Hydrogen Ion Concentration Changes in Mitochondrial Membranes

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SUMMARY

1. Bromthymol blue added to rat liver mitochondria at concentrations in the range 0.4 to 4.0 mmoles per mg of protein is tightly bound as indicated by centrifugation and washing of the mitochondria.

2. The absorbance of the dye is increased on adding Ca++ or Mn++ to the mitochondria in the absence of a permeant anion. The extent of increase is 0.03 cm⁻¹ for 167 μm Ca++ at 618 μm for 0.5 mmole of bromthymol blue per mg of protein.

3. The spectrum of the indicator bound to the mitochondria has shifted only slightly to longer wave lengths than in the solution. The indicator shows very nearly the same absorbance changes on alkalinization of the mitochondrial suspension as it does upon addition of small amounts of calcium to the mitochondria.

4. The absorbance increase is independent of the pH of the suspension medium (6.5 to 7.4) and of its buffer capacity from 2.0 mM to 0.3 M sodium succinate.

5. At low protein concentrations (1 to 2 mg per ml), the kinetics of the absorbance increase is compared with the glass electrode recordings and indicates an equal and opposite pH change in the suspension medium of a similar time course.

6. At higher protein concentrations (5 to 10 mg per ml), a similar phenomenon is observed, but only after several prior additions of Ca++; endogenous material (termed H⁺) present, in terms of Ca++, at levels of approximately 40 mmoles per mg of protein counteracts the initial absorbance increase. A small amount of added phosphate readily serves as H⁺, and endogenous phosphate at 8 mmoles per mg of protein would afford an appropriate source of H⁺.

7. The absorbance increase on calcium additions with bromthymol blue as an indicator can be counteracted and even reversed with a 30-fold excess of bovine serum albumin, suggesting that the indicator can be removed from the mitochondrial membranes.

8. The Ca++-induced absorbance increase is nullified rapidly by additions of permeant anions such as phosphate or acetate. Ammonium ions at a 3 mM concentration do not prevent the acidification caused by acetate, and 0.1 M concentrations only slightly facilitate the alkalinization on addition of Ca++.

9. It is concluded that rapid accumulation of a cation such as Ca++ in the absence of a permeant anion can result in an intramitochondrial alkalinization and a pH gradient of about 1 pH unit across the mitochondrial membrane. This gradient develops only as a consequence of cation accumulation in the absence of a permeant anion. In the presence of permeant anions, particularly phosphate under the conditions of oxidative phosphorylation, there is no evidence of a measurable pH gradient across the mitochondrial membrane.

10. The intramitochondrial location of the indicator and of the site of calcium accumulation is tentatively assigned to the intracristal space because of the insensitivity of the bromthymol blue response to changes of osmolarity from 0.1 to 0.8 M.

11. The reaction mechanism for cation accumulation based upon the reaction with the X ~ I intermediate of energy transfer meets the needs of the available experimental data.

The energy-linked reaction of mitochondria with cations is generally studied in phosphate-containing media, and many properties of this reaction have been observed (1–3). Proton ejection from mitochondrial membranes accompanies cation accumulation and can readily be recorded with sensitive glass electrode techniques (1, 2). More recent studies show cation accumulation and proton ejection in the absence of added phosphate. In the presence of other permeant anions such as acetate (4, 5), proton ejection is diminished and the mitochondria swell considerably due to the accumulation of calcium acetate as a soluble salt and the entrance of water into the mitochondria (4, 5). A current mechanism proposes that the uptake of calcium in the absence of a permeant anion leads to the formation of hydroxide ions in the mitochondria (6) causing alkalinization of their interior and resulting in an inhibited state of ion uptake and respiration (7). If a permeant anion such as acetate is present, the soluble calcium acetate is formed in the matrix space, the alkalinity neutralized, and the respiration reactivated (8, 9). The exploration of the important relationship of external pH changes to the internal pH depends upon the development of a suitable technique for measurements of intramitochondrial pH.

For the measurement of external pH changes in a suspension of guinea pig kidney mitochondria, we have previously used bromthymol blue at about 10 μmoles per mg of protein in a phosphate-containing medium (3). It is now found that
bromthymol blue is tightly bound by rat liver mitochondria at \( \sim \) 5 mmole per mg of protein. Under these conditions BTB acts as a colorimetric indicator of acidification and alkalinization in the interior of the mitochondria. The method gives information on “the other half” of the ion uptake reaction and shows properties of the mitochondrial membrane across which a pH gradient exists.\(^2\)

**PREPARATIONS**

Rat liver and pigeon heart mitochondria were prepared as described for previous experiments (11, 12). The mitochondria were prepared in \( 10^{-4} \text{ M} \) EDTA which was removed by two washings in the last stages of the preparation. The Ca\(^{++}\) content of the mitochondria was \( \sim \) 5 mmole per mg of protein. A supplementary test is used to detect calcium-deficient mitochondria; addition of 20 mm acetate to the suspension in mannitol-sucrose-Tris reaction medium should show no swelling (4, 5). The nature of the buffer system employed is of critical importance in these studies; chloride may be used as the impermeant anion (4, 5) with 0.225 \text{ M} \text{mannitol} and 0.075 \text{ M} \text{sucrose} for osmotic balance. In a few experiments, 0.15 \text{ M} \text{KCl} was also found to be satisfactory for this purpose. It was desirable to vary the buffer strength over a wide range in order to test whether the pH changes inside the mitochondria were dependent upon the buffer strength outside the mitochondria. A simpler reaction medium and the one in which optimal responses to Ca\(^{++}\) were observed consisted simply of sodium succinate; as we have reported, succinate is relatively ineffective as a permeant anion (4, 5). In other cases it is possible that buffer cations or anions participate in the Ca\(^{++}\) accumulation reaction.

The pH of the mitochondrial suspension was monitored by Leeds and Northrup electrodes connected with a special modulating circuit which allowed the measuring circuit to “float” with respect to the circuit for the platinum electrode. Oxygen concentration was continuously monitored by means of a vibrating platinum electrode (13) which provided stirring for the solution as well. The measuring circuit for the platinum electrode was also isolated from “ground” in order to avoid interference with the recording of the glass electrode so that either electrode could be made to “float” electrically. Usually, the reference electrode for the pH circuit was grounded. In the dilute buffers, the excursions of pH were fairly large but were continuously monitored.

**Indicators**—Three indicators were employed; two of them, bromthymol blue and bromcresol purple (14), are colorimetric; the third, 4,1,3-methylumbelliferyl-7-hydroxycoumarin (15), is fluorometric. The colorimetric indicators were the most satisfactory, since their absorption maxima occurred at wave lengths where the cytochrome interference either was minimal or could readily be avoided by choice of an appropriate reference wave length. The fluorometric indicator was excited at 366 mp. This shift is somewhat greater than that caused by binding to mitochondria. The absorption spectra of 3.3 \muM BTB in \( 20 \text{ mm mannitol-sucrose-sodium succinate} \) medium in the presence and absence of rat liver mitochondria. The \( \text{pK} \) of the indicator under our experimental conditions is 7.2 and is shifted to 8.0 in the presence of rat liver mitochondria. This shift is somewhat greater than that caused by binding to three proteins, bovine serum albumin, the coupling factor \( F_1 \), or a mitochondrial supernatant solution. The interpretation of the shift of the \( \text{pK} \) of the indicator in relation to the experiments described here is presented in the “Discussion.”

The possibility that the BTB participates in oxidation reduction under these conditions seems unlikely due to its stability in the presence of a strong reducing agent such as dithionite. Another meaningful control was provided by the fact that the addition of anions such as phosphate or acetate, which would not be likely to participate in oxidation-reduction reactions,

**RESULTS**

The positions of the absorption bands of BTB have been examined under various conditions to determine the extent to which its optical properties are altered on binding to the mitochondria. The absorption spectra of 3.3 \muM BTB in the presence of 3.0 mg of mitochondrial protein per ml were published recently (10), and the spectra show that as the pH was raised from 6.9 to 8.9, corresponding increases in the absorbance of the dye at 618 \text{ mp} were recorded. The isosbestic point is near 500 \text{ mp}.

A comparison of this spectrum with standard curves for the indicator (14) shows no significant change in either the absorption maximum or the isosbestic point between the acid and basic forms of the indicator.

The extinction coefficient of the dye in the presence of the mitochondria is 38 \text{ cm}^{-1} \text{ mm}^{-1}, somewhat increased because of multipath light scattering in the mitochondrial suspensions over the value in solution of 23 \text{ cm}^{-1} \text{ mm}^{-1}.

The \( \text{pK} \) of the indicator is shifted to the alkaline side of pH 7, and this point is amplified by Table I which compares the \( \text{pK} \) of 3.3 \muM BTB in 20 mm mannitol-sucrose-sodium succinate medium in the presence and absence of rat liver mitochondria. The \( \text{pK} \) of the indicator under our experimental conditions is 7.2 and is shifted to 8.0 in the presence of rat liver mitochondria. This shift is somewhat greater than that caused by binding to three proteins, bovine serum albumin, the coupling factor \( F_1 \), or a mitochondrial supernatant solution. The interpretation of the shift of the \( \text{pK} \) of the indicator in relation to the experiments described here is presented in the “Discussion.”

**Table I**

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>( \text{pK} )</th>
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</thead>
<tbody>
<tr>
<td>Mannitol-sucrose sodium-succinate, 20 \text{ mm}</td>
<td>7.2</td>
</tr>
<tr>
<td>+BSA, 10.0 mg per ml</td>
<td>7.6</td>
</tr>
<tr>
<td>+( F_1 ), 1.1 mg per ml</td>
<td>7.5</td>
</tr>
<tr>
<td>+Supernatant solution from mitochondria(^a)</td>
<td>7.3</td>
</tr>
<tr>
<td>+Rat liver mitochondria, 2.5 to 4.8 mg of protein per ml</td>
<td>8.0</td>
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</table>

\(^a\) Obtained by 30-min centrifugation at \( 10^4 \times g \) of sonically disrupted rat liver mitochondria (45 sec at 3 amp).
cause a reversal of the spectroscopic effects resulting from calcium accumulation.

Binding Constants for Indicator—The high affinity of BTB for hemoglobin, myoglobin, and BSA has already been reported by Antonini et al. (17), who used equilibrium dialysis to determine binding constants. In these studies we were hesitant to assume that the mitochondria would be sufficiently stable for the equilibrium dialysis method and instead have employed centrifugation and filtration techniques. The latter method seemed to be the simplest and was studied in some detail. However, it was found that a considerable amount of BTB was retained on the Millipore filters (0.5 μ) and thus the filtrate considerably underestimated the free BTB. For this reason, the Millipore filtration method had limited usefulness in these studies. For small scale centrifugation the Coleman microfuge was employed in a number of cases, and reasonably consistent results were obtained. However, supernatants after 5 min of centrifugation were not completely clear. Clear separations were obtained by a 10-min centrifugation of 3 ml of mitochondrial suspension at 7700 × g at 0°. It is likely, however, that the centrifugation procedure underestimates the binding of BTB due to the packing of the mitochondria in the sediment. Supernatant fluids were then made alkaline to pH 9.0 to give the maximum indicator color. Optical measurements of the BTB absorption in these supernatant fluids were made at 618 μm with the use of 700 μm as the reference wave length in the double beam spectrophotometer. Where sediments were assayed, they were clarified by the addition of Triton X-100 and made alkaline to pH 9.

The results are given in Table II.

At the protein concentrations at which the bulk of the experiments of this paper are carried out (4 to 6 mg of protein per ml), the binding in the absence of calcium is approximately 80% (10). However, addition of calcium will increase this binding to approximately 90%. A binding constant may be computed from the intersections of the two limbs of the curves of Fig. 3A of Reference 10 to be about 2 µmole of BTB per mg of protein, about 10 times the cytochrome c content of the mitochondria (10). It is a matter of interest that the binding is as selective as this, since BTB is known to bind to many types of protein (17). The dissociation constant of BTB at least on the steep portion of the curve of Fig. 3A of Reference 10 is considerably less than 10-4 M.

### Table II

<table>
<thead>
<tr>
<th>Osmolarity</th>
<th>Protein</th>
<th>Type of material</th>
<th>Experimental conditions</th>
<th>Total BTB bound to washed mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>m</td>
<td>mg/ml</td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>0.3</td>
<td>6.0</td>
<td>Washed mitochondria</td>
<td>No added Ca++</td>
<td>82</td>
</tr>
<tr>
<td>0.3</td>
<td>6.0</td>
<td>Washed mitochondria</td>
<td>330 µM Ca++</td>
<td>90</td>
</tr>
<tr>
<td>0.3</td>
<td>6.0</td>
<td>Washed mitochondria</td>
<td>330 µM Ca++ + 1 mM Pi</td>
<td>85</td>
</tr>
<tr>
<td>0.3</td>
<td>6.0</td>
<td>Washed mitochondria</td>
<td>330 µM Ca++ + 3 mM AcO⁻</td>
<td>83</td>
</tr>
<tr>
<td>0.1</td>
<td>1.6</td>
<td>Washed mitochondria</td>
<td>330 µM Ca++</td>
<td>83</td>
</tr>
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<td>0.8</td>
<td>1.6</td>
<td>Washed mitochondria</td>
<td>330 µM Ca++</td>
<td>76</td>
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<tr>
<td>0.3</td>
<td>6.0</td>
<td>Submitochondrial particles</td>
<td>No added Ca++</td>
<td>80</td>
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</table>

The decrease of binding by 7% in 3 mM phosphate is much too small to eliminate the response of the indicator to intramitochondrial alkalinization. Two additional experiments are indicated to show the effect of osmolarity upon the binding of the indicator. Here the protein concentration is dropped to 1.6 mg per ml so that appreciable dissociation occurs; nevertheless the change of binding over this 8-fold range of osmolarity is only 7%.

Comparison of Changes of BTB and BCP—The anomaly which resulted in the content of this paper is shown in Fig. 1, A and B. This figure illustrates serial additions of 167 µM calcium to a suspension of mitochondria (approximately 5 mg of protein per ml suspended in mannitol-sucrose-20 mM sodium succinate buffer, pH 6.5, 0.3 M mannitol-sucrose; 25° (Experiment 1038-8, 6 IV).

![Fig. 1. A comparison of the responses of 8.7 µM BCP (A) and 3.2 µM BTB (B) to serial additions of 167 µM Ca++. Followed by the addition of 1 mM phosphate. Time proceeds from left to right in each diagram. The traces are labeled O₂, pH, BCP or BTB, and L.S. (light scattering). Calibrations are opposite the appropriate trace; absorbance changes for BCP or BTB are given in logarithmic units (0.022 cm⁻¹ per division) while the light scattering data are given as a percentage (34% per division). Increased absorbance at 560 and 618 μm is recorded as an upward deflection of the trace. Light scattering is recorded at 640 and 700 μm, and an increased transmission corresponding to decreased light scattering is recorded as an upward deflection. Rat liver mitochondria, 6.5 mg of protein per ml, 2 mM sodium succinate buffer, pH 6.5, 0.3 M mannitol-sucrose; 25° (Experiment 1038-8, 6 IV).](http://www.jbc.org/)
electrode recording is diminished by 50% by the addition of 1 mM phosphate. Considerable swelling of the mitochondria ensues after 30 sec, as indicated by the upward deflection of the bottom trace (LS) recording a decrease of light scattering at 640 μm. The light scattering change is so great that the 580 to 640 μm trace deflects downward. Here, a total of approximately 130 μmoles of Ca++ per mg of protein has been added to the mitochondria, a relatively heavy load in the absence of a permeant anion. Consequently, phosphate addition leads to irreversible swelling after a short interval.

The experiment is now repeated with 3.3 μM BTB, a concentration roughly one-third that of the BCP used in Fig. 1A, and at wave lengths (618 - 700 μm) appropriate to this indicator. The sensitivity to absorbance change is retained at 0.022 per mM trace deflects downward. Here, a total of approximately 130 μmoles of Ca++ per mg of protein has been added to the mitochondria, a relatively heavy load in the absence of a permeant anion. Consequently, phosphate addition leads to irreversible swelling after a short interval.

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The first, it is unexpected that BTB should act as an indicator at all; its pK is 8.0 under these conditions and the pH of the medium is 6.4; thus, color changes of the indicator would scarcely be expected. Second, the observed absorbance change at pH 6.5 is 10 times that of an equal concentration of BCP. Third, an increasing absorbance at 618 μm is observed. Since all of the conditions except the nature and pK of the indicator were similar in the two experiments, and since the response of BCP is the same as that of the glass electrode (10) and that of BTB opposite to it, we shall interpret the increased absorbance of the BTB on adding calcium as an alkalinization of the interior of the mitochondria, thus balancing the observed acidification of the external medium, an expected relationship in the absence of a permeant anion. It further follows that most of the BTB is within the space in which the cations are being accumulated; that which is in the external medium would respond in the opposite direction. This working hypothesis will be further justified in the discussion of the experimental data which follows. In summary, BTB not only responds in the direction opposite to that of BCP, but also shows a quantitatively greater effect.

The alkalization inside the mitochondria following the first addition of calcium is not constant, but returns in an interval of about 15 sec to almost the same value prior to the addition of calcium. This is attributed to small amounts of permeant anions in the mitochondrial suspension, probably phosphate (see below). Since the substance neutralizes the alkalinity, it is termed the endogenous acid, H+. The alkalization inside the mitochondria following the first addition of calcium is not constant, but returns in an interval of about 15 sec to almost the same value prior to the addition of calcium. This is attributed to small amounts of permeant anions in the mitochondrial suspension, probably phosphate (see below). Since the substance neutralizes the alkalinity, it is termed the endogenous acid, H+.

The second and third additions of calcium show a much more striking response; the alkalization is larger and the H+ reaction is almost completely absent. Other experiments in which a lower protein concentration is employed show even less H+ (see below). It is then possible to titrate the mitochondria with additions of calcium leading to increased alkalinization, until a plateau is approached on the fifth addition.

Following calcium addition, 1 mM phosphate is added. In the case of BCP (8.7 μM) there is a slight jog of the BCP trace in the direction of alkalinization, suggesting that some hydroxyl ions came out of the mitochondria. But there is a net displacement of the trace; phosphate addition returns the trace by only one-fifth of the total change caused by five additions of calcium. With BTB (3.3 μM) the effect is more striking. Addition of phosphate decreases the absorbance to the level obtained prior to the addition of calcium, and there is no net displacement of the trace from the start of the calcium titration. This is consistent with the suppression that practically all of the BTB is indicating pH in the space where the calcium is accumulated, and that phosphate addition has completely neutralized the alkalinity of this space. Respiration, inhibited by the calcium addition, is reactivated by phosphate addition (7). Again, the mitochondria become unstable shortly after the addition of phosphate, and large amplitude swelling occurs, as indicated by the bottom trace (LS) of Fig. 1B.

Fig. 2 affords a graphic illustration of the differences in the responses of BCP and BTB at two buffer strengths. The experimental conditions are similar to those of Fig. 1 except that Fig. 2 provides, in addition to the titration in 2.0 mM sodium succinate (circles), a titration in 20 mM succinate (squares), both at pH 6.5. The stronger buffer holds the external pH within tighter bounds, as indicated by the halving of the absorbance decrease of BCP at 580 μm. The BTB change is larger in the higher, than in the lower, buffer strength, a further indication that it is an "internal" response. This effect is more fully explained below. Thus, the responses of the two indicators are opposite not only in sense, but also in response to a change of buffer strength.

**Spectroscopic Data**—In order to determine whether the indicator color in the calcium-supplemented mitochondria corresponds to a different species of the indicator than that observed in Fig. 2, we have published spectra elsewhere (Fig. 1, A and B, of Reference 10). The split-beam spectrophotometer has been employed to plot the absorbance changes caused by the addition of calcium to mitochondrial suspensions supplemented with BTB as in the case of Fig. 1. The data clearly indicate an increase of BTB absorption with Ca++ addition. The maximum of absorption in the presence of Ca++ is at 622 μm, a shift of approximately 5 μm from spectra in the absence of Ca++. The isosbestic point in the presence of Ca++ is at a somewhat longer wave length, 515 μm, as compared with 500 μm in the absence of Ca++. The correspondence of the two spectra is sufficiently close to indicate that the BTB color change caused by Ca++...
Intramitochondrial pH Changes

is the same as that caused by pH changes. The absorbance shifts are probably due to slight differences in light scattering in the two cases. That there is no direct combination of calcium with the indicator is verified by independent controls which indicate no reaction of calcium and BTB under the conditions of our experiment.

The fact that the indicator color is abruptly diminished by the addition of phosphate (Fig. 1) or acetate (Fig. 8) supports the identification of color change with a pH change.

Effect of pH and Buffer Capacity on Indicator Response—Fig. 3 affords a comparison of the response of BTB at pH 6.5 (20 mM succinate) and pH 7.4 (2 mM and 20 mM succinate) to additions of calcium in a suspension of rat liver mitochondria. The mitochondrial protein concentration was 0.5 mg per ml; the concentration of BTB was 3.3 µM. In the three charts, the four traces record, respectively (top to bottom): oxygen concentration, extramitochondrial pH according to the glass electrode, intramitochondrial pH according to BTB, and light scattering, as indicated by measurements at 700 µm. In Fig. 3A, sodium succinate acts as an effective buffer at pH 6.5, and the first few additions of 167 µM calcium cause an ejection of mitochondrial protons into the suspension medium of approximately 0.05 pH unit each; the H⁺:Ca⁺⁺ value is approximately 0.7. In the three charts, the four traces record, respectively (top to bottom): oxygen concentration, extramitochondrial pH according to the glass electrode, intramitochondrial pH according to BTB, and light scattering, as indicated by measurements at 700 µm. In Fig. 3A, at pH 7.4 where the buffer capacity is much less, the pH change is almost double. In Fig. 3B, in 2 mM sodium succinate at pH 7.4, the corresponding pH change has increased to 0.25 unit. The ratio of buffer capacities is approximately 5:1 in Fig. 3, A to C. In spite of these differences in the external medium, the H⁺:Ca⁺⁺ value is higher (approximately 1.8) at pH 7.4. The response of BTB is very similar in the three cases; the first addition being largely suppressed by the endogenous H⁺; with later additions titrating to a plateau of optical density change of approximately 0.04 cm⁻¹. The internal response seems more nearly invariant than the external response of H⁺. Phosphate addition, in the three cases, brings the optical density change within 10% of that observed in the control experiment. Thus, it is not essential to have the indicator present before the addition of calcium in order to have an indication of the pH in the mitochondrial space.

Effect of External pH Changes—It is apparent in Fig. 3 that the increase of the buffer capacity allows a greater displacement of the intramitochondrial pH due to proton ejection in Ca⁺⁺ accumulation. In Fig. 3, A, B, and C, respectively, Ca⁺⁺ accumulation gives pH changes in the external medium of 0.13, 0.25, and 0.40 pH unit. Additions of 167 µM H⁺ and measurement of the pH changes and the BTB change allow a correction of the BTB traces for the external change during Ca⁺⁺ accumulation to be 0.004, 0.0066, and 0.015 cm⁻¹ for Fig. 3, A, B, and C, respectively. Thus, the optical density change per unit of Ca⁺⁺ addition is very nearly independent of pH and buffer strength over the range studied. In detail, the total changes are 0.053, 0.057, and 0.062 cm⁻¹ (an average of 0.057 cm⁻¹), and the maximum change for 167 µM Ca⁺⁺ is 0.026, 0.083, and 0.029 cm⁻¹ or an average of 0.029 cm⁻¹. It is further apparent from Fig. 3C that an external pH change of 0.26 unit at pH 7.0 is required to shift the intramitochondrial pH as much as does the accumulation of 167 µM Ca⁺⁺ (30 mmoles per mg of protein). The total intramitochondrial shift involved in these Ca⁺⁺ titrations is equivalent to 1 unit of external pH change.

In more highly buffered systems, for example 300 mM sodium succinate (mannitol and sucrose being omitted), or in the presence of 0.1 M ammonium chloride, pH changes recorded by the glass electrode fell in approximately 0.02 unit, but the BTB response was very similar to that indicated in Fig. 1. Thus, no measurable change in the pH of the external medium need accompany the intramitochondrial BTB response.

Effect of Mitochondrial Concentration—Fig. 4 compares (A) the BTB response of dilute mitochondria (0.7 mg of protein per ml) to the first addition of Ca⁺⁺ with (B) that of more concentrated mitochondria (4 mg of protein per ml) to the second

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Fig. 3. The effect of pH and buffer strength upon the response of BTB to serial additions of 167 µM Ca⁺⁺. The conventions are similar to those of Fig. 1; 6.5 mg of protein per ml. The sodium succinate concentrations are indicated in the diagram as are the pH values (Experiment 1039 1, 2, 3 IV).
addition of Ca++. When Fig. 4A is corrected by means of NaOH addition for the effect of external pH change, 167 \( \mu \text{M} \) Ca++ gives 0.035 cm\(^{-1}\) for the BTB change for the two values of protein concentration. Thus, the absorbance change is constant over a range of Ca++ uptake from 100 mmoles per mg of protein to 40 mmoles per mg of protein.

The record (4) further indicates that a large response may be obtained to the first addition of Ca++ if the mitochondrial protein concentration is as low as 1 mg per ml.

These relationships are clarified for a variety of protein concentrations in Fig. 5, where the changing nature of the titration curve with varying protein concentrations is shown. At low protein concentrations, a rectangular hyperbola is obtained, while at higher protein concentrations a sigmoid curve appears. The amplitude of the curves decreases because of acidification of the external medium by H\(^+\) ejection in Ca++ accumulation as illustrated in Fig. 4. The directly determined absorbance changes are plotted here, and when this effect is corrected as above for the external pH changes, the plateau values of the curves and their maximum slopes are similar as in the case of Fig. 4. Fig. 5 affords an opportunity to calculate the amount of H\(^+\) from the intersection of the abscissa and the maximum slope of the curves at the two higher protein concentrations. These intercepts indicate that the endogenous H\(^+\) is equivalent to Ca++ at a concentration of about 40 mmoles per mg of protein (see "Discussion").

The optical measurements of the previous figure give no evidence of extramitochondrial BTB, even at the lowest protein concentration employed (1 mg per ml).

**Effect of Bovine Serum Albumin on Bromthymol Blue Response—**

By binding BTB to added BSA, the indicator can no longer enter the mitochondria (10). Thus, we may reverse the response of BTB on calcium addition and obtain an acidification response with BTB external to the mitochondria. Fig. 6 shows the BSA effect somewhat more clearly than the data of Reference 10. Additions of Ca++ ions are plotted on the abscissa and optical density changes along the ordinate with increased absorbance at 620 nm plotted upward and decreased absorbance at 620 nm plotted downward. The profiles for various concentrations of BSA are included. The control experiment is similar to that of the previous figures, particularly Fig. 4, where the increased absorbance of BTB is observed on the initial calcium addition. Considering first the response to the initial addition of calcium, concentrations of BSA about 15 \( \mu \text{M} \) obliterate the initial alkalinization. However, a suppression of the intramitochondrial alkalinization for all values of Ca++ employed requires 93 \( \mu \text{M} \) BSA. At intermediate values, further additions of calcium enable the mitochondria to sequester the indicator from the BSA as if the affinity of the mitochondria for the indicator was increasing as the calcium was accumulated. BSA addition causes a change in the response of BTB to added phosphate as well; an alkalinization is observed when BSA is present, an acidification is observed in the absence of BSA. While this result suggests interesting details of the mechanism whereby BTB enters the mitochondria, the general result that the internal alkalinization can be converted to an external acidification during calcium accumulation by the addition of BSA provides conclusive evidence regarding the nature of the indicator response recorded in the preceding figures.

**Control of H\(^+\) Level—** It is apparent from the preceding experiments that supplements of calcium increased the sensitivity of the BTB response to subsequent additions of calcium. The possibility that H\(^+\) is being "titrated out" in the course of these additions of calcium is more clearly shown with a supplement of Mn++. The kinetics of BTB responses to mitochondria supplemented with Mn++ are indicated in Fig. 10, but the quantitative aspects of this experiment are relevant here (Fig. 7A).

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The titration is carried out under conditions similar to those of Fig. 3 except that smaller increments of calcium are used. It is apparent that increasing values of Mn++ (30 and 167 \( \mu \text{M} \)) decrease the optical density of extramitochondrial BTB, even at the lowest protein concentration employed (1 mg per ml).
creases the intercept with the abscissa. By extrapolation, 220 
\( \mu M \) Mn\(^{++} \) or 65 \( \mu M \) mmpmole per mg of protein would just "titrate out" the H\(^{+} \). In the control experiment without Mn\(^{++} \), 110 
\( \mu M \) Ca\(^{++} \) (32 mmpmole per mg of protein) was required to titrate out H\(^{+} \). These data and those of Fig. 5 show that H\(^{+} \) can be titrated out with either calcium or manganese, but that calcium is about twice as effective as manganese. On a physicochemical basis, it is apparent that either the stoichiometry of the reaction with manganese or its dissociation is much greater than that for calcium.

Fig. 7B shows that a phosphate supplement increases the calcium titration of the BTB response. Here, titrations are made with 0, 20, and 40 \( \mu M \) phosphate. The intercept of the Ca\(^{++} \) titration with the abscissa increases with phosphate concentration, suggesting that H\(^{+} \) could be phosphate itself. If this is the case, a plot of the abscissa intercepts of Fig. 7B with respect to added phosphate (this graph is not shown) gives by extrapolation 30 \( \mu M \) phosphate (8 mmpmole per mg of protein) as the phosphate equivalent of endogenous H\(^{+} \).

**Effect of Osmolarity on BTB Response** —In order to determine in more detail a binding site for BTB, we have compared the response in hypo- and hypertonic media. If the indicator is located in the osmotically active space of the mitochondria, the volume of this space would be greatly decreased in a hypertonic medium. In experiments described recently an 8-fold (0.1 to 0.8 m mannitol-sucrose) change of osmolarity shows a 35% decrease in the initial response to 167 \( \mu M \) calcium and a similar decrease in the total absorbance change at the end point of the titration in the hypotonic medium (10). Independent optical assays show that BTB is tightly bound to the mitochondria at both 0.1 and 0.8 m mannitol-sucrose (Table II). This result is inconsistent with the location of the indicator in the osmotically active space and suggests instead that the indicator is bound to the more rigid portion of the mitochondrial structure such as the intracristal space. On the basis of these data, the membranes appear to expand and even increase their volume slightly in the hypertonic conditions.

**Effects of Acetate and Phosphate** —Fig. 8 (A and B) illustrates the response of the system to additions of phosphate or acetate. Sodium succinate at pH 7.4, 20 mm, was employed with a mitochondrial protein of 4.3 mg per ml, which gave a clear-cut alkalization of the interior pH on the second addition of Ca\(^{++} \). The H\(^{+}\):Ca\(^{++} \) value of 0.8 and 0.9 for the first two additions of Ca\(^{++} \) is computed using the left-hand scale for H\(^{+} \) concentrations. Following this, 1 mm phosphate, pH 7.4 (Fig. 8A) or 3 mm acetate, pH 7.4 (Fig. 8B) was added. In both cases, the interiors of the mitochondria were rapidly neutralized, more rapidly with phosphate than with acetate. Here we find an example of an indicator color change in the absence of any detectable electron transport activity (18, 19). At the same time, the pH trace returned partially toward the initial level. Thereafter, two further additions of Ca\(^{++} \) were made, which underline the difference between the reaction in the presence of phosphate and that in the presence of acetate. In the presence of phosphate, marked H\(^{+} \) ejection occurred (H\(^{+}\):Ca\(^{++} \) = 0.9 and 1.0, using the right-hand scale for the concentration of H\(^{+} \) because of the added buffer capacity), but the intramitochondrial pH showed no measurable change on adding the Ca\(^{++} \) because of the presence of phosphate in the mitochondria. Irreversible swelling of the mitochondria commenced shortly after the second addition of Ca\(^{++} \) and caused a deflection of the differential spectrophotometric trace. In the acetate-supplemented system, the proton ejection was small (H\(^{+}\):Ca\(^{++} \) = 0.4, 0.2), and the intramitochondrial pH swings initially in the alkaline direction, but it was then partially neutralized, presumably by the uptake of acetate (4, 5). The latter effect is verified by the light scattering trace at 700 m\( \mu \), which shows decreased light scattering following the addition of acetate and after two subsequent additions of Ca\(^{++} \). It is apparent that 1 mm phosphate holds the intramitochondrial pH constant, and that 3.3 mm acetate allows it to swing initially in the alkaline
direction but returns it to neutrality as more acetate is taken into the mitochondria.

**Effects of Ammonium Ion**—In view of the studies of Brandt (20) and ourselves (21) demonstrating the high permeability of yeast cells to ammonia and to substituted amines, we have examined the possibility that the mitochondria are highly permeable to ammonia (see Reference 22) which would be expected to cause increased alkalinity. Thus, we have compared the effects of sodium acetate and ammonium acetate. Fig. 9A represents a control in which the addition of 167 μM Ca++ causes a large alkalinalization as indicated by the DTB measurements in a 20 mM sodium succinate medium at pH 7.4. Here, the mitochondrial protein concentration is sufficiently low that H+ does not interfere with the response of the indicator to the initial addition of Ca++, and a maximal response of 0.044 cm⁻¹ is recorded. When the pH change has reached its maximum (H⁺:Ca++ = 1.0), 3 mM sodium acetate is added (Fig. 9A) and the DTB trace drops to the initial level with a half-time of approximately 10 sec. A net H⁺ change of 35 μM is observed (correcting for the pH inequality of the sodium acetate), and the mitochondrial light scattering decreases by 14%. In Fig. 9B, the experiment was repeated, giving a H⁺:Ca⁺ value of 0.95 and a DTB change of 0.038 cm⁻¹. Instead of sodium acetate, 3 mM ammonium acetate was added, with the same result as that shown in Fig. 9A; the DTB trace returns to the initial level with a half-time of 10 sec, as before. The corrected pH change corresponds to 38 μM H⁺. Less swelling occurs; the light absorption decrease is 5%. Thus, ammonia at higher concentrations can slowly penetrate the mitochondrial space in which Ca++ is accumulated, but the reaction is not rapid enough to interfere with the acidification caused by Ca++ accumulation or the alkalinalization caused by acetate addition.

Following the addition of acetate, two additions of 167 μM Ca++ stimulate respiration in the usual way to give Ca++:O values of 8 to 9 with H⁺:Ca⁺ values of 0.2 to 0.3 in the six experiments. There is less respiratory control with 0.1 M ammonium chloride.

**Effect of Manganese**—Fig. 10 affords a comparison of the effects of Ca++ and Mn++ under conditions where the effects of both cations are measurable. First, the response to a low concentration of Ca++ (35 μM) is indicated by the ejection of 77 μM H⁺ (H⁺:Ca⁺ = 2.4) and a transient alkalinization of the DTB during proton ejection, followed by a neutralization. Then a 5-fold greater Mn++ concentration (167 μM) is added. There is now a slower proton ejection of an extent of over 70 μM H⁺ at a rate of acid formation of one-tenth that observed with 33 μM Ca++. The DTB trace shows a disturbance on adding Mn++, followed by an alkalinization at about one-twentieth the rate observed with Ca++. Apparently the rate of Mn++ uptake under these conditions is so slow relative to the rate of Ca++ uptake that alkalinization is also both small and slow. Nevertheless, the effects are qualitatively similar.

As shown in Fig. 7, titration with Ca++ following Mn++ supplements gives values of H⁺ that are greatly diminished.

**Relation to Electron Transport**—Pretreatment of the mitochondria with 0.3 mM cyanide for a sufficient interval to cause...
into the mitochondria as an anion together with Ca++. Insofar as the indicator following accumulation of Ca++, first, the centrifugation experiments, and second, the competition experiments with a related meets the needs of these experiments. The fact that the indicator is there as the Ca++ is accumulated in the space where the alkalinity due to Ca++ accumulation is formed; the fact that the indicator is in the osmotically inactive intracristal space and, as a consequence, the BTB is located primarily in the matrix space, and it is possible that the BTB is located primarily in the matrix space. 3 Note Added in Proof—It has been found that BTB also indicates acidification in illuminated chloroplasts and chromatophores while the glass electrode shows an alkalinization, suggesting that BTB is presumably in the intravesicular membrane. Apparently BTB binds the hydrophilic portion of many types of membranes.

**DISCUSSION**

**Binding of Indicator**—A number of points indicate that we are dealing with a bound, not a free form of BTB. Prominent among these is the observation that 80 to 90% of the indicator remains bound to the mitochondria even on centrifugation. This binding is apparently due to a constituent of the mitochondrial membrane since centrifugation experiments show that sonically disrupted particles also bind the indicator. Secondly, the binding of BTB to mitochondria and to mitochondrial membranes (Table II) is apparently tight enough so that the dissociation constant corresponds to about $10^{-4}$ M. However, the binding does not appear to affect the spectrum of the indicator greatly, either with respect to the mitochondrial extinction coefficient or position of the maximum. The binding to BSA shifts the pK of the indicator to pH 7.6, and the binding to mitochondria shifts the pK to 8.0 (Table I).

Two experimental results suggest a tighter binding of the indicator following accumulation of Ca++, first, the centrifugation experiments, and second, the competition experiments with BSA. Neither of these effects is large, but they need not be ignored. It is possible that some of the indicator is transported into the mitochondria as an anion together with Ca++. Insofar as the interpretation of this paper is concerned, it does not matter whether or not Ca++ is initially at a maximum concentration in the space where the alkalinity due to Ca++ accumulation is formed; the fact that the indicator is there as the Ca++ is accumulated meets the needs of these experiments. The fact that the pK of the indicator is shifted prior to the addition of Ca++ is consistent with the idea that the BTB is already bound at the "inside" site. Under our experimental conditions, external buffer minimizes the response of external BTB.

**General Location of Indicator**—The following data show that BTB indicates changes of pH concentration in the space or volume in which cations are accumulated. (a) Whereas BCP and the glass electrode show H+ concentration changes in the same sense, BTB shows changes in the opposite sense, not only in cation accumulation but also in response to additions of phosphate or acetate following cation accumulation; (b) titration of BTB with Ca++ at pH values of 7.4 or 6.5 for the suspending medium gives the same extent of BTB change; (c) a 0.5 unit pH shift in the external medium causes an indicator change equal to that caused by the accumulation of 30 mmoles of Ca++ per mg of mitochondrial protein; (d) an increase of the buffer capacity of the suspending medium from 2 mm sodium succinate to 300 mm sodium succinate causes no significant change in the extent of BTB change; (e) the magnitude of the BTB response to addition of excess Ca++ is independent of a variation of mitochondrial concentration from 5 mg of protein per ml to less than 1 mg of protein per ml; and (f) BSA binds the indicator tightly, and, when added to the mitochondria, prevents the alkalinization of the indicator on Ca++ addition.

These data appear to afford sufficient basis for a working hypothesis that the indicator is located in the intramitochondrial space in which Ca++ is accumulated.4

**Specific Location of Indicator**—Electron micrographic evidence indicates that the site of cation accumulation may be in the matrix space, and it is possible that the BTB is located primarily here. But electron micrographs also show Ca++ deposits on or in the cristal membrane (23, 24; for a summary, see Reference 3). The intracristal and matrix spaces may be distinguished by a variation of the osmotically responsive volume. The matrix space should greatly decrease in volume at high osmotic pressures, while the crista should remain unchanged. The 8-fold decrease of the osmotically active (matrix) space caused by an increase of mannitol-sucrose concentration from 0.1 to 0.8 M caused no increase in the RTR response. Presumably, then, the indicator is in the osmotically inactive intracristal space and, as a consequence, is the primary site of cation accumulation. This observation suggests that the energy utilization in the accumulation of calcium occurs near the outer surface of the crista.

The location of the indicator in molecular terms has not yet been elucidated; it is possible that the binding site is on a particular component either of the respiratory chain, of the energy transfer system, or of the ADP phosphorylation system. Preliminary studies indicate that the "stripped membranes" of mitochondria (24) do not bind BTB tightly, nor does an oxidative phosphorylation factor, $F_1$ (Table I) (25).

**Nature of Titration Curve for Calcium**—While at lower protein concentrations (1 to 4 mg per ml), the relationship between added calcium and increased absorbance of BTB approximates a rectangular hyperbola, at higher protein concentration (approximately 6 to 8 mg per ml), a sigmoid curve is observed. In the preceding discussion the failure to attain a maximal response of

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4 Note Added in Proof—It has been found that BTB also indicates acidification in illuminated chloroplasts and chromatophores while the glass electrode shows an alkalinization, suggesting that BTB is presumably in the intravesicular membrane. Apparently BTB binds the hydrophilic portion of many types of membranes.
BTB to initial additions of calcium has been attributed to an internal acid, \( H_2^+ \), of the mitochondria present at concentration levels equivalent to 30 to 40 \( \text{mM} \) moles of \( Ca^{++} \) per mg of protein. It is very probable that the mitochondrial membrane has considerable buffering power, and its constituents, for example, phospholipids, may contribute \( H_2^+ \). Nevertheless, an endogenous acid is certainly not the only explanation of these titration curves. For example, the permeation of an anion would neutralize the alkalinity as indeed is observed when phosphate or acetate is added (see Fig. 8). We find this neutralization to be caused by small concentrations of phosphate; the phosphate already present in mitochondria could easily account for the neutralization; \( 8 \text{mM} \) moles per mg of protein is sufficient. Alternatively, the initial binding of some of the indicator to a soluble protein of the mitochondrial suspension such as BSA would have a similar effect upon the titration curve (see Fig. 6). We have examined these proteins for BTB binding, but have not yet obtained the characteristic shift of pK to 8.0 observed with mitochondrial binding (see Table 1).

**Range of pH Changes**—Fig. 12 relates these studies to the titration curve for BTB bound to the mitochondria and shows the indicator characteristic for BTB in the mannitol-sucrose-succinate medium in the absence of mitochondria (14). Starting under the conditions in Fig. 3 at pH 7.4, the suspension is acidified to pH 6.9, then made progressively alkaline by serial additions of NaOH to the 20 mM sodium succinate medium. A plateau is reached at approximately pH 9.0, and the midpoint of the curve is at pH 8.0. The range of pH changes caused by BTB accumulation in the mitochondrial membrane is therefore 

\[ \text{pH} = 6.9 \quad \text{to} \quad \text{pH} = 8.0 \]

By means of the curve shown in Fig. 12, we may evaluate the range of pH changes caused by \( Ca^{++} \) accumulation in the mitochondria. For example, the maximum optical density change obtained at the end point of \( Ca^{++} \) titration carries the indicator nearly to the alkaline form, a change of about 1 pH unit. A more accurate calculation would be obtained by the absorbance change for a given amount of calcium, for example, 0.025 optical density unit for the addition of 167 \( \mu \)M calcium (see Fig. 5). Reading this increment off the appropriate portion of the curve (starting at pH 7.4), we find the color change corresponds to pH 8.0.

While these experiments do not fix the value of the internal pH, two points may be considered. First, the fact that the internal pH can be altered by altering the external pH is significant and suggests that the two values cannot differ greatly. Second, the absence of a large change in the external or internal pH on adding an uncoupler to the mitochondria indicates that no large pH gradient exists across the mitochondrial membrane (10). It is further apparent that when a pH gradient does exist across the mitochondrial membrane, the addition of a permeant anion such as phosphate or acetate neutralizes this gradient in a few seconds. Particularly in the case of phosphate, no further change of the intramitochondrial pH can be detected following \( Ca^{++} \) additions.

Thus, it seems unlikely that a pH gradient across the mitochondrial membrane would be present in phosphate-supplemented mitochondria.

The neutralization caused by small concentrations of phosphate; the phosphate already present in mitochondria could easily account for the neutralization; \( 8 \text{mM} \) moles per mg of protein is sufficient. Alternatively, the initial binding of some of the indicator to a soluble protein of the mitochondrial suspension such as BSA would have a similar effect upon the titration curve (see Fig. 6). We have examined these proteins for BTB binding, but have not yet obtained the characteristic shift of pK to 8.0 observed with mitochondrial binding (see Table 1).

**Responses of Other Types of Mitochondria**—Identical results have been obtained with guinea pig liver mitochondria, and similar results but smaller absorbance changes with pigeon heart mitochondria have also been obtained. The latter show a 10% step response in the direction of acidification (see Fig. 11). Thereafter, the kinetics is very similar to that illustrated in this paper and has been observed over a wider pH range (6.4 to 8.0). We had previously used BTB as an external pH indicator at about 10 \( \text{mM} \) moles per mg of protein in a suspension of guinea pig kidney mitochondria (3) supplemented with phosphate. At lower BTB concentrations (~1 \( \text{mM} \) per mg of protein), guinea pig kidney mitochondria show the same responses as do rat liver mitochondria.

**Time Relations of External and Internal pH Changes**—The foregoing considerations have shown that the internal pH change does not bear a compulsory relationship to ion uptake. Thus, one need not expect a close time correlation of the two effects, and indeed, considerable discrepancies can exist. For example, Fig. 1B illustrates that there may be very little internal pH change in response to the first addition of \( Ca^{++} \) to a high concentration of mitochondria, while the external pH change is large and rapid. At lower protein concentrations or for the second addition of \( Ca^{++} \) (Fig. 3A), the kinetics of external and internal pH changes is similar, and in Fig. 5B, nearly identical. Thus, the corre-
tion is good when permeant anions and the intramitochondrial proton donor (H^+) are absent. In the presence of acetate, a correlation of internal and external changes is still observable, but both are proportionately reduced in magnitude as the acetate concentration is increased. Phosphate eliminates the internal pH change, but leaves the external changes (Fig. 8a).

**Calcium Accumulation**—Ca^{2+} accumulation by mitochondria in the absence of a permeant anion was shown in previous studies (5). It is probable that Ca^{2+} accumulation is inhibited as the pH gradient across the mitochondrial membrane increases, and further calcium additions cause no further pH changes. Recent studies with a Ca^{2+}-sensitive electrode (Corning Glass Company) support this viewpoint; the Ca^{2+} concentration external to the mitochondria rises to measurable values only as the pH gradient becomes large (26).

**Reaction Mechanism**—Fig. 13 presents an elaborated form of the mechanism presented previously (3). The first row of the diagram represents four steps in the processes of energy conservation and transfer to charge the high energy compound, X \sim I. The second row represents four steps in the process of Ca^{2+} accumulation, and the third row, three steps in the process of anion reaction. In each step, the mitochondrial membrane is pictured schematically and that labeled "outside" is adjacent to the invaginations of the cristae that are in contact with the water phase. The part labeled "inside" is adjacent to the matrix space. Thus, the double walled structure is the cristal membrane and is here shown to include the respiratory carriers and the projecting subunits of Fernandez-Moran (27) and Chance, Parsons, and Williams (25), which have now been functionally identified with the phosphorylation of ADP (28). A diagram of the structure is presented elsewhere (10).

The steps in electron transfer and energy conservation necessary to restore the end product of Ca^{2+} accumulation, the deenergized state, State 3 (second row, fourth diagram), to the energized state, State 4 (first row, fourth diagram), by a recycling process is indicated on the first line. The diagram for the energy conservation and electron transport reactions begins with the energy conservation intermediate in the low energy state (X \sim I). The first step of energy conservation involves the oxidation of reduced cytochrome c (Diagram 2) plus the conservation of energy in the intermediate, denoted by I*. The third diagram represents the reduction of oxidized cytochrome c by reduced cytochrome a, leading to further energy conservation in the electron transport-controlling intermediate (~I). In the fourth diagram the energy is transferred from cytochrome c to form the X \sim I compound.

The over-all reaction for the first three steps of the "charging" reaction is indicated as follows.

\[
e_1^- + a_1^- + H_2X \sim I \rightarrow e_1^- + a_1^- + H_2X \sim I
\]

It is important to note that the oxidation-reduction span available for this reaction is that between a_1 and a_3; not that between c and a. A fourth step of this reaction restores a_1 and a_3 to their initial states.

The four steps of ion accumulation are indicated in the second row. The initial diagram indicates the reaction of the State 4 high energy intermediate with 2 atoms of calcium in an initial state of binding, involving the replacement of 2 protons of the high energy intermediate with 2 calcium ions, resulting in a net positive charge. This particular mechanism suggests proton ejection as a primary event in the reaction with X \sim I; however, it is apparent that proton ejection could occur at any one of the three steps in this reaction. In addition, proton ejection can occur in a reaction with a phospholipid as a constituent of either X \sim I or the membrane itself (29). It is important to note that we picture the outer layer of the cristal membrane to be permeable to calcium and to allow combination of the carrier with the cation. One mechanism which is consistent with the stimulation of the uptake of monovalent cations by valinomycin and gramicidin (21, 30, 31) is that these compounds sensitize the membrane to the monovalent cations which then combine with the high energy intermediate in the same manner as do calcium, strontium, and manganese. Thus, this mechanism combines a carrier molecule of relatively low specificity with a membrane that can be sensitized to various cations with appropriate substances. The type of carrier molecule is obviously beyond the scope of this discussion; one cannot fail to be compelled by the x-ray crystallographic data for ferri-myoglobin on the binding of sulfate to the distal histidine and xenon to the space near the proximal histidine as evidence that proteins can have fairly nonspecific "pockets" for charged and uncharged molecules (32-34).

The transport of calcium half-way across the cristal membrane (approximately 80 A) is indicated in the second diagram; transport is here indicated to occur simply by a mechanical process involving rotation and translation, but obviously other mechanisms are applicable. Such a transport process may well involve energy utilization which for clarity only is indicated here as a separate process, although it may actually be simultaneous with transport. Energy utilization in this case involves the release of calcium from the carrier, a conversion of the high energy intermediate to its low energy form, and the donation of 2 protons to the carrier to equalize the charges across the membrane. These protons can be made available by water molecules on the inside of the mitochondria.

It should be noted that the choice of the outside or the inside of the cristal membrane as the locus of the concentration gradient is so far purely arbitrary. But the experimental data suggest that BTB may be bound in the cristal membrane itself and the concentration gradient may be at the outside layer of the crista.

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**Fig. 13.** A current mechanism for cation and anion reactions with mitochondria. Note that CaOH^+ may be completely dissociated or may be a complex calcium salt.
This is particularly evident in studies of sub mitochondrial particles, in which the indicator is bound to the inside of the vesicles. Thus, the transport only brings calcium into the intracristal space, and the energy utilization occurring at that point need not directly eject calcium into the matrix space. The matrix space may not be equilibrated with the intracristal space until the $H_1^+$ directly ejects calcium into the matrix space. Thus, the transport only brings calcium into the intracristal particles, in which the indicator is bound to the inside of the vesicles.

The over-all chemical reaction for this step is as follows.

$$\text{H}_2\text{X} \rightarrow I + 2\text{Ca}^{2+} + 2\text{H}_2\text{O} \rightarrow$$

$$\text{2CaOH}^+ + \text{H}_2\text{X}^{-} \rightarrow \text{2H}^+$$

The formula, CaOH$^+$, implies a dissociated species or a coordinated form of monobasic calcium hydroxide of a $pK$ of about 8. This compound is formed in the matrix space. At this point, the anion reaction occurs.

In the fourth step of the accumulation reaction sequence, the carrier compound resumes its initial position, and the low energy compound can recycle through the reactions of the first row to achieve the dynamic properties of the high energy compound is acquired. This compound is formed in the matrix space. At this point, the carrier compound resumes its initial position, and the low energy compound can recycle through the reactions of the first row to acquire the dynamic properties of the high energy compound is achieved.

$\text{CaOH}^+$, $\text{H}_2\text{X}^{-}$, $\text{I}$, $\text{2H}^+$ (membrane) (outside) (outside) (inside) (inside)

The latter reaction is entirely analogous to the reaction with acetate in Equation 3. Both Equations 3 and 5 are consistent with the experimental observation that hydroxyl ions are ejected on addition of acetate or phosphate to mitochondria alkalinated with calcium.

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