Effect of Poly-L-lysine on Energy-Linked Chloroplast Reactions*

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ABSTRACT: A synthetic polymer (poly-L-lysine) is a potent uncoupler of photophosphorylation in spinach chloroplasts, increasing the rate of electron transfer while inhibiting adenosine triphosphate (ATP) formation. The mode of uncoupling does not appear to be similar to amine uncoupling, which is considered to require the uncharged form of amines, but seems to be due to a property of the positively charged macromolecule.

In particular, it is the ability of this polycation to bind to the chloroplast grana membrane which apparently gives rise to the uncoupling action. Kinetic studies show that polylysine inhibition of photophosphorylation is competitive with respect to adenosine diphosphate (ADP) and P_i. These data are interpreted as evidence that either (1) a portion of the polymer binds to the grana membrane near the active site of ADP and P_i binding involved in the ATP formation, and that these two cofactors can displace the polymer from the active site, thus overcoming the polylysine inhibition, or (2) polylysine acts to lower the affinity of the coupling site or a transport site for ADP and P_i. Light-induced H^+ uptake as well as electron transport is stimulated by the low concentrations of polylysine which inhibit photophosphorylation. This mode of action is unusual for uncouplers and it provides a potentially important experimental tool for studying the H^+ uptake reaction apart from phosphorylation. High concentrations of polylysine increase chloroplast membrane permeability, leading to apparent inhibition of light-induced H^+ uptake due to a rapid dissipation of the ion gradient. Other energy-linked membrane phenomena such as K^+ transport and volume changes are correspondingly also affected by polylysine.

It has been shown that the light-induced H^+-cation-exchange activity in *Spinacea oleracea* chloroplasts (sometimes referred to as the "H^+ pump") is closely linked to the energy conservation mechanism which can ultimately form ATP[1] (Jagendorf and Hind, 1963; Dilley and Vernon, 1965; Jagendorf and Uribe, 1966; Dilley, 1966; Mitchell, 1966; Hind and Jagendorf, 1965). Unlike chloroplast volume changes, which are competitive with ATP formation (Dilley, 1966; Dilley and Vernon, 1964), the H^+ pump activity appears to be a *prerequisite* for ATP formation (Dilley, 1966). Other evidence was that most of the uncouplers of photophosphorylation also cause a similar degree of inhibition of the H^+ pump (Jagendorf and Neumann, 1965; Dilley, 1966). The mechanism of inhibition of the H^+ pump is not clear in all these cases, *e.g.*, Triton X-100 and Cl-CCP appear to inhibit by making the membrane leaky to H^+ ions, thus not allowing a measurable pH gradient to exist (Jagendorf and Neumann, 1965), whereas quinacrine appears to inhibit the uptake of H^+ ions, possibly by competing with H^+ for a binding site (Dilley, 1966). Other evidence implicating the H^+ uptake as an obligate prerequisite for ATP formation was found in the observation (Dilley, 1966) that in a phosphorylating system, the H^+ uptake reaction occurs prior to the onset of ATP formation. Under these circumstances, light-induced shrinkage is inhibited by ATP formation, as previously reported (Dilley and Vernon, 1964). In addition, Izawa and Hind (1967) concluded from a kinetic study using a rapid flow technique, that the rate of H^+ uptake is sufficient to account for observed PHP rates. These authors also concluded that the H^+ uptake and X_E (defined as the capacity of chloroplasts to form ATP in the dark, after a preillumination) are fundamentally the same thing.

It is also evident that the H^+ pump does not require ongoing phosphorylation, for it is observable under a wide range of conditions which preclude ATP formation, *i.e.*, pH 5.5, absence of added ADP, P_i, and Mg^{2+} (Neumann and Jagendorf, 1964). This is consistent with the H^+ pump acting as a prerequisite to ATP formation, but having a greater tolerance for diverse conditions. In spite of a large amount of data we still do not understand the relationship of electron transport, H^+–cation exchange, and ATP formation. In order to further that understanding, it would be valuable to have an uncoupler which inhibits PHP, but allows H^+ uptake and electron flow to be observed. This report deals with such a compound, poly-L-lysine; a synthetic cationic polymer.

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates; Cl-CCP, M-chlorocarbonyl cyanide phenylhydrazone; DPIP, 2,6-dichlorophenolindophenol; PHP, photophosphorylation; PYO, pyocyanine; $TMQH_2$, trimethylhydrobenzoquinone; IDP and GDP, inosine and guanosine diphosphates.

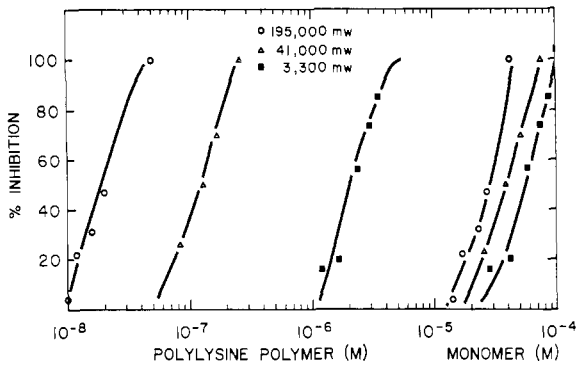

FIGURE 1: Inhibition of photophosphorylation by poly-L-lysine. The reaction mixtures contained 0.1 M KCl, 0.5 mM $MgCl_2$, 0.2 mM ADP, 0.2 mM Na_2HPO_4, 15 μM PYO, 20–25 μg/ml of chlorophyll as chloroplasts, and various concentrations of poly-L-lysine in a total volume of 10 ml. The pH was adjusted to 7.8. The left-hand portion of the abcissa gives the molar concentration of the polymers, and the right-hand portion gives the concentration of the inhibitor expressed on a monomer unit basis. The rate of ATP formation was measured at 1 min after illumination began, by taking the slope of the ΔpH trace.

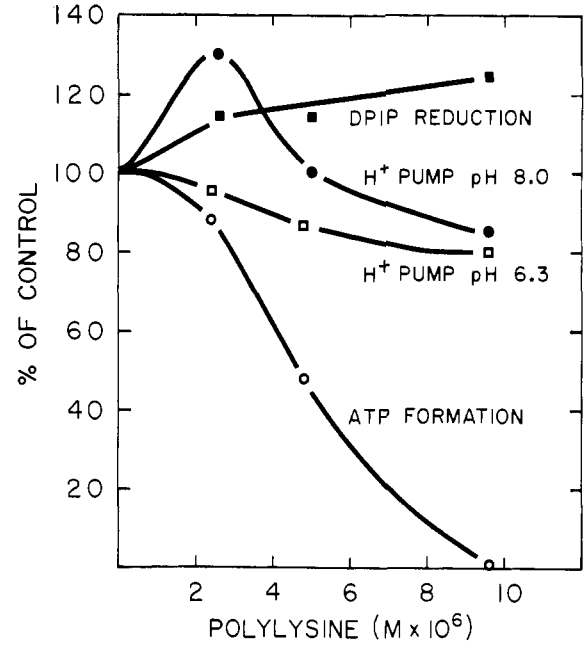

FIGURE 2: Effect of 3300 mol wt polylysine on the rate of ATP formation, H^+ uptake (initial rate), and DPIP reduction. The reaction conditions were as described in Figure 1 for ATP assay and the H^+ uptake. The H^+ uptake rate was obtained at pH 6.3 before the addition of ADP and P_i. After adding ADP and P_i and adjusting the pH to 8.0, the light was again turned on; the initial ΔpH was taken as a measure of the H^+ uptake (*cf.* Dilley, 1966), and the rate of ΔpH at 1 min was taken as the relative rate of ATP formation.

Methods and Materials

Spinach chloroplasts were prepared in a medium of 0.4 M sucrose, 0.02 M Tris-HCl (pH 7.8), and 0.01 M KCl by grinding leaves with a mortar and pestle with a small amount of sand, washed once in the same medium, and resuspended in 0.2 M sucrose, 0.05 M KCl, 2.5×10^{-3} M $MgCl_2$, and 10^{-4} M Tricine-KOH (pH 7.8). Other details of the chloroplast preparation and chlorophyll assay were as given previously (Dilley and Vernon, 1964).

Electron-transport assays using DPIP as an acceptor were previously described (Dilley and Vernon, 1964). Oxygen evolution assays utilizing ferricyanide as the electron acceptor were carried out using a Clark-type oxygen electrode and an amplifier built by the Yellow Springs Instrument Co. Electron transport to ferricyanide was also measured by following the acidification of an unbuffered chloroplast suspension due to the H^+ ions released from water in the Hill reaction. The apparatus for recording pH changes was as described before (Dilley and Vernon, 1967).

Photophosphorylation was assayed by measuring the increase in pH of the suspension due to ATP formation (Nishimura *et al.*, 1962; Dilley, 1966). The assay system was occasionally checked by measuring the disappearance of inorganic phosphate by the Fiske and Subbarow (1929) technique. The H^+ uptake, Mg^{2+} and K^+ transport, and chloroplast shrinkage were measured as described previously (Dilley and Vernon, 1965). Poly-L-lysine of four molecular weights and poly-L-arginine were purchased from Miles Laboratory, Elkhart, Ind.

Results

Inhibition of Photophosphorylation. Figure 1 shows a typical polylysine inhibition curve for photophosphorylation using three samples of polymers of molecular weight 3300, 41,000, and 195,000. The largest polymer inhibits 100% at about 5×10^{-8} M, whereas the smallest polymer requires about 100 times as much (on a polymer basis) for complete inhibition. When the concentration of lysine units was expressed on a monomer basis, however, the polymers gave complete inhibition at more nearly equivalent concentrations between 5×10^{-5} and 10^{-4} M; with the 195,000 mol wt species being about twice as potent as the 3300 mol wt form (Figure 1). Monomeric L-lysine did not show appreciable inhibition even at concentrations of 1 mM suggesting that the mode of inhibition of the polymeric form is due to a property not shared by the monomer.

Poly-L-arginine (28,000 mol wt) gave an inhibition pattern of PHP very similar to polylysine, with 50 and 90% inhibition occurring at 0.25 and 1 μM, respectively. Protamine from Salmon sperm,[2] consisting of 70% arginine, also was inhibitory. Ribonuclease, another

[2] Kindly supplied by Dr. W. Zaugg and Dr. T. Langan.

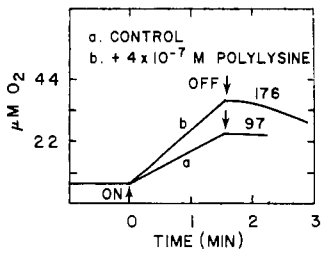

FIGURE 3: Effect of 195,000 mol wt polylysine on oxygen evolution. The reaction mixture contained in a 4-ml total volume: 0.05 M Tricine-KOH (pH 7.5), 0.2 mM ADP, 0.2 mM K_2HPO_4, 2.0 mM $MgCl_2$, 1 mM K_3-$Fe(CN)_6$, and 122 μg of chlorophyll equivalent chloroplasts. Oxygen evolution at 18° was measured with an oxygen electrode as described under Methods. The ordinate gives the O_2 concentration in the medium, the numbers above the traces give the rate of ferricyanide reduction in micromoles per hour per milligram of chlorophyll.

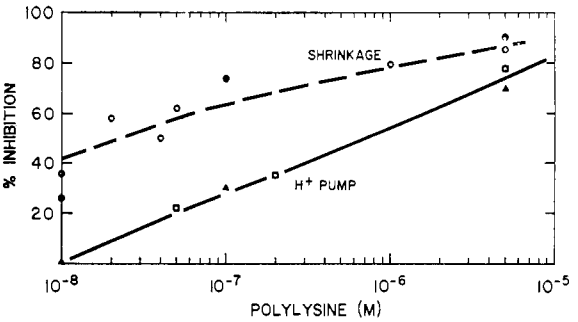

FIGURE 4: Effect of 195,000 mol wt polylysine on light-induced shrinkage and H^+ uptake. (-----) Shrinkage assay: 0.1 M Tris-acetate (pH 6.0), 30 μM PYO, and 20 μg of chlorophyll equivalent chloroplasts/ml. Shrinkage was measured by following changes in turbidity of a 1-ml sample. (○) Extent of turbidity increase. (●) Initial rate of turbidity increase. (———) H^+ uptake assay: conditions as described in Figure 2 for the pH 6.3 assay of H^+ uptake. (□) Relative H^+ uptake extent. (Δ) Initial rate of H^+ uptake.

basic protein, did not inhibit PHP appreciably at 7.6 μM. Spermine, a low molecular weight tetraamine, did not inhibit PHP at 0.5 mM.

Effect on the H^+ Pump and Electron Transport. At concentrations of the 3300 mol wt polylysine completely inhibiting PHP, the H^+ pump is only slightly inhibited while DPIP reduction is stimulated (Figure 2). This behavior places polylysine in the class of uncouplers by definition. The novel feature of the uncoupler's effect, however, is found in the response of the H^+ pump; no other uncoupler except arsenate + ADP shows very slight inhibition of the H^+ pump at concentrations completely inhibiting PHP. The stimulation of H^+ uptake at pH 8.0 (Figure 2) caused by low concentrations of polylysine is most likely due to the uncoupling action; as the rate of electron transport is stimulated, the rate of H^+ uptake could therefore be stimulated. The H^+ pump at pH 6.3 is likewise only slightly inhibited by concentrations of polylysine which completely inhibit PHP at pH 8.0 (Figure 2).

Figure 3 shows the stimulation of electron transport by 195,000 mol wt polylysine using ferricyanide-supported oxygen evolution as the assay. There is nearly a doubling of the rate of O_2 evolution from chloroplasts when 4×10^{-7} M polylysine is added to a complete phosphorylating system at pH 7.5. Lower concentrations of polymer also stimulated oxygen evolution. The higher concentration was chosen here to indicate that oxygen evolution is resistant to polylysine concentrations several times higher than that which inhibits PHP completely. In the absence of ADP, P_i, and $MgCl_2$ (with other conditions as given in Figure 3), similar stimulation of oxygen evolution was seen, although the control rate was lower; *i.e.*, control, 61 μmoles of $Fe(CN)_6^{3-}$/hr mg of chlorophyll, compared to 10^{-7} M polylysine (195,000 mol wt), 106 μmoles of $Fe(CN)_6^{3-}$/hr mg of chlorophyll. These data clearly demonstrate that polylysine acts as a true uncoupler, in that PHP is inhibited while electron transport is stimulated.

In the absence of the complete phosphorylating system, the H^+ pump becomes more sensitive to polylysine (Figure 4) but still electron transport is not inhibited. A study of the rate of the decay of the pH gradient as a function of the extent of the gradient shows that 2×10^{-7} M polylysine (195,000 mol wt) causes a considerably faster decay reaction (Figure 5b). Most uncouplers cause a similar effect (Jagendorf and Neumann, 1965) and this can account for much of the apparent inhibition of the H^+ pump, *i.e.*, the leakage of H^+ out is so fast as to prohibit a large gradient to develop.

Effect on Light-Induced Shrinkage and K^+ Transport. Previous work has shown a direct correlation between light-induced shrinkage, the H^+ pump, and K^+ efflux from chloroplasts (Dilley and Vernon, 1965). Furthermore, these ion-exchange phenomena appear to be related to the energy conservation mechanism in a manner as yet not understood. Therefore it was considered important to check the effect of polylysine on these reactions.

Light-induced shrinkage appears to be inhibited by polylysine to a greater extent than is the H^+ pump when both are assayed in the absence of the phosphate acceptor system (Figure 4). However, the concentration required to partially inhibit shrinkage is considerably higher than that which completely inhibits PHP.

When a high concentration of polylysine is added to a chloroplast suspension in the dark, there is an immediate release of K^+ into the medium (Figure 6), which may be measured with a cation electrode. Figure 6 also shows a typical light-induced K^+ efflux measurement. After the K^+ efflux in the dark is obtained by adding the polymer, there is very little or no light-induced K^+ flux, as expected if the polymer causes a

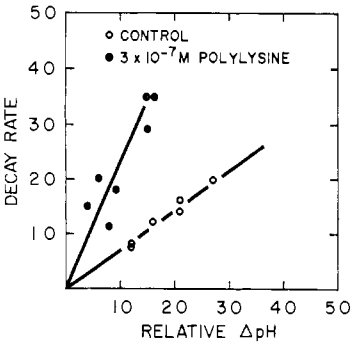

FIGURE 5: Effect of 195,000 mol wt polylysine on the rate of decay of the light-induced H^+ gradient. Reaction conditions similar to those given above for the H^+ uptake reaction. The extent of the reaction was varied by giving illumination times progressively less than that required to give the maximal extent. (○) Control reactions. (●) Plus 3×10^{-7} M 195,000 mol wt polylysine.

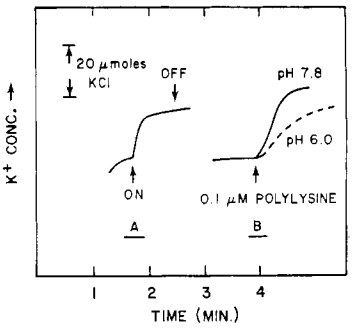

FIGURE 6: Effect of polylysine on K^+ transport in chloroplasts. The reaction contained 0.05 M Tris-Ac (pH 6.0) or 7.8 as indicated, 0.2 M sucrose, and 13 μg/ml of chlorophyll equivalent chloroplasts in a total volume of 6 ml. The K^+ concentration of the medium was measured with a K^+-sensitive electrode as described in Methods. (a) Light-induced K^+ efflux at pH 6.0. (b) Polylysine-induced K^+ efflux in the dark at pH 6.0 (----) and 7.8 (———).

loss of most of the internal K^+ ion. Polylysine also causes Mg^{2+} to be lost from chloroplasts in the dark as shown by assaying the Mg^{2+} concentration (by atomic absorption spectrophotometry) in the supernatant of a centrifuged suspension. Between 0.05 and 0.1 equiv of Mg^{2+}/mole of chlorophyll was lost by this treatment. The light-induced Mg^{2+} efflux reported earlier (Dilley and Vernon, 1965) usually gave in the range of 0.2 equiv of Mg^{2+}/mole of chlorophyll. The K^+ efflux elicited by polylysine in the dark is more nearly equivalent to the light-induced K^+ efflux (Dilley, 1964). The difference between K^+ and Mg^{2+} in response to polylysine could be due to the tighter binding of Mg^{2+} in the chloroplast lamallae. Several other uncouplers including gramicidin and Triton X-100, also induced a similar dark efflux of K^+ and Mg^{2+} from chloroplasts. Another report will consider these effects in greater detail (N. Shavit, R. A. Dilley, and A. San Pietro, to be submitted).

The observation that high concentrations of polylysine induced a dark efflux of K^+ and stimulate the dark decay of the H^+ gradient could explain why light-induced shrinkage is inhibited. A plausible explanation for the inhibition of shrinkage, K^+ leakage and faster H^+ ion gradient decay could be that these concentrations of polylysine make the lamellae membranes more permeable, thus dissipating gradients more readily. This interpretation is corroborated by the fact that the polylysine-induced K^+ efflux is faster at pH 7.8 than at pH 6.0, in keeping with the fact that chloroplasts are generally more permeable at the higher pH (Dilley, 1966; Dilley and Rothstein, 1967).

Concerning the Mechanism of Polylysine Inhibition of PHP. It is known from previous work (Vambutas and Racker, 1965) that the polycation protamine will bind the chloroplast coupling factor (CF_1). This raises the possibility that the polylysine inhibition of PHP reported here may be due to binding at or near the coupling factor site. The experiments discussed below were designed to test this hypothesis.

A necessary condition for the above postulate to hold is that the polymer bind to chloroplast grana membranes. The following experiment, while it appears complex, is in principle quite straightforward. It demonstrates: (1) that under the conditions used in most of our experiments 90% of the polymer from a 10^{-7} M 195,000 mol wt polylysine solution was bound to the chloroplasts, and (2) part of the chloroplast-bound polylysine could be released by adding 0.01 M $MgCl_2$ to the medium used to resuspend chloroplasts which had been centrifuged through a polylysine solution.

The experiment which demonstrates binding was conducted by adding 10^{-7} M of 195,000 mol wt polylysine to a 10-ml mixture of 0.1 M KCl, 5×10^{-4} M $MgCl_2$, and 122 mg of chlorophyll as chloroplasts (this salt solution was routinely used for the PHP assay in which 100% inhibition by 10^{-7} M polylysine may be demonstrated). After 4-min incubation at ice-bath temperature, this suspension and suitable controls were centrifuged for 5 min at 12,000*g*. The supernatants were decanted and aliquots were used as media for a PHP assay using untreated chloroplasts, as described in the legend of Table I. This type of bioassay was considered an appropriate way to measure the amount of polylysine left in the supernatants. It is apparent from Table I that the 10^{-7} M polylysine which was added to tube 1 was 90% removed from the suspension with the chloroplasts (compare line 2 with line 1), and enough remained combined with the chloroplasts even after resuspension of the pellet from tube 1 in 10 ml of 0.01 M $MgCl_2$ and 0.2 M sucrose, centrifugation, and final resuspension in 1.0 ml of 0.2 M sucrose prior to assaying, to give 70% inhibition of PHP (line 8). The $MgCl_2$ in the medium used to wash this pellet apparently released some poly-

TABLE I: Binding of Polylysine to Chloroplasts.[a]

Assay	μmoles of P_i (hr^{-1} $(MgChl)^{-1}$)	% of Control
Supernatant		
1. Control, 5 ml of no. 1 supernatant	233	100
2. 5 ml of no. 2 supernatant (10^{-7} M polylysine initially)	210	90
3. 5 ml of no. 3 supernatant (5×10^{-7} polylysine initially)	0	0
4. 1 ml of no. 3 supernatant, 4 ml of 0.1 M KCl–5×10^{-4} M $MgCl_2$	144	62
5. 5 ml of no. 4 supernatant (no chloroplasts, 10^{-7} M polylysine initially)	0	0
6. 5 ml of supernatent from step 8 below	144	60
Pellet (after one washing)		
7. Control pellet 1	115	100
8. Pellet 2	35	30
	relative to line 7 control	

[a] In this experiment chloroplasts were exposed to two concentrations of polylysine, centrifuged, and the supernatants were used as assay media for PHP assays, after being fortified with the cofactors for PHP. The degree of inhibition of PHP is a measure of the amount of polylysine contained in the supernatant. Similarly, the pellets from the various treatments and controls were assayed after having been resuspended in fresh media. The treatments are given below by number and are referred to by that number in the table. Explanation of treatments: (1) A chloroplast suspension having 10 ml of 0.1 M KCl, 5×10^{-4} M $MgCl_2$, and 122 mg of chlorophyll equivalent chloroplasts was incubated 4 min at ice-bath temperature. (2) Similar to 1 with 10^{-7} M 195,000 mol wt polylysine. (3) Similar to 1, with 5×10^{-7} M polylysine added. (4) Similar to 1 but no chloroplasts and 10^{-7} M polylysine added. Treatments 3 and 4 were controls to check whether the experimental manipulations affected either the inhibitor or the chloroplasts. After a 4-min incubation, all tubes were centrifuged 5 min at 12,000*g* and supernatants were decanted and aliquots were assayed for PHP activity by adding to a total of 5 ml, 77 μg of chlorophyll as fresh chloroplasts, 1.5×10^{-5} M pyocyanine, 2×10^{-4} M ADP, and 2×10^{-4} M K_2HPO_4. Dilutions as indicated were made with a mixture of 0.1 M KCl–0.5 mM $MgCl_2$. The pH was adjusted to 8.0 with standard KOH. The pellets from tubes 1 and 2 were resuspended in 10 ml of 0.01 M $MgCl_2$–0.2 M sucrose and centrifuged again; the pellets from this step were taken up in 1.0 ml of 0.2 M sucrose, 0.01 M KCl, and 10^{-4} M Tricine (pH 7.8). The pellets were assayed for PHP activity (lines 7 and 8) as well as the supernatants from this second centrifugation (line 6).

lysine, since this supernatant was more inhibitory to PHP (line 6) than the control (line 1) or the first supernatant from the 10^{-7} M polylysine treatment. These data demonstrate that under these conditions 10^{-7} M polylysine is at least 90% bound (referring to Figure 1 for the concentration which inhibits PHP 10%), and at 5×10^{-7} M polylysine, less than 90% is bound.

Nobel and Mel (1966) as well as earlier workers (Fishman and Moyer, 1942) have shown that chloroplasts and chloroplast lamellae have a net negative charge above pH 4.5–4.7 (the isoelectric point). It is reasonable that polylysine, being positively charged, would have a tendency to electrostatically bind to the chloroplast membrane. That electrostatic binding does occur is demonstrated by evidence that salt (KCl) partially reverses the inhibition of PHP by polylysine. In general, high salt tends to break electrostatic bonds between polyelectrolytes and oppositely charged surfaces (Tanford, 1961). Table II shows that 0.1 M KCl

TABLE II: Salt Reversal of Polylysine Inhibition of PHP.[a]

KCl (M)	Polylysine	Rel Rate of Phosphorylation	% Inhibn
		122	
	+	50	59
0.1	+	108	7
0.1	+	90	22
0.1, no sucrose		132	
0.1, no sucrose	+	108	20

[a] The reaction mixtures contained 0.2 M sucrose, KCl as indicated, 0.2 mM ADP, 0.2 mM K_2HPO_4, 0.5 mM $MgCl_2$, 20 μg/ml of chlorophyll equivalent chloroplasts in a 10-ml total volume at pH 7.9, and 8.6×10^{-8} M 41,000 mol wt polylysine as indicated.

added to a low ionic strength medium reduces the polylysine inhibition of PHP from about 60 to 22%, consistent with the concept that the higher salt concentration displaced some of the polylysine bound to the chloroplast, leading to less inhibition. A similar pattern of salt ($MgCl_2$) displacement of polylysine was seen in the experiments summarized by Table I. We interpret the above data as evidence that (a) polylysine binds electrostatically to chloroplasts, and (b) that the inhibitory action of this polycation depends in some way on such binding.

Kinetic experiments were carried out to test the nature of polylysine inhibition. As Figures 7 and 8 show, polylysine appears to be a competitive inhibitor with respect to either ADP or P_i. We must consider the possibility that such a pattern could be due to ADP

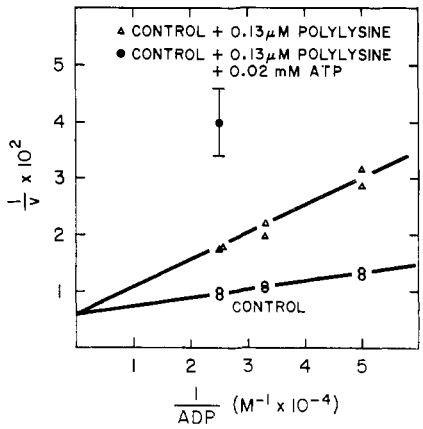

FIGURE 7: Effect of ADP on polylysine inhibition of PHP. The reaction mixture contained 0.1 M KCl, 0.5 mM $MgCl_2$, 15 μm PYO, 0.2 mM Na_2HPO_4, 0.02, 0.03, and 0.04 mM ADP, ±0.13 μM 41,000 mol wt polylysine, and 20 μg/ml of chlorophyll equivalent chloroplasts in a 10-ml total volume at pH 7.9. In some assays 0.02 mM ATP was added to 0.02 mM ADP in the presence of polylysine, the total nucleotide concentration being taken as the substrate concentration. The relative rate of PHP was measured by taking the slope of the ΔpH trace at 1 min after illumination began.

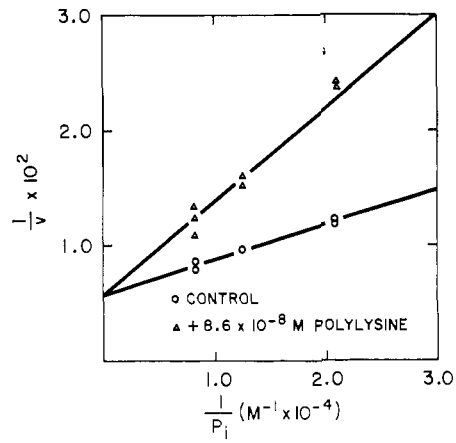

FIGURE 8: Effect of P_i on polylysine inhibition of PHP. The reaction mixtures contained 0.1 M KCl, 0.5 mM $MgCl_2$, 15 μM PYO, 0.2 mM ADP, 0.048, 0.080, and 0.12 mM K_2HPO_4, ±8.6 × 10^{-6} M 41,000 mol wt polylysine, and 20 μg/ml of chlorophyll equivalent chloroplasts in a 10-ml total volume at pH 7.9. The relative rates of PHP were determined as in Figure 7.

and P_i binding the polymer in solution thus lowering the effective concentration of the cofactors, rather than the cofactors and the polymer competing for an active site. The equilibrium dialysis experiments discussed below strongly suggest that under these conditions such binding with ADP does not occur to an appreciable extent. Additional evidence that polymer–nucleotide binding is not the reason for the competitive inhibition pattern is found in the observation from the kinetic experiments (Figure 7) that if ATP is substituted for a portion of the ADP, there is no decrease in the degree inhibition as there is with added ADP. If nucleotide binding to the polymer were causing the competitive inhibition pattern, then ATP should be more effective than ADP in overcoming the inhibition. Turbidity measurements at pH 6.0 and low $MgCl_2$ concentration showed that ATP and ADP were both bound to polylysine (the binding was reversed by adding more $MgCl_2$), suggesting that ATP has the capacity to bind the polycation. However, under the phosphorylating conditions used here, such binding apparently does not occur to a significant extent. IDP and GDP at low concentrations did not act like ADP in overcoming the inhibition of PHP by polylysine (Figure 9), again suggestive that the competitive inhibition pattern is *not* a result of polylysine binding the nucleotide, but rather due to the high affinity of chloroplasts for ADP (and P_i).

However, at higher concentrations of IDP and GDP (0.1–0.4 mM), both nucleotides by themselves partially overcame polylysine inhibition just as ADP does at tenfold lower concentrations. In our experiments the apparent K_m values taken from the Lineweaver–Burk plots, for these three nucleotides in PHP were ADP (1–2 × 10^{-5} M), IDP (4 × 10^{-4} M), and GDP (2 × 10^{-4} M). The K_m for P_i in our experiments was 5 × 10^{-5} M, considerably less than other published values (Avron and Shavit, 1965).

Similarly, SO_4^{2-} did not act like PO_4^{3-} did in over-

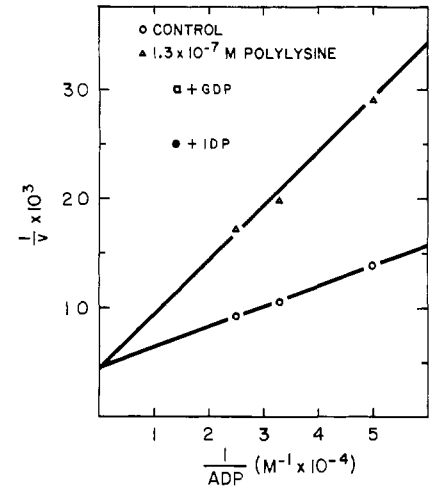

FIGURE 9: Effect of IDP and GDP on polylysine inhibition of PHP.The reaction mixtures and conditions were similar to those of Figure 7. To reaction mixtures containing 1.3 × 10^{-7} M polylysine with 0.03 mM ADP was added either 0.04 mM IDP (●) or 0.04 mM GDP (□), as indicated in the figure; the total nucleotide concentration being taken as the substrate concentration.

coming polylysine inhibition; it might be expected that SO_4^{2-} would be attracted to polylysine as well as PO_4^{3-}. The absence of any SO_4^{2-} reversal of PHP is consistent with the concept that P_i overcomes polylysine inhibition by displacing the polymer from an active site, or that the presence of the polycation causes a decrease in the affinity of the active site(s) for ADP and P_i (Bygrave and Lehninger, 1967).

Equilibrium dialysis experiments were carried out to measure the degree of ADP binding to polylysine under conditions similar to those used in the inhibition studies (except no chloroplasts were used in the dialysis studies). When the concentration ratio of polylysine monomer to ADP was about 1:1, there was 10^3 more free ADP than bound ADP, when the polylysine monomer to ADP concentration ratio was 40:1, the ratio of free to bound ADP was 10^2. Since the inhibition studies usually employed from a 1:1 to 1:10 ratio of polylysine monomer:ADP, it is clear that the mode of inhibition of PHP in these studies could not be due to the polycation binding ADP, for at most only $^1/_{100}$ of the ADP would be removed from the reaction in this manner. The fact that polylysine is strongly bound to chloroplasts would further reduce the amount of polymer available to bind ADP or P_i.

Discussion

This work shows that poly-L-lysine acts as an uncoupler of photophosphorylation in spinach chloroplasts, inhibiting ATP formation while stimulating electron transport (Figures 2 and 3). The inhibitory action appears to be a property of the positively charged polymer inasmuch as (a) polymers of different molecular weight from 3300 (24 monomer units) to 195,000 (1450 monomer units) gave inhibition at about the same concentration (30–60 μM) on a *monomer* basis (Figure 1), but the monomeric form of L-lysine did not give any inhibition at these concentrations, and only slight inhibition at 10^{-3} M. (b) Another cationic polymer, poly-L-arginine (28,000 mol wt) also inhibited PHP 50% at 2.5×10^{-7} or 3×10^{-5} M on a monomer basis. (c) Binding studies indicated that the inhibition is probably a result of the cationic polymer electrostatically binding to the negatively charged lamellae. In accordance with this, a partial reversal of PHP inhibition was observed by increasing the salt concentration in the reaction mixture (Table II), leading to a displacement of the polycation from the lamellae membrane due to the added salt. Concentrations of polylysine which severely inhibit PHP do not inhibit the H^+ pump; rather the rate of H^+ uptake is markedly stimulated. This is probably due to the stimulating effect of polylysine on electron transport. All other uncouplers of PHP except ADP + arsenate (Jagendorf and Neumann, 1965; Dilley, 1966) inhibit the observable H^+ uptake to approximately the same degree that they inhibit PHP. Polylysine is thus a unique and interesting uncoupler, and could serve as a tool for studying the relationship between electron transport, the H^+ pump, and PHP.

It was shown that polylysine causes the thylakoid membranes to become more permeable to K^+ and H^+ (measured by the decay rate of the light-induced H^+ uptake), and as a consequence, light-induced shrinkage is inhibited. At first thought it is somewhat strange that the H^+ uptake reaction is much more resistant to inhibition by polylysine than K^+ efflux and shrinkage (Figure 4). This is explainable if one postulates, as Mitchell (1966) has, that the H^+-transport mechanism is a fundamental and primary aspect of the energy conservation mechanism. If the H^+ transport is linked either to the alternating electron (*viz.* cytochrome) and electron-proton carriers (*viz.* plastoquinone) as suggested by Mitchell, or if it is mediated *via* a membrane carrier as suggested by Dilley (1966) and Dilley and Vernon (1967) one might expect that the active driving force of H^+ uptake could maintain a greater H^+ ion gradient than if the H^+ gradient were merely a passive response to some other, primary, ion-transport process. A similar relative retention of H^+ ions could arise if part of the H^+ ions taken up were bound to negative charge groups of the membrane, as suggested earlier (Dilley and Vernon, 1965). Such a binding of H^+ ions could of course aid in retention for either of the two postulated H^+-transport mechanisms outlined above.

An estimate of the net number of negative charge groups on a chloroplast membrane system gave a result of 5×10^8 negative groups/chloroplast. For this approximate calculation we used the charge density data of Nobel and Mel (1966), that at pH 8.0 and 0.88 M NaCl, negative charges are about 40 Å apart, similar to the surface charge density of red blood cells (Gross *et al.*, 1964). The estimate of total grana surface area per chloroplast was taken to be 2000 μ^2/chloroplast (Thomas *et al.*, 1956). Using a value of 1.5×10^9 chloroplasts/mg of chlorophyll (Tolberg and Macey, 1965), our average chlorophyll concentration of 20 μg/ml, and the concentration of 195,000 mol wt polylysine required to give 100% inhibition of PHP (5×10^{-5} M on a monomer basis), we may calculate a ratio of number of lysine residues to the number of fixed negative charge groups in the plastids. This ratio is estimated at five lysine groups per negative charge. Considering the shape of the PHP inhibition curves (Figure 1), the onset of inhibition occurs at about one lysine group per negative charge. We do not know how many of the lysine residues actually take part in binding interactions with the plastid membrane surface, so the above calculation is of limited utility at present.

If polylysine binds a large proportion of the surface negative charges in chloroplast lamellae, then it seems unlikely that these groups would be involved in the postulated "active H^+ binding" referred to previously (Dilley and Vernon, 1965; Dilley and Rothstein, 1967). It is more reasonable that such negative charge groups would be within the membrane, not in direct contact with the stroma phase, since the H^+ uptake reaction proceeds without appreciable inhibition in the presence of bound polylysine.

Kinetic analysis suggests that both ADP and P_i competitively overcome polylysine inhibition of photo-

phosphorylation (Figures 7–9). $MgCl_2$ does not act as a competitive inhibitor, however. These kinetic data suggest that the cationic polymer may act to mask a site(s) at which ADP and P_i must bind prior to the final esterification step in ATP formation. Other workers have shown that polycations bind the isolated spinach chloroplast coupling factor (Vambutas and Racker, 1965), so it is not unreasonable that polylysine may bind to the lamellae membrane *via* some of the same negative sites. It is also probable that there are many other fixed negative charge groups on the lamellae, not associated with the coupling factor, to which polylysine may bind. It seems likely that the ADP–Mg and P_i–Mg complexes, being anions, may interact with a positive charge group(s) at the binding sites(s), in which case, the polylysine would not interact with these groups, except *via* the carboxyl group. The lack of correspondence between the degree of inhibition and the concentration of carboxyl groups (Figure 1) argues against the latter possibility. On the other hand, the correspondence between the degree of inhibition and the total concentration of monomer lysine residues (using polymers from 3300 to 195,000 mol wt), is consistent with the concept that the inhibition of PHP is due to the polymer binding a certain number of negative charged groups on the lamellae. If this is the case, it is possible that portions of the polymer could sterically mask and/or bind the active center involved in utilizing ADP and P_i in the ATP-formation process.

In view of the fact that in low salt conditions ADP does bind to polylysine it may be worthwhile to summarize the data which led us to reject this as the explanation of polylysine inhibition of PHP (and in particular, the competitive inhibition pattern with respect to ADP and P_i), in favor of the hypothesis that the inhibition is due to the polymer binding the grana membrane so as to either reversibly block the approach of ADP and P_i to an active site(s) involved in some phase of the ATP formation process or to cause a decrease in the affinity of the active site(s) for ADP and P_i (Bygrave and Lehninger, 1967; Vignais *et al.*, 1962). (1) ATP, GDP, and IDP at low concentrations (20 μM) do not reverse polylysine inhibition of PHP as does 20 μM ADP. Reversal would be expected if the inhibition were merely due to the additional nucleotide saturating polymer binding sites, thus freeing more ADP to act as substrate for ATP formation. At higher concentrations GDP and IDP alone also give competitive inhibition patterns with polylysine, consistent with their lower affinities for the chloroplast phosphorylation mechanism. Also SO_4^{2-} did not act like PO_4^{3-} in releasing polylysine inhibition, as might be expected if soluble anion binding to the polycation were responsible for the competitive inhibition pattern. (2) Equilibrium dialysis indicated that at the ratios of polymer to ADP we use to obtain inhibition of PHP, there is less than 1% of the ADP bound to the polymer, suggesting that the inhibition is not due to having the ADP bound to the polymer in solution. (3) Polylysine strongly binds to chloroplast lamellae under the conditions of our experiments, reducing even further the amount of free polymer in solution to interact with ADP and P_i. (4) Given the observation that polylysine stimulates electron flow as do uncouplers in general, it is not easy to rationalize the inhibition of PHP as due to binding ADP and P_i. This fact further suggests that it is the binding of the polymer to the membrane which induces the observed effects on the energy conservation mechanism. In view of the fact that polylysine binds the isolated chloroplast-coupling factor (Vambutas and Racker, 1965; R. A. Dilley, unpublished observation), it is tempting to interpret the polylysine inhibition as being due to direct interference with the coupling site *in vivo*. However, it is also possible that the polycation binding causes a change in the affinity of the coupling site for ADP and P_i, through some other effect on the membrane. These alternatives are currently being tested (R. A. Dilley, N. Shavit, and A. San Pietro, to be published). It does not appear possible to prove either case on the basis of present evidence. In either event, polylysine appears to have a very interesting effect on chloroplast membranes, and should prove to be a useful tool for study of certain membrane-related phenomena.

Schwartz and his colleagues (Schwartz, 1965; Johnson *et al.*, 1967) have found that histones and polylysine have significant effects on energy-linked mitochondrial reactions, but there is no clear indication of the mechanism of action in that system. The mitochondrial interaction with polycations is somewhat complicated since stimulation and inhibition of respiration were both observed under various conditions. Machinist and Crane (1965) have shown that protamine and tetrachlorohydroquinone interact with mitochondrial membranes in a manner which they interpret as due to the polycation binding to a negatively charged pore in place of cytochrome *c*. Such studies indicate that interactions of negative membrane surfaces with added polycations may have profound effects on biochemical functions. It has been previously postulated (Schwartz, 1965; Johnson *et al.*, 1967; Machinist and Crane, 1965) that endogenous histones may interact with cellular membrane systems, thereby exerting a type of control on cellular functions. It is not yet possible to state whether endogenous polycations do play a role in the biochemistry of chloroplasts, but it is now a pertinent question.

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The Metabolism of Bile Acids in the Developing Rat Liver*

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ABSTRACT: The metabolism of cholic acid, deoxycholic acid, 5β-cholestane-3α,7α,12α-triol, and 7α-hydroxycholest-4-en-3-one was studied in homogenates of liver from rat embryos and suckling rats. The specific activities of the enzymes concerned with conjugation of bile acids were found to increase 30- to 40-fold between 15 days after fertilization and 5 days after birth. A similar increase in activity was observed for the enzymes catalyzing the oxidation of 5β-cholestane-3α,7α,12α-triol and the 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one. The results suggest that the activities of the enzymes concerned with the conversion of cholesterol to bile acids increase synchronously in the developing rat liver.

The main pathways for the formation and interconversion of the bile acids (*cf.* Figure 1) have now been elucidated (Bergström *et al.*, 1960; Danielsson, 1963; Mendelsohn and Staple, 1963; Mendelsohn *et al.*, 1965; Danielsson and Einarsson, 1966). As far as is known, the liver is the sole source of these acids. The pattern of accumulation in embryological development of these enzymes has not been defined. Whitehouse *et al.* (1962) demonstrated that the livers from 13-day-old rat embryos catalyzed the formation of labeled carbon dioxide from [26-^{14}C]cholesterol (I) and [26-^{14}C]5β-cholestane-3α,7α,12α-triol (V). Thus at least some of the activities in the pathway are present at a relatively early stage in development. In the present studies, the metabolism of 7-hydroxycholest-4-en-3-one (III), 5β-cholestane-3α,7α,12α-triol (V), cholic acid,

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