the mercurial but is actually activated to nearly the same extent as with the uncoupler CCP. Swelling in the presence of this CMB-dependent elevated rate of respiration is accelerated slightly over that seen when the CMB is added in the presence of CCP (Figure 4). Addition of dithioerythritol after extensive swelling has been obtained results in respiration-dependent contraction. Contraction does not occur in preparations treated with CCP, however. CMS added in place of CMB in these experiments produces accelerated respiration but no swelling.

Addition of CMB (50 μM) to mitochondria respiring with succinate in a medium of 0.1 M KCl (Figure 5) results in (a) a cycle of H^+ extrusion and uptake similar to that seen on addition of valinomycin under these conditions (Brierley, 1970), (b) extensive swelling, and (c) inhibition of respiration. Addition of dithioerythritol results in the initiation of both a high rate of respiration and respiration-dependent contraction. If CMB is added to mitochondria respiring with succinate in the presence of CCP (Figure 5), the high rate of respiration induced by CCP is inhibited completely by CMB. No cycle of pH changes is observed under these conditions,

TABLE I: Differential Uptake of ^{203}Hg-Labeled CMS and CMB by Heart Mitochondria in Various Suspending Media.[a]

Expt	Suspending Medium	mμmoles of Mercurial Bound/mg CMB	CMS
1	KCl (0.12 M)	38	15
	K^+-acetate (0.12 M)	18	18
	NaCl (0.12 M)	36	18
	NH_4Cl (0.12 M)	34	18
	NH_4-acetate (0.12 M)	20	16
	K^+-phosphate (0.12 M)	17	16
	$MgCl_2$ (0.08 M)	33	16
2	Sucrose (0.25 M)–KCl (10 mM)	41	18
	Sucrose (0.25 M)–K^+-acetate (10 mM)	22	18
	Sucrose (0.25 M)–K^+-formate (10 mM)	21	
	Sucrose (0.25 M)–KF (10 mM)	24	
	Sucrose (0.25 M)–KBr (10 mM)	42	
	Sucrose (0.25 M)–K^+-HEPES (10 mM)	37	
	Sucrose (0.25 mM)–KNO_3 (10 mM)	25	

[a] Binding was evaluated in the medium indicated (buffered at pH 7 with 5 mM Tris salt of the appropriate anion) by incubating the labeled mercurial (60 mμmoles/mg available) for 3 min at 25°. The mitochondria were removed by filtration and mercurial binding was estimated from the decrease in radioactivity of the filtrate.

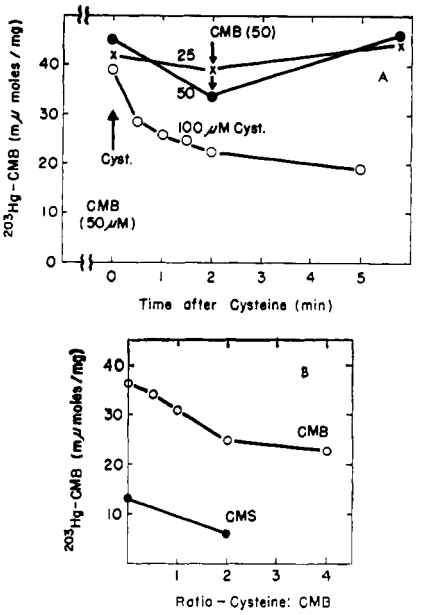

FIGURE 6: Removal of bound CMB by treatment with cysteine. The reaction was carried out under the conditions specified in Figure 1 with 50 μM [^{203}Hg]CMB present. (A) After 4-min incubation either 25, 50, or 100 μM neutralized cysteine was added and samples were filtered through Millipore filters at the indicated times. The bound mercurial remaining on the membrane was calculated from the decrease in radioactivity in the filtrate. In the two experiments indicated a second addition of 50 μM labeled CMB was made 2 min after the addition of cysteine. (B) Effect of increasing cysteine concentration on the removal of bound CMB and CMS from the mitochondrial membrane. The experimental conditions were identical with those of part A with 50 μM CMS or CMB interacted for 4 min followed by the addition of the indicated amount of cysteine. Samples were filtered after 2 min of further incubation and the bound mercurial was calculated as previously described.

and the passive swelling is delayed to some extent compared with that in the absence of uncoupler. Addition of dithioerythritol under these circumstances restores the high rate of respiration but does not induce contraction (Figure 5). Addition of CMS instead of CMB in the protocol of Figure 5 produces inhibition of respiration, and in the absence of CCP, no swelling. Succinate respiration in the presence of CCP is inhibited only by high concentrations of CMS under these conditions, and the high rate of respiration is not completely restored by dithioerythritol.

Addition of cysteine to mitochondria which have bound CMB under these conditions removes about one-half of the bound mercurial (Figure 6). The amount of mercurial removed is a function of the time of the reaction (Figure 6A), but the bulk of the loss occurs in the first minute. The reversal also depends on the molar ratio of cysteine to CMB (Figure 6B). Optimal reversal requires at least a twofold excess of thiol over the mercurial. Dithioerythritol removes about the same amount of mercurial under these conditions. A large proportion of bound CMS is also removed by treatment with cysteine or dithioerythritol, so that the total bound mercurial is very low after treatment with CMS followed by cysteine (Figure 6B).

Effect of CMB and CMS on Swelling in Isotonic K^+-Acetate. Heart mitochondria take up large amounts of K^+ and swell spontaneously by an energy-linked reaction when suspended in isotonic K^+-acetate (Brierley *et al.*, 1968b). We have previously reported that CMB and CMS are equally effective in activating this energy-linked reaction in the presence of ascorbate–TMPD respiration and that both reagents fail to induce swelling in the absence of a source of energy (Knight *et al.*, 1968; Brierley *et al.*, 1968a). More extended times of incubation, however, show that CMB has a somewhat greater tendency to induce passive swelling in isotonic K^+-acetate at pH 7.0 than does CMS at equal concentrations. The qualitative differences in effectiveness of CMB relative to CMS in a Cl^- medium as opposed to the rather similar response of these two reagents in an acetate medium was unexpected. A possible explanation for these results is apparent, however, from the data summarized in Table I, which show that CMS and CMB are bound to the same extent in a medium of isotonic K^+-acetate in contrast to the marked discrepancy between the binding of CMS and CMB in a Cl^- medium. CMS binding is limited to less than 20 mμmoles/mg of protein in either Cl^- or acetate media when compared at a constant incubation time of 3 min. Under these conditions, CMB binding in an acetate medium also approaches a value of 20 mμmoles of mercurial bound per mg of protein, but in a Cl^- medium the binding is consistently closer to 40 mμmoles/mg. The uptake of mercurial is relatively independent of the type of cation present in the medium, since isotonic K^+-, Na^+-, NH_4^+-, and Mg^{2+}-acetate all give low values (Table I). The binding of the mercurial is also not dependent on the initial state of swelling of the mitochondria, since particles

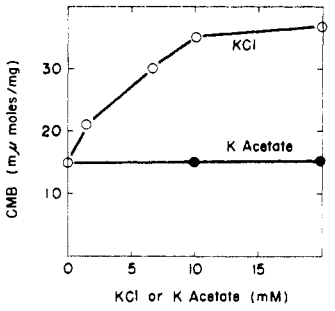

FIGURE 7: Effect of increasing concentrations of KCl and K^+-acetate on the binding of ^{203}Hg-labeled CMB in a medium of 0.25 M sucrose. The labeled mercurial was present at a level of 60 mμmoles/mg of protein. Time of incubation was 3 min. Other conditions were identical with those of Figure 6.

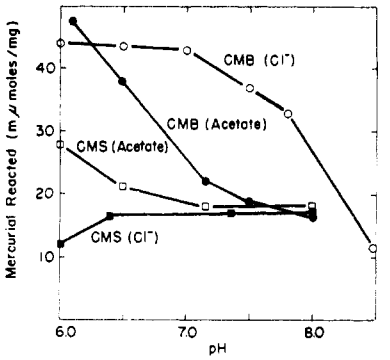

FIGURE 9: Binding of mercurials by heart mitochondria as a function of pH. Labeled CMB or CMS (60 mμmoles/mg) was reacted in 0.1 M KCl or K^+-acetate containing 5 mM Tris buffer (either the Cl^- or acetate salt) at the indicated pH for 2 min. Binding of mercurial was calculated from the decrease in radioactivity of filtrates.

which are extensively swollen in isotonic NH_4^+-acetate show lower uptake than the corresponding unswollen particles in NH_4Cl. In general, salts of permeant anions (acetate, formate, F^-, and phosphate) show the low level of binding, whereas salts of nonpermeant anions (Cl^-, Br^-, HEPES) show higher levels of CMB uptake (Table I). In contrast to the binding of CMB, it is apparent that the binding of CMS is not affected by changes in the predominant anion of the suspending medium.

The elevated binding of CMB is a function of the Cl^- content of the medium (Figure 7) with about 10 mM KCl being sufficient to support maximum uptake of the mercurial. The presence of acetate does not depress the level of mercurial binding in media which contain Cl^-.

Kinetics of Mercurial Uptake. Studies of the rate of uptake of CMS and CMB by suspensions of heart mitochondria show that the bulk of the uptake of CMS in both Cl^- and acetate media and CMB in an acetate medium occurs in the first few seconds of incubation (Figure 8). The more extensive uptake of CMB in a Cl^- medium also occurs rapidly. A large proportion of the total uptake under these conditions takes place in the first 30 sec. The presence or absence of endogenous respiration does not appear to alter the rate of uptake of CMB in the Cl^- medium. Since the binding of mercurial does not change to a great degree in the time range of 3–5 min, valid comparisons of binding can be made at a constant time of incubation (*cf.* Table I). Prolonged incubation with high concentrations of either CMB or CMS (1 hr or more) result in uptake of a limiting amount of about 70 mμmoles of mercurial/mg of protein.

pH Dependency of Mercurial Uptake. The binding of CMS to the mitochondrial membrane is relatively insensitive to the pH of the suspending medium in the range from 6.5 to 8.0 (Figure 9). The extent of CMB interaction, on the other hand, shows a marked dependence on pH with optimal uptake from a Cl^- medium at pH 7.0 and a striking decrease in reactivity with increasing pH. In an acetate medium, the uptake of CMB remains relatively constant and at a low level in the range from pH 7 to 8.

Effect of Mercurials on Swelling in KCl at pH 8.2. It is apparent from the data of Figure 9 that at pH 8.2 the binding of CMS and CMB in a Cl^- medium should be of similar

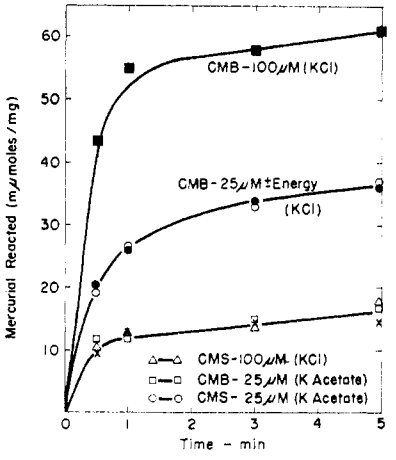

FIGURE 8: Kinetics of mercurial binding by heart mitochondria. The indicated concentrations of labeled mercurial were incubated with 5 mg of protein in a total volume of 8 ml of either 100 mM KCl containing 5 mM Tris-Cl (pH 7.2) or 100 mM K^+-acetate buffered with Tris-acetate. Samples were filtered at the times shown and the uptake of the mercurial was calculated from the decrease in radioactivity.

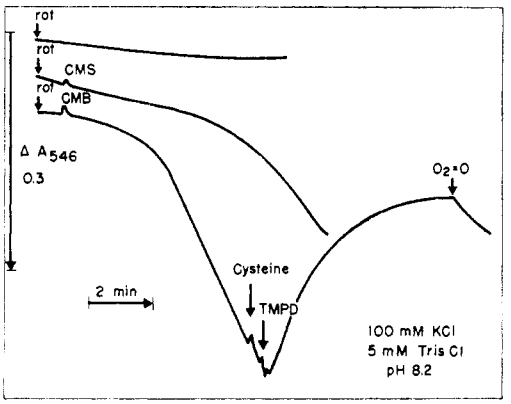

FIGURE 10: The effect of CMB and CMS (50 μM) on the passive swelling of heart mitochondria suspended in 0.1 M KCl at pH 8.2. The experimental conditions and concentrations of reagents were the same as for Figure 1, except that the pH was maintained at 8.2.

TABLE II: Conditions Affecting Uptake of CMB by Mitochondria and Submitochondrial Particles.[a]

Suspending Medium (0.12 M)	Additions	CMB Bound (mμmoles/mg) (of 120 Available)		Maximum Binding Sites (mμmoles/mg)		K_{CMB} (μM)	
		pH 7.1	pH 8.3	pH 7.1	pH 8.3	pH 7.1	pH 8.3
Mitochondria							
KCl	None	44	23	67	40	50	50
	EDTA (0.3 mM)	18	14	26	20	20	22
	Dithioerythritol (0.3 mM added 4 min after CMB)	20	15				
K^+-acetate	None	22	16	30		27	
	ATP (3 mM)	32		45		45	
	CCP (3×10^{-5} M)	50		71		67	
Submitochondrial particles							
KCl	None	77	75	100		80	
	EDTA	56	46	33		30	
	Dithioerythritol (as above)	50	50				
K^+-acetate	None	76					
	CCP	76					
	ATP	56					

[a] Mitochondria (2.5 mg) or submitochondrial particles (3 mg) were reacted with 100 μM [^{203}Hg]CMB in 3 ml of the medium indicated for 4 min at 25° and filtered through Millipore filters as already described. Dithioerythritol was added after a 4-min incubation with CMB and the suspension was incubated for an additional 3 min before filtration. The maximum binding was evaluated from the plots shown in Figure 12 as the reciprocal of the intercept on the ordinant ($1/CMB_{max}$). K_{CMB} was taken as the negative reciprocal of the intercept with the absicisca ($-1/K_{CMB}$).

magnitude. Accordingly, the swelling of heart mitochondria induced by these reagents was evaluated in a medium of 0.12 M KCl at pH 8.2 (Figure 10). Untreated heart mitochondria do not swell or take up ions under these conditions in the presence or absence of respiration or in the presence of an uncoupler, even though the mitochondrion appears to be permeable to Cl^- at this pH (Azzi and Azzone, 1967). In the absence of respiration, both CMB and CMS promote swelling under these conditions, although CMB is the more effective (Figure 10). In this medium, as at the lower pH, energy-linked contraction occurs if the swollen mitochondria are treated with cysteine and a source of energy such as TMPD–ascorbate respiration. At this pH, some respiration-dependent contraction can be detected even in the absence of cysteine.

When CMS or CMB are added in the presence of TMPD–ascorbate respiration in this medium at pH 8.2, the results shown in Figure 11 are obtained. Both mercurials induce only a slight swelling under these conditions and strongly activate respiration. If an uncoupler is added during this phase of the reaction in the presence of either CMS or CMB, a rapid passive swelling results. This swelling also occurs at anaerobiosis with CMB but not with CMS. Addition of cysteine prevents the swelling which results when the supply of energy is interrupted under these conditions (Figure 11). These results indicate that TMPD–ascorbate respiration is coupled to a contracting reaction under these conditions, which opposes the passive influx of ions and the resulting osmotic swelling.

Additional Factors in the Uptake of CMB by Mitochondria. The high levels of CMB binding which are routinely observed in Cl^- media are independent of the metabolic state of the mitochondrion and remain relatively constant in the presence or absence of respiration, exogenous ATP, high levels of CCP, and oligomycin. The binding of CMB is strongly inhibited by EDTA, however (Table II). In contrast, the binding of CMB in K^+-acetate can be increased considerably by the addition of ATP or of high concentrations of CCP. The effect of exogenous ATP is not duplicated by the presence of respiratory substrates. It also should be noted that the absolute value for CMB uptake in acetate varies to a greater extent from experiment to experiment than does the binding in the presence of Cl^-. The explanation for the rather unusual response to alteration of the metabolic state of the mitochondrion is not available at present.

Saturation curves for the binding of mercurials to the membrane, such as that shown in Figure 2, can be plotted as a double reciprocal (1/amount of mercurial bound *vs.* 1/amount of mercurial available) and since in most cases straight-line plots formally equivalent to Lineweaver–Burk plots for enzyme activities are obtained, estimates of the maximum number of binding sites and the K_{CMB} (concentration of mercurial required for half-saturation of these sites)

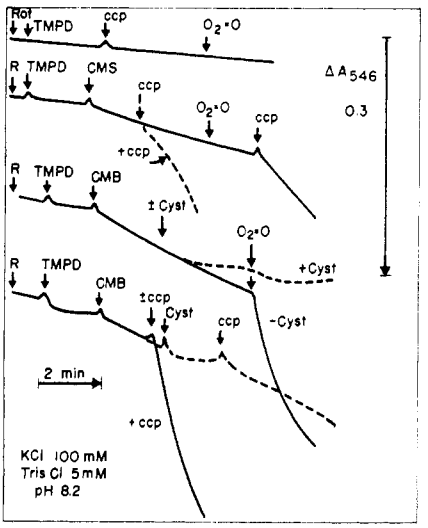

FIGURE 11: Effect of mercurials and uncouplers on swelling of heart mitochondria suspended in 0.1 M KCl at pH 8.2. The experimental conditions were identical with those of Figure 10 with the following additions at the points indicated: rotenone (4 μg/mg), TMPD (0.1 mM), Tris-ascorbate (3.5 mM), CMS and CMB (50 μM), CCP (5×10^{-6} M), and cysteine (100 μM).

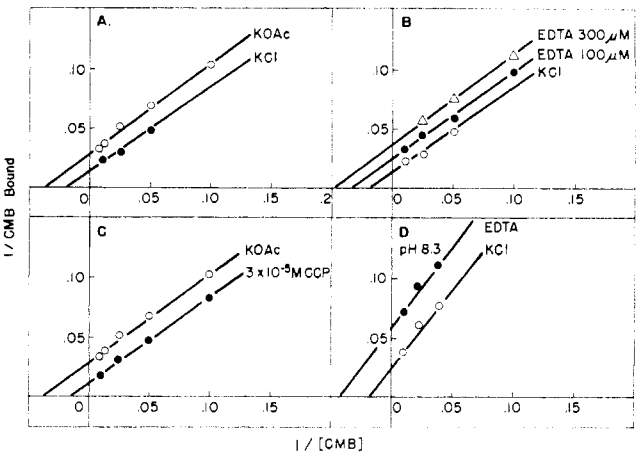

FIGURE 12: Double-reciprocal plots of the uptake of CMB as the concentration of CMB available under a variety of conditions. Mitochondria (2.5 mg of protein) were reacted with the indicated concentration of ^{203}Hg-labeled CMB in 3 ml of either KCl (0.12 M) or K^+-acetate (0.12 M) buffered at either pH 7.1 or 8.3 with 5 mM Tris-chloride or -acetate. The concentration of EDTA was 100 or 300 μM; CCP, 3×10^{-5} M. After 4 min, the suspensions were filtered and binding of mercurial estimated from the decrease in radioactivity of the filtrates.

can be made for each condition. Binding data for CMB under a number of experimental conditions have been analyzed in this way. The resulting plots are reproduced in Figure 12, and the maximum number of binding sites and K_{CMB} are summarized in Table II.

This analysis shows that fewer binding sites are available to the mercurial in the acetate medium, but that the K_{CMB} for these sites is lower than that required for half-saturation of the larger number of sites available in the Cl^- medium. At pH 8.3, the number of available sites in Cl^- is also lower than at pH 7.1, but these sites require 50 μM CMB for half-saturation. Binding of mercurial is strongly inhibited by EDTA as is the mercurial-dependent passive swelling in 0.1 M Cl^-. The data of Table II show that in the presence of the chelator the maximum number of reactive sites is reduced to about 26 mμmoles/mg and that these sites are half-saturated by 20 μM CMB. The increased binding of CMB in an acetate medium which is elicited by ATP and by high concentrations of CCP is also reflected in an increase in the maximum number of binding sites available to the mercurial and in increased K_{CMB} (Table II). Submitochondrial particles show no difference in mercurial binding in KCl as opposed to an acetate medium. These particles bind about 76 mμmoles of CMB/mg of protein when exposed at pH 7.1 to a level of 100 mμmoles of available CMB per mg in either medium. This is reduced to 55 by the presence of EDTA and to about the same level by 3 mM ATP. Treatment of particles which have bound 76 mμmoles of CMB/mg with dithioerythritol results in the removal of about one-third of the mercurial. Binding of mercurial to submitochondrial particles does not appear to be as dependent on pH as is the binding in intact mitochondria (Table II).

Lipid Solubility of CMB and CMS in Various Media. A rough approximation of the lipid solubility of the mercurial species present in the various media employed in the binding and permeability studies can be made by estimating the fraction of the total label which can be extracted into an organic phase. The data of Table III establish that 10–12% of the total mercurial can be extracted into butanol–toluene (Pressman *et al.*, 1967) in 1 min at 25°. In the presence of EDTA, only about one-quarter of this amount can be extracted. At pH 8.3 in Cl^- or in acetate media at either pH 7 or 8.3, no count over background was detected and no CMS was extracted under any condition tried (Table III). Addition of cysteine prevented the extraction of labeled CMB into the organic phase in the Cl^- medium and, if cysteine was added to the aqueous phase after the initial distribution, it removed the mercurial from the organic phase. In contrast, dithioerythritol neither prevented extraction nor removed the mercurial from the organic layer.

Relationship of the Amount of Mercurial Bound to Other Mitochondrial Transport Reactions. It has recently been established that the entrance and exit of inorganic phosphate from the mitochondrial matrix can be blocked by low levels of mercurials (Tyler, 1969; Fonyo and Bessman, 1968). The study shown in Figure 13 establishes that this facet of mercurial activity in the mitochondrial membrane (as evaluated by the inhibition of passive swelling in isotonic ammonium phosphate) involves the uptake of less than 12 mμmoles of mercurial/mg of protein. In agreement with previous studies (Tyler, 1969), we find that this inhibition can be relieved by addition of cysteine or dithioerythritol. In contrast to the level of mercurial uptake which affects the phosphate transporter system, higher levels of bound mercurial are associated with the activation of K^+-acetate accumulation. This energy-dependent uptake was evaluated as a function of CMB bound under the conditions of Brierley *et al.* (1968a) and is also plotted in Figure 13. These data establish that the phosphate transporter system is inhibited 50% by the binding of 7 mμmoles of CMB/mg, whereas the energy-linked K^+-acetate uptake is activated to 50% of maximum by the binding of

TABLE III: Organic Solubility of [^{203}Hg]CMB and CMS.[a]

Medium	pH	Per Cent Total Radioactivity Extracted into Butanol–Toluene (3:7)	
		CMB	CMS
KCl	7.0	10	0
K-acetate	7.0	0	0
KCl	8.3	0	0
K-acetate	8.3	0	
KCl + EDTA (0.3 mM)	7.0	2.5	
NH_4Cl	7.0	12	

[a] The ^{203}Hg-labeled mercurial (100 μM) was added to 3.0 ml of a 100 mM solution of the indicated salt buffered at the pH shown. An equal volume of 1-butanol–toluene (3:7 v/v) was added and the stoppered tubes were shaken for 1 min on a Vortex shaker. The radioactivity of the organic phase was determined and expressed as per cent of the total counts in the tube.

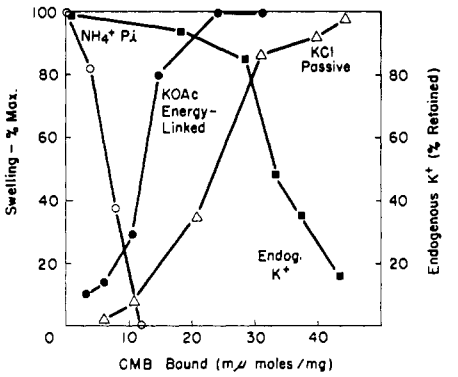

FIGURE 13: Relationship between the extent of mercurial uptake and inhibition or activation of mitochondrial swelling and release of endogenous K^+. Binding of CMB was evaluated in each case by the uptake of the ^{203}Hg-labeled reagent. The open circles show the decreased passive swelling in 0.1 M NH_4^+-phosphate (pH 7.0) as a function of CMB uptake (*cf.* Tyler, 1969). The closed circles show the effect of CMB binding on the uptake of K^+-acetate supported by TMPD–ascorbate (*cf.* Brierley *et al.*, 1968a, Figure 3). The open triangles are the data of Figure 2 plotted as a function of mercurial binding and show the effect of increased CMB uptake on passive swelling in 0.1 M KCl. The squares show the release of endogenous K^+ (initial level 70 mμmoles/mg) as a function of mercurial uptake in a medium of 0.15 M sucrose containing 15 mM Tris-Cl. Mitochondria (5 mg of protein) were treated with rotenone and incubated in a 3-ml total volume for 3 min at 25°, centrifuged rapidly, extracted with 0.5 N $HClO_4$, and the K^+ content of the residue was estimated by atomic absorption.

12 mμmoles of CMB/mg. A comparable titration of the activation of passive swelling in 0.1 M KCl (an indication of permeability to Cl^- as well as to K^+) shows that this reaction is activated to 50% of maximum by the uptake of about 24 mμmoles of CMB/mg.

Release of Endogenous K^+. Beef heart mitochondria suspended in buffered K^+-free 150 mM sucrose in the absence of respiration retain their endogenous K^+ when low levels of mercurial are bound (Figure 13). Little, if any K^+ is released when the particles are reacted with 50 or 100 μM CMS for 3 min in acetate or Cl^- media and little K^+ is lost when low levels of CMB interact with the membrane under these conditions. About 50% of the endogenous K^+ is released, however, when 33 mμmoles of CMB/mg of protein has reacted with the membrane and higher amounts of bound mercurial result in nearly quantitative loss of endogenous K^+.

Discussion

It has been known for some time that CMB and other mercurials can promote swelling of isolated mitochondria (Tapley, 1956; Dickens and Salmony, 1956; Arcos *et al.*, 1967) and result in increased turnover and net loss of endogenous K^+ from mitochondria (Gamble, 1957; Scott and Gamble, 1961). In addition, mercurials have been found to inhibit respiration, uncouple phosphorylation, and interfere with the process of energy transport in submitochondrial particles (Kurup and Sanadi, 1968; Kielley, 1963; Boyer *et al.*, 1966; Minakami *et al.*, 1963). Despite these well-documented pathological effects of mercurials on mitochondrial activities, it has been established in our laboratory that under certain conditions mercurials activate the energy-linked accumulation of Mg^{2+} and K^+ salts by intact mitochondria (Brierley *et al.*, 1967, 1968a). The present studies indicate that rather large differences exist in the amount of a given mercurial which interacts with the mitochondrion under a given set of conditions and that the net response to the addition of one of these reagents can be closely related to the extent of this interaction. The binding of various mercurial reagents to the mitochondrial membrane is highly dependent on (a) the polarity of the reagent, (b) the pH, (c) the anionic composition of the suspending medium, and possibly, (d) on the metabolic state of the mitochondrion as well. This degree of interaction with the membrane is closely related to alterations in membrane permeability and energy-linked ion fluxes. At lower levels of interaction, mercurials block the action of the phosphate transporter system of the membrane (Tyler, 1969; Fonyo and Bessman, 1968). At intermediate levels of binding, the energy-linked accumulation of K^+-acetate is activated; at higher levels of binding, the membrane becomes permeable to Cl^- and releases endogenous K^+. The effects of low and intermediate levels of mercurial binding are largely reversible when cysteine or dithioerythritol is added to compete with the membrane for mercurial binding and it has been established that a portion of the bound mercurial is actually removed from the mitochondrion by these reagents.

The degree of interaction of an organic mercurial with a biological membrane system such as the mitochondrion will depend on the ability of the reagent to penetrate into the membrane. In the present case, the more polar CMS appears to penetrate the membrane to a rather limited extent, since its maximum binding in short-term incubations is consistently on the order of 20 mμmoles/mg of protein or less. A similar situation exists in the red blood cell membrane (Sutherland *et al.*, 1967), in which CMS reacts with only a portion of the membrane thiols in short-term incubations, whereas CMB reacts with a considerably larger number of thiols and affects permeability to cations. The polarity of CMS seems to be the

TABLE IV: Summary of Mitochondrial Responses to the Binding of Organic Mercurials.

Addition	Case I, K^+-Acetate, pH 7, CMS or CMB	Case II, KCl, pH 7, CMS	Case III, KCl, pH 7, CMB	Case IV, KCl (pH 7) + Cysteine (CMB 50% Remov)	Case V, KCl, pH 8.2, CMB or CMS
Amount of mercurial bound (mμmoles/mg)	20	20	40	20	20
Location of mercurial	Exterior	Exterior	External and internal	Largely internal	External
Succinate respiration[a]	Inhibited	Inhibited	Inhibited	Activated	Inhibited
TMPD–ascorbate respiration	Activated	Activated	Activated	Activated	Activated
Passive swelling	No	No	Yes	Yes	Yes
Active swelling	Activated	No	No	No	No
Active contraction	No	No	No	Yes	Yes
Permeable to K^+	*b*	?	Yes	Yes	Yes
Permeable to Cl^-		No	Yes	Yes	Yes[c]
H^+/K^+ antiport	Circumvented by HoAc influx[d]	Active	Circumvented by H^+ or OH^- permeability	Active[e]	Active

[a] In all cases succinate respiration is restored by addition of cysteine. [b] Active accumulation increased, but passive permeability develops only slowly. [c] Permeable to Cl^- in the absence of mercurial at this pH. [d] Similar to the situation outlined in Figure 14 of Brierley (1970). [e] Similar to the situation outlined in Figure 13 of Brierley (1970).

overriding feature of its reaction with the mitochondrial membrane, since its ability to interact is not affected by the anionic composition of the medium, by the metabolic state of the mitochondrion, or by pH in the range from 6.5 to 8.0. This reaction of CMS with mitochondrial thiols can be readily reversed by addition of cysteine, a nonpenetrating thiol compound, and can be presumed to involve interaction with the most exposed surfaces of the membrane. The outer membrane of the mitochondrion appears to contribute very little to this reaction, since very low titers of mercurial binding affect the phosphate transporter system which is localized in the inner membrane (Tyler, 1969). Submitochondrial particles take up nearly as much CMS as CMB under a variety of conditions, however, a result which indicates that there is little barrier to the penetration of the reagent in these particles. The thiols on the exterior of the coupling membrane which are available to CMS may be equivalent to the "fast-reacting" thiols noted in the experiments of Riley and Lehninger (1964), which were closely related to mitochondrial swelling.

The interactions of CMB with the mitochondrial membrane is a much more complex reaction. The strong dependence of the binding of CMB on the composition of the suspending medium suggests that changes in the predominant ligands present around the Hg atom determine its reactivity with potential sites in the membrane (for a review of the chemistry of organic mercurials, see Webb, 1966). The present results certainly indicate that the reaction in the presence of at least 10 mM Cl^- or Br^- is considerably more extensive than in other suspending media. One striking property of media which promote high reactivity is the enhanced lipid solubility of the CMB (Table III). It appears possible that the Cl^- complex permits much greater penetration of the reagent through the lipid barrier of the membrane. However, further work will be necessary to establish the exact significance of the altered binding of CMB to the mitochondrion as a function of the anionic composition of the medium.

Regardless of the chemical explanation for the altered reactivity with these various conditions, certain responses to the increased alteration imposed by the higher binding of CMB can be clearly defined. The situation is again analogous to that of the erythrocyte (Sutherland *et al.*, 1967) in that increased permeability to cations is observed with increased mercurial binding. At higher levels of interaction the membrane also becomes permeable to anions such as Cl^-. The level of bound mercurial also has characteristic effects on energy-linked swelling and contraction of mitochondria on respiration rates, and, as will be developed in detail in a later manuscript (K. M. Scott and G. P. Brierley, in preparation), on ATPase activity.

Several different experimental situations have been defined with regard to respiration-dependent mitochondrial swelling and shrinking. As was the case for valinomycin-induced swelling in KCl (Brierley, 1970) and uncoupler-induced swelling in NH_4Cl (Brierley and Stoner, 1970), the chemiosmotic hypothesis of Mitchell (1966; Mitchell and Moyle, 1969a,b) has proven most helpful in explaining the observed results and will be used as the model in the discussion which follows. It should be emphasized, however, that the present results are by no means conclusive enough to rule out other models. Five different responses can be defined experimentally and are summarized in Table IV.

Case I. ACETATE MEDIUM AT NEUTRAL PH. Under these

conditions, both CMS and CMB in concentrations of 100 μM and below result in low levels of mercurial binding (20 mμmoles or less) and the binding appears to be largely with the most exposed population of thiols. Addition of mercurial to mitochondria respiring with succinate or endogenous substrates in this medium results in first an activation of energy-linked K^+ and acetate accumulation which is followed by inhibition of respiration (Brierley *et al.*, 1968a), and consequent inhibition of ion movements. TMPD–ascorbate respiration is activated by mercurials and supports extensive ion accumulation in this medium (Brierley *et al.*, 1968a). Cysteine reverses the inhibition of succinate respiration and the activated uptake of ions. The mercurial appears to cause an increased permeability to K^+ in this set of experiments which is analogous to that induced by the artificial ion carrier valinomycin (*cf.* the model in Figure 14 of Brierley, 1970) and the modification can be reversed upon removal of the mercurial to reestablish the normal low permeability to K^+.

Case II. CHLORIDE MEDIUM AT NEUTRAL PH WITH LOW MERCURIAL UPTAKE. Under these conditions CMS or very low concentrations of CMB (15 mμmoles of bound mercurial/mg or less) result in inhibition of succinate respiration, activation of TMPD–ascorbate respiration, and little swelling in the presence or absence of respiration. The situation here is quite like that of case I, except that the failure of Cl^- to penetrate limits the swelling. The excess entering K^+ could exchange for metabolic H^+ on the exchange diffusion system (K^+/H^+ antiport of Mitchell, 1966) and result in activated respiration with TMPD–ascorbate.

Case III. CHLORIDE MEDIUM, NEUTRAL PH, HIGH MERCURIAL UPTAKE. Under these conditions the membrane becomes permeable to both K^+ and Cl^- after a lag period which can be shortened by the energy-dependent uptake of K^+ in the initial stages. TMPD–ascorbate respiration is activated but does not appear to be coupled to ion movements in either direction after the first minute or so of incubation. In this condition the mitochondrion may be regarded as uncoupled in every sense since no respiration or ATP-dependent reactions can be demonstrated. It appears possible that this level of mercurial produces a permeability to H^+ or OH^- which would be equivalent to the presence of a proton conductor such as dinitrophenol.

Case IV. CYSTEINE ADDED TO SWOLLEN MITOCHONDRIA OF CASE III. Under these conditions the exterior or most exposed mercurial is removed from the membrane by the thiol but a large portion (about 50%) of the thiols of the membrane remain modified. This treatment results in energy-linked ion extrusion and contraction which can be supported by either succinate or TMPD–ascorbate respiration. The blocks of respiration and the supposed H^+ permeability of case III are removed by this treatment. The membrane remains permeable to both K^+ and Cl^-, however, since passive swelling predominates as soon as respiration ceases. Addition of either CMS or CMB to the exterior thiols reestablishes the conditions of case III. The respiration-dependent contraction and ion extrusion resemble those seen in the contraction cycle of valinomycin-treated mitochondria (Brierley, 1969a,b) and can be explained by the action of the K^+/H^+ antiport bringing the H^+ generated by respiration into the interior as shown in Figure 13 of Brierley (1969b).

Case V. CHLORIDE MEDIUM, PH 8.3. Under these conditions the response is similar to that of case II with the increased permeability of Cl^- at high pH superimposed. Low levels of mercurial binding are observed, as is activated respiration with TMPD–ascorbate, but swelling does not occur until respiration is cut off. It appears that the passive influx of K^+ and Cl^- is balanced by the extrusion of K^+ and influx of H^+ by exchange diffusion through the H^+/K^+ antiport. If respiration ceases, or if a proton conductor is added, this respiration-dependent contracting reaction would be circumvented and the high permeability of the membrane to K^+ and Cl^- would result in passive osmotic swelling.

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Quantitative Dissolution of the Membrane and Preparation of Photoreceptor Subunits from *Rhodopseudomonas spheroides**

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ABSTRACT: An effective and optimal combination of three reagents commonly used for modifying the structure of proteins has been developed for causing quantitative dissolution of membrane structure. Alkaline solution, 1.5% Triton X-100, and 6 M urea at 0° for about 2 hr causes quantitative conversion of chromatophores from wild-type *Rhodopseudomonas spheroides* into Triton–protein complexes having particle weights of less than 200,000. No trap activity is lost as evidenced by light-induced absorbance and electron paramagnetic resonance changes. Good rates for photoxidation of exogenous cytochrome *c* also are demonstrated. Especially significant is the maintenance of a near-normal absorbance spectrum in both the bacteriochlorophyll and carotenoid regions in spite of the marked conversion into smaller units. Also, of possible importance and utility is the ability to cause reaggregation of the small units into chromatophore-like structures by the removal of excess detergent. Evidence is given for nearly total lipid displacement by the alkaline–urea–Triton treatment and subsequent sucrose density gradient centrifugation.

In the past 10 years much progress has been achieved in understanding the *in vivo* nature of the functioning light trap in bacterial photosynthesis. The following characteristic physical parameters can be associated with a functioning trap.[1] (1) Photoxidation of the primary electron donor molecule is attended by a loss in absorbance at wavelengths in the near-infrared region where bacteriochlorophyll has an intense band (Duysens, 1952; Duysens *et al.*, 1956; Clayton, 1962a,b, 1966), and by the formation of an unpaired electron (Androes *et al.*, 1962; Calvin and Androes, 1962) characteristic of a chlorophyll free radical (Mauzerall and Feher, 1964; Mauzerall, 1968; Felton and Linschitz, 1966; Fuhrhop and Mauzerall, 1968, 1969) which is easily measured by electron paramagnetic resonance. (2) Both measurable parameters referred to above can be easily reproduced in the dark by chemical oxidation (Goedheer, 1959a,b; Kuntz *et al.*, 1964; Loach *et al.*, 1963) with a midpoint potential of +0.44 V (Kuntz *et al.*, 1964; Loach *et al.*, 1963). (3) These normal light responses shown by all photosynthetic systems of bacterial origin, as well as "system I" of green plants and algae (Kok, 1956, 1961; Witt *et al.*, 1961; Beinert *et al.*, 1962; Beinert and Kok, 1964; Loach *et al.*, 1963), are also reversibly quenched at lower potentials (Kuntz *et al.*, 1964; Loach, 1966; Loach *et al.*, 1963, 1968). (4) The quantum yield for photoxidation of the primary electron donor has been accurately established by both measurable parameters and is the idealized value of 1.0 (Loach *et al.*, 1967, 1968; Parson, 1968; Beugeling, 1968; Bolton *et al.*, 1969); (5) The same light responses can be demonstrated to occur with good efficiency at temperatures so low that few molecular motions are possible (Arnold and Clayton, 1960; Androes *et al.*, 1962; Calvin and Androes, 1962), and the light-induced absorbance changes occur as rapidly as measuring devices can follow (Parson, 1968; Ke, 1969).

It now seems appropriate, therefore, to use these measurable parameters as an assay for a functioning light trap during simplification and purification procedures. In the past, less direct assays had to be used which admitted to the possibility of measuring nonbiological activities. The high concentrations of very reactive light absorbers such as carotenoids and chlorophylls can always complicate the measurement of physiologically authentic secondary reactions. Careful measurements of the quantum yields for such secondary reactions as pyridine nucleotide reduction are necessary before a normal biological role can be ascribed to them.

For the studies reported in this paper, we attempted to apply all of the following criteria as our assay for a functional

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[1] Abbreviations and definitions used are: AUT is used to denote those particles prepared using a combination of alkaline solution, high urea concentration, and the detergent Triton X-100. Trap is defined as that molecular site where absorbed light energy first causes the formation of oxidized and reduced molecular species. Photoreceptor subunit is defined as a protein–bacteriochlorophyll–trap (and possibly carotenoid) complex thought to exist as one of many different kinds of subunits in the membrane and isolable as a distinctive entity.