Packham, M. A., Rathbone, R. L., Guccione, M. A., Lloyd, J. V., Nishizawa, E. E., and Mustard, J. F. (1970), *Proc. Can. Fed. Biol. Soc. 13*, 107.
Papahadjopoulos, D., and Hanahan, D. J. (1964), *Biochim. Biophys. Acta 90*, 436.
Papahadjopoulos, D., Yin, E. T., and Hanahan, D. J. (1964), *Biochemistry 3*, 1931.
Seegers, W. H., and Landabaru, R. H. (1960), *Can. J. Biochem. Physiol. 38*, 1405.
Surgenor, D. M., and Wallach, D. F. H. (1961), *in* Blood Platelets, Johnson, S. A., Monto, R. W., Rebuck, J. W., Horn, R. C., Jr., Ed., Boston, Mass., Little Brown & Co., p 289.
Yin, E. T., and Wessler, S. (1968), *J. Biol. Chem. 243*, 112.

Kinetic Characterization of Oxidative Phosphorylation in *Alcaligenes faecalis**

Robert Adolfsen and E. N. Moudrianakis†

ABSTRACT: Monovalent cations were found to be capable of replacing divalent cations in the preparation of phosphorylating particles by centrifugation from cell-free crude extracts of *Alcaligenes faecalis*. Optimal phosphorylating activity was observed when the membrane fragments were sedimented in the presence of 0.1 M KCl. A comparison of particles prepared in $MgCl_2$ to particles prepared in KCl showed that the specific activity of phosphorylation was about the same, that ADP and DPNH requirements were nearly absolute, that inhibition by 2,4-dinitrophenol and KCN were almost total, and that the only product of the reaction was terminally labeled ATP. Respiratory control of DPNH oxidase activity by P_i and ADP, in contrast to previous studies on this system, was totally absent in both types of particles. Three roles for metal ions were found in this system. (1) They were required in the preparation of the particles, probably for the binding of coupling factors to membrane fragments. (2) They were required, along with 0.25 M sucrose, to stabilize the membranes to storage at −20°. (3) Divalent cations activated the phosphorylating reaction in a biphasic manner. The first phase of the activation was attributed to the formation of the Mg–ADP complex and was insensitive to K^+. The second phase was attributed to activation by free Mg^{2+}, and antagonism by K^+ was observed.

Oxidative phosphorylation has been studied in *Alcaligenes faecalis* for a number of years by Pinchot. The emphasis in that work was on the characterization of coupling factors, primarily the heat-stable factor (Pinchot, 1957; Shibko and Pinchot, 1961b), and then on a proposed high-energy intermediate (Pinchot, 1960; Pinchot and Hormanski, 1962). The purpose of this paper is to characterize the basic kinetic properties of electron transport and oxidative phosphorylation in this system. These studies are meant to provide a firm foundation for future studies on this system, which will include coupling factor purification and characterization and also probes into the mechanism of oxidative phosphorylation.

During the course of the kinetic characterization, several roles for metal ions were found. These related especially to the preparation of the phosphorylating particles, to stabilizing the activity of the system, and to activating the phosphorylating reaction. A striking independence of the phosphorylating system from the electron transport system was also observed. Variables that affected electron transport, such as DPNH concentration, affected phosphorylation similarly—as would be expected. However, variables that affected the rate of the phosphorylating reaction, such as ADP, P_i, $MgCl_2$, and KCl, had no effect at all on the rate of electron transport.

Materials and Methods

Preparation of Phosphorylating Particles. The culture medium for the growth of *A. faecalis* contained 0.2 g/l. of NaCl, 0.1 g/l. of $MgSO_4 \cdot 7H_2O$, 0.1 g/l. of $CaCl_2 \cdot 2H_2O$, 0.0038 g/l. of $Na_2MoO_4 \cdot 2H_2O$, 0.0225 g/l. of $FeSO_4 \cdot 7H_2O$, 0.15 g/l. of citric acid, 0.5 g/l. of yeast extract, and 30 g/l. of CasAmino acids. Medium (12 l. at a time) was prepared and NaOH was added to bring the pH to 8.0. The medium was autoclaved in 1-l. volumes in 6-l. flasks for 20 min at 20 psi. Slants of *A. faecalis* grown on medium containing 2% nutrient agar in addition to the usual components were stored at 4° until needed. The cells were suspended in about 15 ml of sterile medium and 1 ml of suspension was used to inoculate each flask. The cultures were grown for 21 hr at 37° on a shaker to an OD_{660} of approximately 3 (late log phase). The cells were then harvested in a refrigerated Sharples centrifuge and washed once in about 2 l. of 0.1 M KCl–0.25 M sucrose (KCl–sucrose). The wet weight yield of cells per liter of culture medium was approximately 11 g. After suspending the washed cells as a 20% (w/v) slurry in fresh KCl–sucrose, they were ruptured in an Aminco French pressure cell at a pressure of about 6 tons. Debris was removed by centrifugation at 20,000*g* for 10 min (15K in the Spinco No. 30 rotor), yielding the cell-free crude extract. This was

* From the Department of Biology, The Johns Hopkins University Baltimore, Maryland 21218. *Received August 17, 1970.* This work was supported by Grant AI-02443 from the National Institutes of Health and Grant GB-12128 from the National Science Foundation to Dr. G. B. Pinchot and by National Institutes of Health Grant GM-13518 to one of us (E. N. M.).

† To whom correspondence should be addressed.

spun at 78,000*g* for 1 hr (30K in the Spinco No. 30 rotor), and the resulting supernatant fractions were discarded. The pellets consisted of membrane fragments capable of oxidative phosphorylation.[1] These particles were resuspended in 360 ml of KCl–sucrose with the aid of a tissue homogenizer and then sedimented again at 78,000*g* for 1 hr. The supernatant fractions were discarded again, and the pellets were resuspended in about 240 ml of KCl–sucrose. Protein concentration was determined, and enough KCl–sucrose was added to give a final protein concentration of 4 mg/ml. The yield of particles was usually about 1.5 g. Aliquots (2 ml) of particles were stored at −20° until needed.

Assay of Oxidative Phosphorylation. Reagents stored at −20° were 5 mM ADP (Sigma, Grade I), 0.5 M glucose, and 50 E.U./ml of hexokinase (Calbiochem, A grade). Reagents stored at 4° were 0.08 M $MgCl_2$, 0.75 M sucrose, and 0.10 M potassium phosphate buffer, pH 7.4. DPNH (Sigma, Grade III) was prepared fresh daily, 20 mM in 0.01 M Tricine buffer, pH 8.0. Carrier-free [^{32}P]P_i was obtained from Tracerlab. The final concentrations of reagents in the reaction mixture, after the addition of 0.10 ml of phosphorylation particles to 0.90 ml of reaction mixture, were: 2 mM DPNH, 0.25 mM ADP, 5 mM P_i, 8 mM $MgCl_2$, 5 E.U. of hexokinase, 50 mM glucose, 0.10 M sucrose, and approximately 5 μCi of [^{32}P]P_i. The mixture was prepared immediately before use because DPNH was found to be slightly labile at pH 7.4. Aliquots of the reaction mixture were warmed to 30° for about 3 min before the addition of 0.10 ml of the suspension of phosphorylating particles. After 1 min the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. A control was always run in which particle protein was added after the 1 ml of trichloroacetic acid. Precipitated protein was removed by centrifugation, and 0.5 ml of the supernatant was used for the determination of esterified P_i by the method of Berenblum and Chain (1938), modified as follows: 3 ml of water saturated with 2-methyl-1-propanol–benzene (1:1), containing 1 μmole of carrier P_i, was added to 0.5 ml of the supernatant. Ammonium molybdate (1 ml, 5%) in 5 N H_2SO_4 was added, and 5 min were allowed to elapse for the formation of the phosphomolybdate complex. The complex was extracted with three 5-ml volumes of 2-methyl-1-propanol–benzene (1:1, saturated with water), followed by one extraction with 5 ml of ether. A volume of the remaining aqueous phase was plated, dried, and counted in an end-window, gas-flow Nuclear-Chicago counter. The recorded radioactivity (cpm) was corrected first for background counts and then for unextracted [^{32}P]P_i in the control system in which particle protein was added after acid. The unextracted phosphate was usually about 1 nmole out of 5000, giving an efficiency of extraction of 99.98%.

Assay of DPNH Oxidase Activity. The reaction mixture was identical with the one used in the assay of oxidative phosphorylation, except that no [^{32}P]P_i was present. The reaction was carried out in the same manner except that it was terminated with 1 ml of 0.1 N KOH. The precipitate, $Mg(OH)_2$, was removed by centrifugation and a fivefold dilution was made on the supernatant. The OD_{340} of DPNH, stable at this alkaline pH, was read and subtracted from a blank in which particle protein was added after KOH. The amount of DPNH oxidized was determined by using $a_M = 6.22 \times 10^{-3}$ (Pabst Laboratories, 1956).

This method of measuring DPNH oxidase activity had the advantage of employing conditions identical with those used in the measurement of phosphorylation. It had the disadvantage that computation of the amount of substrate oxidized involved taking the difference of two large numbers. Running duplicate systems and doing duplicate dilutions on each system minimized the error to about ±5%, when the initial rate of the reaction was 200–300 nmoles/min. The smaller the initial rate was, the larger the error problem became.

Protein Determination. The method of Lowry *et al.* (1951) was used, modified as follows: 2 ml of 12.5% Na_2CO_3 were added to 1 ml of the sample. Then 0.5 ml of 0.1% $CuSO_4$ was added, and 10 min later 0.5 ml of Folin reagent–H_2O (1:2) was added. The OD_{660} was read after 30 min at room temperature, and protein concentration was computed using bovine serum albumin as a standard.

TABLE I: Comparison of Phosphorylating Particles Prepared in 0.1 M KCl to Particles Prepared in 0.04 M $MgCl_2$.

	Phosphorylation		Oxidation			
	Specific Act.[b]	Total eu	Specific Act.[b]	Total eu	P_i/DPNH	Protein Sedimented[c]
$MgCl_2$[a]	191	1535	2055	19,750	0.09	1.20
KCl[a]	213	1835	1435	10,330	0.15	0.90

[a] KCl or $MgCl_2$ was added to aliquots of crude extract in 0.25 M sucrose to a final concentration of 0.1 or 0.04 M, respectively. The particles were sedimented at 10^5g for 30 min, then washed once and resuspended in 0.1 M KCl–0.25 M sucrose or 0.04 M $MgCl_2$–0.25 M sucrose, respectively. Oxidation and phosphorylation were assayed as described in Methods. [b] Specific activity is reported in nmoles/min per mg. [c] Protein sedimented is reported in mg/ml of crude extract.

Results

Substitution of KCl for $MgCl_2$ in the Preparation of Phosphorylating Particles. It has been previously reported that $MgCl_2$ was essential for the preparation of phosphorylating particles from crude extracts of *A. faecalis* (Shibko and Pinchot, 1961a). Other divalent cations were found to be capable of partially substituting for Mg^{2+}, but monovalent cations were not tried. The data in Table I show that sedimentation of membrane fragments in 0.1 M KCl gave phosphorylating particles that were just as good as those sedimented in 0.04 M $MgCl_2$. This suggested that the most important factor in the preparation of the particles was ionic strength, not the nature of the cation. The oxidase activity of Mg particles[1] was significantly higher than that of K particles. Preparations were slightly variable in this regard, and in general a higher level of activity was not observed unless both KCl and $MgCl_2$ were present during the preparation of the particles. The variability may have been related to a better sedimentation of very small membrane fragments as a result of the much more extensive aggregation of membrane fragments in the presence of divalent cations.

[1] Abbreviations used are: Mg particles, phosphorylating particles prepared from cell-free crude extract by centrifugation in the presence of 0.04 M $MgCl_2$–0.25 M sucrose; K particles, phosphorylating particles prepared by centrifugation in 0.1 M KCl–0.25 M sucrose; KCl–sucrose, 0.1 M KCl–0.25 M sucrose.

TABLE II: Characterization of the Reactions of Phosphorylating Particles Prepared in $MgCl_2$ or in KCl.

	Type of Particle		
	Mg (%)	K (%)	O[b] (%)
Phosphorylation[a]			
ADP requirement	93.0	95.8	
DPNH requirement	99.5	99.4	
DNP sensitivity	98.9	95.5	
KCN sensitivity	98.9	99.1	
Oxidation			
KCN sensitivity	69	78	63
ADP sensitivity	0	0	0
P_i sensitivity	0	0	0

[a] DPNH and ADP requirements were assayed by their respective omissions from the reaction mixture. The P_i sensitivity of oxidation was assayed by replacement with tricine buffer of the same concentration and pH. DNP was 5 mM and KCN was 10 mM when present. KCN was added immediately before the addition of the particles, since a precipitate began to form after a few minutes. The specific activity of phosphorylation in the control Mg particles and K particles was 206 and 285 nmoles per min per mg, respectively. The specific activity of oxidation was 1125 and 1245 nmoles per min per mg in the Mg and K particles, respectively. [b] The O particle consisted of membrane fragments sedimented in the absence of cations; it had no ability to catalyze oxidative phosphorylation.

TABLE III: Effect of the Hexokinase–Glucose Trap on the Charcoal Adsorbability of the Product of the Phosphorylating Reaction.[a]

Charcoal Treatment	Phosphate Esterification (nmoles/min)	
	ATP Trap Present	ATP Trap Inactivated
No	44.8	39.1
Yes	46.8	0.4
Charcoal-adsorbable product (ATP)	−2.0	+38.7

[a] Norit A (acid washed) charcoal was suspended in water, 25 mg/ml, and used without further activation. This slurry (1 ml) was added to an equal volume of the trichloroacetic acid terminated reaction system. This amount was just enough to adsorb all of the nucleotide. Glucose 6-phosphate was not adsorbable under these conditions. Precipitated protein and charcoal were removed by centrifugation, and aliquots of the supernatant fraction were assayed for organic phosphate as described in Methods. The values represent organic phosphate left in solution after the treatment indicated. Where listed as inactive, the hexokinase solution was boiled for 5 min before it was added to the reaction mixture. The assay system contained 0.20 mg/ml of particle protein.

Characterization of the Oxidation and Phosphorylation Reactions. Table II shows that the sensitivity of the phosphorylating reaction to DNP and the requirement for DPNH were essentially total. The slight activity seen in the absence of ADP may indicate the presence of a small amount of endogenous, or bound, ADP. The sensitivity to cyanide was complete for phosphorylation but only partial for oxidation, raising the possibility that there may be more than one DPNH dehydrogenase in the system. There was absolutely no respiratory control of oxidation either in Mg particles or in K particles. This was found using the DPNH oxidase assay described by Scocca and Pinchot (1968). The data in Table III show that the product of the phosphorylating reaction was adsorbable on charcoal and, therefore, nucleotide in character when the hexokinase–glucose trap was not functional. Since an active ATP trap gave complete conversion of this material to a nonadsorbable product (glucose 6-phosphate) the original product may be taken as entirely terminally labeled ATP. This has been verified by Dowex-formate chromatography (B. Salmon, unpublished data).

A claim has been made that the enzymatic activities of the crude extract were stable for several months when the extract was stored at −20° in water (Pinchot, 1967). In the present studies significant lability was noted (Table IV). The coordinate lability of oxidation and phosphorylation suggested that most of the lability was in some portion of the electron transport apparatus or something else in the membranes that could affect electron transport. The specific activity of phosphorylation stabilized after about 5 days at about 30 nmoles/min per mg. Most of the previous work on this system has been done with particles having a specific activity of 20–30 nmoles/min per mg. Freezing the crude extract in KCl–sucrose was found to reduce the lability to a few per cent in a month. Phosphorylating particles stored in KCl–sucrose at −20° lost no activity for at least a month.

Optimal Conditions for Particle Preparation. Figure 1a shows that the ability of the particles to catalyze phosphorylation was critically dependent on the concentration of KCl during the preparation of the particles. Membranes sedimented from the crude extract in the absence of KCl had no activity at all. Figure 1B shows that a slightly acidic pH gave optimal phosphorylating activity. Since the pH of the unbuffered crude extract was 6.6–7.0, no buffer was necessary for preparing the particles.

Optimal Conditions for the Assay of Oxidation and Phosphorylation. The effect of pH and concentrations of the three substrates of the system on oxidation and phosphorylation is shown in Figure 2. The studies were done at optimal or saturating concentrations of the other components, which were those described in Methods. Noteworthy are the atypical saturation curves for DPNH (Figure 2B) and the complete absence of respiratory control by ADP and P_i (Figure 2C,D).

A nearly total dependence of phosphorylation on $MgCl_2$ was found, with saturation occurring at about 8 mM (Figure 3A). In the presence of 0.1 M KCl the saturation was still at about 8 mM, but the magnitude of the rate was only about half as great as in the absence of KCl. Oxidase activity did not vary at all with $MgCl_2$ concentration (data not shown), this indicating that the response of phosphorylating activity to $MgCl_2$ concentration was metal ion activation occurring in the phosphorylating system. Note that the rate of phosphorylation was not affected by 0.1 M KCl at 0 or 0.5 mM $MgCl_2$. The activation was therefore biphasic, consisting of an early phase not sensitive to KCl and a later phase which was sensitive to KCl. Figure 3B shows the lack of variation

TABLE IV: Stability of the Crude Extract in 0.25 M Sucrose at −20°.[a]

Days of Storage	% Phosphorylating Activity Remaining	% Oxidase Activity Remaining
1	100	100
2	55	65
3	43	46
5	28	23

[a] Aliquots of crude extract frozen in 0.25 M sucrose were thawed and KCl was added to a final concentration of 0.1 M. The particles were sedimented, then washed once and resuspended in 0.1 M KCl–0.25 M sucrose and assayed as described in Methods. The activity remaining after 1 day of storage was arbitrarily set at 100% to facilitate comparison of subsequent losses of activity. Extrapolation of the rate of loss to 0 days suggested that more than half of the activity had already been lost by this time.

of oxidase activity with KCl concentration and the pattern of inhibition of phosphorylating activity by KCl at the optimal $MgCl_2$ concentration. The lack of effect on oxidase activity indicated that the site of action of KCl was also on the phosphorylating system.

Time Courses of Oxidation and Phosphorylation. Time courses were performed using the optimal conditions for assaying phosphorylation (Figure 4). The rate of both oxidation and phosphorylation decreased drastically after about 2 min. The limiting factor in the system had its primary action on the electron transport system and not the phosphorylating system, since the shapes of the curves were similar. A time course of DPNH oxidation in a reaction mixture consisting only of 10 mM Tricine, pH 7.4, and 2 mM DPNH showed the same pattern. The decrease in rate was, therefore, not caused by any of the other components in the standard reaction mixture. The effect could then be attributed to one of two possible causes: (1) DPN was accumulating to a critical level at which it became drastically inhibitory to the further oxidation of DPNH, or (2) something was happening in the membranes themselves as a consequence of DPNH oxidation. To determine which alternative was the correct one, a time course of DPNH oxidation was performed in a reaction mixture containing Tricine buffer and a combination of DPN and DPNH equivalent to that observed after the transition to the slower rate in the previous experiment (0.6 mM DPN–1.4 mM DPNH). The initial rate of oxidation was lower, which was attributable to competitive inhibition of oxidation of DPN, but the decrease in rate still occurred after 500–600 nmoles of DPNH had been oxidized. This suggested that the latter of the two alternatives was the correct one. The shape of the time course was modified by pH. The reaction was linear for about 4 min at pH 6.5, but a decrease to a lower rate was still observed after about 500 nmoles of substrate had been oxidized. Further studies on this phenomenon are in progress.

Relationship of Reaction Rate to Enzyme Concentration. The initial velocities of the reactions may be taken from the time course as the amount of ATP synthesized or DPNH oxidized in 1 min. Linearity in the response of the initial velocity of oxidation to enzyme concentration was observed only until the decrease in the rate of oxidation became important (Figure 5). The more protein present in the system, the sooner the decrease in rate occurred, and at 0.4 mg/ml the reaction was not linear for even 1 min. The response of

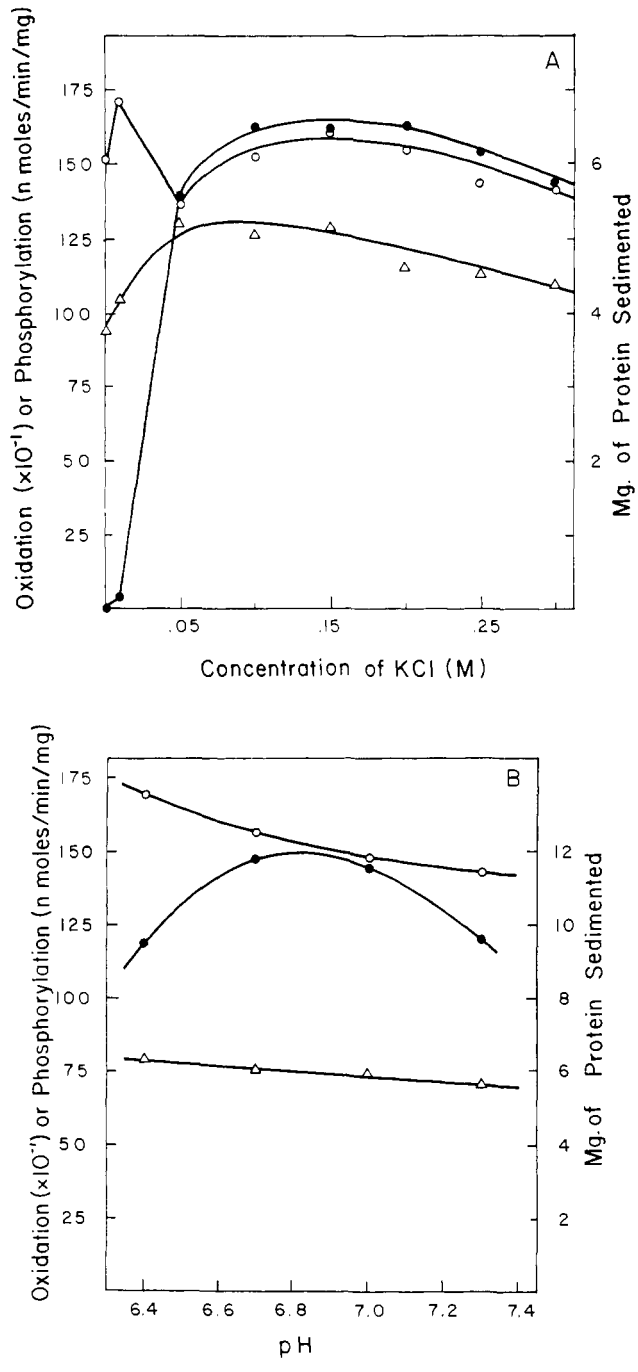


FIGURE 1: The effect of KCl and pH on the preparation of phosphorylating particles. (A) Aliquots of crude extract in 0.25 M sucrose were brought to varying final concentrations of KCl by the addition of an equal volume of KCl in 0.25 M sucrose that was twice the desired final concentration. The particles were then sedimented, washed once, and resuspended in 0.25 M sucrose and KCl of the same concentration in which the sedimentation was performed. Activity was assayed as described in Methods at a particle concentration of 0.2 mg/ml. (B) Aliquots of crude extract were made 0.05 M in phosphate buffer and 0.1 M in KCl by the addition of an equal volume of 0.2 M KCl and buffer that was twice the desired final concentration. The particles were sedimented, washed once, and resuspended in 0.25 M sucrose–0.05 M phosphate buffer–0.1 M KCl and then assayed at a concentration of 0.2 mg/ml: ●, phosphorylation; ○, oxidation; △, protein sedimented.

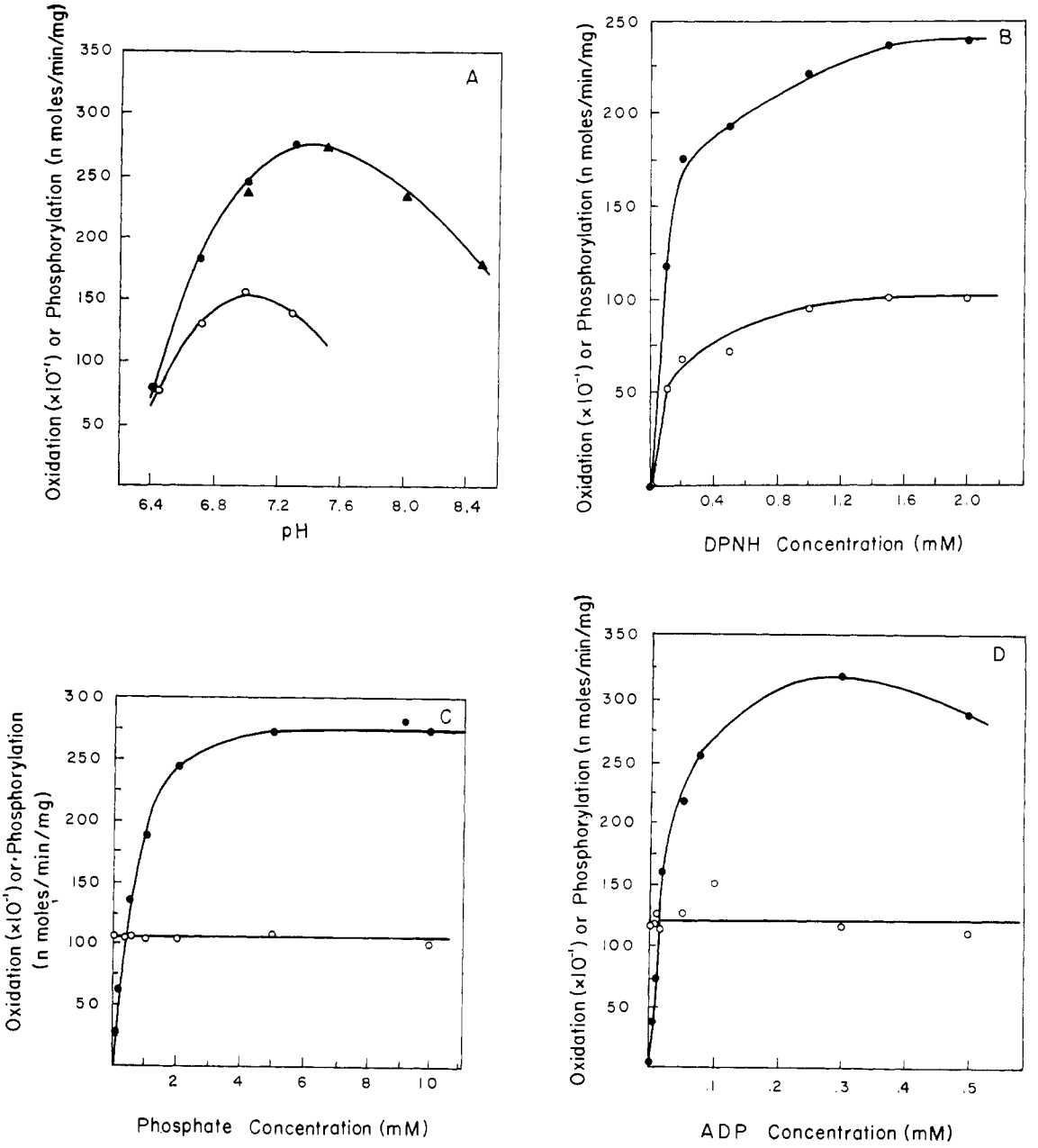


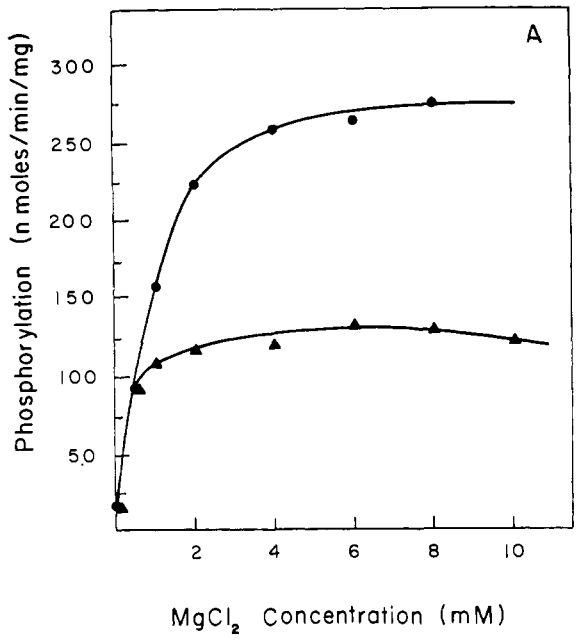
FIGURE 2: Optimal conditions for oxidative phosphorylation. (A) K particles were prepared as described in Methods. The reaction mixture contained the usual components except that phosphate buffer was 10 mM. The higher series of pH values had 10 mM phosphate–10 mM Tricine. (B) DPNH concentration was varied. Each system had its own blank in the oxidase determination. (C) The reaction mixture contained 10 mM Tricine, pH 7.4, and the phosphate concentration was varied. Each system had its own blank in the phosphorylation experiment, since the small background count of [^{32}P]P_i remaining after the extraction of unesterified phosphate depended upon the amount originally present. (D) ADP was varied: ○, oxidation; ●, phosphorylation in phosphate buffer; ▲, phosphorylation in phosphate–Tricine buffer.

phosphorylating activity to units of enzyme showed a definite nonlinearity. The slight curvature of the line was reflected by the increasing values for the specific activity of phosphorylation. The fact that the curvature was seen for phosphorylation and not oxidation indicated that the phenomenon was characteristic of the phosphorylating system, not the electron transport system.

Discussion

Several roles for metal ions were found in this system during the course of the kinetic characterization. The first role was in the preparation of phosphorylating particles from the cell-free crude extract. Previous work on this system (Shibko and Pinchot, 1961a) indicated that the coupling factors were not bound to the membrane fragments unless Mg^{2+} or other divalent cations were present. Since K^+ was capable of substituting for Mg^{2+}, it may be concluded that the important factor in keeping the coupling factors on the membrane fragments was the ionic strength of the environment. This view will receive direct support in the following paper (Adolfsen and Moudrianakis, 1971).

The binding of the coupling factors to the membrane fragments may be fairly weak, even at ionic strengths favoring the association. This was suggested by the nonlinear response of the initial velocity of phosphorylation to units of enzyme



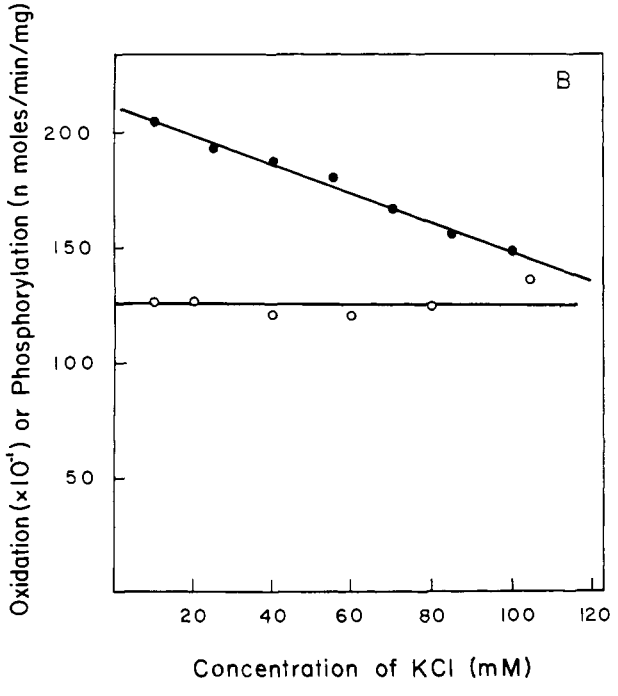


FIGURE 3: Effects of $MgCl_2$ and KCl on rates of oxidation and phosphorylation. (A) $MgCl_2$ was varied. The particle concentration was 0.2 mg/ml. This was 0.1 ml of a particle suspension containing 0.1 M KCl, which contributed an additional 10 mM KCl to the reaction mixture: ●, phosphorylation; ▲, phosphorylation with 0.1 M KCl present. (B) $MgCl_2$ was 8 mM and KCl was varied: ●, phosphorylation; ○, oxidation.

(Figure 5). The methodology of the experiment employed serial dilutions of the particle suspension in KCl–sucrose (see figure legend). If there were an equilibrium between factors bound to membranes and factors free in solution, serial dilutions would result in a decrease of the factor concentration in the aqueous phase of the system. More factors might then dissociate from the particles until the equilibrium was established once again. With fewer factors on the membranes, the measured initial rates and specific activities of phosphorylation would be lower.

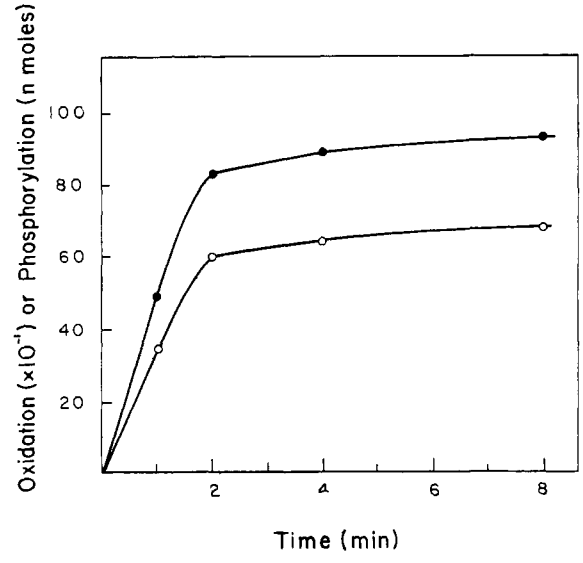


FIGURE 4: Time course of oxidation and phosphorylation. Assays were performed as described in Methods at a particle protein concentration of 0.20 mg/ml: ●, phosphorylation; ○, oxidation.

A second role of ions, or ionic strength, was in the stabilization of DPNH oxidase activity. If the crude extract was frozen at low ionic strength, the membranes lost 90% of their oxidase activity within 5 days storage at −20°. No activity was lost if the material was stored in KCl–sucrose.

A third role for the metal ions was an enzymatic one: Mg^{2+} activated the phosphorylating reaction. The first phase of the activation was attributable to the formation of the Mg–ADP complex. The stability constant for the formation of the complex has been calculated as 3100 M^{-1} (Colomb *et al.*, 1969). The combination of equimolar $MgCl_2$ and ADP

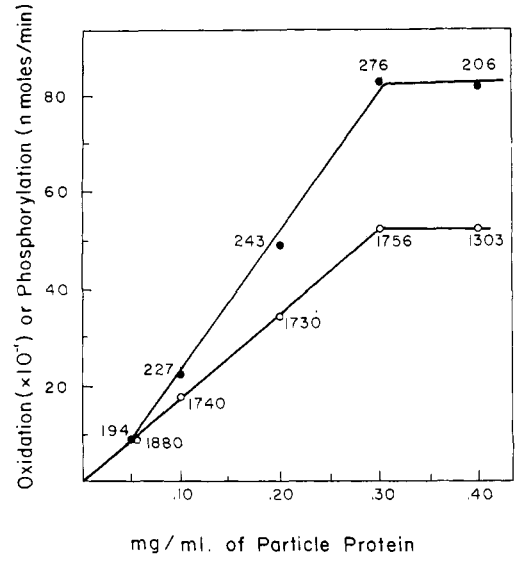


FIGURE 5: Response of rates of oxidation and phosphorylation to enzyme concentration. Serial dilutions of a particle suspension that was 4 mg/ml were made in 0.1 M KCl–0.25 M sucrose. A constant volume of each suspension (0.1 ml) was added to the reaction mixture so that the amount of KCl and sucrose added with the particles did not vary. The numbers beside the points are specific activities of oxidation or phosphorylation in nmoles/min per mg: ●, phosphorylation; ○, oxidation.

would then give better than 98% conversion into the complex. The fact that phosphorylation was stimulated by conversion of ADP into the complex indicated that the complex was a better substrate than free ADP. The second phase of the activation was taken as activation by free Mg^{2+}. Ion antagonism by K^+ was observed for the second but not the first phase of the activation. Whether the activation and antagonism occurred at an active site in the phosphorylating system or through changes in conformation in the phosphorylating system is not known.

The particles used in these studies possessed absolutely no respiratory control by either ADP or P_i. This was somewhat surprising in view of some previous work on this system in which control as high as 50% was reported (Scocca and Pinchot, 1968). In those studies Mg particles were prepared from crude extracts which had been stored frozen in water, so 90% or more of the electron transport activity had probably decayed. In view of this fact and the total absence of control in the present studies, the validity of the previous observations must be seriously questioned. If respiratory control were truly an inherent property of oxidative phosphorylation, then it should be observed in all cases, with no exceptions. It was not observed here. Two explanations are possible for cases in which it was seen. Control was conferred by components in the particles other than the immediate electron transport and oxidative phosphorylation systems, or it was an artifact which somehow resulted from the low oxidase activity of the particles.

Acknowledgments

We wish to thank Dr. Gifford B. Pinchot, whose grants supported these studies in part and in whose laboratory these studies were done. We also acknowledge the technical assistance of Mr. William E. Burleigh in the preparation of some of the cell-free crude extracts.

References

Adolfsen, R., and Moudrianakis, E. N. (1971), *Biochemistry 10*, 440.

Berenblum, J., and Chain, E. (1938), *Biochem. J. 32*, 295.

Colomb, M. G., Cheruy, A., and Vignais, P. V. (1969), *Biochemistry 8*, 1926.

Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Pabst Laboratories (1956), Circular OR-10.

Pinchot, G. B. (1957), *J. Biol. Chem. 229*, 1, 25.

Pinchot, G. B. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 929.

Pinchot, G. B. (1967), *Methods Enzymol. 10*, 147.

Pinchot, G. B., and Hormanski, M. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1970.

Shibko, S., and Pinchot, G. B. (1961a), *Arch. Biochem. Biophys. 93*, 140.

Shibko, S., and Pinchot, G. B. (1961b), *Arch. Biochem. Biophys. 94*, 257.

Scocca, J. J., and Pinchot, G. B. (1968), *Arch. Biochem. Biophys. 124*, 206.

Reconstitution of Oxidative Phosphorylation in *Alcaligenes faecalis**

Robert Adolfsen and E. N. Moudrianakis†

ABSTRACT: When phosphorylating particles from *Alcaligenes faecalis*, prepared by centrifugation from cell-free crude extracts in 0.1 M KCl–0.25 M sucrose, were sedimented and resuspended in sucrose in the *absence* of KCl, all phosphorylating activity was lost. This was shown to be due to a release of coupling factors from the membrane fragments. The factors could be separated from the membrane fragments at this point by centrifugation. A reconstitution assay was developed in which the reappearance of phosphorylating activity in the membrane fragments had an *absolute* dependence on the addition of the coupling factor supernatant fraction. Recovery of activity was close to 100%. The binding constants of the heat-labile and heat-stable coupling factors to the membrane fragments in the presence of KCl were estimated to be approximately 10 and 30, respectively. In the absence of KCl the constant for at least one of the factors was approximately zero. When KCl was added back to the system the factors bound back onto the membrane fragments. Binding was found to be temperature independent, but very little phosphorylating activity reappeared unless the system was incubated at 30°. Reconstitution therefore appeared to be a two-step process. The first step was the binding of factors to membranes and the second probably involved some conformational rearrangements which the system had to go through before regaining its ability to function enzymatically.

In the previous paper (Adolfsen and Moudrianakis, 1971), it was shown that membrane fragments from *Alcaligenes faecalis* exhibited optimal phosphorylating activity when they were prepared from cell-free crude extracts in the presence of 0.1 M KCl–0.25 M sucrose. Shibko and Pinchot (1961) claimed that *divalent* cations were required to keep coupling factors of oxidative phosphorylation bound to

* From the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218. *Received August 17, 1970.* This work was supported by Grant AI-02443 from the National Institutes of Health and Grant GB-12128 from the National Science Foundation to Dr. G. B. Pinchot and by the National Institutes of Health Grant GM-13518 to one of us (E. N. M.).

† To whom correspondence should be addressed.