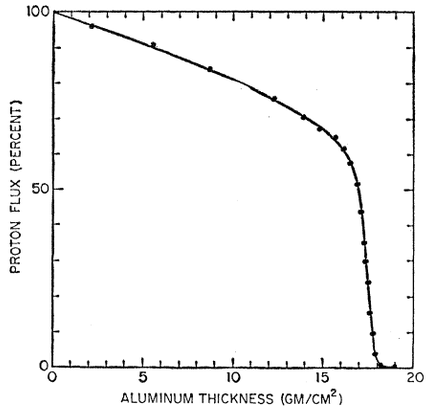

Fig. 2. Proton flux as a function of depth in aluminum. The steeply falling portion of the curve near 18 g/cm² is used to obtain the high contrast of Fig. 1.

in Fig. 2. Near total thickness of 18 g/cm² the flux is changed rapidly by small changes in thickness. A test object with average thickness equivalent to 18 g/cm² of aluminum, but with local voids, transmits a greater flux through the portion containing the voids; this variation can be recorded on photographic film pressed against the exit face of the test object. A thin object can be built up to equivalence to 18 g/cm² by placement of additional absorber ahead of it. Irregular objects can be radiographed by immersing them in an appropriate fluid contained in a parallel-sided box.

The proton exposure required to produce a radiograph depends on the photographic film and the thickness of the absorber used. A surface exposure of 2×10^9 proton/cm² was required for Fig. 1, with relatively insensitive Polaroid type-52 film (*2*). Induced radioactivity has not been a problem at such low exposures. Substantially lower exposures are sufficient if more-sensitive film is used in contact with an intensifying screen.

The smallest change in thickness that can be detected depends on the steepness of the flux-depth curve and on the characteristics of the photographic film. With Polaroid type-52 film and the proton beam just described, the minimum detectable change is now 0.01 g/cm², or about 0.05 percent of the total thickness examined. Since the spatial resolution is so poor by comparison, the technique is best suited to detection of thin cracks or inclusions having considerable extent transverse to the beam, or to detection of density changes as small as 0.01 g/cm³ extending over a volume of 1 cm in diameter, or greater, within a nearly homogeneous object. Applications to detection of flaws in technical materials and to medical radiography are possible.

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15 January 1968

Sodium Ion Influences on Phosphorylations Associated with Oxidation of Succinate by Turnip Root Mitochondria

Abstract. *Sodium ions (10^{-3} mole/liter) cause a marked increase in the ratio of phosphate esterified to oxygen utilized when turnip root mitochondria oxidize succinate. Separate study of the two phosphorylation sites associated with succinate oxidation indicates that the observed effect is a summation of differential responses of these sites to sodium ions. The activity of the first site, that associated with the reduction of cytochrome* c, *is stimulated about threefold by 10^{-3} molar NaCl, whereas phosphorylation at the second site, coupled with the oxidation of ferrocytochrome* c, *is slightly inhibited by the same concentration of NaCl.*

Although the yield of certain crops is increased as a result of the application of sodium salts (*1*) and although *Atriplex vesicaria* has a specific requirement for sodium when cultured in water carefully purified of Na^+ (*2*), sodium is more commonly considered to be either not required by plants or specifically toxic to them. The search for explanations of the variety of responses elicited from plants by Na^+ has only recently turned to study of the effects of this ion on cell-free systems. Pea seedlings grown 6 days in $75 \times 10^{-3}M$ NaCl produce mitochondria which have a higher rate of oxidation but the same P/O ratios [phosphate esterified (micromoles per hour) to oxygen utilized (microatoms per hour)] as mitochondria from untreated seedlings (*3*). However, NaCl added to mitochondria during and after preparation has no effects on either oxidation or phosphorylation (*3*), but the experimental procedures indicate the presence of substantial concentrations of K^+, which in our experiments can mask the effects of added Na^+.

We have tried to determine the effects of Na^+ in a wide range of concentrations on the oxidation of succinate by plant mitochondria and the phosphorylations which accompany this oxidation.

Fresh turnips (*Brassica rapa, L.*) were obtained from a local market. Mitochondria were prepared from 200 g of root tissue by the method of Wedding and Black (*4*), the mitochondrial pellet being suspended after the second wash in 2 ml of 0.4*M* sucrose in 0.05*M* tris-Cl buffer [tris(hydroxymethyl)aminomethane] at *p*H 7.0.

The oxidative and phosphorylative capacity of mitochondria is usually measured in experiments where no particular effort is made to eliminate or restrict monovalent cations. In addition to the endogenous ions contributed by the tissues from which mitochondria are prepared, many substrates and cofactors are available only as Na or K salts. To provide for a minimum concentration of monovalent cations in the reaction mixture, we used all substrates and cofactors as tris or magnesium salts as indicated in the legends for the figure and tables. These salts were prepared either by neutralization of commercially available free-acid preparations, or by passage of Na or K salts of commercial products through Dowex 50 (8 percent cross-linkage, 400 mesh) ion-exchange resin followed by adjustment of the *p*H with tris base or MgO. The Na^+ content of flasks receiving no added NaCl was $5 \times 10^{-5}M$.

The oxidation of succinate was measured in a Warburg respirometer at 27°C. At the end of a 10-minute equilibration period, the system was closed and the mitochondria were tipped in from a side arm. Oxygen uptake was measured for the subsequent 30 minutes, after which the flasks were removed and placed in an ice bath. For measurement of phosphate esterification, 0.5 ml of the reaction mixture was pipetted into 2.0 ml of 10 percent trichloroacetic acid and centrifuged at 500*g*, and 0.2-ml samples of the clear supernate were used for determination of residual inorganic phosphate (*4*).

The "P/O" ratios relating to the re-

duction of cytochrome *c* by succinate were measured by a modification of the method of Bogström, Sudduth, and Lehninger (*5*). The appearance of ferrocytochrome *c* was followed at 540 nm after the reaction was initiated by the addition of ferricytochrome *c* to the cuvette. The reactions were followed in a recording spectrophotometer with the cell compartment controlled at 27°C. After a suitable reaction interval, usually 10 minutes, when 20 to 40 nmole of cytochrome *c* had been reduced, the reaction was halted by the addition of 1 ml of 20 percent trichloroacetic acid, the preparation was centrifuged at 500*g*, and the amount of unesterified phosphate remaining was determined with a 4-cm cell for measurement of phosphomolybdate absorbance. Samples of 2 ml from each cuvette were reacted without dilution to produce the phosphomolybdate color. With this technique it was possible to reproducibly measure a difference of 1 nmole in P_i concentration in the 3-ml reaction mixture.

Measurement of the oxidation of ascorbate and its coupled phosphorylation was carried out in a Warburg respirometer by the method of Maley and Lardy (*6*). After a 10-minute temperature equilibration, the reaction was initiated by tipping in the mitochondrial suspension from a side arm; oxygen uptake was measured for 45 minutes. The reaction was stopped by the addition of 1 ml of the reaction mixture to 2 ml of 10 percent trichloroacetic acid, and phosphate esterification was determined on a 1.2-ml sample of the centrifuged solution, with the absorbance read in a 1-cm cell.

A slight increase in succinate oxidation occurs when $10^{-3}M$ NaCl is added to the reaction mixture; a larger decrease occurs as the concentration of Na^+ is raised to 10^{-1} mole/liter (Fig. 1). A pronounced stimulation of phosphorylation is brought about by $10^{-3}M$ NaCl, amounting to a 70 percent increase in the rate of adenosine triphosphate formation as compared with the control containing no added NaCl. The P/O ratios calculated from these values progress from 0.73 for the control, to a maximum of 1.11 at $10^{-3}M$ NaCl and decline to 0.67 for the mitochondria treated with $10^{-1}M$ NaCl.

Experiments of the same type with KCl demonstrate a similar, although less pronounced, stimulation of phosphorylation, with a maximum effect at 10^{-3} mole/liter and an inhibition at higher concentrations. The increase in phosphate esterification induced by $10^{-3}M$ K^+ is approximately 30 percent, but the curves for both succinate oxidation and phosphorylation as a function of K^+ concentration otherwise resemble the lines in Fig. 1. Data for other monovalent cations are not available, but the stimulatory effect demonstrated for Na^+ and K^+ is probably not limited to these ions.

Experiments including both Na^+ and K^+ in which the concentration of KCl was maintained constant while that of NaCl varied over the range 10^{-5} to 10^{-1} mole/liter showed that a KCl concentration between 10^{-3} and 10^{-2} mole/liter was sufficient to completely mask any stimulatory effect of Na^+ on phosphorylation.

Repetition of the experiment summarized in Fig. 1 with Na_2SO_4 used instead of NaCl gives essentially identical results, indicating that the observed responses are not due to the chloride ion.

The two phosphorylations which accompany the oxidation of succinate, one associated with the reduction of ferricytochrome *c* and the other with the oxidation of ferrocytochrome *c*, have been experimentally isolated by the use of appropriate substrates and inhibitors (*6*, *7*). The methods employed, developed for use with rat liver mitochondria, have proven effective with turnip root mitochondria as well, and have been utilized in determining the effects of Na^+ on the activity of these two phosphorylation sites individually.

The first site, in terms of the sequence of transport of electrons from succinate, is isolated by preventing the oxidation of ferrocytochrome *c* through the addition of high concentrations of CN^-. The rates of cytochrome *c* reduction and accompanying phosphorylation when turnip root mitochondria are supplied with succinate in the presence of a range of NaCl concentrations are given in Table 1.

It may be seen that $10^{-3}M$ NaCl causes a threefold stimulation of phosphate esterification. The maximum "P/O" ratios occur between 10^{-4} and $10^{-3}M$ NaCl, with a peak at 1×10^{-3} mole/liter, when the value of 0.75 approaches the theoretical value of 1, which may be an indication that an appropriate concentration of monovalent cations is actually a requirement for optimum activity of this phosphorylation site. There is little effect of NaCl on cytochrome *c* reduction except at

Table 1. Effect of added NaCl on the reduction of cytochrome *c* and coupled phosphorylation in turnip root mitochondria. Cytochrome *c* reduction and phosphate esterification were measured as described in the text. Each cuvette contained: magnesium succinate, 15 μmole; cytochrome *c*, 0.15 μmole; tris-ADP, 0.3 μmole; tris-PO_4, phosphate 1 μmole; tris-CN, 4 μmole; sucrose, 600 μmole; tris-Cl (*p*H 7.0), 75 μmole; glucose, 50 μmole; hexokinase, 0.2 units; NaCl as indicated; and 0.1 ml of mitochondrial suspension. The total volume was 3.0 ml, the temperature 27°C. Values given are means of three separate determinations. "P/O" ratios were calculated as P_i esterified divided by one half the cytochrome *c* reduced.

Added NaCl (mole/liter)	Cytochrome *c* reduced (nmole)	Phosphate esterified (nmole)	"P/O"
0	57.6	8.8	0.31
1×10^{-5}	55.2	8.8	.32
1×10^{-4}	59.2	11.0	.37
3×10^{-4}	67.0	14.2	.42
6×10^{-4}	58.4	16.9	.58
1×10^{-3}	60.6	22.7	.75
3×10^{-3}	64.0	20.5	.64
6×10^{-3}	60.0	12.3	.41
1×10^{-2}	58.4	10.1	.35
1×10^{-1}	20.2	2.5	.25

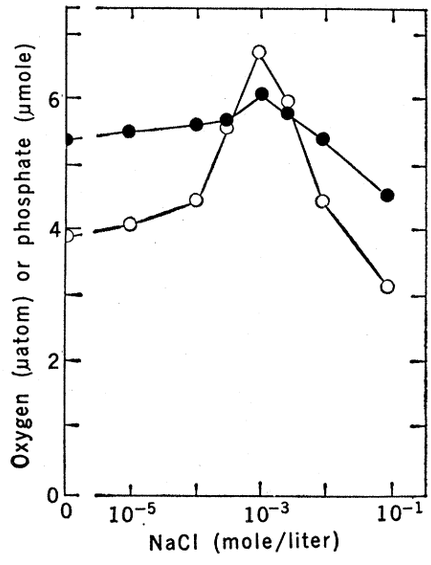

Fig. 1. Oxidation of succinate and coupled phosphorylation by turnip root mitochondria as affected by added NaCl. The "zero" concentration of NaCl is actually $10^{-5}M$. Oxygen uptake and phosphate esterification measured by methods described in the text. Each flask contained: magnesium succinate, 30 μmole; tris-PO_4, 30 μmole; tris-ADP, 0.5 μmole; sucrose, 644 μmole; tris-Cl (*p*H 7.0), 81 μmole; glucose, 50 μmole; hexokinase, 0.2 unit; NaCl as indicated; and 0.25 ml mitochondrial suspension. Total volume was 3.0 ml; the center well contained 0.2 ml of 5*N* KOH; the gas phase was air; the temperature was 27°C. (●) Oxygen uptake, microatoms per 30 minutes; (○) phosphate esterified, micromoles per 30 minutes. Each point is the mean of six determinations.

Table 2. Effect of added NaCl on the oxidation of ascorbate and coupled phosphorylation in turnip root mitochondria. Oxygen uptake and phosphate esterification were measured as described in the text. Each flask contained: tris ascorbate, 6 μmole; cytochrome *c*, 0.005 μmole; $MgSO_4$, 30 μmole; tris-ADP, 0.5 μmole; tris-PO_4, 4 μmole; sucrose, 600 μmole; tris-Cl (*p*H 7.0), 75 μmole; glucose, 50 μmole; NaCl as indicated; hexokinase, 0.2 units; and 0.5 ml of mitochondrial suspension. The total volume was 3.0 ml; the center well contained 0.2 ml of 5*N* KOH; the gas phase was air; and the temperature was 27°C. Values given are means of four determinations.

NaCl (mole/liter)	Oxygen uptake (μatom)	Phosphate esterified (μmole)	P/O
0	1.39	1.26	0.91
1×10^{-5}	1.43	1.26	.88
1×10^{-4}	1.55	1.24	.80
3×10^{-4}	1.59	1.10	.69
6×10^{-4}	1.65	1.02	.62
1×10^{-3}	1.70	0.97	.57
3×10^{-3}	1.67	.98	.58
6×10^{-3}	1.60	1.10	.69
1×10^{-2}	1.46	1.10	.75
1×10^{-1}	1.32	1.11	.83

$10^{-1}M$ NaCl, where the rates of both cytochrome *c* reduction and phosphorylation are diminished to about half of the control values.

The second site, separated from the other by the use of ascorbate as a reductant for cytochrome *c*, the oxidation of which is coupled to the phosphorylation, and molecular oxygen as a terminal acceptor for the electrons, is affected by NaCl in the manner detailed in Table 2. In this case, oxygen uptake is somewhat increased (about 20 percent) by $10^{-3}M$ NaCl, but phosphorylation is inhibited to a similar degree by the same concentration. The result is to reduce the P/O ratio from 0.91 to a value only slightly greater than ½ of the theoretical 1. Because the amount of mitochondria and the times of measurement differ for the experiments reported in Tables 1 and 2, it is not possible to calculate accurately the P/O ratios for the summation of the activity of both sites measured separately. But an estimation of these values, corrections being made for differing volumes, amounts of mitochondria, adenosine diphosphate and reaction time, gives results in general agreement with the data reported in Fig. 1, with the calculated P/O for the sum of both sites at $10^{-3}M$ NaCl being 80 percent higher than that for the preparations with no added NaCl as compared with a 50 percent increase in the P/O ratios actually observed in the experiments detailed in Fig. 1. It therefore seems reasonable to assume that the observed effect on phosphorylation when both sites are in operation is actually a summation of the effects of Na^+ observed on the two sites separately.

One might assume that the effect of Na^+ on phosphorylation may be related to the ($Na^+ + K^+$)-activated adenosine triphosphatases which have been reported from a variety of sources (*7*, *8*). A report of stimulation by K^+ of oxidative phosphorylation in brain mitochondria (*9*) has been explained on this basis. However, our evidence gives little support to such an assumption, particularly since the optimum concentration of Na^+ found here is one or two orders of magnitude lower than those reported for the adenosine triphosphatases. In addition, there appears to be no requirement for both cations. In experiments involving both Na^+ and K^+, the effect of the two ions in combination was simply additive.

Although it might be possible that Na^+ or other monovalent cations are required as an activator for the phosphorylation accompanying the reduction of cytochrome *c*, it seems improbable that the mechanism of this activation is through binding of the ion at a specific point, either the active site of the phosphorylating enzyme, or at an allosteric site. It is, of course, possible that the influence of the cations on the activity of both the phosphorylation sites studied is not exerted through an effect on the configuration of a single enzyme, but instead through influences on the architecture of the mitochondrion.

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7 March 1968

Cytotoxic Action of Stimulated Lymphocytes on Allogenic and Autologous Erythrocytes

Abstract. *Fowl erythrocytes are lysed when exposed to an excess of fowl blood lymphocytes in the presence of phytohemagglutinin. No significant cell damage is seen in the absence of phytohemagglutinin, or when the lymphocytes are replaced by malignant lymphoid cells, thymus cells, or nonlymphoid cells. The lymphocytes remain viable during the reaction. Differences in histocompatibility between lymphocytes and erythrocytes are not required. Autologous lymphocytes are cytotoxic to the same extent as allogenic lymphocytes over a wide range of experimental conditions.*

Lymphocytes are assumed to play an important role in tissue-damaging immune reactions, such as those occurring in delayed hypersensitivity, in various homograft and graft-versus-host interactions and in autoimmunity. Evidence for their possible direct participation in cell destruction comes from experiments in vitro in which antigen-carrying cells in tissue culture are damaged when exposed to lymphocytes from sensitized animals. The reactions are immunologically specific, do not require addition of complement, and are not correlated with titers of cytotoxic antibody in the blood of the lymphocyte donors (*1*). These reactions appear also when lymphocytes and target cells are of syngenic or even autologous origin (*2*). Furthermore, lymphocytes from normal donors may become cytotoxic for tissue culture cells, provided they have been stimulated by phytohemagglutinin (PHA) or antigen (*3–5*). Cytotoxicity requires living, immunologically competent lymphocytes whose energy-supplying metabolism is intact (*6*).

The use of nondividing target cells offers many advantages in studying the problem of the mechanism of lymphocyte cytotoxicity. We now report that fowl erythrocytes can easily be used for this purpose. In contrast to what has been found with tissue culture cells, lack of histocompatibility between lymphocytes from normal donors and fowl erythrocytes is not necessary for the PHA-induced cytotoxic reaction.

Twenty milliliters of blood from White Leghorn fowls (3 to 12 months old) were taken up in a syringe containing 100 I.U. of heparin in 3 ml of a 1:1 mixture of Hanks's balanced salt