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INTRAMITOCHONDRIAL PH CHANGES IN CATION ACCUMULATION

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One aspect of mitochondrial function of considerable current interest is the reaction of the mitochondria with monovalent and divalent cations.[1, 2] The addition of small concentrations of Ca^{++} (100 μM) causes an intense stimulation of respiration, large shifts in the steady state of the respiratory carriers,[1] and eventual accumulation of nearly all of the Ca^{++}.[3, 4] A number of other reactions accompany cation accumulation, for example, ejection of protons,[3] the uptake of anions,[5, 6] and particularly the establishment of an inhibited state of electron transport when none of the appropriate species of permeant anion is present.[7]

Electron transport is identified as a membrane-bound phenomenon by the isolation of submitochondrial particles in which the electron transport occurs as effectively as it does in the intact mitochondria,[8–10] leading generally to the idea of a membrane-bound respiratory assembly or oxysome which embraces the functions of forward and reversed electron transport, oxidative phosphorylation, and more recently, ion accumulation.

Demonstration that ion accumulation takes place directly in the cytochrome-containing membrane is a problem of considerable importance in the development of ideas on reaction mechanisms. However, the only evidence available so far on this point is the rapid response of the respiratory carriers to Ca^{++} addition[1] and suggestions from electron microscopy that Ca^{++} is accumulated not only in the matrix space of the mitochondria but also in the cristal membrane (see ref. 1). The description in this communication of a cation responsive indicator bound within the cristal membrane opens new possibilities for the investigation of reaction mechanisms of cation uptake.[11, 12]

Experimental Methods.—Preparations: The results presented here are obtained with rat liver mitochondria,[13] but pigeon heart mitochondria have been used as well (Chance and Mela, in preparation). The suspension medium for the mitochondria has been reduced to its simplest ingredients and contains 0.225 *M* mannitol, 0.075 *M* sucrose for osmotic balance, and sodium succinate to serve as substrate and a "minimal" buffer; all other cations and anions that would permeate the mitochondria and interfere with the observation of intramitochondrial events are omitted. It is important to note that our preparation method for rat liver mitochondria involves the use of 10^{-4} *M* versene in the first two centrifugations. The mitochondria are subsequently washed twice to free them of the versene. Mitochondria prepared in this way contain a minimal amount of cations (5 mμmoles Ca^{++}/mg protein).

The technology of simultaneous measurements of pH of the external medium, oxygen concentration, absorbance changes of the indicator, and light scattering is described elsewhere[1, 14] (Chance and Mela, in preparation), and the diagrams presented here are principally summaries of these data emphasizing the absorbance changes of the indicator.

Bromthymol blue (BTB) is employed in the concentration of 3.3 μM (usually 0.4 mμmoles BTB/mg protein). Changes in absorbance are measured near the maximum of 620 mμ most conveniently by the dual wavelength method, employing 618 mμ as a measuring wavelength and 700 mμ as a reference wavelength. Bromcresol purple (BCP) was employed at concentrations of 1–2 mμmoles/mg protein and was measured at 580 and 640 mμ.

BTB has previously been used at higher concentrations (~10 mμmoles/mg protein) to measure extramitochondrial pH changes caused by Ca^{++} uptake in the presence of phosphate in guinea pig kidney mitochondria.[1] Here we employ rat liver mitochondria, a phosphate-free medium, and ~0.4 mμmoles BTB/mg protein.

Experimental Results.—Spectral changes of BTB in the presence of mitochondria: Figure 1*A* illustrates the expected result that BTB even in the presence of mitochondria would show a response to NaOH qualitatively similar to that observed in the solution in which increasing alkalinity causes increased absorption at 620 mμ. In the presence of mitochondria the absorption maximum is at the same wavelength and the molecular extinction coefficient has approximately the same value as that in solution; however, a quantitative difference is observed in the NaOH concentration for half maximal indicator color—pH 8.1 is found in this case as compared with pH 7.2 in the absence of the mitochondria. This shift of pK is similar to that observed with hemoglobin binding.[15]

When Ca^{++} is added to the BTB-supplemented mitochondria, instead of the expected acidification observed with the glass electrode, an unexpected result is obtained; small concentrations of Ca^{++} (330 and 670 μM) increase the indicator color, indicating an alkalinity. Ca^{++} itself has no effect upon the indicator nor upon the pH of the adequately buffered solution. Instead, it is apparent that we are observing alkalinization of the interior of the mitochondrion that is complementary to the acidification of the medium exterior to the mitochondrion. It is probable that the indicator is bound to the opposite side of the membrane across which calcium is accumulated; the BTB is "inside" the mitochondria.

Kinetic and equilibrium aspects of the indicator response to Ca^{++} addition: The lower trace of Figure 2*A* indicates the external responses of mitochondria to Ca^{++}.[16]

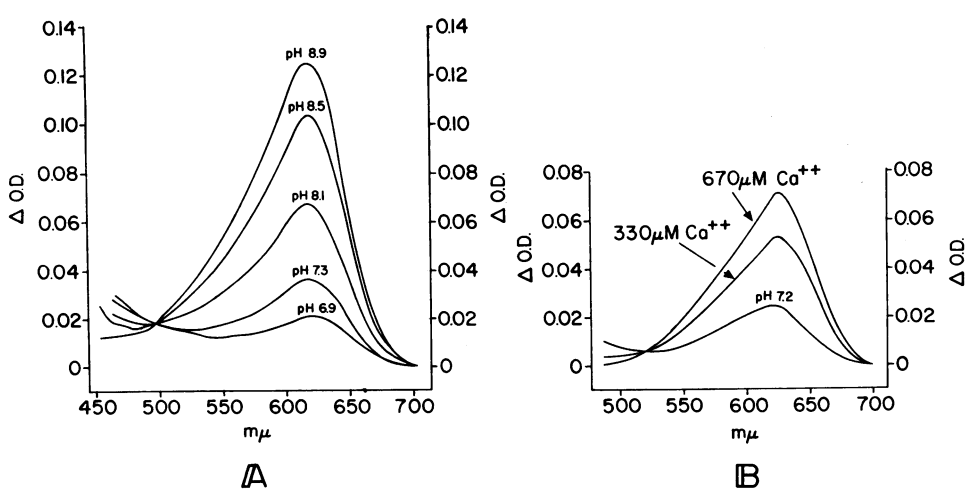

FIG. 1.—(*A*) Absorption spectrum of 3.3 μM BTB bound to rat liver mitochondria; 2.5 mg protein/ml; 0.3 *M* mannitol-sucrose; 20 mM sodium succinate buffer. (LM-8)
(*B*) Effect of 330 and 670 μM Ca^{++} on the BTB spectrum. 3.0 mg protein/ml. (LM-7)

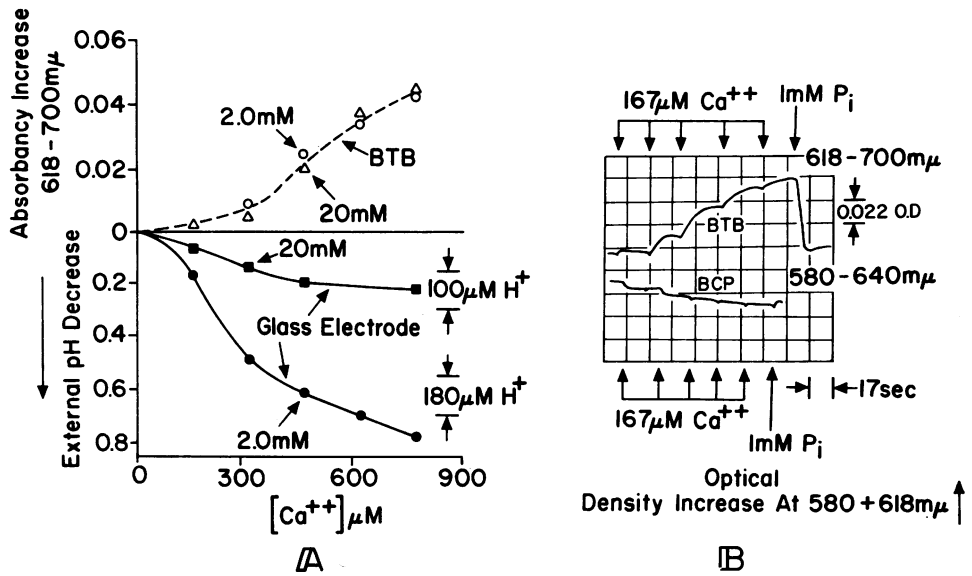

FIG. 2.—(*A*) Effect of buffer strength upon the response of 3.3 μM BTB to serial additions of 167 μM Ca^{++}. Rat liver mitochondria; 6.5 mg protein/ml. The sodium succinate concentrations are 2 and 20 mM. Initial pH is 7.4 and pH changes are indicated in the diagram. The 100 μM H^+ calibration is for the 2 mM titration, and the 180 μM H^+ calibration is for the 20 mM titration. (1039-1,2,3)

(*B*) Comparison of the responses of 3.3 μM BTB and 8.7 μM BCP to serial additions of 167 μM Ca^{++}. 6.5 mg protein/ml; 2 mM sodium succinate; pH 6.5. (1038-8,6)

Each addition of Ca^{++} causes a proton ejection from the mitochondrial membranes. Since the 2 mM medium is lightly buffered, the pH changes are large (approximately 0.8 unit), and those of the 20 mM medium are smaller. On the upper portion of the graph we have indicated the absorbance increases of the indicator BTB occurring in the same mitochondrial suspension. In agreement with Figure 1*B*, increased absorbance of the indicator occurs with each addition of Ca^{++}, although a greater response is observed in the later than in the earlier additions. A comparison of these two graphs indicates that BTB is showing an alkalinization of a degree independent of the buffer strength while the glass electrode shows an acidification dependent upon the buffer strength. Our interpretation of this result is that the two pH indicators are operating on opposite sides of the mitochondrial membrane, the glass electrode on the outside and the BTB on the inside.

The experiment of Figure 2*A* can in principle be duplicated by using, instead of the glass electrode, another color indicator which is not bound on the inside of the mitochondria (Fig. 2*B*). We have found that BCP is such an indicator. Its optical characteristics are similar to those of BTB, except that the maximum of absorption is at 580 instead of 618 mμ. In this chart the progress of the reaction is followed as a function of time after addition of Ca^{++}, and five successive additions of Ca^{++} give absorbance changes of the two indicators registered on the same optical density scale. It should be noted, however, that BCP is present in approximately three times the concentration of BTB (8.7 μM compared with 3.3 μM). The time for half maximal response to each addition of the Ca^{++} is about 10 sec. BTB shows absorbance increases as before. BCP, on the other hand, shows absorb-

ance changes of the opposite sign to those of BTB as indicated by the downward deflection of the traces on the successive additions of Ca^{++}. Thus, the response of BCP agrees with that of the glass electrode and with the previously established measurements of proton ejection on Ca^{++} addition to mitochondria. BTB clearly shows a different response from those recorded previously. The explanation of the smaller response of BCP in spite of its larger concentration is that it is indicating pH in a large volume external to the mitochondria, while BTB is indicating pH in a small volume inside the mitochondria. The BTB trace duplicates the results of previous experiments and records larger increments of absorbance for the later additions of Ca^{++}.

Included in Figure 2*B* is an illustration of the effect of phosphate upon the absorbance of BTB; the addition of 1 mM phosphate returns the BTB trace almost exactly to the absorbance prior to the addition of Ca^{++}. Thus, the entry of P_i into the space to which BTB is bound neutralizes the alkalinity formed there, presumably by converting calcium hydroxide to calcium phosphate with the ejection of a hydroxyl ion caused by a phosphate-hydroxyl ion exchange. Further additions of Ca^{++} are rapidly accumulated, yet no further pH changes in the membrane are observed. It is of considerable importance to note that the pH gradient across the mitochondrial membrane is apparent only when no permeant anions are present.

Mitochondrial binding of BTB: Figure 3 illustrates three basic aspects of the binding of BTB by mitochondria. Figure 3*A* indicates the profile for BTB binding prior to the addition of Ca^{++} and following the addition of 330 μM Ca^{++}. The BTB absorption found in the supernatant of a high-speed centrifugation of the mitochondria is subtracted from the initial amount (3.3 μM BTB) and plotted as percentage on the scale of the ordinate. The abscissa represents the mg protein in the initial suspension. Other conditions are the same as in Figure 2. In the region of our experiments, 4–6 mg protein/ml, the BTB is approximately 80 per cent bound, and following the addition of calcium, is over 90 per cent bound, even after centrifugation, which would be expected to release some of the indicator. Thus, the centrifugation results support the spectroscopic observations that we are unable to detect any acidification of the indicator; our results show only an alkalinization on adding Ca^{++}.

Numerous other experiments are reported elsewhere (Chance and Mela, in preparation). Among them is the observation that fragments of mitochondria obtained by ultrasonic disruption of the mitochondria also bind the indicator roughly to the extent indicated here. Thus, the binding of BTB to the mitochondrial membranes is due to a chemical affinity and not simply to a "physical enclosure" of the indicator in the interior of the mitochondria.

Figure 3*B* illustrates the successful competition of bovine serum albumin (BSA) with the mitochondrial binding site for BTB. The experiments are carried out as in Figure 2, namely, by sequential additions of Ca^{++} to a suspension of mitochondria in the presence of BTB; the absorbance changes are recorded as increased absorption upwards and decreased absorption downwards. The curve labeled zero corresponds to a control without BSA, and the usual increase of absorbance with Ca^{++} concentration due to intramitochondrial alkalinization is observed. With 93 μM BSA the response is completely reversed; instead of a large increased absorption of the indicator, there is a small decrease. Intermediate concentrations of BSA give

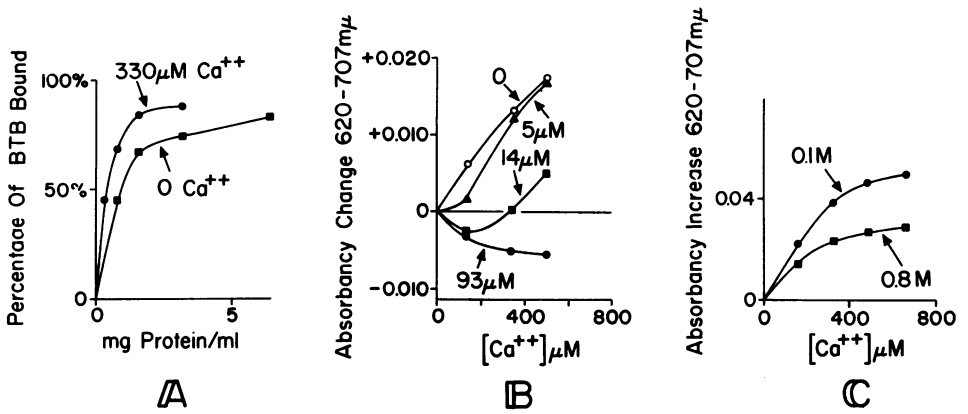

FIG. 3.—(*A*) Effect of protein concentration upon BTB binding as determined by centrifugation; rat liver mitochondria. [Ca^{++}] on diagram; 20 mM sodium succinate; pH 7.4. (LM-20)
(*B*) Effect of BSA upon the response of BTB to serial additions of Ca^{++}. Rat liver mitochondria; 2.6 mg protein/ml; 20 mM sodium succinate; pH 7.4. BSA concentrations indicated on the diagram. (LM-15)
(*C*) Effect of osmolarity upon the response of BTB to additions of Ca^{++}. 2.5 mg protein/ml; 0.1 and 0.8 *M* mannitol-sucrose; 20 mM sodium succinate; pH 7.4. (LM-12)

results in accordance with those of Figure 3*A* which indicate that the binding constant of the mitochondria for BTB increases with the amount of Ca^{++} added. The initial addition of Ca^{++} at 14 μM BSA shows an extramitochondrial acidification, while further additions of Ca^{++} show intramitochondrial alkalinization. Thus, the indicator BTB can indicate pH changes in the intra- or extramitochondrial spaces, depending upon where it is bound. This experiment lends considerable support to the conclusion that BTB indicates intramitochondrial pH changes.

Figure 3*C* gives important evidence on the location of the indicator in the mitochondrial structure. The data so far show that BTB is in the space in which primary accumulation of Ca^{++} occurs and from which H^+ ions are ejected into the space surrounding the mitochondria. In view of the above-mentioned data which show that the indicator is tightly bound to fragments of mitochondria, it is presumed that the indicator is tightly bound to the cristal membranes. It is important to know whether or not the indicator is exposed to an aqueous phase of volume dependent upon the osmotic pressure, or is in the more rigid structure of the mitochondria whose volume is not critically dependent upon osmotic pressure, e.g., crista. Figure 3*C* represents an experiment similar to that of Figure 2, involving serial additions of Ca^{++} to a suspension of mitochondria supplemented with BTB. The usual titration curve is obtained in hypotonic medium (0.1 *M*) showing increasing alkalinity with increasing Ca^{++} concentration. An eightfold increase of osmolarity causing an eightfold decrease of the volume of the osmotically active space gives a titration curve which is no steeper than that obtained in the hypotonic conditions; in fact, it is less steep by about 40 per cent. This result suggests that the space in which the indicator is retained has not decreased in volume in the hypertonic conditions but may have slightly increased in volume. We interpret this result to show that the indicator is bound to the rigid structure of the mitochondria and this is the primary site of ion accumulation and proton ejection.

Discussion.—Evidence that BTB is acting as an intramitochondrial pH indicator:

To summarize briefly, the points of the previous experiment which allow us to interpret the response of BTB on the basis that it is an indicator of intramitochondrial pH are the following: (*a*) whereas BCP and the glass electrode show pH changes of the same sense, BTB shows changes in the opposite sense, not only to cation accumulation but also in response to additions of phosphate or acetate following cation accumulation; (*b*) the response of BTB to titration of the mitochondrial suspension with Ca^{++} is independent of the pH of the external medium between 6.5 and 7.4 and of buffer strengths from approximately 2 to 300 mM in sodium succinate; (*c*) a decrease of the mitochondrial protein from 5 to 1 mg/ml causes only a small decrease in the amplitude of BTB response in the Ca^{++} titration; (*d*) BSA can bind BTB and can change the alkalinization of BTB due to Ca^{++} addition to an acidification. These data, together with centrifugation data of intact mitochondria and submitochondrial membranes, are consistent with the view that BTB is tightly bound to the mitochondria, and is on the "inside" of the space within which Ca^{++} is accumulated.

Structural considerations: Since we identify the indicator with the primary site of cation accumulation and find, furthermore, that this site is the osmotically unresponsive space, it is appropriate to consider current knowledge on the components of the cristal membrane. The diagram of the cristal membrane, while derived from the ideas of Estabrook and Holowinski[17] and Lehninger,[4] includes as well the dimensions proposed by Sjöstrand,[18] where the width of the inner membrane is given as 185 Å. This diagram resolves the problems of fitting the protein molecules into the available width of the crista by a model where the proteins are intimately mingled with the lipids, and the idea of separate protein and lipid layers is not essential to this diagram. (These ideas were greatly stimulated by discussions with F. Sjöstrand and R. B. Park.)

Figure 4 indicates a schematic model of the crista and the oxysome—the functional unit for electron transport in oxidative phosphorylation.[19] Generally, the respiratory chains consist of electron carriers, the energy-conserving intermediates, and the enzymes for phosphorylating ADP to ATP.[20] While the exact location of these components is not known, the ADP phosphorylating enzyme is now tentatively identified with the projecting subunits of the inner membrane, which are exposed on phosphotungstic acid staining of the surface-spread components of the mitochondrial structure.[21, 22] In surface-spread mitochondria, the subunits appear to project outwards, and unless artifactual reorganization of the structure has occurred in surface spreading in phosphotungstic acid staining (which is indeed possible), we may tentatively consider these structures as a part of the intact cristal membrane. There, however, no satisfactory phosphotungstic acid staining can be obtained, and osmium tetroxide does not reveal the subunits. Most workers in the field, however, accept the fact that the subunits exist but are unstainable.[23] Since the 90-Å diameter of these subunits and the respiratory enzymes can scarcely be accommodated to within the observed width of the crista (approximately 180 Å), we have arbitrarily indicated that the ADP phosphorylating enzymes are elliptical in the membrane and occupy one half the membrane, while respiratory enzymes occupy the other half. Obvious intermingling of the two could occur, in order to control electron transfer in the resting and active states of mitochondria. At best, the figure represents a very distorted view of the actual situation, since it is only a two-

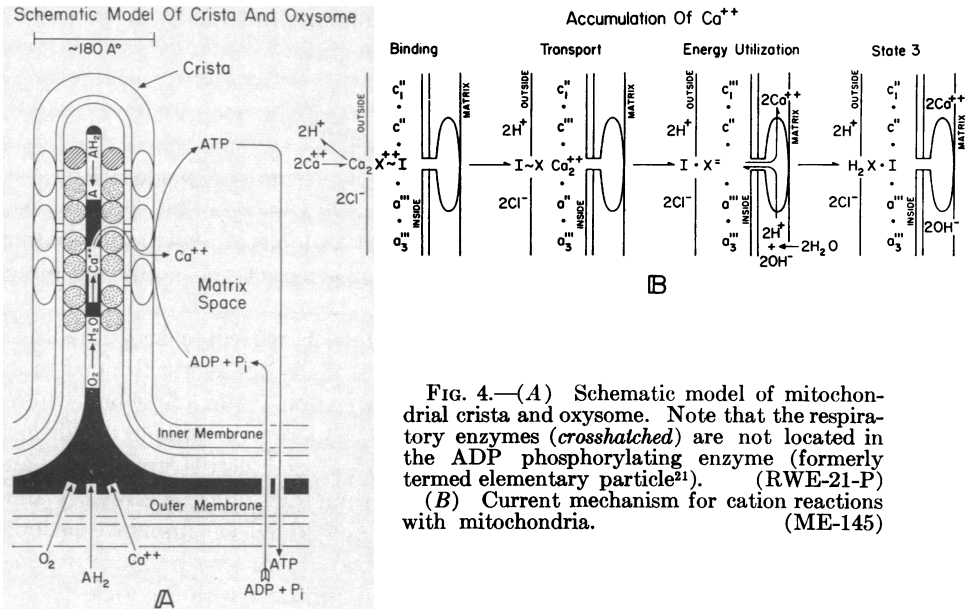

FIG. 4.—(*A*) Schematic model of mitochondrial crista and oxysome. Note that the respiratory enzymes (*crosshatched*) are not located in the ADP phosphorylating enzyme (formerly termed elementary particle[21]). (RWE-21-P)
(*B*) Current mechanism for cation reactions with mitochondria. (ME-145)

dimensional view and does not show necessary elongations in the third dimension which would accommodate the molecular weights of the various components.

The invaginations of the crista and the space between the inner and outer membranes are believed to be highly permeable to substrates, anions, cations, and adenine nucleotides. The diffusion of oxygen, substrates (AH_2), and cations is depicted. The active energy-dependent accumulation is across the cristal membrane as indicated above, and the primary site of active accumulation may lie within this membrane. Ca^{++} accumulated in this way can be released into the matrix space (particularly on addition of acetate) where alterations of the osmotic pressure in this space may cause water exchange by the mitochondria with consequent volume and light-scattering changes.

According to this mechanism, the pathway of phosphorylation of ADP is complex; it must be phosphorylated initially in the matrix space. This result is, however, consistent with the data which indicates that atractyloside can inhibit phosphorylation of external adenine nucleotides but not of internal nucleotides.[24]

Reaction mechanism: Figure 4*B* summarizes four steps[1, 11, 12] in Ca^{++} accumulation based upon a chemical species ($X \sim I$) as the energy source for this and other energy-dependent activities of mitochondria. The over-all chemistry of the reaction is given in equation (1). The cation transport mechanim is of the rotational carrier type as proposed for electron transport.[9, 25]

$$\underset{\text{membrane}}{H_2X \sim I} + \underset{\text{outside}}{2Ca^{++}} + 2H_2O \rightleftharpoons \underset{\longleftarrow\text{membrane}}{2Ca^{++}} + \underset{\text{membrane}\longrightarrow\text{outside}}{2OH^-} + H_2X \cdot I + 2H^+. \quad (1)$$

BTB responds to the OH^- in the membrane. The stoichiometry of the reaction is approximately one H^+/Ca^{++}, but may vary somewhat with Sr^{++} (C. Wenner, personal communication). In any case, the ejection of protons is an early step in the reaction, associated with a primary binding of the cation to the membrane or

to the carrier ($X \sim I$) as indicated here. The reaction is completed by an exchange diffusion reaction with a permeant anion such as acetate:

$$\underset{\text{inside}}{2OH^-} + \underset{\text{outside}}{2Ac^-} \rightleftharpoons \underset{\text{outside}}{2OH^-} + \underset{\text{inside}}{2Ac^-}. \quad (2)$$

Other mechanisms for these three processes are actively under consideration, and one which has received a good deal of attention recently has been derived from the earlier thinking of Davies, Conway, Lundegårdh, and Mitchell,[26-29] where a proton gradient across the mitochondrial membrane is proposed as the basic driving force for these reactions.

These experimental results are highly significant with regard to such theories. Figures 1 and 2 indicate that as a pH gradient across the mitochondrial membrane increases due to successive additions of Ca^{++}, a saturation value is reached where Ca^{++} causes no further response in the absence of a permeant anion. Addition of a permeant anion such as phosphate (or acetate) reduces the pH gradient to an immeasurable value (from about 1 pH unit to less than 0.04 units, see Figs. 1 and 2). Yet it is well known that the mitochondria are now able to accumulate cations rapidly[1, 5, 6] and in the absence of a measurable pH gradient across the mitochondrial membrane, an apparent inconsistency with the proton pump theories.

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*MODIFICATION OF TADPOLE LIVER CHROMATIN BY THYROXINE TREATMENT**

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Administration of thyroxine to tadpoles induces precocious metamorphosis which is accompanied by a number of biochemical changes in the liver. Carbamyl phosphate synthetase is one of the enzymes induced early and ahead of gross morphological changes by the hormone treatment.[1] Experiments using both whole animals[2] and liver slices[3, 4] established that the increase in carbamyl phosphate synthetase activity is the result of *de novo* net synthesis of the enzyme. It has also been found that thyroxine treatment increases the rate of synthesis of various types of RNA in tadpole liver.[5–7]

Inhibition of new RNA synthesis with actinomycin D abolishes carbamyl phosphate synthetase induction,[8] suggesting that new RNA synthesis is required for the enzyme synthesis.

The increased rate of synthesis of both RNA and carbamyl phosphate synthetase, after thyroxine treatment, could be due either to an increase in the amount or activity of RNA polymerase, or to other effects such as the modification of the template efficiency of chromatin.

The experiments reported in this study deal with the preparation of chromatin from tadpole liver nuclei free of endogenous RNA polymerase activity. Thyroxine treatment of tadpoles was found to modify the template efficiency of chromatin preparations.

Materials and Methods.—Animals and thyroxine treatment: Tadpoles, *Rana catesbeiana*, weighing between 6 and 7 gm, were purchased from Lemberger Co., Oshkosh, Wisconsin. Thyroxine treatment was carried out at 25°C for 11 days as described by Paik and Cohen.[1]

Preparation of liver nuclei and chromatin: The livers were removed and washed with frog Ringer solution and then homogenized in 5 vol of 0.25 *M* sucrose solution in standard buffer (500 mM Tris pH 7.4, 5 mM $MgCl_2$, and 25 mM KCl). The homogenates were filtered through four layers of cheesecloth and centrifuged at 700 × *g* for 10 min. The sedimented crude nuclear fraction was resuspended in 3 ml of original homogenizing medium and layered over 5 ml of 0.34 *M* sucrose in standard buffer. The nuclear fraction was sedimented again by centrifugation at 700 × *g* for 6 min. The washing procedure was repeated twice again. The washed nuclear fractions were further purified by suspension in 4 ml of 2.4 *M* sucrose in standard buffer and centrifugation for 30 min at 25,000 rpm in a Spinco SW39 rotor. Pure nuclei, which sedimented to the bottom of the tube, were washed once again with the original homogenizing medium.

Chromatin was prepared by the method of Dingman and Sporn[9] with slight modification. After removing the traces of cytoplasmic material by washing as described, the nuclear fraction was homogenized in a volume of solution F (0.2 mM trisodium EDTA, pH 7.1), and dialyzed