BBA 45720

THE STOICHIOMETRIC RELATION OF PHOSPHORYLATION TO ELECTRON TRANSPORT IN ISOLATED CHLOROPLASTS

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(Received April 22nd, 1968)

SUMMARY

Electron transport in chloroplasts can proceed by two pathways. One pathway is dependent on the presence of ADP and orthophosphate and is responsible for photophosphorylation. This pathway is sensitive to the photophosphorylation inhibitor phlorizin, to antimycin A and to *p*-chloromercuribenzoate (PCMB). The other pathway is independent of phosphorylation, insensitive to phlorizin, relatively insensitive to antimycin A and insensitive to PCMB. Apparently amines increase the electron transport rate by uncoupling the antimycin-sensitive phosphorylating pathway while carbonylcyanide phenylhydrazones uncouple by increasing the activity of the antimycin-resistant non-phosphorylating pathway.

During photophosphorylation the electron transport rate normally exceeds the corresponding rate measured in the absence of phosphate and, under many conditions, the amount of ATP formed is proportional to the additional electron transport which occurs in the presence of phosphate. Indeed there is often a rather precise mole for mole correspondence between ATP formation and the accompanying increase in ferricyanide reduction. This is true when phosphorylation conditions are optimal and it is also true when the phosphorylation rate is varied over a wide range by limiting concentrations of phosphate, by the addition of antimycin A, by the addition of PCMB, or by the addition of carbonylcyanide phenylhydrazones. It is not true unless the rate of electron transport is controlled at the level of the phosphorylation reaction. Thus the correspondence between phosphorylation and extra electron transport is no longer observed when electron transport is limited by low light intensities or by the addition of phenylureas. Under these conditions phosphorylation may greatly exceed the increase in electron transport associated with phosphorylation. Moreover the correspondence is not always found when the chloroplasts have been partially uncoupled by amines such as Tris or methylamine.

Our observations suggest that, at high light intensities and in the absence of amine uncouplers, the phosphorylating electron transport is superimposed on a constant non-phosphorylating electron transport. If this is so the phosphorylating process must yield two molecules of ATP for every pair of electron it transfers.

* Abbreviations: PCMB, *p*-chloromercuribenzoate; CCCP, carbonylcyanide 3-chlorophenylhydrazone, *i.e.*, ketomalononitrile 3-chlorophenylhydrazone; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; P/e_2, the ratio of ATP molecules formed to pairs of electrons transported; TES, *N*-Tris(hydroxymethyl)methyltaurine; tricine, *N*-Tris(hydroxymethyl)methylglycine.

INTRODUCTION

It has been widely accepted that the theoretical maximum efficiency of "non-cyclic" photophosphorylation is 1 ATP molecule formed for each pair of electrons transported[1,2]. This implies that "non-cyclic" phosphorylation is coupled to a single oxidation–reduction reaction involving 2 electrons. Moreover the ratio of 1 ATP to 1 electron pair means that only 1 ATP molecule can be formed in the reduction of a molecule of $NADP^+$, an amount of ATP which is insufficient for reduction of CO_2 by the Calvin–Benson cycle[3] or by any plausible modification thereof. Consequently much effort has been expended in a search for a second kind of phosphorylation which must perforce utilize a "cyclic" (*i.e.* an as yet unmeasured) electron flow.

Recent observations have shown that the value of $P/e_2 = 1.0$ is probably wrong and therefore most of the reasons for postulating a "cyclic" phosphorylation mechanism *in vivo* have disappeared. In the first place, overall values of P/e_2 much higher than 1.0 are routinely observed in our laboratories[4] and in other laboratories*. This in itself suggests that the theoretical maximum value may be 2.0. In the second place, the overall values do not take into account the fact that two kinds of electron transport, phosphorylating and non-phosphorylating, may occur simultaneously in chloroplasts. We know that isolated chloroplasts are capable of very considerable rates of electron transport in the absence of phosphorylation and there is no reason for supposing that this process must completely cease during phosphorylation. Therefore the overall stoichiometry may represent the average efficiency of two processes, only one of which phosphorylates ADP. In the absence of any knowledge of the relative magnitudes of these two concurrent processes, the efficiency of the phosphorylation process itself cannot be deduced. Nor does the average efficiency in isolated chloroplasts tell us anything about the *in vivo* efficiency of phosphorylation since we cannot assume that the non-phosphorylating and phosphorylating pathways of electron transport have the same relative activities *in vivo* and *in vitro*. Indeed it is possible that the whole of the non-phosphorylating electron transport *in vitro* results from a partial uncoupling of the chloroplasts which occurs during their isolation. It follows that the overall *in vivo* efficiency of photophosphorylation might be as high as the *in vitro* efficiency of the phosphorylating part of the electron transport of isolated chloroplasts.

This paper describes experimental conditions under which the phosphorylating and non-phosphorylating electron transport are probably additive. In other words the phosphorylating electron transport seems to be superimposed on a independent and constant non-phosphorylating process. By taking advantage of these conditions we have attempted to determine the real efficiency of the phosphorylating component of the electron transport.

MATERIALS AND METHODS

Chloroplasts

Leaves of spinach (*Spinacea oleracea* L.), obtained from a local market, were ground in a Waring Blendor for 5 sec in a medium containing 0.3 M NaCl, 2 mM

* Personal communications from M. AVRON and H. BALTSCHEFFSKY.

$MgCl_2$ and 0.04 M tricine adjusted to pH 7.8 with NaOH. The homogenate was squeezed through cheesecloth and centrifuged at 3000 × *g* for 4 min. The pellet was resuspended in a medium containing 0.2 M sucrose, 3 mM $MgCl_2$ and 0.03 M *N*-Tris-(hydroxymethyl)methylglycine (tricine) adjusted to pH 7.3 with NaOH. The suspension was centrifuged at about 1200 × *g* for 1 min to remove cell debris, then centrifuged again at 3000 × *g* for 4 min. The washed chloroplasts were finally taken up in a small amount of the same sucrose–tricine–$MgCl_2$ medium. (Chloroplasts used in *p*-chloromercuribenzoate (PCMB) experiments were prepared and tested in *N*-Tris-(hydroxymethyl)methyltaurine (TES) instead of tricine since tricine complexes mercuric ions very strongly.) All operations were conducted in a cold room at or as near to 0° as possible. The stock suspension of chloroplasts thus prepared was very stable and could be stored at 0° for more than 12 h without serious loss of activity.

In one experiment (see Table I), chloroplasts of white mustard (*Brassica hirta* Moench) and tobacco (*Nicotiana tobacum* L.) were employed. These were prepared in the same manner except that the leaves were ground for 20 sec.

Chemicals

Phlorizin was recrystallized from aqueous ethanol after charcoal (Norit) treatment in order to remove impurities which reduced a significant amount of ferricyanide and had weak uncoupling effects. The purified phlorizin (0.01 M) was dissolved in tricine buffer (0.02 M, pH 8.0) immediately before use. Ethanolic solutions of phlorizin were avoided since the combination of phlorizin and traces of ethanol often produced a slight uncoupling or an unspecific inhibition of electron transport. 3(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) and carbonylcyanide 3-chlorophenylhydrazone (CCCP) were dissolved at appropriate concentrations in 50 % aqueous ethylene glycol.

TABLE I

RATES OF FERRICYANIDE REDUCTION AND ATP FORMATION AS AFFECTED BY LIGHT INTENSITY, AMINES AND CHLOROPLAST SOURCE

The rates are given in μmoles/h per mg chlorophyll. Observed values of P/e_2 represent the average efficiencies of the phosphorylating and non-phosphorylating transport systems. The computed value of P/e_2 is the efficiency of the phosphorylating part of the transport, calculated by assuming the rate of non-phosphorylating transport to be the same in the presence and absence of phosphorylation ($+P_i$ and $-P_i$). As explained in the text this assumption is not valid at low light intensities or in the presence of amines. Control conditions as described in MATERIALS AND METHODS.

Chloroplast source	*Reaction conditions*	*Ferricyanide reduction*			*ATP formation*	P/e_2	
		$+P_i$	$-P_i$	$(+P_i/-P_i)$		*Observed*	*Calculate*
Spinach	Control	403	175	(2.30)	221	1.10	1.95
Spinach	+0.5 mM methylamine	504	418	(1.29)	200	0.74	3.27
Spinach	Low light*	172	140	(1.34)	81	0.94	5.05
Mustard	Control	564	242	(2.33)	310	1.10	1.92
Mustard	Assayed in 0.05 M Tris–HCl (pH 8.2)	545	340	(1.60)	264	0.97	2.58
Mustard	Aged and assayed in 0.05 M Tris–HCl**	215	168	(1.28)	75	0.70	3.20
Tobacco	Control	468	210	(2.23)	264	1.13	2.05

* Light intensity approx. 14 kergs/cm² per sec.
** Chloroplasts aged in Tris buffer (pH 7.4) for 5 h at 0°. Assayed at pH 8.2.

Antimycin A was dissolved in a methanol–ethylene glycol mixture (2:3, v/v). In all cases the final concentrations of the organic solvents were kept as low as possible.

Measurements

The standard reaction mixture (2.0 ml) contained, in μmoles: sucrose, 300; tricine (pH 8.4) (or TES, pH 8.2, for PCMB tests), 80; $Na_2H^{32}PO_4$, 20 (if used); ADP, 2; $MgCl_2$, 8; potassium ferricyanide, 0.8; and chloroplasts containing 40 μg chlorophyll. Reactions were carried out at 19° in a standard rectangular 1.0-cm cuvette illuminated with red light (approx. 600–700 mμ). Preincubation for temperature equilibration was for 3 min unless otherwise noted. The actinic light was obtained by passing the beam of a 500 W (incandescent) projector lamp through a dilute $CuSO_4$ solution and a pair of Corning glass filters (Nos. 2424 and 4600). The intensity of the actinic beam was about 570 kergs/cm^2 per sec. This intensity was enough to saturate the ferricyanide reducing system. Lower light intensities were obtained by placing calibrated screens in the light beam. Ferricyanide reduction was continuously monitored by recording changes in the $A_{420\,m\mu}$ of the reaction mixture. Scattered red actinic light was kept from entering the spectrophotometric device by inserting appropriate blue filters before the photomultiplier tube. In all instances the total change in $A_{420\,m\mu}$ agreed very closely with the amount of ferricyanide present at the beginning of the period of illumination. Therefore it seems safe to equate absorbance change to ferricyanide reduction. ATP was measured as residual radioactivity after extraction of the remaining orthophosphate as phosphomolybdate by the method of AVRON[5], either as soon as all of the ferricyanide had been reduced (3–5 min) or when the reaction was very slow, after a known portion of the ferricyanide had been reduced*. There were negligible amounts of radioactivity in the dark controls and in DCMU-treated, illuminated controls.

RESULTS

Conditions under which the number of moles of ATP formed is equal to the number of additional moles of ferricyanide reduced during phosphorylation (Figs. 1–6)

A consistent correspondence between the rate of ATP formation and additional electron transport associated with phosphorylation strongly implies that the phosphorylating electron transport is superimposed on a constant non-phosphorylating electron transport. This, in turn, implies that the two processes are parallel but do not compete. Such absence of competition is to be expected if the overall rate of transport is determined by the combined capacities of the two processes to carry electrons. The initial carrier enzymes of both systems should then be saturated and therefore the two processes should be additive. Figs. 1–6 illustrate a series of experiments which seem to satisfy these conditions. The potential activity of the chloroplasts was such

* This technique does not actually measure the amount of ATP produced but rather the amount of orthophosphate converted into other forms during illumination. It therefore measures electron transport-dependent phosphorylation but not necessarily the phosphorylation of ADP. However, the phosphorylation reactions described herein were completely dependent on the addition of ADP and, on the basis of chromatography of the reaction mixture, yielded ATP as the preponderant radioactive product. We cannot yet preclude the possibility that a very small proportion of the radioactivity of the orthophosphate was incorporated into ADP instead of ATP as claimed by DEL CAMPO, RAMIREZ AND ARNON[6].

that the rate of electron transport increased several-fold when uncouplers were added. Therefore the rate of electron transport must indeed have been controlled at the level of the phosphorylation reaction.

Phlorizin inhibits both ATP synthesis and that part of the electron transport which is induced by the addition of ADP and phosphate, without in any way affecting the rate of electron transport in the absence of phosphate[7,8,12]. Regardless of the concentration of phlorizin and the rate of phosphorylation, 1 molecule of ATP is formed for every extra ferricyanide ion reduced in response to the addition of phosphate (see Fig. 1). We may also state this relationship in another way which begs the question of the activity of the non-phosphorylating pathway of electron transport during phosphorylation: for every ferricyanide ion which is not reduced because of phlorizin, a molecule of ATP is not formed.

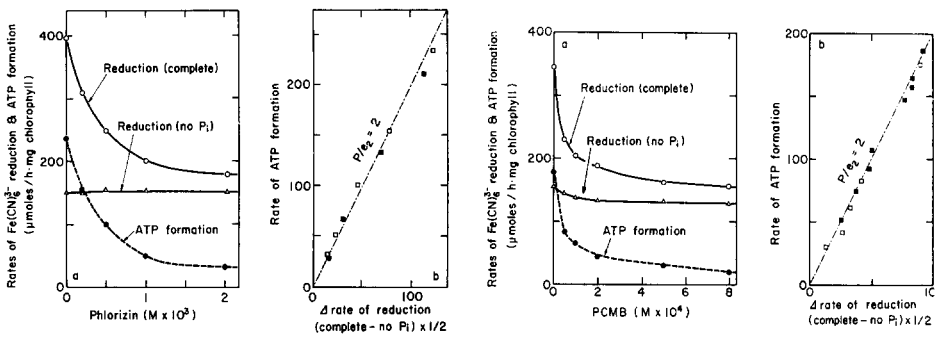

Fig. 1. Effects of phlorizin on electron transport and phosphorylation in spinach chloroplasts. For details of reaction conditions see MATERIALS AND METHODS. Phosphorylating electron transport (complete) was measured in the presence of ADP, phosphate (P_i) and Mg^{2+}. Non-phosphorylating electron transport (no P_i) was measured in the same system lacking only phosphate. b. □, values computed from the data of (a); ■, values computed from a duplicate experiment. The dotted line is not drawn through the points but rather represents the location of the points predicted by assuming that each additional pair of electrons carried during phosphorylation results in the formation of 2 molecules of ATP.

Fig. 2. Effects of PCMB on electron transport and phosphorylation. Data are presented as in Fig. 1.

PCMB is known to inhibit photophosphorylation[9] and it has been suggested that the phosphorylation reaction is more sensitive to this sulphydryl reagent than is (uncoupled) electron transport[10]. We find that PCMB at low concentration behaves much as does phlorizin: it inhibits photophosphorylation and the phosphorylation-associated part of the electron transport (Fig. 2). And, as with phlorizin, 1 ATP molecule is always found for every additional electron transferred to ferricyanide when the rate of phosphorylation is varied with PCMB.

As was pointed out in a earlier publication[11], phosphorylation may be reduced to any desired extent by the use of sub-optimal concentrations of phosphate, and the varying amounts of ATP formed are then equal to the varying amounts of extra ferricyanide reduced as the result of the addition of the phosphate. Fig. 3 confirms the earlier observation and extends it by showing that the same relationship is observed when phosphorylation rates are controlled by combinations of phlorizin and limited phosphate.

Antimycin A can also inhibit photophosphorylation and the phosphorylation-

associated increase in electron transport without inhibiting the non-phosphorylating transport (see Fig. 4). (Antimycin A also has some uncoupling action since it causes a slight increase in the rate of electron transport in the absence of phosphate.) However, antimycin differs from phlorizin in that it also inhibits amine-uncoupled electron transport[12]. Again at all levels of inhibition the amount of ATP formed is equal to the extra ferricyanide reduced during phosphorylation.

Carbonylcyanide phenylhydrazones prevent ATP synthesis in a different way, neither inhibiting phosphorylation directly nor inhibiting phosphorylation-coupled electron transport. Rather they seem to open an alternative, non-phosphorylating

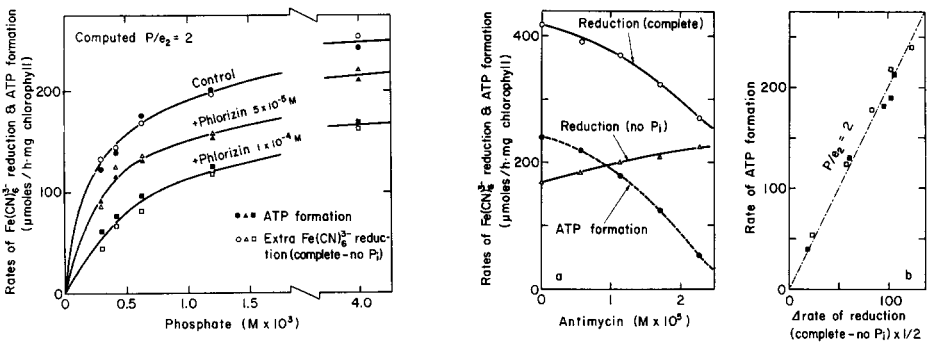

Fig. 3. Effects of phosphate concentration on electron transport and phosphorylation in the presence and absence of phlorizin. Methods as in Fig. 1. Under all of these conditions and at all levels of activity of the phosphorylation system, the number of molecules of ATP formed closely approximated the number of additional ferricyanide ions reduced during phosphorylation ($P/e_2 = 2.0$).

Fig. 4. Effects of antimycin A on electron transport and phosphorylation. Conditions were as in Fig. 1 except that the chloroplasts were preincubated with the inhibitor for 5 min. Data are presented as in Fig. 1.

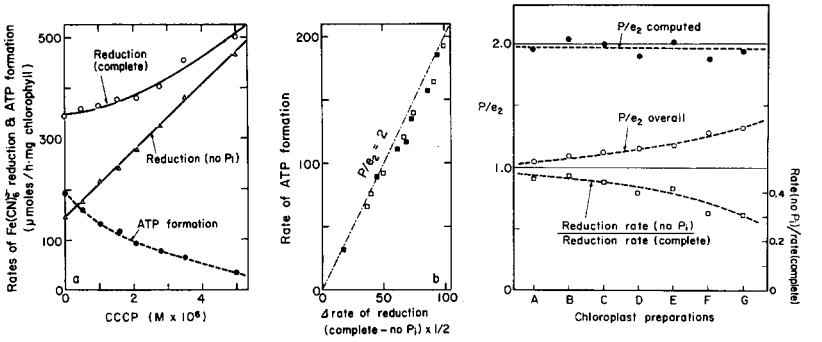

Fig. 5. Effects of CCCP on electron transport and phosphorylation. Data are presented as in Fig. 1.

Fig. 6. Variations among chloroplast preparations in overall efficiency of phosphorylation (P/e_2 overall), the ratio of non-phosphorylating (no P_i) electron transport to total transport during phosphorylation (complete), and the computed efficiency of the phosphorylating part of the electron transport (P/e_2 computed). Computation is based on the assumption that the non-phosphorylating electron transport observed in the absence of phosphate continues at the same rate during phosphorylation. Values are plotted in order of increasing overall efficiencies. Despite considerable differences in overall P/e_2, the computed efficiency of the phosphorylating component of the electron transport is almost constant at a value of P/e_2 close to 2.0.

pathway for electron transport. The induced by-pass of the phosphorylation site resembles the endogenous "basal" electron transport process in being relatively insensitive to antimycin A (ref. 12). Once again the amount of ATP formed is equal on a molar basis to the extra ferricyanide reduced during phosphorylation, no matter how severely the phosphorylation is curtailed by CCCP (Fig. 5). Details of the differential effects of antimycin A on amine- and CCCP-uncoupled electron transport will be described elsewhere.

The amount of inadvertent uncoupling probably depends on the method of preparation of the chloroplasts and on the condition of the leaves from which the chloroplasts have been prepared. For historical reasons the resulting non-phosphorylating electron transport is referred to as the "basal" transport—perhaps a happy choice of terms since it does seem that this process forms a base on which phosphorylating electron transport is superimposed. In any event, the degree of uncoupling and hence the "basal" non-phosphorylating electron transport varies considerably from one preparation to another (see Fig. 6). As a result the average efficiency of phosphorylation (the observed or overall P/e_2) also varies considerably. However, in every instance the amount of phosphorylation corresponds to the extra electron transport which occurs during phosphorylation. The ratio of ATP molecules to extra electrons transported is close to 1.0 (or $P/e_2 = 2.0$) in each preparation.

Conditions under which the number of moles of ATP formed is greater than the additional number of moles of ferricyanide reduced during phosphorylation (Figs. 7–9)

Ratios of P/e_2 "corrected" in the manner described above for non-phosphorylating electron transport are not always consistent and are sometimes unacceptably high. Indeed, as STILLER AND VENNESLAND[13] and PUNNETT[14] have pointed out, values thus computed can be infinite! Consequently it is incumbent on us to explain why the computation is sometimes invalid if we are to assert that it is sometimes valid. As we have already suggested, non-cyclic electron transport in active chloroplasts at high light intensities must be controlled at the level of the phosphorylation reaction since uncouplers of phosphorylation can produce dramatic increases in electron transport rates. It is probable that under such conditions the carriers supplying the phosphorylating and non-phosphorylating pathways are both saturated by a more than adequate supply of photochemically produced electrons, and therefore one would not predict any interaction between the two processes. However, the situation is completely changed if the supply of electrons becomes for any reason a rate-determining factor. Low light intensities, DCMU-poisoning, and the ageing of chloroplasts under unfavorable conditions all inhibit electron transport at or near the level of the photochemical reactions. The phosphorylating and non-phosphorylating processes are then forced to compete for the limited supply of electrons and, under these conditions, the introduction of the phosphorylating process does decrease the activity of the non-phosphorylating process. This is particularly obvious in the limiting case where light intensities are so low or other conditions are so unfavorable that the rate of electron transport is not at all enhanced by situations which permit phosphorylation. Since phosphorylation at such low light intensities may still proceed with reasonable overall efficiency it is clear that the phosphorylation-coupled process must occur at the expense of the non-phosphorylating process which is correspondingly diminished. Computations based on a supposedly constant rate of non-phospho-

rylating electron transport are patently invalid if the rate-determining process is not associated with phosphorylation.

Fig. 7 shows the effects of various light intensities on rates of phosphorylation, and on rates of ferricyanide reduction in the presence and absence of phosphorylation. (Our results differ from those of other workers[15–17] in that we find quite high overall phosphorylation efficiencies even at the lowest intensities. Possibly the mild uncoupling effect of the Tris buffer employed by others becomes more significant as electron transport rates are drastically reduced.) Clearly the non-phosphorylating component of the electron transport is not constant under these conditions and therefore it is not possible to estimate the activity of the phosphorylating part of the electron transport except in terms of ATP formed.

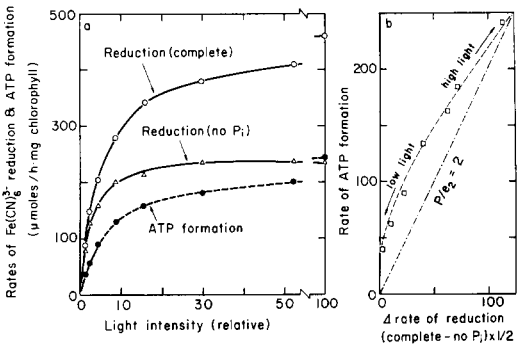

Fig. 7. Effects of different light intensities on electron transport and phosphorylation. The intensity at relative value 100 was approx. 570 kergs/cm² per sec (600–700 mμ). Other conditions as in Fig. 1. At low light intensities the overall efficiency of phosphorylation is almost as high as at high light intensities, yet there is little or no increase in the rate of electron transport on the addition of phosphorylation substrates. Consequently phosphorylating electron transport must occur at the expense of the non-phosphorylating process which is correspondingly decreased. Thus "correcting" by subtracting the basal electron transport as in (b) is not a valid procedure and yields meaningless high values for the computed P/e_2.

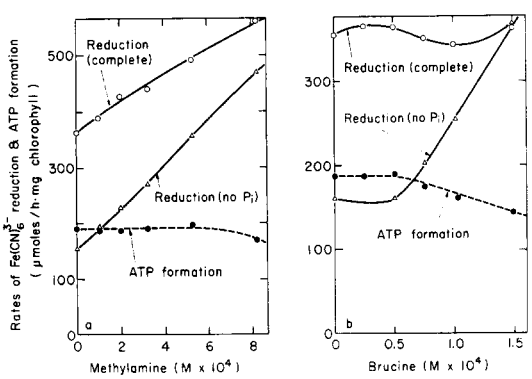

Fig. 8. Effects of methylamine hydrochloride (a) and brucine (b) on electron transport and phosphorylation. Conditions as in Fig. 1. At the relatively low concentrations employed in the experiment, these amine uncouplers do not seriously inhibit phosphorylation. Nevertheless they do greatly enhance the non-phosphorylating (no P_i) electron transport. Thus the amine-uncoupled component of the non-phosphorylating electron transport seems to compete very unfavorably with the phosphorylating electron transport. It is therefore not surprising that the number of extra electrons transported during phosphorylation falls far short of the number of molecules of ATP formed.

Since adding DCMU to chloroplast suspensions is tantamount in terms of electron transport to reducing the light intensity, it is not surprising that computations based on the erroneous assumption of a constant rate of non-phosphorylating transport are also meaningless when phosphorylation is limited by this inhibitor[6].

Uncoupling by amines also renders invalid our procedure of subtracting a supposedly constant non-phosphorylating electron transport. Fig. 8 shows the effects of methylamine and the new uncoupler brucine, a complex alicylic amine, on electron transport and phosphorylation. In this case the interaction between the phosphorylating and non-phosphorylating processes is more complicated. Since low concentrations of amines enhance the rate of non-phosphorylating electron transport (rate without phosphate) but do not greatly decrease phosphorylation, it may be deduced that amine-uncoupled electron transport competes very poorly with phosphorylating electron transport. (In contrast see CCCP-uncoupling, Fig. 5.) When we consider, further, that the site of amine-uncoupling lies on the same antimycin-sensitive pathway as phosphorylation[12], we can readily imagine that amine-uncoupled and phosphorylating electron transport share a common rate-limiting step which is not involved in the "basal" or CCCP-uncoupled transport. For some such reason—a shared bottleneck and competition which favors the phosphorylation process—it seems that electron transport by the amine-uncoupled pathway is much slower when phosphorylation occurs. This partial suppression of amine-induced electron transport during phosphorylation is shown very clearly by a corresponding suppression of the large chloroplast volume changes which are always associated with amine-uncoupled electron transport (S. IZAWA, unpublished observations). Computations of the kind which yielded consistent values for the efficiency of phosphorylating electron transport in Figs. 1–6 therefore cannot be used when amines are present since the non-phosphorylating component of the electron transport is conspicuously not constant. This applies even to Tris buffer, which is a highly polar amine with a weak uncoupling action[18]. Chloroplasts aged and assayed in Tris buffer probably cannot be used for computation of the "corrected" P/e_2 for two reasons: Tris-induced uncoupling and Tris-induced inhibition of electron transport[19] may both invalidate the procedure (see Table I).

DISCUSSION

Fig. 9 summarizes our hypotheses regarding the various pathways for electron transport. Chloroplasts as prepared by current methods always exhibit a "basal" electron transport. This antimycin-resistant, non-phosphorylating transport represents a variable proportion of the total transport capacity. Under optimal conditions (active chloroplasts, high light intensities and no uncoupling amines) ATP formation is closely proportional to the difference between the capacity of the basal transport system, as determined in the absence of phosphate, and the total transport capacity as determined in the presence of phosphate. This relationship is most readily interpreted in terms of a continuing non-phosphorylating electron transport on which the phosphorylating transport is superimposed. In other words the two processes do not compete under the optimal conditions enumerated. Such absence of competition could arise in two ways: saturation of potentially competing enzymes, or a physical separation of the sites of the two processes which precluded competition. However,

examination of the behavior of chloroplasts at low light intensities or in the presence of DCMU shows that competition for the same electrons is indeed possible. Therefore the concept of separate and effectively isolated phosphorylating and non-phosphorylating sites must be abandoned.

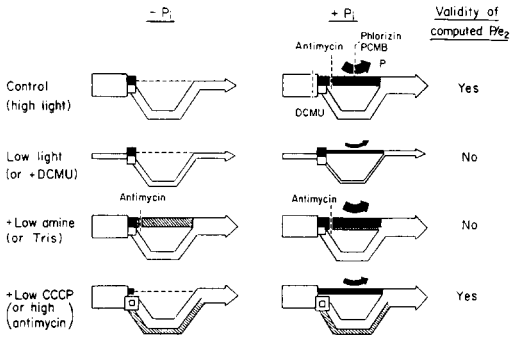

Fig. 9. Diagrammatic representation of the various electron transport processes discussed. Width of arrows is intended to represent electron transport rates. Solid squares represent electron-accepting sites associated with phosphorylating transport while open squares represent similar (damaged?) sites associated with non-phosphorylating transport. At high light intensities both types of sites are saturated with the result that the phosphorylating and non-phosphorylating processes are additive. At low light intensities the supply of electrons becomes limiting, the two types of sites are forced to compete, and the processes are no longer additive. Amines uncouple the antimycin A sensitive phosphorylating pathway but the amine-uncoupled transport competes unfavorably with the phosphorylating process and may be largely suppressed during phosphorylation. Hence the non-phosphorylating and phosphorylating electron transport are not additive when amines are present. In contrast, the uncoupler CCCP seems to convert phosphorylating sites into non-phosphorylating sites, but both types of sites remain saturated at high light intensities; hence the phosphorylating and non-phosphorylating processes are once more additive.

Naturally we cannot expect to estimate the activity of the phosphorylating pathways on the basis of a constant non-phosphorylating transport when the two processes are quite evidently competing; we should not expect to and do not find ATP formation proportional to the additional electron transport induced by phosphorylation conditions when the electron transport rate is controlled at some site other than the phosphorylation site. This situation typically arises when sub-optimal light intensities are used and when the chloroplasts have been badly treated in various ways. Another typical but often overlooked instance sometimes occurs when $NADP^+$ is employed as electron acceptor instead of ferricyanide or flavins or viologens; the much lower rate of electron transport and the poor stimulation of electron transport by uncouplers or ADP and phosphate point to the fact that the terminal steps in $NADP^+$ reduction often limit the overall process.

Our tentative conclusion regarding the maximum efficiency of photophosphorylation is based on two independent considerations: (1) Most models of phosphorylating mechanisms predict a stoichiometry which is represented by a whole number and it is unlikely that the overall value of P/e_2 in photophosphorylation is as low as 1.0. And (2) over a wide range of conditions 2 ATP molecules are formed for each additional pair of electrons transported during phosphorylation. It would be well to consider each of these points in some detail.

(1) Our first point has been challenged by DEL CAMPO, RAMIREZ AND ARNON[6]

on the ground that 5 % or more of the radioactivity assumed to be in ATP may be associated with ADP. However, we would like to point out that almost 30 % of the radioactivity would have to be in ADP to explain our data[4] if the true value of P/e_2 is 1.0. In view of the virtual absence of ADP–P_i exchange reactions in chloroplasts reported by others[20] this seems exceedingly unlikely. Moreover, if ADP does become labeled to a significant extent, the incorporation of orthophosphate therein is totally dependent on electron transport and thus represents but another (and very minor) electron transport-coupled phosphorylation. However, there is a much more serious objection to all computations of the stoichiometry of photophosphorylation. This resides in the difficulty of being sure that all of the electron transport has been measured, that no hidden "cyclic" or "pseudocyclic" electron transport has occurred. Indeed, it may well be that the existence of a considerable amount of unrecognized electron transport was responsible for the high P/e_2 ratios observed by LYNN AND BROWN[21]. Nevertheless, it should be stressed at this point that the phosphorylation reactions we have discussed in this paper and in ref. 4 were ferricyanide-dependent. That is to say, one can subtract the endogenous cyclic or pseudocyclic phosphorylation which occurs in the absence of ferricyanide (5–7 % of the ferricyanide rate) and still measure ratios of P/e_2 which are consistently in excess of 1.0. Therefore, if one is to invoke cyclic phosphorylation to explain the excess over 1.0 one must postulate a ferricyanide-dependent cyclic phosphorylation. In view of the fact that all known types of cyclic and pseudocyclic phosphorylation seem to be inhibited by substrate level concentrations of ferricyanide[4,22,23], this seems improbable.

(2) It is difficult to explain the linear relation between phosphorylation and the phosphorylation-associated increase in electron transport except in terms of additive phosphorylating and non-phosphorylating electron transport processes. If the correspondence between phosphorylation and increased electron transport had been observed over a narrow range of phosphorylation rates, or if it had only been encountered when the phosphorylation was varied in a single way, one might reasonably have attributed the correspondence to a fortuitous balance of several factors. In fact the correspondence is observed over a wide range of phosphorylation rates when the various rates have been obtained in a wide variety of demonstrably different ways. Consequently it is difficult to plead happenstance. On the other hand, if we accept the fact that the non-phosphorylating and phosphorylating processes are additive, it follows that the non-phosphorylating electron transport observed in the absence of phosphate may be legitimately subtracted from the total electron transport in order to compute the activity of the phosphorylation process. And if one accepts the validity of such operations it follows that the phosphorylating efficiency of the phosphorylating part of the electron transport must be 2 ATP molecules formed for every electron pair transported.

ACKNOWLEDGEMENTS

This work was supported in part by Grant (GB 4568) from the National Science Foundation of the U.S. and in part by a grant (A-3743) from the National Research Council of Canada and is published as Journal Article 4373 of the Michigan Agricultural Experiment Station.

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