Histones and Mitochondrial Ion Transport*

Carl L. Johnson, Charles M. Mauritzen, Wesley C. Starbuck, and Arnold Schwartz†

ABSTRACT: Very low concentrations of histones stimulate the release of K^+ from mitochondria by an energy-dependent process. Either electron transport or adenosine triphosphate can serve as the energy source. The histone-induced K^+ efflux is blocked by inhibitors of electron transport (antimycin A, cyanide, and amytal) or by energy-transfer inhibitors or uncouplers (octylguanidine, 2,4-dinitrophenol, and oligomycin). The phenomenon is also inhibited by chlorpromazine, atractyloside, and the divalent cations, Mg^{2+}, Sr^{2+}, and Mn^{2+}. Inorganic phosphate, in concentrations significantly lower than that required for oxidative phosphorylation, is necessary for optimal histone-induced K^+ efflux.

Investigations concerning mechanisms of mitochondrial ion transport have received impetus by the recent discovery of a number of agents which stimulate transport *via* energy-dependent processes. Valinomycin (Moore and Pressman, 1964; Harris *et al.*, 1966) and parathyroid hormone (Rasmussen *et al.*, 1964), *e.g.*, induce an uptake of K^+ by mitochondria, with the expenditure of energy. Under certain conditions these agents also cause an efflux of K^+ (Harris *et al.*, 1966; Rasmussen and Ogata, 1966) presumably by an energy-*independent* process. A preliminary report from this laboratory indicated that certain basic proteins are also capable of inducing a K^+ efflux from mitochondria (Johnson *et al.*, 1966), an event which appears to be associated with a stimulation of oxygen consumption, ATPase[1] activity, and swelling (Schwartz, 1965a,b; Schwartz *et al.*, 1966). Rasmussen and Ogata (1966) have recently reported that a basic polypeptide isolated from parathyroid glands induces a number of effects which are similar and some that are dissimilar to parathyroid hormone. The possible significance of mitochondrial ion transport in maintaining ionic balance in the cell (Harris *et al.*, 1966) and the fact that the cell contains appreciable amounts of basic proteins in the form of histones prompted further investigation into the mechanism of histone-induced K^+ efflux.

Materials and Methods

The sources of chemicals employed in this study are listed in a previous paper (Schwartz *et al.*, 1966). Tris salts of the chemicals were made by passing the particular Na^+ salt through a Dowex 50 (H^+) column. The content of Na^+ in the resultant salt was insignificant. The histone fractions f2a, whole β, and β-7 were isolated from ox thymus or rat liver and characterized by described procedures (Busch and Mauritzen, 1967). All three fractions are arginine rich, or "moderately" arginine rich; β-7 is electrophoretically pure.

Rat liver mitochondria were isolated by the method of Schneider (1948) and suspended in unbuffered 0.25 M sucrose in a final concentration of about 50 mg of protein/ml. Protein was determined by the biuret method (Jacobs *et al.*, 1956).

Potassium ion movements were estimated by means of a Beckman cationic electrode (no. 39137) connected to a Beckman research pH meter and the recorder portion (millivolt setting) of a Sargent XV polarograph. In general, the experiments were performed in a medium consisting of 125 mM choline chloride, 20 mM Tris-chloride, pH 7.4, and 3.3 mM Tris-phosphate. The data were programmed, automatically computed, and plotted by the Computational Research Center of Baylor University College of Medicine.

Atractyloside was generously donated by Dr. A. Bruni, the Public Health Research Institution, New York City. Appreciation is extended to Dr. B. C. Pressman, Johnson Research Foundation, Philadelphia, for generous supplies of valinomycin.

Results

K^+ Efflux Induced by Histones. Both histone fractions f2a and β-7 produce a marked efflux of K^+; the β-histone fraction appears to be more active than f2a (Figure 1). It should be noted that, until recently, no

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[1] Abbreviations used: ATPase, adenosine triphosphatase; ADP, adenosine diphosphate; TMPD, tetramethyl-*p*-phenylenediamine; NOQNO, 2-*N*-heptyl-4-hydroxyquinoline *N*-oxide; *m*-Cl-CCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol tetraacetate.

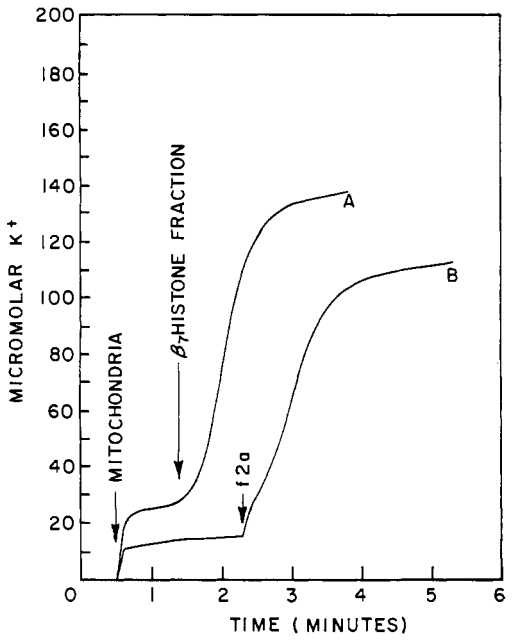

FIGURE 1: K^+ efflux induced by two histone fractions. The medium in each case consisted of 10 ml containing 125 mM choline-Cl, 20 mM Tris (pH 7.4), and 3.3 mM Tris-phosphate (final concentrations). The mitochondrial concentration was 1.1 mg of protein/ml. At the indicated points, 125 μg of β_7 histone (11 μg/mg of mitochondrial protein) was added to A and 125 μg of f2a histone (11 μg/mg of mitochondrial protein) to B. Values on the ordinate represent K^+ concentration in the external medium. In this and subsequent figures the data are representative of numerous reproducible experiments.

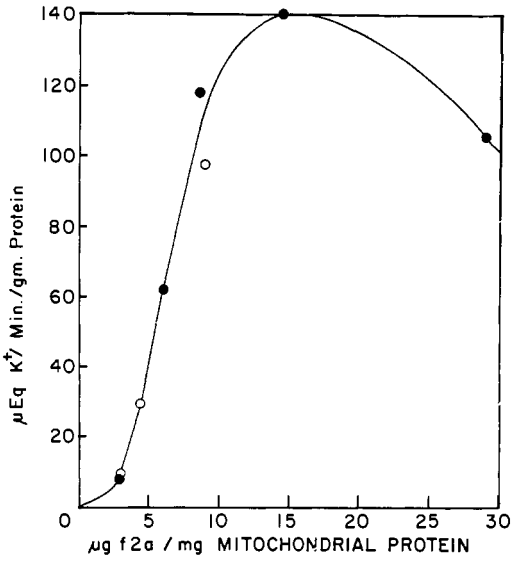

FIGURE 2: Effect of varying the f2a histone to mitochondrial protein ratio on the rate of K^+ efflux. The data were obtained by using a constant amount of histone and varying the mitochondrial concentration (O—O) or by using a constant amount of mitochondria and varying the histone concentration (●—●). The medium was the same as in Figure 1.

homogeneous histones have been available and previous studies in this laboratory have utilized relatively complex fractions; f2a, for example, consists of at least ten components. The β-7 fraction, however, appears to be a homogeneous, arginine-rich protein of molecular weight about 10,000.

The effect of varying f2a histone and mitochondrial concentrations is shown in Figure 2. A linear relationship exists between the rate of K^+ efflux and the amount of histone added, in the range of 3–9 μg of histone/mg of mitochondrial protein. Half-maximal efflux (K_m) is induced at approximately 6.5 μg of histone/mg of mitochondrial protein.

Requirements for Histone Stimulation of K^+ Efflux. As shown in Figure 1, the histone-induced K^+ efflux occurs in the absence of added substrate. Addition of exogenous substrate does not appreciably affect the efflux rate. After depletion of endogenous substrate, however, by a 4-min preincubation with ADP plus inorganic phosphate (3.3 mM), the β-histone-induced K^+ efflux decreased from 50 to 14.4 μequiv of K^+/min per g of mitochondrial protein, a drop of 72%. The latter is almost at the level of the passive K^+

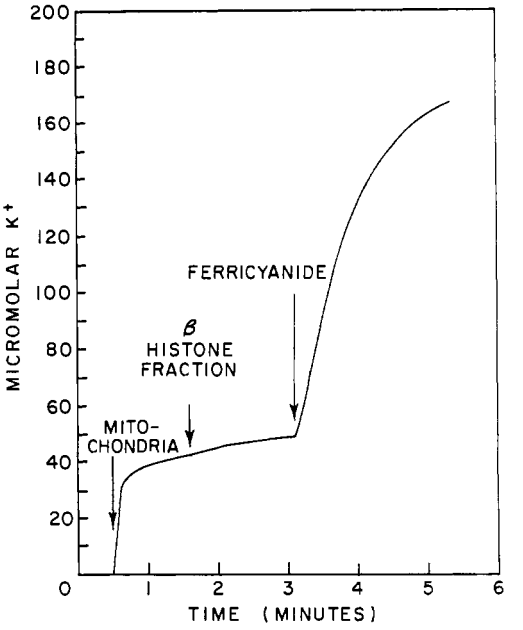

FIGURE 3: Effect of cyanide on histone-induced K^+ efflux. The medium was the same as in Figure 1 with the addition of 1 mM Tris-cyanide. The mitochondrial concentration was 1.7 mg of protein/ml. The additions were 250 μg of β-histone fraction (14 μg/mg of mitochondrial protein) and 1 mM Tris-ferricyanide.

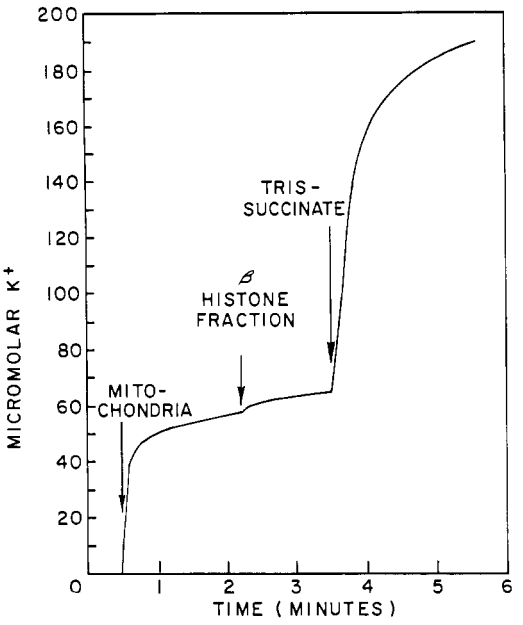

FIGURE 4: Effect of amytal on histone-induced K^+ efflux. The medium was the same as in Figure 1 with the addition of 2.5 mM Tris-amytal. The mitochondrial concentration was 1.7 mg of protein/ml. The additions were 250 μg of β-histone fraction (14 μg/mg of mitochondrial protein) and 6 mM Tris-succinate.

efflux rate (Figure 1). Oligomycin, which inhibits phosphorylation but not electron transport, prevents the effect of ADP plus phosphate; that is, histone-induced K^+ efflux is unchanged from control. The inhibition of histone-induced K^+ efflux caused by preincubation with ADP is overcome by subsequent addition of substrate (Tris salts of glutamate plus malate, succinate, or α-ketoglutarate).

The histone requires exogenous phosphate for *maximal* efflux rate. In a typical experiment, the K^+ efflux rate was 17.1 μequiv of K^+/min per g of protein in the absence of phosphate and 54.2 in the presence of 4 mM phosphate. A similar requirement for phosphate was observed for the histone stimulation of oxygen consumption (Johnson *et al.*, 1966). As in the case of histone-stimulated oxygen consumption (Johnson *et al.*, 1966) there appears to be no requirement for specific added cations.

The histone-induced K^+ efflux occurs in a medium containing 0.25 M sucrose and 20 mM Tris-Cl or 0.225 M mannitol, 75 mM sucrose, and 20 mM Tris-acetate.

Effect of Electron Chain Inhibitors. In an effort to define the site(s) of action of the histone, a number of inhibitors and artificial electron acceptors and donors were employed. As previously reported (Johnson *et al.*, 1966), antimycin A completely inhibits the histone-induced K^+ efflux. The inhibition is reversed by addition of ATP or TMPD. The latter presumably feeds elec-

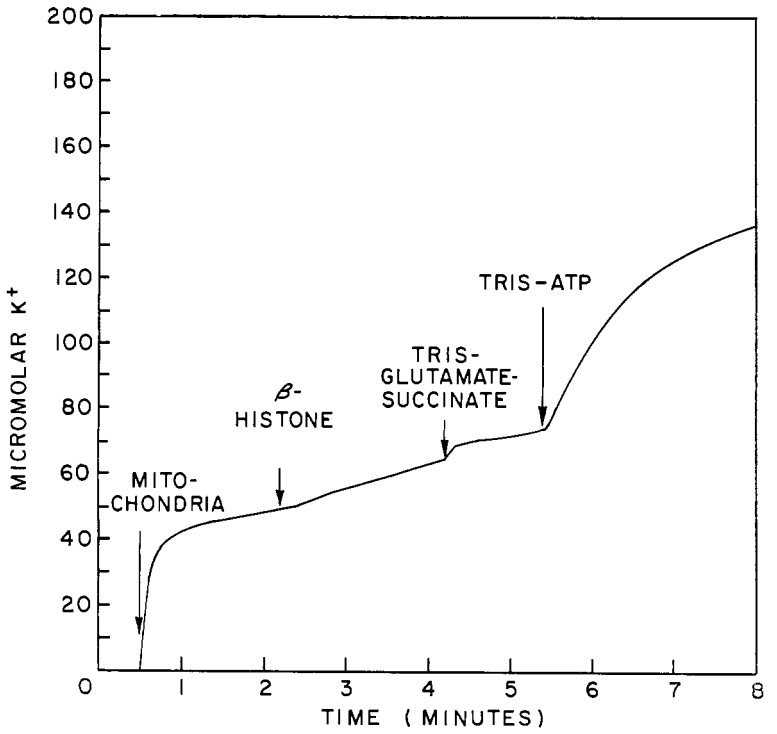

FIGURE 5: Effect of NOQNO on histone-induced K^+ efflux. The medium was the same as in Figure 1 with the addition of 20 μg of NOQNO (2 μg/ml). The mitochondrial concentration was 1.7 mg of protein/ml. The additions were 250 μg of β-histone fraction (14 μg/mg of mitochondrial protein), 6 mM Tris-succinate plus 6 mM Tris-glutamate, and 0.5 mM Tris-ATP.

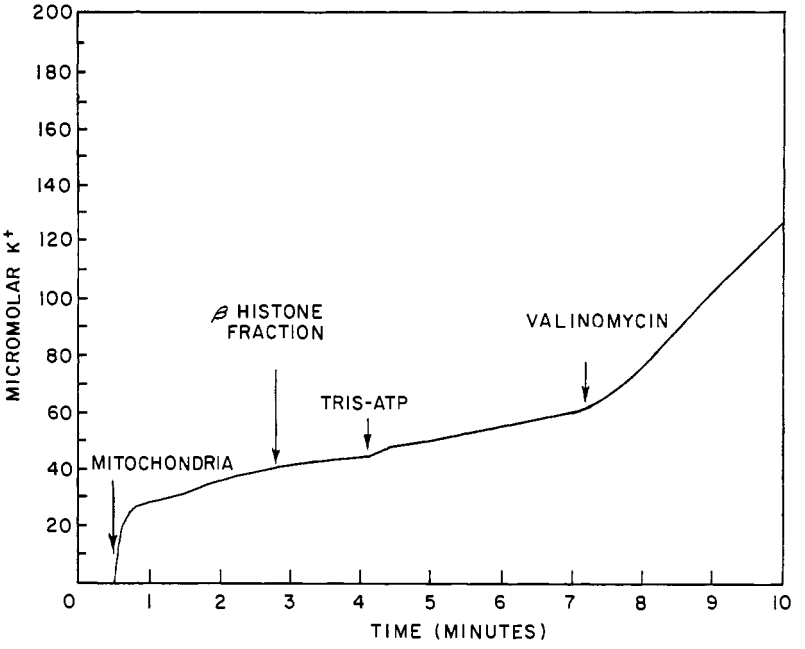

FIGURE 6: Effect of octylguanidine on histone-induced K^+ efflux. The medium was the same as in Figure 1, with the addition of 100 μM octylguanidine. The mitochondrial concentration was 1.6 mg of protein/ml. The additions were 250 μg of β-histone fraction (15 μg/mg of mitochondrial protein), 0.5 mM Tris-ATP, and 0.4 μg of valinomycin (0.025 μg/mg of mitochondrial protein).

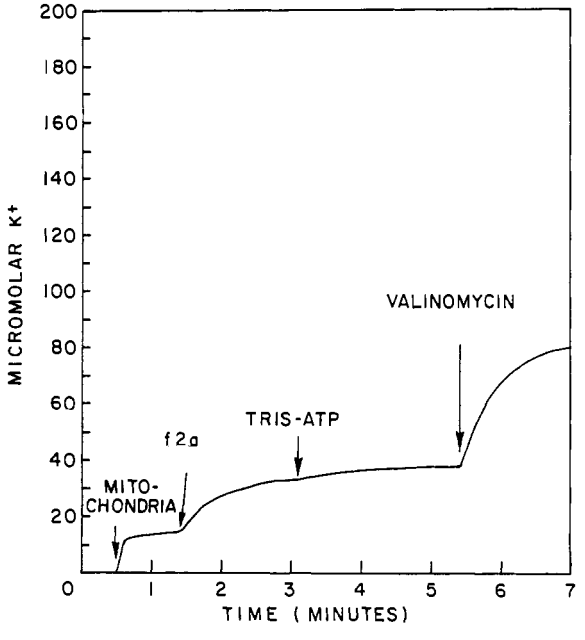

FIGURE 7: Effect of atractyloside on histone-induced K^+ efflux. The medium was the same as in Figure 1 with the addition of 150 μg of atractyloside (potassium salt and 25 μg/mg of mitochondrial protein) and 10 μg of antimycin A (1.5 μg/mg of protein). The mitochondrial concentration was 0.6 mg of protein/ml. The additions were 100 μg of f2a histone fraction (17 μg/mg of mitochondrial protein), 0.5 mM Tris-ATP, and 0.4 μg of valinomycin (0.07 μg/mg of mitochondrial protein).

trons into the respiratory chain beyond the antimycin A sensitive site (Tyler *et al.*, 1966; Packer and Mustafa, 1966).

The inhibitory effect of cyanide is shown in Figure 3. Addition of ATP or ferricyanide (the latter serving as terminal electron acceptor in place of oxygen) restores the ability of the histone to cause the release of K^+.

Amytal completely blocks the histone-induced efflux (Figure 4). The inhibition is reversed by addition of succinate; succinate-supported reversal is in turn prevented by malonate. K^+ efflux stimulated by histone is also strongly inhibited by rotenone. The inhibitory effect of NOQNO is shown in Figure 5. Like antimycin and amytal, the inhibition caused by this agent is also reversed by ATP.

Effect of Uncoupling Agents and Energy Transfer Inhibitors. The uncoupling agents 2,4-DNP and *m*-Cl-CCP cause a slight increase in K^+ efflux. However, they prevent any further stimulation of K^+ release by the histone, and the effect of these agents is not reversed by ATP.

Octylguanidine completely blocks the effect of the histone. Furthermore, the inhibition cannot be reversed by the addition of ATP (Figure 6). Oligomycin, aurovertin, and atractyloside are ineffective inhibitors since electron transport still supports the histone-induced K^+ efflux. In the presence of antimycin A or cyanide (with ATP as energy source), however, both oligomycin and atractyloside strongly inhibit the histone-induced efflux (Figure 7 and Johnson *et al.*, 1966). Aurovertin only partially blocks the histone effect. It is note-

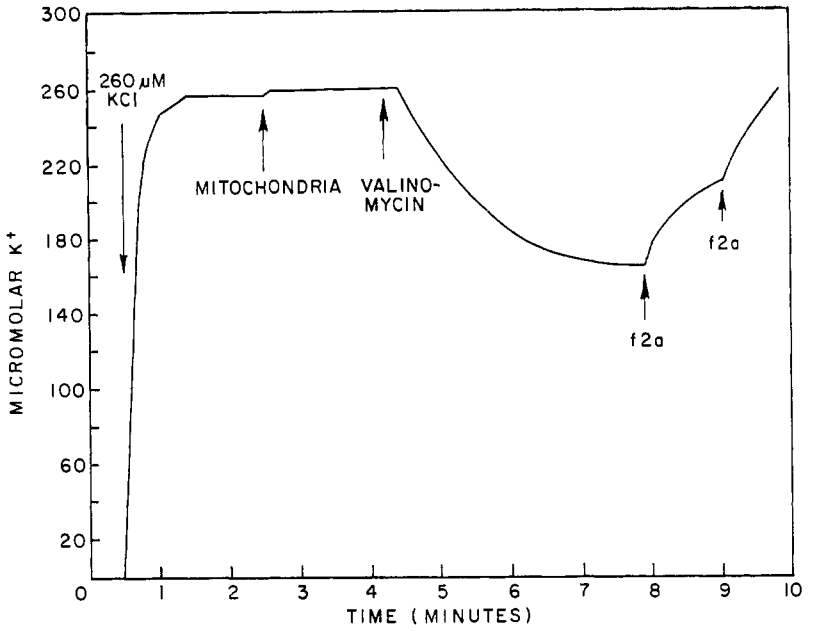

FIGURE 8: Effect of histone addition after K^+ uptake induced by valinomycin. The medium consisted of 10 ml containing 0.25 M sucrose, 3.3 mM Tris-phosphate (pH 7.4), and 3 mM Tris-glutamate plus 3 mM Tris-malate. The mitochondrial concentration was 2.5 mg of protein/ml. The additions were 0.4 μg of valinomycin (0.015 μg/mg of mitochondrial protein) and 125 μg of f2a histone fraction (5 μg/mg of mitochondrial protein).

worthy that valinomycin is still capable of inducing an efflux of K^+ even in a completely energy-blocked system (Figures 6 and 7).

Effect of Valinomycin. The uptake of K^+ induced by valinomycin in the presence of 260 μM exogenous K^+ and a suitable energy source is shown in Figure 8. Subsequent additions of histone result in the usual loss of K^+. It is also possible to show a partial uptake of K^+ by valinomycin after K^+ ejection induced by the histone.

Effect of Divalent Cations and Miscellaneous Inhibitors. The divalent cations Mg^{2+}, Mn^{2+}, and Sr^{2+} are potent inhibitors of the β-histone-induced K^+ efflux. Half-maximal inhibition by these agents occurs at concentrations of 8 mM Mg^{2+} and 0.25 mM Mn^{2+} or Sr^{2+}. Chlorpromazine at a concentration of 3×10^{-5} M produces 60% inhibition; EDTA, EGTA (8 mM), or ouabain (10^{-5} M) do not significantly affect the histone-induced K^+ efflux.

Effect of Low Temperature. An extensive study of the effect of temperature on the histone-induced K^+ efflux has not yet been made. However, preliminary results indicate that at 2° the histone effects are almost completely blocked. Rasmussen *et al.* (1964) reported that parathyroid-induced K^+ uptake occurs at 4°.

Discussion

The results presented in this paper clearly indicate the energy-dependent nature of histone-induced K^+ efflux. There appears to be no specific energy conservation site for the histone effects. Efflux occurs, *e.g.*, equally well with energy derived from electron transport at each of the three sites of phosphorylation.

When substrate oxidation is prevented, ATP may serve as a secondary energy source for ion movements and the ATP-supported histone-induced K^+ efflux is blocked by those agents which prevent ATP utilization, formation, or transport such as oligomycin, aurovertin, and atractyloside. It should be noted that the inhibition by aurovertin, in contrast to that by oligomycin, is incomplete, supporting the suggestion of Connelly and Lardy (1964) concerning a difference in sites of action of the two antibiotics.

It has been postulated that the intermediate(s) involved in ion transport lie off the main pathway of oxidative phosphorylation (Figure 9) (Moore and Pressman, 1964; Connelly and Lardy, 1964) and that the intermediate(s) common to both pathways lies between the octylguanidine (Pressman, 1963) and oligomycin-sensitive sites. Octylguanidine would be expected, therefore, to completely prevent the histone-induced K^+ movements with substrate (electron transport) as energy source.

There are two probable explanations for the results obtained after preincubation of the mitochondria with phosphate and phosphate acceptor, ADP. First, a depletion of endogenous substrate occurs which leads to a diminution of the energy-rich intermediate(s) required for the histone effects. Second, a competition may exist between ATP synthesis and ion transport for a common high-energy intermediate ("X ~ I"?) (see Figure 9). Excess ADP shifts equilibrium toward ATP synthesis and away from the ion-transport pathway.

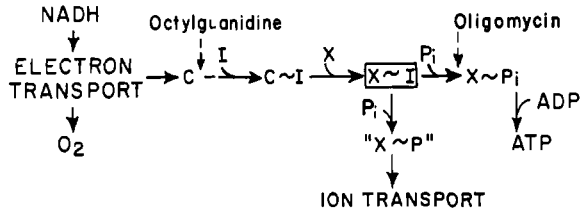

FIGURE 9: Possible energy intermediates in electron and ion transport.

Oligomycin, incidentally, prevents the formation of ATP and thus allows the energy intermediate(s) to be utilized for ion transport. Oligomycin might also prevent the rapid depletion of endogenous substrates by blocking the induction of state 3 respiration by ADP. K^+ efflux under these conditions would still be supported by substrate oxidation since the histone-induced oxygen consumption is oligomycin insensitive (Johnson *et al.*, 1966).

The requirement for inorganic phosphate for maximal efflux rates deserves some comment. Since histone-induced efflux occurs in the absence of added phosphate, attempts were made to deplete the endogenous phosphate with a glucose–hexokinase trap. Such attempts failed, however, because hexokinase alone strongly inhibits the histone effect. This may be due to the initiation of a state 3 respiration. It has not been possible as yet, therefore, to demonstrate a minimal requirement for phosphate. Several attempts to determine the K_m for phosphate on ion efflux were carried out. Although the results showed considerable variability, all values were significantly lower than the K_m for phosphate on oxidative phosphorylation measured under the same conditions as those employed for K^+ transport. Similar results have been reported for the respiratory release and K^+ uptake induced by valinomycin (Moore and Pressman, 1964). These authors postulate an oligomycin-insensitive, phosphate-dependent, ion-transport pathway off the main pathway of oxidative phosphorylation. The results presented here lend further support to the side-pathway theory.

Divalent cations (Mg^{2+} and Sr^{2+}) inhibit the histone-induced K^+ efflux in concentrations which have also been shown to inhibit histone-induced oxygen consumption, ATPase, and swelling (Schwartz *et al.*, 1966; Johnson *et al.*, 1966; Laseter and Schwartz, 1967). The data of Harris *et al.* (1966) indicate approximately 50% inhibition of valinomycin-induced K^+ influx by 9 mM Mg^{2+}. The present results show a 50% inhibition of histone-induced K^+ efflux by 8 mM Mg^{2+}. Similar inhibitory effects of Mg^{2+} on parathyroid hormone induced K^+ uptake and efflux have been reported (Rasmussen *et al.*, 1964; Rasmussen and Ogata, 1966).

Under the conditions of the present experiments, we were unable to measure any changes in H^+ concentration due to the strong buffering capacity of our system. We therefore examined the effect of the histone in a weakly buffered medium consisting of 0.25 M sucrose and 40 mM choline-Cl. No change in H^+ concentration was detected.

The requirement of energy for the induction of K^+ efflux, which represents a movement in the direction of the chemical potential gradient, appears to be an anomaly. However, it is possible that the histone effects are realized only when the mitochondrion is in a specific structural configuration, the latter requiring a particular energy state (Hackenbrock, 1966). Another and equally plausible interpretation is that histone uptake directly requires energy. This would also be consistent with the changes, noted previously, on oxygen consumption, ATPase, and swelling (Schwartz, 1965a,b; Schwartz *et al.*, 1966; Johnson *et al.*, 1966). The basic molecule may exchange for K^+, the latter being extruded in the usual energy-independent manner. This might be analogous to the NH_4^+ and substituted amine induced efflux of K^+ reported recently by Chappell and Crofts (1966). It is important to emphasize, however, that contraction of the mitochondria and H^+ extrusion accompany the amine uptake, while swelling and no apparent H^+ extrusion are characteristic of histone uptake.

The possible relationship between the effects induced by histone and those reported for valinomycin and parathyroid hormone are of interest. With regard to valinomycin-induced K^+ *efflux* a major difference exists: the histone event is energy dependent while the antibiotic-stimulated efflux is completely independent of an energy source. A comparison of histone and parathyroid hormone is also of current interest in view of some recent suggestions that some of the effects of the hormone may be due to the presence of a basic protein (Aurbach *et al.*, 1965). We have as yet been unable to carry out a detailed comparative study in this laboratory; however, some interesting aspects are available in the current literature. Both histone- and parathyroid hormone induced K^+ efflux are completely inhibited by Mg^{2+} (see Rasmussen and Ogata, 1966, Figure 16), an effect which incidentally is not shared by the basic peptide "peak 2" (Rasmussen and Ogata, 1966). It is not yet known whether the hormone-stimulated K^+-*efflux* is energy dependent.[2] The requirement for Mg^{2+} and K^+ in the case of the hormone-induced *oxygen uptake* (Rasmussen and Ogata, 1966) appears to be specific since neither the histone nor the basic peptide "peak 2" requires either cation for respiratory effects, thus differentiating the histone from the hormone. Histone-induced K^+ uptake has not as yet been demonstrated as it has for parathyroid hormone (Rasmussen *et al.*, 1964), although the efflux appears to be significantly reduced with increasing external K^+ (up to 1.2 mM; B. Safer and A. Schwartz, manuscript in preparation). It is evident that more detailed studies are required to elucidate the possible interesting relation-

[2] Very recent experiments in this laboratory have confirmed that purified parathyroid hormone effects an *energy-dependent* K^+ extrusion, under the conditions employed in this paper (note added in proof).

ships among all of the agents which effect significant changes in mitochondrial ion transport.

References

Aurbach, G. D., Houston, B. A., and Potts, J. T. (1965), *Biochem. Biophys. Res. Commun. 20*, 592.
Busch, H., and Mauritzen, C. M. (1967), *Methods Cancer Res.* (in press).
Chappell, J. B., and Crofts, A. R. (1966), *Biochim. Biophys. Acta 7*, 293.
Connelly, J. L., and Lardy, H. A. (1964), *Biochemistry 3*, 1969.
Hackenbrock, C. R. (1966), *J. Cell Biol. 30*, 269.
Harris, E. J., Cockrell, R., and Pressman, B. C. (1966), *Biochem. J. 99*, 200.
Jacobs, E. E., Jacob, M., Sanadi, D. R., and Bradley, L. B. (1956), *J. Biol. Chem. 223*, 147.
Johnson, C., Safer, B., and Schwartz, A. (1966), *J. Biol. Chem. 241*, 4513.
Laseter, A. H., and Schwartz, A. (1967), *Texas Rept. Biol. Med.* (in press).
Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.
Packer, L., and Mustafa, M. G. (1966), *Biochim. Biophys. Acta 113*, 1.
Pressman, B. C. (1963), *J. Biol. Chem. 238*, 401.
Rasmussen, H., Fischer, J., and Arnaud, C. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1198.
Rasmussen, H., and Ogata, E. (1966), *Biochemistry 5*, 733.
Schneider, W. C. (1948), *J. Biol. Chem. 176*, 259.
Schwartz, A. (1965a), *J. Biol. Chem. 240*, 939.
Schwartz, A. (1965b), *J. Biol. Chem. 240*, 944.
Schwartz, A., and Johnson, C. (1965), *Life Sci. 4*, 1555.
Schwartz, A., Johnson, C., and Starbuck, W. (1966), *J. Biol. Chem. 241*, 4505.
Tyler, D. D., Estabrook, R. W., and Sanadi, D. R. (1966), *Arch. Biochem. Biophys. 114*, 239.

Chemistry, Microscopy, and Performance of Methylated Glycerol-Treated Muscle Fibers*

William J. Bowen and Virginia L. S. Field

ABSTRACT: This investigation was made to learn more of the procedures, reactions, and products involved in the methylation of glycerol-treated rabbit psoas muscle fibers. It is known from earlier research that dimethyl sulfate methylated glycerol-treated muscle fibers lose their adenosine triphosphatase (ATPase) activity but retain two-thirds of their ability to shorten when ATP is applied. Shortening is also brought about by adenosine diphosphate (ADP), adenosine monophosphate (AMP), and sodium pyrophosphate (PP) which makes it appear that this shortening is due to the binding of the anions of these phosphate compounds to cationic sites of the methylated protein. Anionic sites of the protein are covered by methyl esterification and the fibers become predominantly cationic. Thus this type of shortening is anion induced rather than ATP induced. This appears not to be a general protein phenomenon because methylated collagen fibers did not shorten. Phase-contrast microscopy revealed that the structure of the fibers is reversibly distorted by methylation and subsequent hydration. When ATP or PP are applied to them, the fibers recover from the distortion and show clear I and A bands as well as H zones. Methylation with ^{14}C-labeled dimethyl sulfate revealed that carboxyl groups were implicated in only about two-thirds of the sites methylated. Attempts to identify the other one-third were made by means of chromatography and autoradiography and the results indicate that histidine and lysine residues also are sites of methylation.

It has been reported that methylated glycerol–water extracted fibers of rabbit psoas muscle retain ATP[1]-induced contractile ability and lose ATPase activity (Bowen and Martin, 1964). In those experiments fibers were methylated by exposure to dimethyl sulfate in aqueous buffered solution. Contractility was induced by ATP, ADP, AMP, and PP, all of which possess a large anion. Small anions also caused shorten-

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[1] Abbreviations used: DMS, dimethyl sulfate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; PP, sodium pyrophosphate; PMB, *p*-mercuribenzoate.