The Stoichiometry of Photophosphorylation[1]

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Early investigations of non-cyclic photophosphorylation in chloroplasts indicated that one ATP molecule was formed for each pair of electrons transferred to NADP or ferricyanide (Arnon et al., 1958; Krogmann et al., 1959). Subsequent studies (Good, 1960; Stiller and Vennesland, 1962) have tended to confirm these observations and it is now widely believed that the theoretical limit of the P/2e ratio is 1.0 ---- presumably one phosphorylation reaction coupled to one oxido-reduction reaction involving a pair of electrons. However, during recent years much has been learned about factors which uncouple electron transport from phosphorylation and, on the basis of this newer knowledge, we suspected that reported measurements of the stoichiometry of phosphorylation had not been made under the best conditions. Our current investigations have shown that the previously reported pH optimum of 8.0 and P/2e ratio of 1.0 are only found when Tris-HCl is used as buffer. With other buffers, pH optima between 8.6 and 8.9 and P/2e ratios greater than 1.0 are regularly observed.

Chloroplasts were prepared from fresh market spinach (*Spinacia oleracea*), from garden-grown peas (*Pisum sativum*) and from wild pokeweed (*Phytolacca americana*). The chloroplasts were isolated by two different procedures. For most experiments the leaves were ground for 5 seconds in a Waring Blendor in a medium consisting of 0.3 M NaCl, 1.0 mM $MgCl_2$, 1.0 mM EDTA and

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0.04 M tris(hydroxymethyl)methylaminoethanesulfonic acid buffer (TES; Good et al., in press) adjusted to pH 7.3 with NaOH. The chloroplast pellet (2000 x g for 5 min.) was washed twice in a medium consisting of 0.2 M sucrose, 0.01 M NaCl, 0.5 mM $MgCl_2$ and 0.02 M tris(hydroxymethyl)methylglycine buffer (Tricine; Good, 1962) adjusted to pH 7.3 with NaOH and suspended in the same medium. For some experiments chloroplasts were isolated and suspended in a more conventional medium consisting of 0.4 M sucrose, 0.01 M NaCl and 0.05 M tris(hydroxymethyl)aminomethane buffer (Tris) adjusted to pH 7.8 with HCl.

Ferricyanide reduction was measured spectrophotometrically by the method of Izawa and Good (1965). The measured ferricyanide additions were checked both by noting the absorbance of the reaction mixture at 420 mμ and by determination of the change in absorbance when the ferricyanide had been reduced. ATP formation was determined by trapping incorporated $^{32}PO_4$ in glucose-6-phosphate, extracting the excess orthophosphate as phosphomolybdic acid, and measuring the residual radioactivity of the aqueous phase (Avron, 1960) in a GM immersion tube. The precision of the procedures was such that replicated estimates of the P/2e ratio never differed by more than 4%.

The results are presented in Figures 1 and 2 and in Table I. When the chloroplasts were prepared and the reaction was carried out in media containing Tris-HCl, the data we obtained agreed with the reports of other workers. The rates of electron transport and phosphorylation were highest at pH 8.0 and decreased rapidly on either side of this optimum. The P/2e ratio was also highest (0.95 to 1.03) at this pH and declined precipitously as the pH was raised. However these responses to pH are not a true expression of the effect of hydrogen ion concentration on the phosphorylation mechanism. Four other buffers (Tricine, glycylglycine, glycine and carbonate-bicarbonate) gave entirely different results. With Tricine and glycylglycine the maximum rates of electron transport and phosphorylation were obtained near pH 8.6 and the highest P/2e ratios (1.20 to 1.30) were obtained close to pH 8.9.

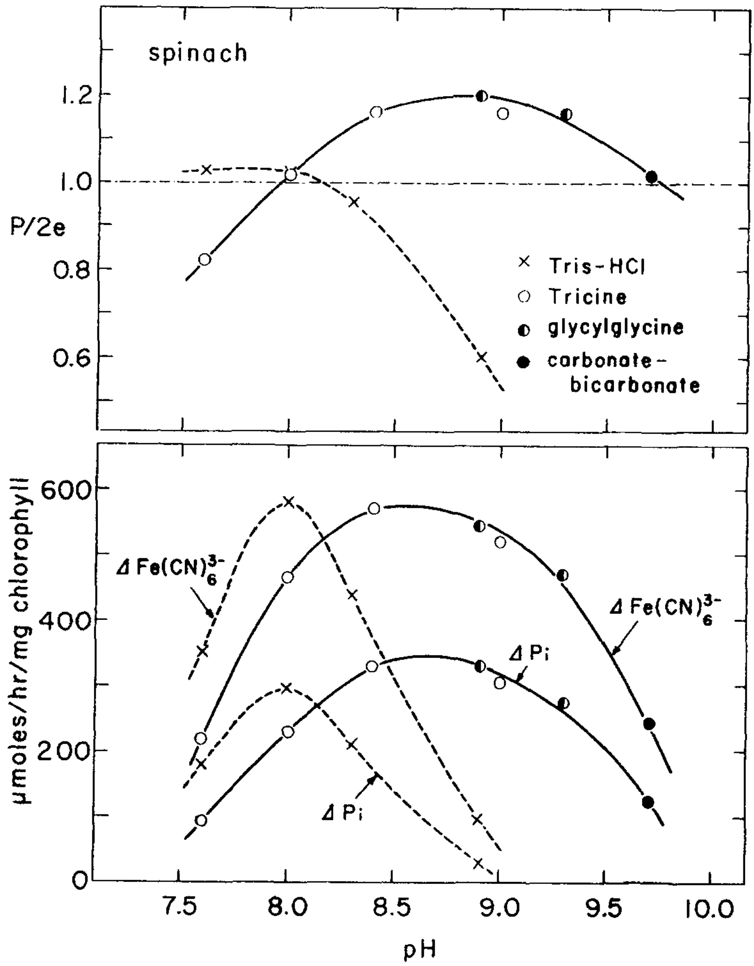

Fig. 1 A comparison of Tris-HCl with three other buffers in the phosphorylation-coupled reduction of ferricyanide by spinach chloroplasts. The 2.0 ml reaction mixture contained in µmoles, $K_3Fe(CN)_6$ 0.8, ATP 1.0, $Na_2HP^*O_4$ 30 (about 10^6 c/m), $MgCl_2$ 2, glucose 20, sucrose 300, and the indicated buffer 100 (adjusted to give pH shown); hexokinase 1 mg and chloroplasts (40 µg chlorophyll). Temperature 19°. Saturating actinic light >560 mµ. Immediately after reduction of the ferricyanide was complete, the reaction mixture was analyzed for glucose-6-phosphate by the method described in the text.

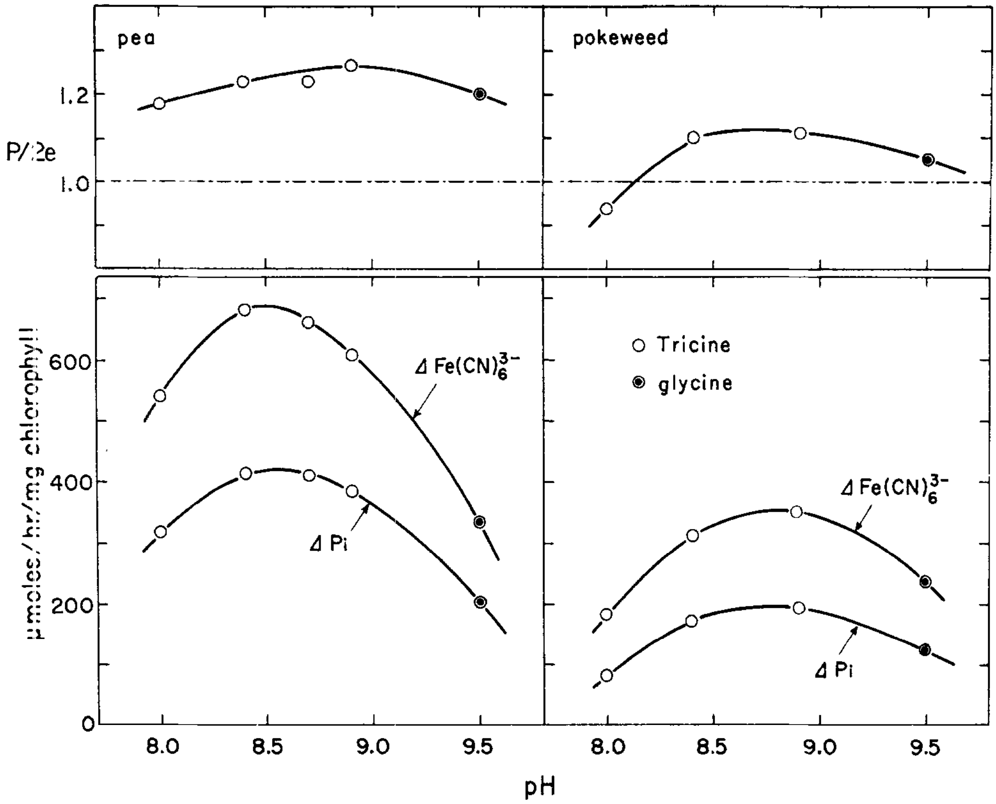

Fig. 2 Phosphorylation and electron transport in pea and pokeweed chloroplasts. For reaction conditions, see fig. 1.

In these experiments dark controls and illuminated controls containing 5×10^{-6} M DCMU showed no phosphate incorporation. For instance in one experiment the background was 16 c/m, the dark control was 25 c/m, the DCMU control was 30 c/m, and phosphorylation gave 6510 c/m, while total counts per minute before orthophosphate extraction would have been 379,000. On the other hand chloroplasts illuminated in the absence of ferricyanide or other exogenous electron acceptors did produce ATP at significant rates -- 7 to 8% of the rate with ferricyanide. This endogenous phosphorylation is associated with electron transport via a slow Mehler reaction (Good and Hill, 1955) and probably does not occur in the presence of ferricyanide, since ferricyanide seems to completely suppress the known types of cyclic and pseudocyclic

Table I. Rates of Ferricyanide Reduction and ATP Formation with Chloroplasts from Various Plants in Various Buffers. The chloroplasts illuminated in Tris-HCl were also isolated in this buffer. The chloroplasts illuminated in Tricine and glycylglycine were isolated in TES and suspended in Tricine as described in the text. Reaction conditions were as described for Fig. 1.

Exp. No.	Material plant	Reaction medium buffer	pH	Rates (µmoles/hr/mg chlorophyll) $K_3Fe(CN)_6$ reduction	ATP formation	P/2e
1	pokeweed	Tricine	8.9	350	194	1.11*
2	spinach	Tricine	8.4	438	252	1.15**
3	spinach	Tricine	8.9	526	334	1.27
4	spinach	glycylglycine	8.9	545	327	1.20
5	pea	Tricine	8.9	607	385	1.27
6	pea	Tricine	8.4	742	475	1.28
7	pea	Tricine	8.4	836	540	1.29
8	pea	Tricine	8.7	884	574	1.30
9	spinach	Tris-HCl	8.0	432	205	0.95
		Tris-HCl	8.3	243	62	0.73
		Tris-HCl	8.7	110	22	0.40
10	spinach	Tris-HCl	8.0	582	300	1.03
		Tris-HCl	8.3	456	218	0.96
		Tris-HCl	8.9	95	29	0.60

* Plants conspicuously injured by frost.
** Leaves stored for 7 days at 3-4°.

phosphorylation. In our experiments neither PMS- nor FMN-catalyzed phosphorylation occurred when ferricyanide was present.

Summary

Using chloroplasts from three different plant species in the presence of four different buffer systems we found that the P/2e ratio was consistently above 1.0 if the pH was between 8.4 and 9.4. At pH 8.9 the ratio was usually above 1.25 and indeed no single determination of the ratio fell below 1.1 at this pH unless Tris-HCl was present. Even if the endogenous phosphorylation were subtracted (a procedure which is probably not valid) no single determination would yield a value of P/2e as low as 1.0. Consequently it seems very probable that the theoretical maximum efficiency of photophosphorylation is higher than has been thought.

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