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Transport of Phosphate across the Osmotic Barrier of *Micrococcus pyogenes*: Specificity and Kinetics

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SUMMARY: The system coupling inorganic phosphate exchange across the osmotic barrier of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) strain Duncan is highly specific for phosphate, probably for the H_2PO_4^- ion. Arsenate, however, can replace phosphate. The exchange is inhibited by thiol reactors, certain anions and certain uncouplers of oxidative phosphorylation. The specificity and kinetics of the exchange suggest that the phosphate is carried in the osmotic barrier as R-phosphate groups moved to and fro across the barrier by thermal agitation, the heat of activation for the movement being 37,400 cal./mole, of which at least 17,700 cal./mole is an entropy component. The exchange of the phosphate of the R-phosphate groups in the barrier with inorganic phosphate on either side may be enzymically catalysed, the free energy of the R-phosphate bond being conserved. Alternatively, the R-phosphate groups may dissociate or hydrolyse, forming free H_2PO_4^- ion and R, the latter then being unable to return across the barrier until R-phosphate is again formed by the spontaneous uptake of H_2PO_4^- ion.

In resting suspensions of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) strain Duncan, it has been shown that inorganic phosphate molecules can exchange across the osmotic barrier of the cells, but that a net transfer of phosphate across the barrier cannot occur (Mitchell, 1953). It has therefore been suggested that the movement of phosphate molecules across the osmotic barrier is regulated by a carrier system similar to that which Ussing (1947) proposed to account for the exchange-diffusion of cations across a cell membrane. The present paper describes work designed to give more information about the mechanism of exchange of phosphate across the osmotic barrier of *M. pyogenes*. It deals in particular with the specificity of the exchange system for phosphate and with certain aspects of the kinetics of the exchange reaction.

METHODS

Growth and preparation of organism. Cultures of *Micrococcus pyogenes* were grown as previously described (Mitchell & Moyle, 1953): The cells were harvested at a concentration equivalent to 2.5 mg. dry weight/ml., washed twice with distilled water and suspended in distilled water at a dry-weight concentration of c. 100 mg./ml. The pH value of the suspensions, measured with a glass electrode, was c. 5.5 after washing, it was adjusted to the appropriate value with dilute HCl or NaOH immediately before each experiment.

Estimation of P and ^{32}P . The methods used were as previously described (Mitchell & Moyle, 1953).

Estimation of P in presence of As. Samples containing 3–30 μg . phosphorus in the presence of up to 75 μg . arsenic could be estimated by adding the Fiske & SubbaRow (1925) reagents in the reverse order, the arsenate thus being

reduced to arsenite by the reducing reagent before the addition of the molybdate. To 0.5 ml. samples in stoppered tubes, 0.05 ml. 10N-H₂SO₄ and 0.2 ml. of the reducing reagent were added. The samples were incubated at 25° for 48 hr., made up to 4.5 ml. with distilled water, and 0.5 ml. of the acid molybdate reagent was added. After 40 min. at 25°, the extinction was determined at a wavelength of 800 mμ, in the Beckman model DU spectrophotometer and compared with standard phosphate + arsenate solutions. The extinction values were reproducible with a standard deviation of $\pm 1\%$, were linear with respect to the phosphate content and independent of the arsenate content of the samples.

Estimation of P + As. Samples containing 0.1–1.0 μmole of arsenate + phosphate could be estimated using stannous chloride in place of the aminonaphtholsulphonic acid reducing reagent in the Fiske & SubbaRow (1925) method. The samples were made up to a volume of 4.3 ml. with distilled water, 0.5 ml. of 2.5% (w/v) ammonium molybdate in 5N-H₂SO₄ was added, followed by 0.2 ml. of 0.2% (w/v) stannous chloride in 0.05N-HCl. After 40 min. at 25°, the extinction was compared with that of standard phosphate + arsenate solutions as described above. The extinction values were reproducible with a standard deviation of $\pm 1\%$ and were linear with respect to both phosphate and arsenate content. The phosphate content of the sample having been determined as described in the previous paragraph, the arsenate content could be obtained by difference.

Manometry. Warburg manometers were used according to the methods recommended by Dixon (1934). The residual fermentation was determined at 25°, using the equivalent of 50 mg. dry-weight cells in 2.5 ml. 100 mM-NaCl containing 2 mM-sodium phosphate and 10 mM-sodium bicarbonate under a gas phase of 95% (v/v) N₂ and 5% CO₂, giving a pH value of 7. Fermentation of glucose was measured by tipping in 2.5 μmole glucose in 0.25 ml. water from the side bulb.

Estimation of dry weight. The approximate concentrations of the washed cell suspensions were adjusted turbidimetrically, the exact dry-weight concentrations were measured gravimetrically as previously described (Mitchell, 1953).

Measurement of $\dot{P}$. The rate of exchange ($\dot{P}$) of inorganic phosphate across the osmotic barrier was measured in μmole/g. cell dry weight/min. (written μmole/g./min.) by methods similar to those described by Mitchell (1953). The value of $\dot{P}$ was given by the equation

$$\dot{P} = \frac{1}{t_2 - t_1} \frac{P_E \cdot P_I}{P_E + P_I} \ln \left(\frac{1 - R_{t_1}}{1 - R_{t_2}} \right)_{I \text{ or } E}, \quad (1)$$

where R_{t_1} and R_{t_2} represent the relative specific activities of the phosphate with respect to ³²P either inside (I) or outside (E) the cells at times t_1 and t_2 , and P_I and P_E represent the total amounts of phosphate inside and outside the cells.

The routine measurements of $\dot{P}$ were made at 15° at pH 7 using a cell dry-weight concentration of 20 mg./ml. in suspension media containing 100 mM-

NaCl and 2 mM-phosphate marked with ^{32}P . In experiments where the external phosphate concentration was 10 mM or more, $\dot{P}$ was measured by following the rate of movement of ^{32}P from cells to medium. The time interval $t_2 - t_1$ was adjusted to allow between 20 and 70 % equilibration of the ^{32}P between the internal and external media in all experiments.

RESULTS

Anion specificity and inhibition of phosphate exchange. When added as the sodium salts to give a final concentration of 2 mM (the same as the external phosphate concentration), the following anions had no effect on $\dot{P}$ at 15° at pH 7 and did not cause any change in P_E or P_I : acetate, arsenite, azide, bicarbonate, bromide, chromate, cyanide, ethylenediaminetetra acetate, fluoride, glutamate, glutamine, iodide, molybdate, nitrate, nitrite, oxalate, pyro-antimonate, succinate, sulphate, thiosulphate, *p*-toluenesulphonate, tungstate. None of these anions could therefore substitute for phosphate in the system responsible for phosphate exchange. On the other hand, the anions shown below, when present at a final concentration of 1 mM at 15° at pH 7, depressed $\dot{P}$ by the following percentages: permanganate (71), 2:4-dinitrophenate (41), aureomycin (39), chlorate (30), tetraborate (29), dichromate (26), vanadate (23), sulphite (17), arsenate (17). With the exception of arsenate, however, none of these anions caused any change in P_E or P_I , and could not therefore substitute effectively for phosphate in the exchange reaction.

Table 1. Movement of P, ^{32}P and As across the osmotic barrier at pH 7 in 30 min. at 15° : External medium containing 100 mM-NaCl and a trace of $^{32}\text{PO}_4$ with additions shown

External medium containing	% entry of ^{32}P	% entry of As	% exit of P	$\dot{P}$
2 mM- PO_4	14.0	—	0	0.75
2 mM- PO_4 + 2 mM- AsO_4	9.5	7.1	7.3	0.48
1 mM- PO_4 + 1 mM- AsO_4	14.8	10.5	10.7	0.39
2 mM- AsO_4	15.9	11.0	11.1	0

Exchange of phosphate for arsenate. In the suspensions containing arsenate there was a rise in the external phosphate concentration, suggesting that arsenate entered the cells in exchange for phosphate. Moreover, the depression of $\dot{P}$ recorded above for arsenate is about that which would apparently have been given by adding 1 mM-phosphate in place of arsenate and failing to take account of the addition in calculating $\dot{P}$ from equation (1). Exchange experiments were therefore done in which the changes in the amounts of ^{32}P , total P and total As were followed simultaneously in the suspension medium and internal medium of the cells. The total As + P remained constant in both the internal and external media, but the ^{32}P , ^{31}P and As were mutually interchangeable across the osmotic barrier. Table 1 shows a typical set of results. Although $\dot{P}$ was depressed by adding arsenate, the substitution of arsenate for phosphate in the external medium caused the percentage of ^{32}P entering the cells in 30 min. at 15° to increase slightly. Also, when As and ^{32}P were both added to

the external medium, the percentage entry of As was always less than that of ^{32}P . It may be concluded that arsenate and phosphate compete in the exchange reaction, and that the probability of reaction of arsenate is somewhat less than that of phosphate under the conditions of these experiments.

Oxidizing agents as inhibitors of exchange. The inhibitory action of permanganate, chlorate, dichromate and vanadate described above might be due to oxidation. Other oxidizing agents were therefore tested. Perchlorate, persulphate, ferricyanide, hydrogen peroxide, iodosobenzoate, benzoquinone, and chlorine did not inhibit phosphate exchange at a concentration of 1 mM, even when incubated with the cells at pH 5 for 15 min. at 25° before $\dot{\text{P}}$ was measured at 15° in the usual way. On the other hand, 1 mM-bromine and 1 mM-iodine, although only slightly inhibitory (less than 10 %) after reacting with the cells at pH 7, decreased $\dot{\text{P}}$ by 36 and 91 % respectively after reacting with the cells at pH 5.5 for 15 min. at 25°. Since bromine and iodine are more effective inhibitors in acid than in neutral solution, it is probable that they inhibit by oxidation rather than by substitution. However, the inhibitory action of the oxidizing agents appears to depend upon more specific factors than what one would normally regard as their strength as oxidizing agents. The explanation which suggests itself is that the degree of inhibition given by the oxidizing agent depends upon the proportion of its oxidizing capacity which is directed towards the groups essential to the exchange reaction.

Thiol reactors as inhibitors of exchange. In view of the inhibitory action of iodine and bromine described above, and the inhibitory action of mercury and other heavy metals previously described (Mitchell, 1953) the sensitivity of the exchange reaction to substances which alkylate thiol groups was measured. The substances were allowed to react with the cells at pH 7 for 15 min. at 25° before the percentage depression of $\dot{\text{P}}$ (shown respectively in the following brackets) was measured at 15° in the usual way: 100 mM-sodium iodoacetate (O), 10 mM- and 100 mM-chloroacetophenone (25 and 70 %), 10 mM- and 100 mM-N-ethylmaleimide (13 and 62 %). Evidently, groups which behave like thiol groups of low reactivity are essential to the system which controls phosphate exchange.

Possible triggering of phosphate exchange by residual metabolism. The inhibition of phosphate exchange by the uncouplers of oxidative phosphorylation, 2:4-dinitrophenate and aureomycin (Loomis & Lipmann, 1948; Loomis, 1950) and by reagents which react with thiols suggest that, although the exchange of phosphate across the osmotic barrier does not in itself involve a net free energy change, a small residual metabolism might be necessary to allow the exchange reaction to occur.

The routine exchange measurements were always done in narrow test-tubes as previously described (Mitchell, 1953) to minimize residual respiration. The suspensions contained 20 mg. dry-weight cells/ml. and it is unlikely that enough oxygen was available for significant respiration except in the top few millimetres of the suspension. However, it was desirable to suppress respiration completely. When this was done by passing a stream of pure nitrogen through cell suspensions in long narrow test-tubes tightly plugged with cotton-wool

$\dot{P}$ remained unchanged. Moreover, $\dot{P}$ was not depressed by 10 mM-KCN, nor was it affected by vigorous aeration of the suspensions. It therefore seemed unlikely that the exchange of phosphate across the osmotic barrier could depend upon residual respiration. It might, however, depend upon residual fermentation.

The residual fermentation was determined at pH 7 at 25° using the equivalent of 50 mg. dry-weight cells. Less than 1 μ l. CO₂ (or acid)/hr. was produced. Although the residual fermentation was so low, the cells could rapidly ferment glucose, for the addition of 2.5 μ mole glucose caused the production of 110 μ l. CO₂, equivalent to 5 μ mole acid within 10 min., after which there was no further gas production. This fermentation, however, was completely inhibited when 10 mM-iodoacetate was present. The residual fermentation was thus shown to be so low as to make it unlikely that phosphate exchange could depend upon it. Moreover, even when the system which can ferment added glucose was inhibited by 10 mM-iodoacetate, $\dot{P}$ was unaffected. It therefore seems justifiable that the substances which inhibit the exchange of phosphate across the osmotic barrier do not do so by affecting the residual metabolism, but act directly upon the exchange mechanism. This being the case, kinetic studies should be amenable of interpretation.

Kinetics of phosphate exchange

The effect of external phosphate concentration on $\dot{P}$. The dependence of $\dot{P}$ on the external phosphate concentration ($[P]_E$) was determined at pH 6.8 in 100 mM-NaCl at 25°. In view of the hyperbolic appearance of the plot of $[P]_E$ against $\dot{P}$, the reciprocals of $\dot{P}$ and $[P]_E$ were plotted and found to give the straight-line relationship of Fig. 1. It is convenient to describe the straight-line relationship in the form

$$1/\dot{P} = 1/\dot{P}_{\max.} + [P]_E/[P]_E \cdot \dot{P}_{\max.}, \quad (2)$$

$\dot{P}_{\max.}$ denoting the maximal value of $\dot{P}$ and $[P]_E$ denoting the value of $[P]_E$ at which $\dot{P}$ is half maximal. The value of $[P]_E$, given by the intercept of the straight line of Fig. 1 on the $1/[P]_E$ axis, is 1.6 mM.

Temperature dependence of $\dot{P}$. The effect of temperature on the relationship between $1/\dot{P}$ and $1/[P]_E$ is shown in Fig. 2. The data represent experiments similar to that of Fig. 1, done at 5° (○) and 15° (●) at pH 6.85. Within experimental error (corresponding to *c.* 0.2 mM- $[P]_E$), the intercept of the straight lines on the $1/[P]_E$ axis is the same as at 25°, and gives a value of 1.6 mM for $[P]_E$. Thus, the temperature coefficient of $[P]_E$ is practically negligible between 5 and 25°. On the other hand, Fig. 2 shows that $\dot{P}_{\max.}$ (equal to the value of $\dot{P}$ when $1/[P]_E=0$) has a very large temperature coefficient. Since the temperature coefficient of $[P]_E$ is very small, the variation of $\dot{P}_{\max.}$ with temperature is the same as that of $\dot{P}$ at a fixed value of $[P]_E$. It was therefore convenient to determine the temperature dependence of $\dot{P}_{\max.}$ by measuring $\dot{P}$ at an external phosphate concentration of 2 mM in 100 mM-NaCl at pH 7 over a range of temperatures. The results are plotted in Fig. 3 as $\log \dot{P}$ against $1/T$, T being the absolute temperature. The points fall

on a straight line over the temperature range from 5 to 20°. The slope of this straight line may be represented by the Arrhenius equation, written in the form

$$\frac{d \ln \dot{P}}{d(1/T)} = -\frac{E^*}{R},$$

R being the gas constant and E^* , the activation energy of Arrhenius, having the value 38,000 cal./mole.

Fig. 1

Fig. 2

Fig. 1. Dependence of $\dot{P}$ on the external phosphate concentration ($[P]_E$) at pH 6.8 in 100 mM-NaCl at 25°.

Fig. 2. Dependence of $\dot{P}$ on the external phosphate concentration ($[P]_E$) at pH 6.8 in 100 mM-NaCl at 5° (O) and 15° (o).

Fig. 3

Fig. 4

Fig. 3. Dependence of $\dot{P}$ (logarithmic scale) upon temperature at pH 7 in 100 mM-NaCl containing 2 mM-phosphate.

Fig. 4. Dependence of $\dot{P}$ upon pH at 25° in external media containing 100 mM-NaCl with 1 mM-phosphate (O) or 2 mM-phosphate (●) or 10 mM-phosphate (●). Curves 1 and 2 were calculated for 1 and 2 mM-phosphate respectively from curve 3, drawn through the points for 10 mM-phosphate.

pH dependence of $\dot{P}$. The values of $\dot{P}$ at 25° in external media containing 100 mM-NaCl and 1, 2 and 10 mM-($\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$) over a pH range from 5 to 9 are shown in Fig. 4. The points representing the measurements in 10 mM-

phosphate (●), through which the upper curve is drawn, give an optimum value of $\dot{P}$ at pH 7.35, while in 2 mM-phosphate (●) and 1 mM-phosphate (○) the points in the alkaline range are depressed more than those in the acid range and the optimum value of $\dot{P}$ moves across pH 7. This suggests that the $\text{H}_2\text{PO}_4'$ ion might be more reactive in the exchange system than the HPO_4' ion. The pK'_2 of phosphoric acid in 100 mM-NaCl is pH 6.8 at 25°, having only a small temperature coefficient (Harned & Owen, 1943), and the value 1.6 mM for $[\text{P}\frac{1}{2}]_{\text{E}}$ therefore represents 0.8 mM- $\text{H}_2\text{PO}_4'$ + 0.8 mM- HPO_4' . Assuming that only the $\text{H}_2\text{PO}_4'$ ion reacts in the exchange system, equation (2) may be written

$$1/\dot{P} + 1/\dot{P}_{\text{max.}} + 0.8/[\text{H}_2\text{PO}_4']_{\text{E}} \cdot \dot{P}_{\text{max.}}, \quad (3)$$

expressing $[\text{H}_2\text{PO}_4']_{\text{E}}$ in mM. From this equation and from the dependence of $[\text{H}_2\text{PO}_4']$ upon pH and $[\text{P}]_{\text{E}}$, namely

$$\text{pH} = 6.8 + \log_{10} \frac{[\text{P}]_{\text{E}} - [\text{H}_2\text{PO}_4']_{\text{E}}}{[\text{H}_2\text{PO}_4']_{\text{E}}}, \quad (4)$$

the change of $\dot{P}$ with $[\text{P}]_{\text{E}}$ at any given pH can be calculated. Thus, the curves 1 and 2 of Fig. 4, representing the values of $\dot{P}$ at $[\text{P}]_{\text{E}} = 1$ mM and $[\text{P}]_{\text{E}} = 2$ mM respectively, have been calculated from the curve (3) drawn through the points representing $\dot{P}$ at $[\text{P}]_{\text{E}} = 10$ mM. The curves approximate reasonably well to the experimental values, and it follows that the data of Fig. 4 are adequately explained if only the $\text{H}_2\text{PO}_4'$ ion reacts in the exchange system and $[\text{P}\frac{1}{2}]_{\text{E}} = 0.8 \pm 0.1$ mM- $\text{H}_2\text{PO}_4'$, independent of pH. The falling alkaline arm of the pH curves of Fig. 4 is accordingly attributed to the depletion of the $\text{H}_2\text{PO}_4'$ concentration with increasing pH, and consequent desaturation of the exchange system. The acid arm of the pH curve, on the other hand, shows a dependence of $\dot{P}_{\text{max.}}$ on pH. It follows that, at a phosphate concentration sufficiently high to keep the exchange system nearly saturated with $\text{H}_2\text{PO}_4'$ ion, the dependence of $\dot{P}$ on pH should nearly represent that of $\dot{P}_{\text{max.}}$ on pH. In an external medium containing 100 mM-phosphate without added NaCl, at 25°, the dependence of $\dot{P}$ on pH is described in Fig. 5. The application of equations (3) and (4) indicates that up to pH 7, $\dot{P}_{\text{max.}}/\dot{P}$ is not greater than 1.02, but at pH's 7.5, 8.0, 8.5 and 9.0 the values of $\dot{P}_{\text{max.}}/\dot{P}$ are 1.05, 1.13, 1.41 and 2.10 respectively. Hence, even at an external phosphate concentration of 100 mM, the falling alkaline arm of the pH curve may probably be attributed to desaturation of the exchange system with $\text{H}_2\text{PO}_4'$ ion, the value of $\dot{P}_{\text{max.}}$ increasing with pH over the whole range from pH 5 to 9.

DISCUSSION

The system which couples the exchange of phosphate across the osmotic barrier of *Micrococcus pyogenes* is highly specific for phosphate: it behaves as if it were specific for $\text{H}_2\text{PO}_4'$ ion, the other ionic species of orthophosphate apparently being unable to react. These facts suggest that the phosphate molecules pass across the barrier in combination with a specific carrier group belonging to one of the components of the osmotic barrier. Equation (3) shows that the rate

of exchange of the phosphate ($\dot{P}$) depends upon the external concentration of H_2PO_4' ion ($[H_2PO_4']_E$) as though it were a linear function of the degree of saturation with H_2PO_4' ion of a carrier group which is moved to and fro across the barrier by thermal agitation with a frequency independent of $[H_2PO_4']_E$. The value of $[H_2PO_4']_E$ at which $\dot{P}$ is half maximal (0.8 ± 0.1 mM) would thus correspond to the apparent dissociation constant of the carrier group-phosphate compound, and the maximum value of $\dot{P}$ would correspond to the effective rate of shuttling of the carrier group. If the carrier group (R) were in equilibrium with free H_2PO_4' ion and R-phosphate compound on both sides of the osmotic barrier, and both R and R-phosphate could shuttle across the barrier,

Fig. 5. Dependence of $\dot{P}$ on pH at 25° in an external medium containing only 100 mM-phosphate.

unsaturation of R on one side of the barrier would result in a net movement of phosphate into the medium on the unsaturated side. However, even when $[H_2PO_4']_E$ was as low as 0.08 mM (an order of magnitude less than the apparent dissociation constant of the R-phosphate compound), no net transfer of phosphate could be observed. Thus, if the phosphate were to react directly with the R group, it must be supposed that R could move across the barrier only as R-phosphate. Alternatively, R might always be present as R-phosphate in company with a transferring enzyme which catalyses the reaction

on either side of the osmotic barrier (* being a marker for phosphorus). In this case, the value of $[H_2PO_4']_E$ at which $\dot{P}$ is half maximal would represent the apparent Michaelis constant of the enzyme- H_2PO_4' complex. It will be noted that the exchange of phosphate across the osmotic barrier of *M. pyogenes* is too strictly coupled to be accounted for by the simple exchange-diffusion

model proposed by Ussing (1949) to explain cation exchange across the muscle membrane.

It has been observed in this and in an earlier paper (Mitchell, 1953) that the phosphate exchange is specifically inhibited by reagents known to attack thiol groups of low reactivity. This does not prove that the groups attacked are thiols, but it demonstrates that the exchange reaction is dependent upon the integrity of groups with specific chemical properties. These groups might either be the R groups of the carrier compound which react with the H_2PO_4^- ion, or they might be groups essential to the activity of the enzyme postulated to catalyse the exchange of H_2PO_4^- with the phosphate on R-phosphate.

The temperature coefficient of $\dot{P}_{\text{max}}$ at pH 6.85 represents an Arrhenius energy of 38,000 cal./mole over the temperature range from 5 to 20°. The heat of activation of the exchange process (equal to the Arrhenius energy $-RT$) is therefore 37,400 cal./mole. This heat of activation (ΔH^*) represents the increase in total heat required for the movement of R-phosphate across the osmotic barrier in the two alternative systems proposed above. Measurements of the inhibition of phosphate exchange with phenyl-HgCl (Mitchell, 1953) have shown that the number of sites which would have to bind phenyl-Hg⁺ to give complete inhibition is 3 % of the number of internal inorganic phosphate molecules, or *c.* 4 $\mu\text{mole/g.}$ This figure gives an upper limit to the number of units operating in the exchange system, and makes possible the calculation of a minimum figure for the absolute reaction rate. The value of $\dot{P}_{\text{max}}$ at pH 6.85 at 20° was *c.* 4 $\mu\text{mole/g./min.}$, and the absolute rate is therefore at least $4/4 = 1 \text{ min.}^{-1}$. In other words, one phosphate group is carried across the osmotic barrier in each direction at least once a minute by each unit of the exchange system. This implies that in the carrier systems proposed above, the R-phosphate group moves across the barrier at least twice a minute. The relationship between the absolute reaction rate and the free energy of activation (ΔF^*), described by Glasstone, Laidler & Eyring (1941) can be written

$$\Delta F^* = RT(\ln(kT/h) - \ln \dot{P}),$$

ln standing for natural logarithm, k for Boltzman's constant and h for Planck's constant. Substituting a value of at least 1 min.^{-1} for $\dot{P}$ (since the energy required to activate the movement of the carrier across the barrier in one direction may be much greater than that in the other direction), the free energy of activation for the movement of the R-phosphate group across the barrier is found to be not more than 19,700 cal./mole. Since $\Delta H^* = \Delta F^* + T\Delta S^*$, a large part (at least 17,700 cal./mole) of the heat of activation (37,400 cal./mole) may be attributed to the entropy change (ΔS^*) as in reactions like the denaturation of proteins (Glasstone, Laidler & Eyring, 1941). The thermodynamic data are thus in accord with the view that the osmotic barrier, across which the carrier effects the movement of phosphate and of which the carrier forms an integral part, is a well organized internally bonded structure, the disturbance caused by the movement of the carrier within the barrier being analogous to reversible protein denaturation.

It was suggested by Mitchell & Moyle (1951) that the lipo-protein particles

which can be obtained from disintegrated *Micrococcus pyogenes* represent fragments of the cell membrane which, in the intact cells, would form a sheet *c.* 15 m μ . thick. It is possible that this sheet represents the osmotic barrier for phosphate and that the system responsible for phosphate transport may be found in the lipo-protein particles.

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