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.).

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membrane fragments. The fact that monovalent cations could be substituted for divalent cations in the preparation of the particles suggested that the most important variable in keeping the coupling factors bound was the ionic strength of the environment. The mechanistic importance of ionic strength is studied in detail in the present paper, and attempts are made to gain an understanding of the system in terms of interactions of its macromolecular components.

The coupling factors were found to be reversibly dissociable from the membrane fragments simply by removing KCl and then adding it back. This phenomenon was utilized in the development of a reconstitution assay. Many reconstitution assays suffer from having high levels of residual activity left in the particles after having been partially depleted of coupling factors. These reconstitution assays are then stimulations of base lines of residual activity in which it may not always be clear whether the material added back is really a coupling factor or stimulates the residual activity by some other means. In the reconstitution assay reported here, the appearance of phosphorylating activity in the membrane fragments had an *absolute* dependence on the addition of a coupling factor fraction.

Materials and Methods

Phosphorylating particles were prepared as previously described (Adolfsen and Moudrianakis, 1971) and stored in aliquots in KCl–sucrose[1] at −20°. Phosphorylation, DPNH oxidation, and protein concentration were also assayed as previously described.

Reconstitution. An aliquot of phosphorylating particles was thawed, and the particles were sedimented at $10^5 g$ for 15 min, then resuspended in 0.25 M sucrose (no KCl). This resulted in a total loss of phosphorylating activity. Activity could be recovered at this point by making the particle suspension 0.1 M in KCl and incubating at 30° for 15 min. Alternatively, the membrane fragments could be sedimented again and resuspended in 0.25 M sucrose. The coupling factors were left behind in the supernatant fraction of this second centrifugation. Reconstitution was accomplished by incubating 0.05 ml of membranes (approximately 200 μg of particle protein) with 0.30 ml of the supernatant fraction and 0.05 ml of 1.0 M KCl–0.25 M sucrose for 15 min at 30°. Phosphorylating activity was assayed after the completion of this incubation. The control system, in which 0.25 M sucrose replaced the coupling factor fraction, had no activity at all.

Results

Loss and Recovery of Phosphorylating Activity by Changing the Ionic Strength of the Environment. A loss of the ability of the phosphorylating particles to catalyze oxidative phosphorylation was observed when the particles were washed in solutions of decreasing ionic strength (Figure 1). The stimulation of oxidase activity appeared to be explainable in classical terms as a result of uncoupling of phosphorylation. Reversibility of the loss of phosphorylating activity was observed upon the readdition of KCl (Figure 2), and it was found to be markedly temperature dependent. Time courses of activity in the control particles showed a partial lability of phosphorylating activity at 30°. This appeared to be attributable to a partial lability of DPNH oxidase activity, since time courses for oxidase activity both in the control and experimental particles showed a similar pattern of stability at 0° and partial lability at 30°. Recovery of phosphorylating activity was found to be 95% by comparison of the activity in the experimental system to the level of activity remaining in the control particles after 30 min at 30°.

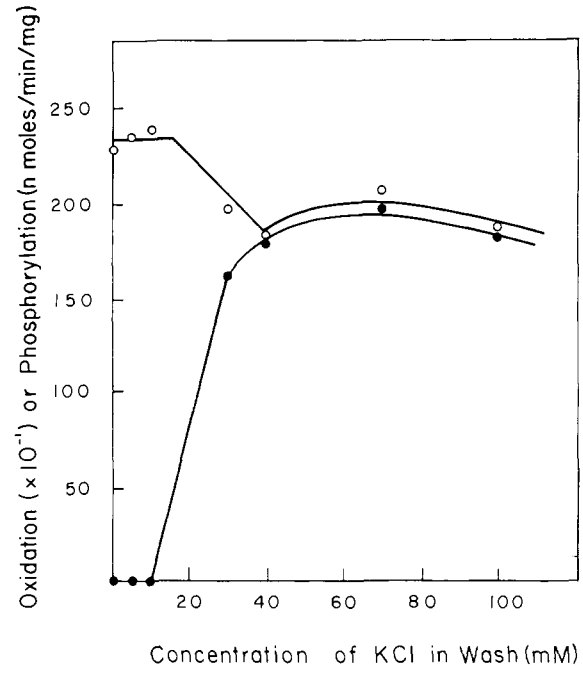

FIGURE 1: The effect of washing native phosphorylating particles in varying KCl concentrations on enzymatic activities. Native particles were sedimented and resuspended in 0.25 M sucrose and KCl of varying concentrations. After 30 min at 0° the particles were resedimented and resuspended in KCl–sucrose. The protein concentration was adjusted to 2.0 mg/ml and oxidation and phosphorylation were assayed at a concentration of 0.20 mg/ml: ○, oxidation; ●, phosphorylation.

The pH was uncontrolled in the above experiments, the suspensions being at approximately 6.5. Figure 3 shows that pH 6.8 was optimal for recovery. From the similarity in the shapes of the oxidation and phosphorylation curves, it was concluded that most of the effect of pH was through lability in the oxidase system.

The effect of varying concentrations of three different salts on recovery of activity is illustrated in Figure 4. The sensitivity of the system to the salts tested was $CaCl_2 > MgCl_2 >$ KCl, and the concentrations at which 50% of the maximal activity was recovered were 2.0, 2.8, and 30 mM, respectively. On the basis of the ionic strength of solutions of mono- and divalent cations of equal concentration, divalent cations should have functioned about 2.5 times as well as monovalent cations in bringing about the recovery of phosphorylating activity. This would be anticipated if ionic strength were the only important variable. Since divalent cations gave

[1] Three types of membrane fragments, or particles, are referred to: (1) native particles, phosphorylating particles prepared by sedimentation from cell-free crude extracts in 0.1 M KCl–0.25 M sucrose; (2) stripped particles, native particles sedimented and resuspended in sucrose in the absence of KCl, then sedimented again—they are essentially free of coupling factors; (3) reconstituted particles, stripped particles incubated with coupling factors in 0.1 M KCl at 30°. Other abbreviations: ionic shock (ionic strength shock treatment), sedimentation of native particles and resuspension in sucrose in the absence of KCl; KCl–sucrose, 0.1 M KCl–0.25 M sucrose; HEPES = *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; MES = (2-*N*-morpholino)ethanesulfonic acid.

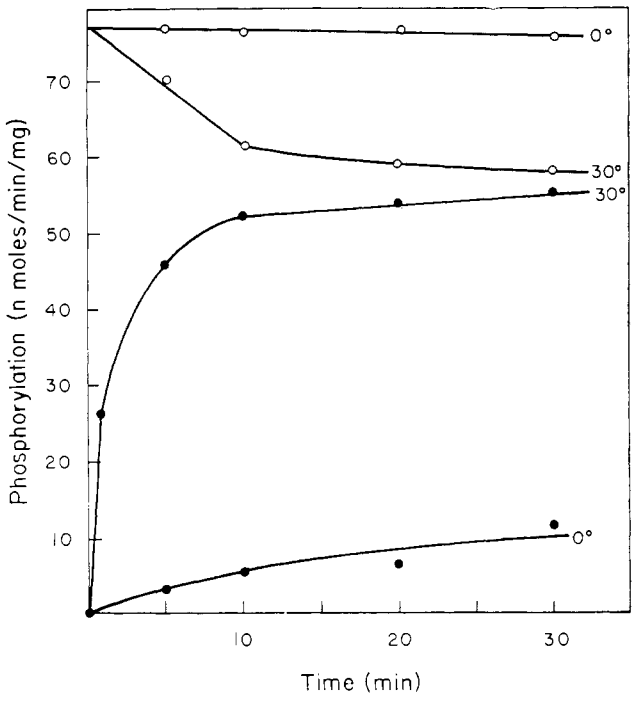

FIGURE 2: Recovery of phosphorylating activity after ionic shock. Native particles were sedimented and resuspended in 0.25 M sucrose in the absence of KCl. At t = 0 min, 1 M KCl–0.25 M sucrose was added to bring the concentration up to 0.1 M. Assays were done as a function of time at 0 and 30°. The controls (upper two curves) were native particles. All assays were done at 0.2 mg/ml of particle protein: ●, experimentals; ○, controls.

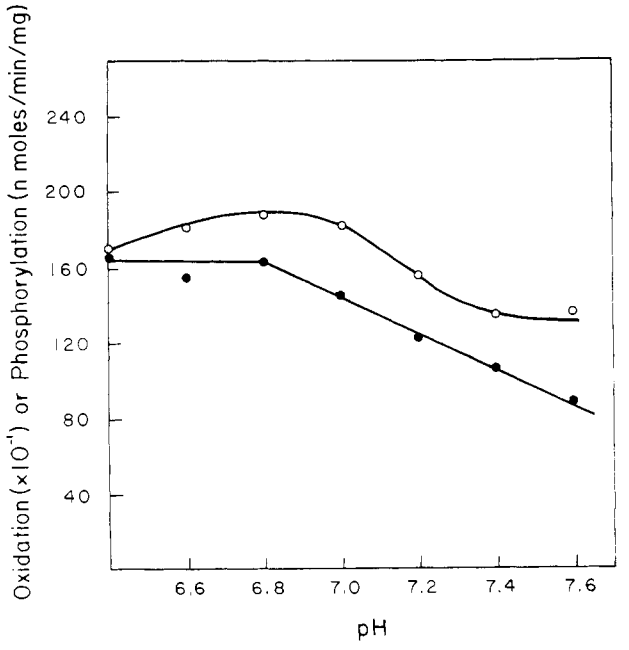

FIGURE 3: Effect of pH on recovery of phosphorylating activity and on oxidase activity. Native particles were sedimented and resuspended in 0.25 M sucrose. Equal volumes of particle suspension and 10 mM phosphate buffer–0.2 M KCl–0.25 M sucrose were combined. After 15 min at 30° oxidase and phosphorylating activities were assayed, using 0.1 ml of particle suspension. This contributed only 0.5 mM phosphate to the reaction mixture, which was 5 mM in phosphate buffer, pH 7.4: ○, oxidation; ●, phosphorylation.

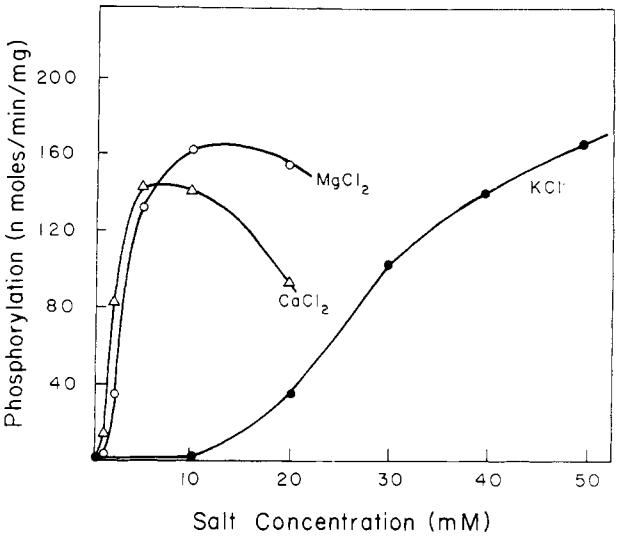

FIGURE 4: Effect of different cations on the recovery of phosphorylating activity. Native particles were sedimented and resuspended in 0.25 M sucrose. Equal volumes of particles and 0.25 M sucrose–$CaCl_2$, $MgCl_2$, or KCl that was twice the desired final concentration were combined. Optimal recovery for KCl was reached between 0.10 and 0.15 M KCl and the overall shape of the curve was the same as for the other two salts. Phosphorylating activity was assayed after 10 min at 30°: △, $CaCl_2$; ○, $MgCl_2$; ●, KCl.

50% maximal recovery of activity at tenfold lower concentrations than monovalent cations, and also since the curves for $MgCl_2$ and $CaCl_2$ were not superimposable, individual types of cations may have specific, subsidiary roles in the process.

Elution of Coupling Factors from Phosphorylating Particles. The fact that the loss of phosphorylating activity reflected a dissociation of the coupling factors from the membrane fragments was easily demonstrable. Phosphorylating particles that had been sedimented and resuspended in sucrose in the absence of KCl and then sedimented again recovered no phosphorylating activity when incubated in 0.1 M KCl–0.25 M sucrose at 30° for 15 min. When some of the supernatant fraction from this second centrifugation was added back before the incubation, complete recovery was observed.

Preliminary indications that both the heat-labile and heat-stable coupling factors originally reported by Pinchot in 1953 were present in this coupling factor fraction were obtained in the experiment shown in Table I. The addition of boiled coupling factor fraction to *native* particles resulted in some increase in the activity of these particles. The stimulation may be taken as an indication of the presence of the heat-stable factor in the coupling factor fraction. When the unboiled coupling factor fraction was added to *native* particles, the level of activity was stimulated by about twice as much. This suggested the presence of more than one coupling factor in this fraction. The presence of the heat-labile factor in the coupling factor fraction was more clearly demonstrated in the second half of the experiment. Activity was restored to *stripped* particles when the unboiled coupling factor fraction was added, but no activity at all was restored when the fraction was boiled before its addition. In recent experiments these two coupling factors have been separated and purified from this crude coupling factor fraction and characterized as distinct molecular entities. The details of the purification and characterization are the subject of a future

TABLE I: Demonstration of the Existence of Two Coupling Factors in the Supernatant Fraction of Native Particles Subjected to Ionic Shock.

Additions to Native Particles[a]	Phosphorylation Specific Act. (nmoles/min per mg)	Additions to Stripped Particles[b]	Phosphorylation Specific Act. (nmoles/min per mg)
None	185	None	0
Unboiled factors	234	Unboiled factors	223
Boiled factors	208	Boiled factors	0

[a] Native phosphorylating particles were washed once in 0.25 M sucrose to remove coupling factors and then resuspended in 0.25 M sucrose. Half of the coupling factor fraction was boiled for 10 min. Equal volumes of native particles and coupling factor fraction, both in 0.1 M KCl–0.25 M sucrose, were combined, and phosphorylating activity was assayed after 15 min at 30°. [b] Reconstitution was carried out by mixing 0.35 ml of factor fraction with 0.10 ml of stripped particles and 0.05 ml of 1 M KCl–0.25 M sucrose. The particle protein concentration in the assay was approximately 0.2 mg/ml. The protein concentrations used in the computation of specific activities of phosphorylation did not include the extra coupling factor protein added to the system.

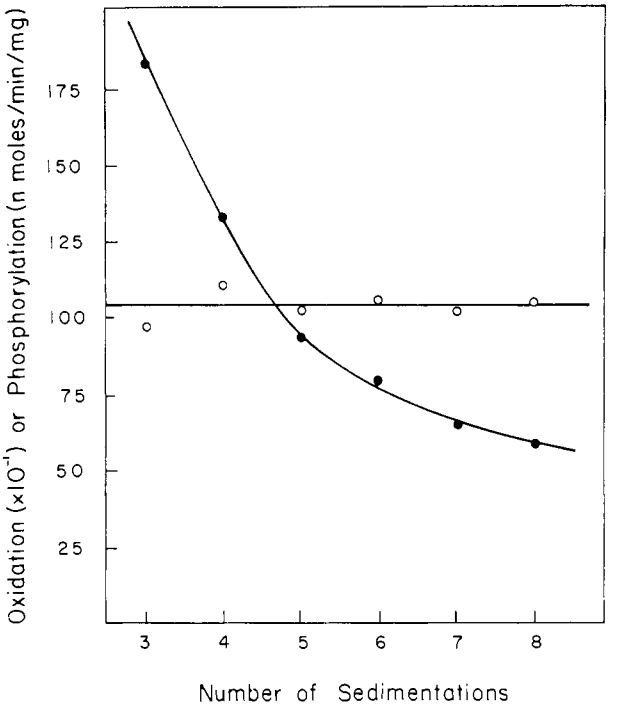

FIGURE 5: Effect of repeated washing in KCl-sucrose on oxidase and phosphorylating activities. Native particles were sedimented and resuspended in KCl-sucrose the indicated number of times. Oxidation and phosphorylation were assayed at 0.2 mg/ml of particle protein: O, oxidation; ●, phosphorylation.

communication (R. Adolfsen and E. N. Moudrianakis, submitted for publication).

An interesting question with respect to the loss of phosphorylating activity as a function of ionic strength (Figure 1) was whether a direct correlation could be made between the loss of activity and the elution of coupling factors from the membrane fragments. To answer this question the supernatant fractions from native particles washed in varying KCl concentrations were tested for coupling factor activity by incubating with stripped particles under reconstitution conditions and then assaying reconstituted phosphorylating activity. A fairly close correlation between the loss of activity and the elution of factors was observed. Half-maximal coupling factor activity was eluted in 20 mM KCl, which agreed quite well with the concentration of KCl at which half-maximal activity was lost in Figure 1. Maximal activity, however, was eluted only when the KCl concentration during the wash was 5 mM or less, even though all activity in the native particles was lost by washing in 10 mM KCl.

Coupling factors could also be eluted to some extent by washing in the presence of KCl. The experiment reported in Figure 5 shows that phosphorylating activity was lost slowly by successive washings in KCl–sucrose. Since oxidase activity was stable, the loss of activity was due to some event in the phosphorylating system. Particles resuspended in the same supernatant from which they were sedimented did not show this loss of activity. It was therefore possible to conclude that the coupling factors were slowly being eluted by these repeated washes.

The experiment reported in Figure 6 was performed to distinguish between elution of the heat-stable factor and the

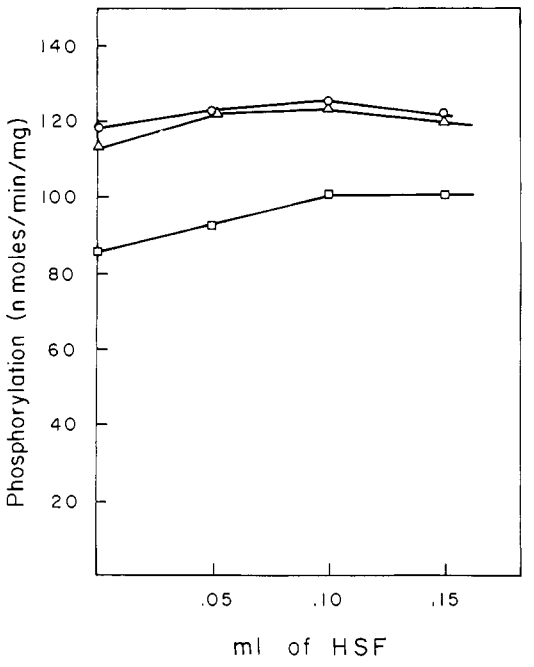

FIGURE 6: Elution of coupling factors by washing native particles in KCl-sucrose. Native particles were washed twice in KCl-sucrose. Native particles of a second aliquot were sedimented and resuspended twice without changing the supernatant solution. A third aliquot of particles was untreated. The coupling factor fraction was obtained from a fourth aliquot as described in Methods, and the heat-labile factor was inactivated by boiling for 10 min: O, untreated particles; △, particles sedimented and resuspended twice without changing the supernatant solution; □, particles washed twice in KCl–sucrose.

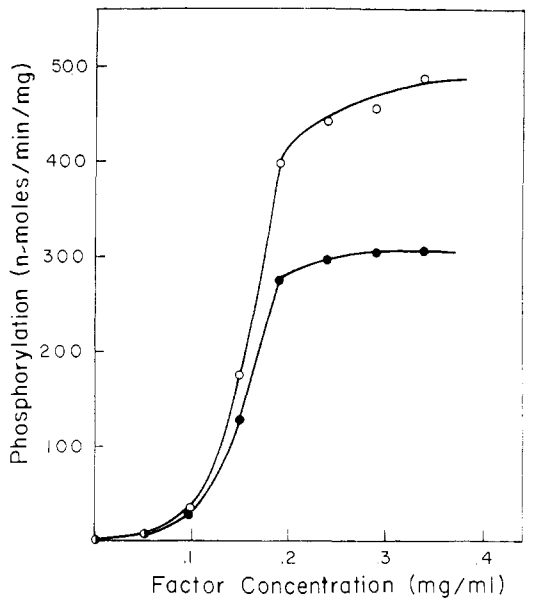

FIGURE 7: Saturation of membrane fragments with coupling factors. Native particles were sedimented and washed once in 0.25 M sucrose at a concentration of about 8 mg/ml to remove coupling factors. Reconstitution was accomplished by using factors and 0.25 M sucrose in varying amounts adding up to 0.40 ml, then adding 0.10 ml of particles, followed by 0.06 ml of 1.0 M KCl–0.25 M sucrose to give a final concentration of 0.1 M KCl. Phosphorylation was assayed after 15 min at 30° at a protein concentration of about 0.06 mg/ml: ●, specific activity computed on the basis of total membrane and factor protein in the system; ○, specific activity computed on the basis of membrane protein alone. The specific activity of the control particles (native particles) was about 200 nmoles/min per mg.

TABLE II: Effect of Temperature and Ionic Strength on the Distribution of Coupling Factors between Membrane Fragments and the Aqueous Phase of the System after Ionic Shock.[a]

System	Supernatant Fraction From	Phosphorylation Specific Act. (nmoles/min per mg)
1	Membranes spun out in sucrose alone	115
2	Membranes spun out after incubation at 0° in 0.1 M KCl	17
3	Membranes spun out after incubation at 30° in 0.1 M KCl.	10

[a] Native particles were sedimented and resuspended in 0.01 M KCl–0.25 M sucrose. KCl was then added back to 0.1 M in systems 2 and 3. The membrane fragments in system 2 were resedimented after 10 min at 0°, and those in system 3 were resedimented after 10 min at 30°. The membrane fragments in system 1 were resedimented without adding KCl back up to 0.1 M. Coupling factor activity in the three supernatant fractions were assayed as described in Methods.

heat-labile factor from the membrane fragments during washes in KCl–sucrose. If the heat-stable factor were being eluted, the addition of increasing amounts of boiled coupling factor fraction to the native particles washed in KCl–sucrose should have brought the activity back up to the original level. The activity did not return to the original level, indicating that the heat-labile factor was the factor being eluted. However, the stimulation of activity observed in the control particles as a result of bringing the heat-stable factor to a saturating level was 8–9% while it was about 15% in the particles washed twice in KCl–sucrose. This indicated some slight loss of heat-stable factor was occurring, in addition to the loss of heat-labile factor.

In view of the slow rate of recovery of phosphorylating activity at 0° observed in Figure 2, it was of interest to determine whether the factors required higher temperatures to reassociate to the membranes or whether they could rebind at low temperatures, the effect of higher temperatures being on something else. The data in Table II show that the factors bound back onto the membrane fragments when KCl was added back at 0°. Reconstitution therefore appeared to be separable into two events: (1) the binding of factors to membranes, which was dependent on KCl but independent of temperature, and (2) the reappearance of phosphorylating activity, which was greatly accelerated by increasing the temperature to 30°. The second step may involve conformational rearrangements of the system which have fairly high activation energies.

Saturation Studies with Membranes and Coupling Factors. The saturation curve of stripped particles with coupling factors is shown in Figure 7. The specific activity of phosphorylation of native particles was about 200. The specific activity of the reconstituted particles was about 300, indicating that the native particles were operating at about 70% of their maximal capacity. With the slow elution of coupling factors by successive sedimentations in KCl–sucrose during the preparation of the native particles, this percentage of activity was about what would have been expected if the membrane fragments in the crude extract had been completely saturated with coupling factors.

Figure 8 shows the results of the reciprocal saturation experiment, in which the coupling factor concentration was the constant quantity and the membrane protein concentration was varied. The membranes may be taken as completely saturated with factors in the first part of the curve because doubling the membrane protein concentration caused a doubling of the initial velocity of the reaction. The second part of the curve may be taken as a reflection of the initial lag in the curve in Figure 7.

There was one more perspective from which the saturation phenomenon could be studied. The concentration of both coupling factors and membrane fragments could be varied simultaneously, with the constant quantity being the ratio of the two. The resulting curve should reflect the distribution of coupling factors bound to membrane fragments and free in solution. The saturation curve observed (Figure 9) was biphasic. It was considered possible that one phase represented saturation by the heat-stable factor and the other represented saturation by the heat-labile factor. This possibility will be discussed in more detail below.

"Uncoupled" Rates of Oxidase Activity. The stimulation of oxidase activity in Figure 1 could be explained classically as a result of uncoupling of phosphorylation. The results of the following experiment make this explanation totally

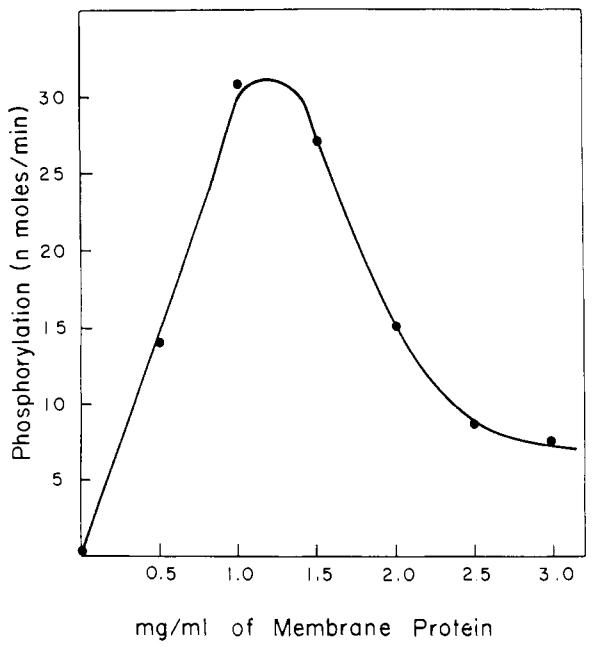

FIGURE 8: Saturation of coupling factors with membrane fragments. Native particles were sedimented and washed once in 0.25 M sucrose to remove coupling factors. The suspension of stripped particles and the coupling factor fraction were both made 0.1 M in KCl. The stripped particles were then diluted to varying degrees with KCl–sucrose, and equal volumes of coupling factor fraction and membrane suspension were combined. Phosphorylation was assayed after 15 min at 30°. The membrane protein concentration denoted in the figure is the concentration during reconstitution. An aliquot of 0.1 ml of each system was used in the assay.

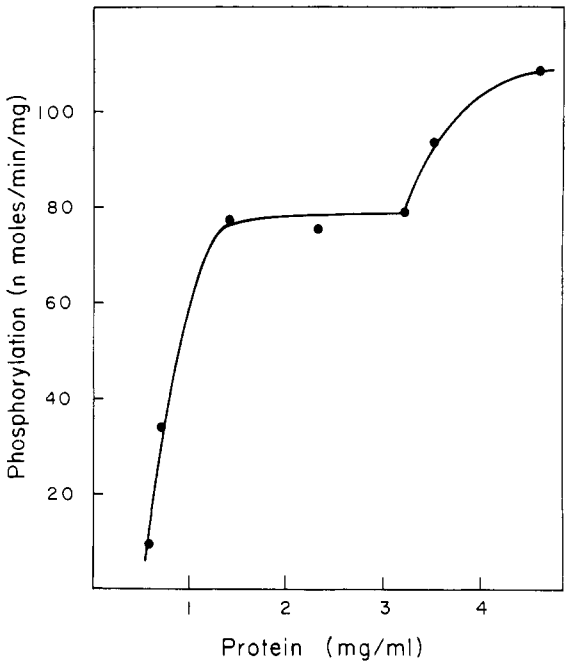

FIGURE 9: Dependence of recovery of phosphorylating activity on total protein concentration. Native particles were sedimented and resuspended in 0.25 M sucrose at approximately 10 mg/ml, and a series of dilutions was made. Recovery of activity was obtained by adding an equal volume of 0.2 M KCl–0.25 M sucrose and incubating at 30° for 15 min.

unacceptable. Particles which had been washed up to three times in the absence of KCl (stripped particles) and then stored at −20° in KCl–sucrose–0.01 M HEPES buffer, pH 6.8, all showed about 20% stimulation of oxidase activity when they were sedimented and resuspended in sucrose in the absence of KCl. This stimulation occurred in the absence of coupling factors, clearly showing that it was the result of the removal of KCl only. In addition, the data in Table III show that the stimulation was completely reversible by the readdition of KCl. When stripped particles suspended in KCl–sucrose were diluted with an equal volume of coupling factor fraction also in KCl–sucrose, no suppression of oxidase activity was observed as a reflection of the binding of coupling factors to the membranes. Oxidase activity was therefore entirely unaffected by the presence or absence of coupling factors associated with the membrane fragments.

Discussion

A reconstitution assay which has an absolute dependence on the addition of a coupling factor fraction to a membrane fraction has been developed. Under proper conditions and with proper controls, close to 100% reconstitution was routinely obtainable. The procedure for reconstitution consisted of incubating membrane fragments and coupling factors in 0.1 M KCl–0.25 M sucrose at 30° for approximately 15 min. When rigorous pH control was desired, 0.01 M MES buffer at pH 6.8 was included in the incubation medium. A number of problems were found that had to be compensated for in order to properly estimate the percentage of reconstituted activity. These are enumerated as follows.

(1) Phosphorylating activity in the control particles was partially labile at 30°. This was found to be a reflection of partial lability of oxidase activity and probably was not important with respect to events occurring in the reconstitution of oxidative phosphorylation. The problem was circumvented by comparing the reconstituted level of activity to the level of activity remaining in native particles which had been incubated at 30° for the same length of time.

TABLE III: Control of Oxidase Activity by Incubation in 0.1 M KCl But Not by Coupling Factors.[a]

System	Oxidation Specific Act. (nmoles/min per mg)
Stripped particles	1390
Stripped particles resuspended in the absence of KCl	1800
Stripped particles resuspended in the absence of KCl, after 30 min at 0° in 0.1 M KCl	1275
Stripped particles and coupling factors, after 30 min at 0° in 0.1 M KCl	1437

[a] Native particles were washed once in 0.25 M sucrose–1 mM HEPES buffer, pH 6.8, and stored at −20° in 0.1 M KCl–0.25 M sucrose–0.01 M HEPES buffer, pH 6.8. Aliquots were thawed and subjected to the treatments described in the table. Coupling factors were obtained fresh from an aliquot of native particles as described in Methods.

(2) Coupling factors were found to be slowly eluted from native particles when washed repeatedly in KCl–sucrose. This problem was compensated for by sedimenting the control particles the same number of times as the experimental particles, resuspending them each time in KCl–sucrose instead of sucrose alone.

(3) A dependence of reconstitution on protein concentration was observed (see Figure 9). The optimal protein concentration for reconstitution was about 5 mg/ml.

From the observed rate of loss of activity as a result of washing native particles in KCl–sucrose (Figure 6) it was possible to estimate the binding constants of the two coupling factors to the membrane fragments. The difference in activity observed in the control and experimental particles at saturating levels of heat-stable factor was about 20%, amounting to a loss of about 10% per wash due to the elution of the heat-labile factor. From this fact alone, ignoring peculiarities in saturation studies with coupling factors and membrane fragments (Figure 7), the binding constant for the heat-labile factor was estimated to be approximately 10. The stimulation of activity as a result of bringing the heat-stable factor to a saturating level (Figure 6) in the particles washed twice in KCl–sucrose was 15%, compared to the 8–9% in the unwashed control particles. The 6% difference, 3% per wash, gave a binding constant for the heat-stable factor of approximately 30.

The difference in the binding constants of the two coupling factors may help to explain the biphasic saturation curve in Figure 9. Since the heat-stable factor had a larger binding constant than the heat-labile factor, the first phase of the curve may have been saturation by the heat-stable factor and the second, saturation by the heat-labile factor. Also, since the preparation of the native particles involved two sedimentations in KCl–sucrose, the particles were probably more deficient in heat-labile factor than in heat-stable factor. This would be expected to accentuate differences observed as a result of the factors having different binding constants.

The nonlinearity of the saturation curve of stripped particles with coupling factors (Figure 7) presents great difficulty in attempts to quantitate reconstitution by defining any simple unit of coupling factor activity. The lag may be suggestive of an inhibitor in the membrane which was titrated by the coupling factor solution. If this were the case, then once the inhibitor had been titrated a doubling of reconstituted phosphorylating activity with a doubling of coupling factor concentration should have been observed. The slope of the linear portion of the curve was greater than unity. An alternate hypothesis which was capable of explaining the sigmoid shape of the curve was that cooperative interactions occurred between the coupling factors and the membrane fragments during reconstitution.

By comparing native particles to particles which had been reconstituted in the presence of saturating amounts of coupling factors, it was estimated that the native particles were about 70% saturated with factors. This suggested that the system had undergone relatively little damage as a result of cell breakage. It was previously found, however, that the P_i:DPNH ratio for native particles was only 0.15–0.20 (Adolfsen and Moudrianakis, 1971). This suggested that a low P_i:DPNH ratio may not be a good indicator of the integrity of bacterial systems. A better estimate may be how close the particles are to saturation with factors.

The stimulation of oxidase activity observed as a result of incubating phosphorylating particles at low ionic strength was found to be in no way related to the presence or absence of coupling factors on the membrane fragments (Table III). This may have significant implications with respect to the mechanism of uncoupling of phosphorylation. The mechanism may involve action on the membranes rather than on the phosphorylating system. Changes in the membranes may alter the ability of the coupling factors to interact properly with the electron transport apparatus. This hypothesis may be applicable to mitochondria and chloroplasts as well. Gross *et al.* (1969) have found that suspending chloroplasts in the absence of cations resulted in faster ferricyanide reduction than when cations were present. ATP production was also lower. The readdition of cations gave slower rates of electron transport and better ATP production. The effect of adding salts was reversed by adding known uncouplers of phosphorylation. Some evidence for the uncoupling of 2,4-dinitrophenol being on the membrane and not the phosphorylating system has recently been found by Eisenhardt and Rosenthal (1968). Their "ATP jump" caused by the accumulation of high-energy intermediates was not affected by 2,4-dinitrophenol and thus suggested that the site of action of 2,4-dinitrophenol was prior to the site of formation of the intermediates. Also, Pinchot (1967) has found that 2,4-dinitrophenol seems to act in *A. faecalis* by preventing the binding of coupling factors to the membrane fragments.

Oxidase activity appeared to be completely uncontrollable in this system. In addition to the inability of the coupling factors to control the rate of oxidation (Table III), there is the previously reported lack of respiratory control by ADP or P_i (Adolfsen and Moudrianakis, 1971). One possible way in which respiration might be controlled in bacteria is through unrelated processes in the cell which keep the DPNH concentration low (Smith, 1968). The resulting lower rates of oxidation could, perhaps, obviate the need for coupling factors or other materials to slow down the rate of oxidation in order to prevent gross wastage of cellular energy.

Acknowledgments

We wish to thank Dr. Gifford B. Pinchot for the use of his laboratory and research funds, and for his encouragement in the undertaking of these studies to examine this experimental system from a different viewpoint.

References

Adolfsen, R., and Moudrianakis, E. N. (1971), *Biochemistry 10*, 434.

Eisenhardt, R. H., and Rosenthal, O. (1968), *Biochemistry 7*, 1327.

Gross, E., Dilley, R. A., and San Pietro, A. (1969), *Arch. Biochem. Biophys. 134*, 450.

Pinchot, G. B. (1953), *J. Biol. Chem. 205*, 65.

Pinchot, G. B. (1967), *J. Biol. Chem. 242*, 4577.

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

Smith, L. (1968), *in* Biological Oxidations, T. Singer, Ed., New York, N. Y., Interscience Publishers, p 55.