period of high oxidation is marked in our core (2.45 m, or 9.6 feet). Thus it is reasonable to assume a connection between the subsequent cooling indicated by the increase of conifers at the expense of oak, a slower rate of ice melt, and a more gradual rise of sea level.

In short, the pollen profile is consistent with the hypothesis that climatic change is responsible for the change in rate of submergence from 0.6 foot per century (7000 to 3000 years ago) to half that figure subsequently (*2*).

I am indebted to Bloom for the reminder that the pollen profile of the upper 6 to 7 feet of the Wellington Marsh, Medford, Mass., as reported by Knox (*4*), is strikingly similar to our profile. But while Knox associates pine maxima and oak minima with xeric conditions and the converse with moist conditions for the Boston area, the weight of present information about conditions between about 1500 B.C. and A.D. 1750 supports, in my judgment, the interpretation given above for the Guilford site.

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18 February 1963

Participation of an Intermediate of Oxidative Phosphorylation in Ion Accumulation by Mitochondria

Abstract. *Heart mitochondria accumulate massive amounts of Mg^{++} and phosphate or Ca^{++} and phosphate when incubated under appropriate conditions. Studies with inhibitors of respiration, oligomycin, and uncouplers of oxidative phosphorylation indicate that the energy necessary for these reactions may be provided either in the form of ATP in the absence of electron transport, or in the form of a high-energy intermediate of oxidative phosphorylation, which operates even though ATP is not produced.*

The transport and accumulation of various ions by isolated mitochondria has been recently reviewed (*1*). It is generally recognized that oxidative metabolism supplies the energy for these reactions, but hitherto it has not been possible to discern whether ion accumulation is supported directly by the oxidative phosphorylation system or by reactions contingent on the production of adenosine triphosphate (ATP). Huijing and Slater (*2*) have suggested the use of oligomycin, a potent inhibitor of the production of ATP by way of oxidative phosphorylation, to distinguish between these two possible sources of energy for reactions in mitochondria. We have observed that heart mitochondria can accumulate massive amounts of Mg^{++} or Ca^{++} phosphate when incubated under appropriate conditions, and these reactions have been studied under the four sets of experimental conditions listed in Table 1 (systems I–IV). System I has been described in some detail (*3*), and we now present experimental evidence bearing on the accumulation of Mg^{++} by system II and of Ca^{++} by systems III and IV. The weight of evidence supports the view that one or more high-energy intermediates of oxidative phosphorylation can participate in the accumulation of the phosphates of both Mg^{++} and Ca^{++}.

Table 1. Four systems for the accumulation of ions, in heart mitochondria.

System	Ions accumulated	Energy source
I	Mg^{++}, P_i	Substrate
II	Mg^{++}, P_i	ATP
III	Ca^{++}, P_i	Substrate (+ ATP)
IV	Ca^{++}, P_i	ATP

The accumulation of magnesium phosphate proceeds best in the presence of substrate (system I). System I is inhibited by uncouplers of oxidative phosphorylation and inhibitors of electron transport but is not affected by concentrations of oligomycin which prevent the synthesis of ATP by oxidative phosphorylation (*3*). The addition of adenine nucleotides [especially adenosine diphosphate (ADP) and to a lesser extent ATP] lowers the observed accumulation, but this inhibition is largely overcome by addition of oligomycin (Fig. 1). That lower levels of magnesium phosphate can be accumulated in the absence of added substrate if ATP is added as the source of energy (System II) is also shown in Fig. 1.

In system II there may be a limited contribution by endogenous substrate, but the accumulation is largely insensitive to cyanide, antimycin, and other inhibitors of electron transport. However, system II is extremely sensitive to oligomycin. This behavior can best be explained by a scheme such as that shown in Fig. 2. It is postulated that the observed ion accumulation is supported by some intermediate in the process of oxidative phosphorylation. Inhibition of ion accumulation by ADP (and a potential source of ADP such as ATP) would be expected since the high energy intermediate, which may be symbolized by HEC (for high energy compound), would be discharged during ATP synthesis from ADP—a reaction which is favored when the two processes, oxidative phosphorylation and ion accumulation, proceed simultaneously (*3*). Reaction II is depicted as proceeding by way of a reversal of oxidative phosphorylation (*4*), but other oligomycin-sensitive pathways for the utilization of the energy of ATP in the accumulation reaction cannot be ruled out.

Evidence for the participation of HEC can also be obtained from a study of the accumulation of Ca^{++} by mitochondria. Kidney mitochondria can accumulate large quantities of Ca^{++} by a process which requires ATP (*5, 6*), and Vasington and Murphy (*6*) have suggested that an intermediate such as we are considering may be involved in Ca^{++} accumulation.

Table 2. The effect of inhibitors, uncouplers, and aging on the rate of Ca^{++} accumulation. The incubation conditions are described in the legend for Fig. 3. Mitochondria designated "aged" were shaken for 30 minutes at 30°C in 0.25*M* sucrose containing 0.01*M* tris-chloride, *p*H 7.5.

Additive (*M*)	Ca^{++} bound (μmole min^{-1} mg^{-1} of protein)	
	System III (ATP + succinate)	System IV (ATP)
Fresh mitochondria		
No addition	0.56	0.33
Antimycin (10^{-6})	.31	.30
Dicumarol (10^{-5})	.04	.04
Dinitrophenol (2×10^{-4})	.05	.04
Aged mitochondria		
No addition	0.40	0.01
Antimycin (10^{-6})	.02	.01
Oligomycin (10^{-5})	.35	.00

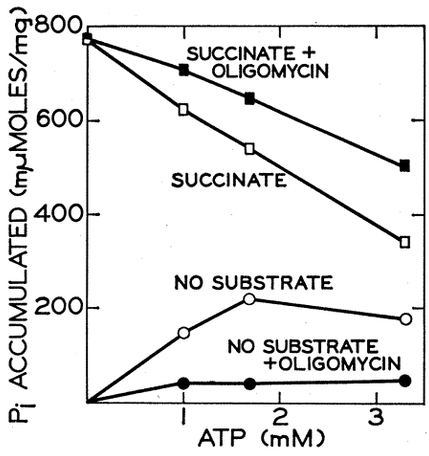

Fig. 1. Effect of ATP concentration on the accumulation of magnesium phosphate. The incubations were carried out at *p*H 7.4 as described previously (*3*) in a medium consisting of 0.25*M* sucrose, 17 m*M* $MgCl_2$, 3.3 m*M* potassium phosphate, 10 m*M* tris-chloride, and 1.7 mg mitochondrial protein per milliliter. After 15 minutes the mitochondria were separated by centrifugation, and the intramitochondrial inorganic phosphate (P_i) was determined. Open circles, no added substrate; solid circles, same with oligomycin ($10^{-5}M$); open squares, succinate (3.3 m*M*); solid squares, succinate with oligomycin.

A similar accumulation of Ca^{++} also proceeds in heart mitochondria. The binding of Ca^{++} is accompanied by an accumulation of inorganic phosphate (P_i), and the molecular ratio of Ca^{++} to P_i is usually about 1.5 to 1.0. Lesser amounts of Mg^{++} (about 15 percent of the Ca^{++}) also are accumulated along with small and variable amounts of K^+. Preliminary electron-microscope studies of mitochondria which have accumulated Ca^{++} (*7*) show large electron-dense deposits (presumably calcium phosphate) within the mitochondria. Coincident with ion accumulation, ATP is hydrolyzed and H^+ is released into the medium. The amount of H^+ released is considerably in excess of that which would be expected as a result of ATP hydrolysis. We have suggested (*3*) that the large H^+ production in system I could result from precipitation of $Mg_3(PO_4)_2$, and it is likely that precipitation of calcium phosphate is responsible for the H^+ produced when Ca^{++} is accumulated.

When substrate is present in addition to ATP (system III) the rate of accumulation (*8*) is much greater than when ATP alone is supplied as the source of energy (system IV). The data of Fig. 3 show that the effect of substrate is very pronounced at low concentrations of ATP. System IV is inhibited by oligomycin but is not affected appreciably by inhibitors of electron transport (*9*). System III is inhibited by cyanide and antimycin and the rate of accumulation that results corresponds well with the rate obtained in system IV. The experiments described in Fig. 3 also show that system III is inhibited to some extent by oligomycin; the rate corresponds closely to that which would be predicted if the rate induced by ATP alone were subtracted.

The effect of eliminating the ATP-supported accumulation can also be seen in aged mitochondria. These mitochondria (aged 15 to 30 minutes at 30°C in the absence of substrate) no longer accumulate Ca^{++} in presence of ATP alone. This loss of activity (Table 2) cannot be explained entirely by depletion of endogenous substrate, since a large portion of the accumulation by system IV in fresh mitochondria is insensitive to antimycin and cyanide. The absence of system IV in aged mitochondria makes the requirements of the substrate-supported portion of system III much more readily discernible. System III in aged mitochondria is almost completely inhibited by antimycin, while oligomycin has relatively little effect (Table 2).

Thus, either ATP or substrate can supply the energy for Ca^{++} accumulation, and the two reactions can be studied separately in the presence of the appropriate inhibitor. In order to evaluate the relative contribution to Ca^{++} accumulation of the two sources of energy, the following points must be considered. The concentration of Ca^{++} employed completely suppresses net oxidative phosphorylation. The mechanism of this suppression is obscure and may be related to the accumulation phenomenon. Regardless of the exact mechanism of uncoupling, ATP formation cannot be observed in intact mitochondria treated with 3 m*M* Ca^{++}.

It is therefore very unlikely that the substrate can affect accumulation by way of ATP production in any of the Ca^{++} reactions considered here. In system IV ATP supplies the energy for accumulation since there is negligible oxidation and large amounts of ATP are hydrolyzed. When ATP hydrolysis is inhibited by oligomycin, low concentrations of azide, or aging, Ca^{++} accumulation by system IV also disappears. In system III both oxidation and ATP hydrolysis occur and both substrate and

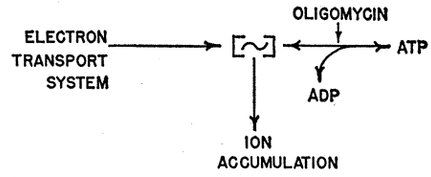

Fig. 2. Relation of the postulated intermediate, HEC (represented by the wiggle sign), to the synthesis of ATP and to ion accumulation.

ATP probably contribute to the observed accumulation. The Ca^{++} accumulation which occurs when substrate is oxidized in the presence of oligomycin, azide, or in aged mitochondria appears to result almost exclusively from the oxidation, since ATP hydrolysis is negligible and the reaction is sensitive to inhibitors of electron transport. It remains to be explained why small amounts of ATP are required for high rates of accumulation in the systems in which the accumulation is supported by substrate oxidation. In these cases the ATP seems to serve a secondary function. In the presence of substrate, but in the absence of ATP, some Ca^{++} is accumulated in the first 1 to 2 minutes of reaction. Unless ATP is added, this accumulated Ca^{++} rapidly diffuses back into the medium. It is well known (*10*) that Ca^{++} affects mitochondrial integrity, and it is possible that small amounts of ATP may

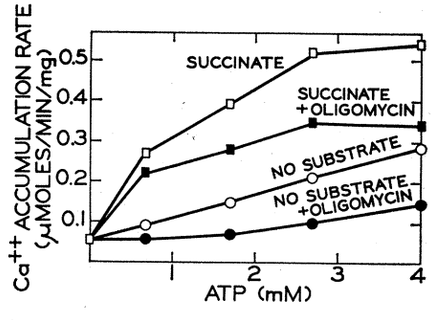

Fig. 3. Effect of ATP concentration on the accumulation of calcium phosphate. The incubation medium contained 0.25*M* sucrose, 10 m*M* $MgCl_2$, 3.3 m*M* $CaCl_2$ (labeled with Ca^{45}), 3 m*M* potassium phosphate (*p*H 7.0), 3 m*M* imidazol buffer (*p*H 7.0), and 2.5 mg mitochondrial protein in a total volume of 3.0 ml. After incubation for 2 minutes at 36°C the mitochondria were separated by rapid filtration (Polypore filter, Gelman Instrument), and the Ca^{++} accumulation rate was determined by radioactivity. Open circles, no substrate; solid circles no substrate with oligomycin ($2 \times 10^{-6}M$); open squares, succinate (3.3 m*M*); solid squares, succinate with oligomycin.

be required to maintain mitochondrial structure so that the bound Ca^{++} can be retained.

These considerations permit us to postulate that Ca^{++} accumulation can also be supported by an intermediate such as that proposed in Fig. 2 for Mg^{++} accumulation. Again in Ca^{++} accumulation as in the Mg^{++} reaction, the ATP-supported reaction may involve a reversal of the enzymatic mechanism for ATP production by way of oxidative phosphorylation, but other oligomycin-sensitive energy transfer mechanisms appear to be more likely. One piece of evidence against a simple reversal of oxidative phosphorylation is provided by the observation that aged mitochondria which cannot bind Ca^{++} in the absence of substrate retain rather high P/O ratios.

Although the accumulation of Mg^{++} and Ca^{++} has many features in common, it is not yet clear whether these ions are accumulated in the mitochondrion by the same system, or whether several mechanisms for accumulation exist. It should also be made clear that more than one high-energy intermediate may be involved. The only criterion which must be met by such a compound is its location with respect to the site of oligomycin inhibition.

It is also not yet clear whether participation of high-energy compounds other than ATP is a feature of ion-transport systems in general or is unique in mitochondrial reaction such as those described (*11*).

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8. The accumulation of Ca^{++} is a very rapid reaction; in an incubation medium similar to that of Vasington and Murphy (*6*) heart mitochondria take up about 75 percent of the available Ca^{++} (3.3 m*M*) in 5 minutes. For this reason we have evaluated the effect of inhibitors and uncouplers on the initial rate of Ca^{++} uptake and not on net accumulation. At low Ca^{++} concentrations (*5*) the rate must be determined in the first 30 to 40 seconds of reaction to be meaningful.
9. Endogenous respiration is inhibited to a large extent by the concentrations of Ca^{++} used.
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11. Supported by National Heart Institute grant HE-00458 (USPH) and by Atomic Energy Commission contract AT (11-1)-909. We thank Oscar Mayer and Co., Madison, for meat by-products, D. G. Hadley for technical assistance, Dr. H. F. DeLuca for many discussions, and Dr. E. Bachmann for ATPase determinations.

10 January 1963

Superconducting Indium Antimonide

Indium antimonide transforms from a semiconducting to a metallic state at 22.5 kb at room temperature (*1*). X-ray powder photographs taken at high pressures show that the metallic phase has the white-tin structure with a random distribution of the In and Sb atoms (*2*). As In and Sb are virtually indistinguishable by ordinary x-ray techniques, no information about possible ordering of the atoms has been reported.

At atmospheric pressure the lattice constant of zinc-blende type InSb is nearly the same as that of gray tin, and it appeared probable that if the metallic phase of InSb could be obtained in a metastable state at 1 atmosphere the volume of the unit cell would be very nearly that of white tin. On considering especially the importance of the volume (*3*) and the lesser importance of the electron-atom ratio, we thought that the metallic form of InSb would be a superconductor with a transition temperature near that of white tin.

The high pressure transition in InSb is reported to be very sluggish, especially if a single crystal is compressed hydrostatically (*4*). Our attempts to recover the metallic form by the use of a Teflon cell and a 40-centistoke silicone oil as the pressure cell and transmitting medium respectively, in a piston-cylinder apparatus designed by Boyd and England (*5*) and Kennedy (*6*) failed.

Work done at the University of California, Los Angeles (*7*), indicated that the metallic form of InSb could be recovered at liquid-nitrogen temperatures. Accordingly, we made an opposed-anvil apparatus from hardened tool steel. The sample cell was similar to the Bridgman (*8*) design. This cell has a retaining ring of isomica (*9*) that is 0.016 inch thick and has an outside diameter of ½ inch and an inside diameter of $\frac{3}{16}$ inch. The sample cell has a silver chloride disk 0.012 inch thick by $\frac{3}{16}$ inch in diameter. The InSb sample itself is 0.012 inch thick by $\frac{3}{32}$ or $\frac{3}{16}$ inch in diameter. The anvil assembly was placed in a stainless-steel beaker with a Styrofoam jacket and was partially insulated from the press by insulating blocks composed of alternating sheets of stainless steel and mica.

The resistivity was monitored by measuring the drop in voltage across the anvils per unit of applied current. As the transition on a single crystal sample is sluggish under quasi-hydrostatic conditions, the samples were kept above the transition pressure for 3 to 4 days. Then liquid nitrogen was added to the beaker and the pressure was released.

Three consecutive experiments were performed in this apparatus: (i) the nitrogen was evaporated and the resistivity was monitored as the sample warmed to room temperature, which proved that the metallic form had been stabilized at the temperature of liquid N_2 and even higher; (ii) an attempt was made to take an x-ray powder photograph at low temperatures to verify the volume and structure of the metallic form, but as a result of the difficulty of loading the camera at low temperatures the experiment failed; (iii) the sample was transferred to a helium-3 cryostat and was tested for superconductivity by the alternating-current method of Schawlow and Devlin (*10*). This sample was superconducting at 2.1 ± 0.2°K. Measurements of critical fields were made down to 0.3°K with the plane of the disk-shaped sample parallel to the magnetic field. The results can be extrapolated to a critical magnetic field at $T = 0$°K of 1.1 kgauss. This high value is undoubtedly due to strains (*11*).

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