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REDUCTION OF FERRICYANIDE BY CHLOROPLASTS IN THE PRESENCE OF NITROGENOUS BASES

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SUMMARY

1. A variety of nitrogenous bases, in addition to amines, can uncouple the reduction of ferricyanide by illuminated chloroplasts.

2. Changes in pH optimum on uncoupling ferricyanide reduction may account for variability in the observed enhancement of electron flow.

3. The efficiency of uncoupling is a function of the ionization constant of the base used.

4. Activity is limited to the unionized molecular species. There is evidence of uncoupling by the fully undissociated form of compounds having two basic functions.

5. There are certain features of resemblance between the effects of uncoupling and inhibitory concentration ranges of bases on ferricyanide reduction rates.

INTRODUCTION

An essential difference between the process of photosynthetic phosphorylation in isolated chloroplasts and oxidative phosphorylation in mitochondria can be demonstrated by the use of inhibitors. 2,4-Dinitrophenol cannot uncouple photosynthetic phosphorylation in isolated chloroplasts[1]. On the other hand KROGMANN *et al.*[2] were able to show in illuminated chloroplasts an inhibition of ATP formation, and a concomitant rise in the rate of ferricyanide reduction on addition of NH_4^+.

At the same time GOOD[3] showed an enhancement of the rate of ferricyanide reduction and of O_2 evolution by chloroplasts in the light upon addition of unidentified naturally occurring bases or of a whole series of monofunctional amines[3]. Estimates of ATP formation confirmed that uncoupling had occurred. However some monofunctional amines *e.g.* aniline proved to be ineffective.

The concentration of NH_3 required to give almost complete uncoupling is somewhat variable and at higher concentrations inhibition of ferricyanide reduction occurs[2]. VERNON AND ZAUGG[4] were able to show that this second effect is due to inhibition of electron flow close to the site of O_2 evolution.

The present work re-examines the uncoupling action of nitrogenous bases and the relationship, if any, which exists between this phenomenon and the ability to inhibit O_2 evolution.

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METHODS

Spinach beet, a cultivar of *Beta vulgaris*, was grown under glass at approx. 18° but without controlling other factors. Leaves were gathered just before use and illuminated under cold water for 1 h at 10–15 klux. The leaf laminae were then ground in a mortar at 0° with "grinding medium": 0.35 M NaCl, 0.03 M $MgCl_2$, 0.01 M ascorbate, and 0.10 M Tris–chloride buffer (pH 7.4, 0°). A neutralized 0.1 M ascorbate solution was prepared by dissolving 0.35 g ascorbic acid in 20 ml water at 0°, and adding 0.26 g Tris.

The chloroplast pellet obtained by centrifugation (1500 × *g*, 7 min) of the filtered leaf macerate was washed once in the above grinding medium but without ascorbate. The final pellet was suspended in the washing solution diluted 10 times. This resulted in fragmentation of the chloroplasts.

The reaction mixture used to study ferricyanide reduction was: 3.0 μmoles potassium ferricyanide, 60 μmoles Tris–chloride buffer (pH 8.0), 50 μg chlorophyll, plus water or additive to a total volume of 1.5 ml. Phosphorylating reagents when added were 6.0 μmoles ADP, 2.5 μmoles ^{32}P-labelled sodium phosphate, 5.0 μmoles $MgCl_2$.

Reaction mixtures in 15-ml centrifuge tubes were illuminated in an apparatus previously described by WALKER[5]. A temperature of 5.0° ± 0.2° was maintained by circulation through a cooling coil of aqueous ethanol at —9.0°. The wall of the glass trough was encased in aluminium foil and a removable red perspex disc covered the base. The tungsten lamps situated under the tank provided "saturating" red light of wavelengths above 595 mμ. Up to 32 reaction tubes could be clipped to the rotor disc (100 rev./min) and illuminated simultaneously. After switching off the light the reaction tubes were either frozen quickly in the dark, or denatured by addition of 2.0 ml 2 % (v/v) trichloroacetic acid. Unused ferricyanide in the denatured supernatants was estimated by determining the absorbancy at 400 mμ. Labelled ATP was determined by adsorption on activated charcoal.

The organic bases investigated were first converted to their hydrochloride salts and recrystallized.

The amounts of unprotonated base present in reaction mixtures were calculated from the expression

$$[\text{total base}] = [\text{unionized base}]\cdot\left[\frac{K_b\cdot[H^+]}{K_w} + 1\right] \qquad (1)$$

For a weak base with ionization constant $K_b \ll 10^{-8}$, if $[H^+] = 10^{-8}$ then

$$[\text{total base}] \approx [\text{unionized base}] \qquad (2)$$

The dissociation constants for morpholine and β-bromoethylamine were determined by titration.

RESULTS

The action of NH_3

The effect of increasing concentrations of NH_4^+ on the reduction of ferricyanide by illuminated chloroplasts is shown in Table I.

By independent variation of pH and methylamine concentration, GOOD was able to show a relation between enhancement of ferricyanide reduction rate and concentration of unionized (rather than total) amine. We have found this to be the case for almost all nitrogenous bases active as uncoupling agents. Fig. 1 illustrates its application to the cyclic base piperidine, which proved to be an efficient uncoupler. The rates are plotted as a function of the piperidinium ion concentration (in this case almost equal to the total added base), and of the unionized piperidine at the three pH values used. The closest relationship is clearly between the enhancement of reduction rate and the concentration of unionized base.

TABLE I

EFFECT OF AMMONIA CONCENTRATION ON FERRICYANIDE REDUCTION

Basal reaction mixture minus P_i and ADP.

	NH_3 concentration (mM)								
	—	*0.005*	*0.020*	*0.050*	*0.100*	*0.200*	*0.500*	*1.000*	*3.000*
Ferricyanide reduced (µmoles/h)	5.0	5.6	8.1	9.6	11.1	12.1	10.2	8.3	4.7

It seemed probable that the inhibition of ferricyanide reduction by high concentrations of NH_3 might be a further effect of the unionized molecules. The pH was varied from 6.8 to 8.3 at intervals of 0.3 units, and the NH_4^+ concentration varied between 25, 50 and 100 mM. Such conditions gave slight to severe inhibition of the uncoupled reduction rate. From Fig. 2, in which activity is plotted against the logarithm of both the NH_3 and NH_4^+ concentration, it is evident that only in the first case is a normal inhibition curve observed.

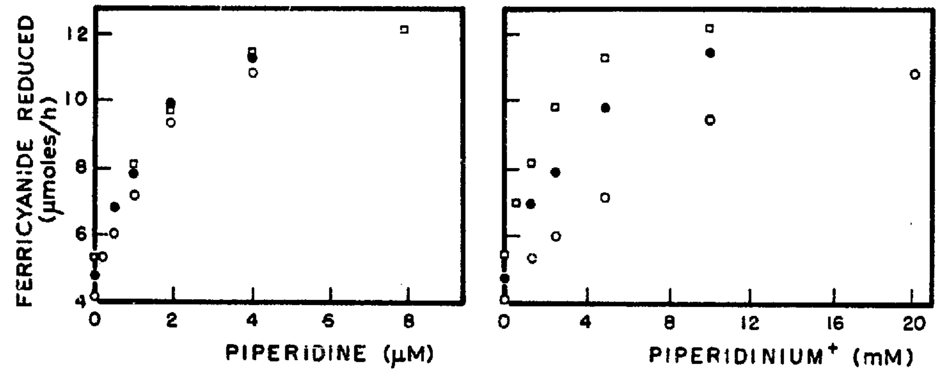

Fig. 1. Uncoupling activity of free piperidine. Basal ferricyanide reduction at three pH values in presence of piperidinium and calculated piperidine. pH 7.4, □; pH 7.7, ●; pH 8.0, ○.

This finding raised the possibility that uncoupling and inhibitory effects might have other features in common. Reversibility of the uncoupling effect by washing had been demonstrated by GOOD. In checking for the reversibility of the inhibition effect, difficulties were encountered since the washing treatment caused much loss of phosphorylating capacity. This could be avoided by the use of whole chloroplasts, prepared by resuspending the once-washed particles in undiluted grinding medium. The whole chloroplasts were then incubated, at equal chlorophyll concentration, in the following media at 0°: A, grinding medium, pH 8.0; B, grinding medium, pH 8.0 + 1.8 mM NH_4Cl; C, grinding medium, pH 8.0 + 75.0 mM NH_4Cl.

The NaCl content of the grinding medium was varied so as to give the same total salt concentration in A, B and C. After 10 min incubation the chloroplasts were centrifuged and resuspended in a large excess of grinding medium, centrifuged again and finally suspended in grinding medium to give 100 μg chlorophyll per 0.1 ml.

TABLE II

EFFECTS OF NH_3 REVERSED BY WASHING

	Column				
	1	*2*	*3*	*4*	*5*
Chloroplast treatment	A	A	A	B	C
Ferricyanide reduced*	8	61	63	61	75
ATP formed*	0	6	16	15	16
Free NH_3 in assay (mM)	4.5	0.1	0.0	0.0	0.0

* μmoles/h/mg chlorophyll. Reaction mixture contained in 1.6 ml (μmoles), ADP, 6.0; P_i, 3.5; $MgCl_2$, 5.0 and Tris buffer (pH 8.0), 40.

Coupled phosphorylation was assayed using the three types of chloroplasts and the results are given in Table II. Columns 1, 2 and 3 show the effect of illuminating A chloroplasts in the presence of the same NH_3 concentrations as were used for the dark pre-incubation. In the presence of 0.1 mM free NH_3 the P:2e ratio is less than the control and indicates uncoupling; with a higher concentration (4.5 mM free NH_3) inhibition of electron flow was confirmed. Chloroplast treatments B and C led to phosphorylation rates the same as the control (Column 3) indicating full reversibility of both base effects. Ability to reduce ferricyanide was likewise close to the control rate, though a significant and reproducible enhancement was observed with C chloro-

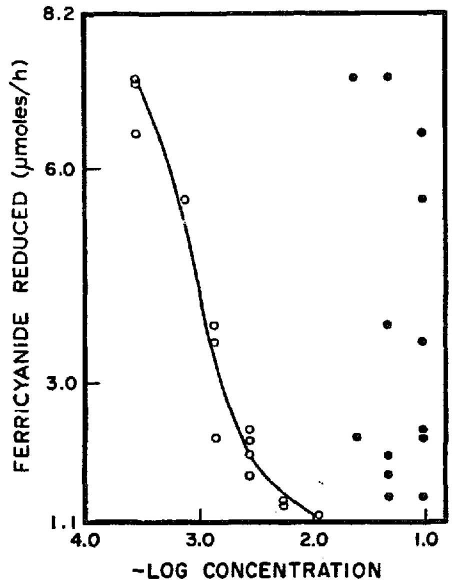

Fig. 2. Inhibition by high free NH_3 concentration. Basal ferricyanide reduction plus NH_4Cl at pH 6.8, 7.1, 7.4, 7.7, 8.0, 8.3. Free and protonated base calculated from Eqn. 1. Optimally uncoupled rate: 8.2 μmoles ferricyanide reduced per h. ○, $-\log [NH_3]$; ●, $-\log [NH_4^+]$

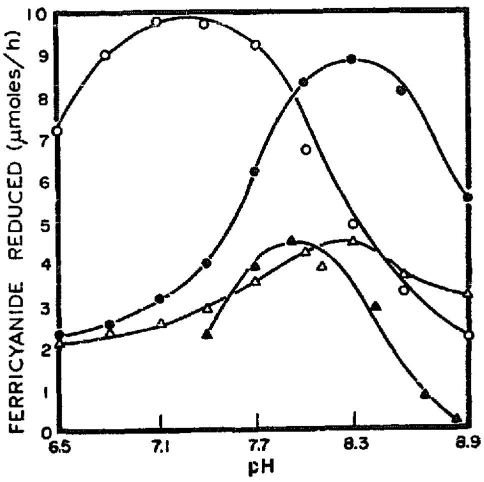

Fig. 3. Optimum pH for ferricyanide reduction. ○—○, in presence of 0.22 mM NH_3. △—△, control (no additions). ●—●, with 25 mM P_i and 2.5 mM ADP. ▲—▲, with 2.8 mM NH_3. Chlorophyll, 30 μg.

plasts for which at present we have no explanation. Columns 2 and 3 illustrate unaltered reduction rates at a point where uncoupling has inhibited phosphorylation (compare also Table III in ref. 6).

In other experiments we observed that unchanged reduction rates were not found if the chloroplasts were treated at a lower pH than 8.0 and this prompted investigation of the pH relations of low and high base concentration effects. The NH_4Cl needed to give a fixed NH_3 concentration was calculated from Eqn. 1 and added to a series of buffers of different pH. For purposes of comparison, the basal rate and the effect of phosphate and ADP on the same preparation of chloroplasts was determined.

A distinct change in pH response is seen when the reduction of ferricyanide is uncoupled by NH_3 (Fig. 3). The optimum shifts from the region of pH 8.1–8.4 for basic and coupled systems to the region of pH 7.1–7.4. In addition the uncoupled chloroplasts are markedly less active at the higher pH values. Two consequences of this behaviour are (1) The percentage rate increase on adding uncoupler to the basal system will be highly pH-dependent. For example at pH 7.2, the optimum NH_3 concentration increases the rate of reduction of ferricyanide by $+270\,\%$ but only by 53 % at pH 8.0. (2) Addition of NH_3 may reduce the rate of electron transport at high pH. The exact points of intersection and peak positions shown in Fig. 3 vary somewhat with the chloroplast preparation and conditions of illumination. AVRON *et al.*[7] have found an almost pH-insensitive basal rate, and an optimum for coupling near pH 7.8.

The curve for the pH relationship of ferricyanide reduction in the presence of excess NH_3 provides some evidence that under these conditions the limiting step is not that at which uncoupling occurs. The inhibited chloroplasts, Curve D, are seen to have a pH optimum nearer to that of the basal than that of the uncoupled system. This suggests that another stage in the electron-transport pathway is controlling the overall reaction rate. In order to find whether these rate-limiting steps are related to light or dark processes, the effect of light intensity was determined. In all cases rates were corrected to allow for chloroplast decay under each type of reaction condition studied.

The effect of light intensity (L) on the rate of ferricyanide reduction (v) follows the relationship (LUMRY AND SPIKES[8])

$$\frac{L}{v} = \frac{L}{K_D} + \frac{1}{K_L} \qquad (3)$$

When the results are plotted directly, a hyperbolic form is obtained which is plotted in the reciprocal form in Fig. 4. A common intercept on the $1/v$ axis was observed for uncoupled and inhibited chloroplasts suggesting that the effect of both high and low NH_3 concentrations is to modify the dark reaction constant. KROGMANN found that on uncoupling by "dilution" a major change in K_D could be shown[6] though the limiting light step was also somewhat modified.

The action of other bases

Active uncouplers can be classed as either aliphatic or alicyclic amines. Having found uncoupling induced by the heterocyclic base piperidine other compounds of this type as well as two aromatic amines, aniline and benzylamine, were investigated.

VALUES FOR PER CENT ENHANCEMENT OF BASAL FERRICYANIDE REDUCTION RATE, CORRECTED FOR RELATIVE SENSITIVITIES OF THE DIFFERENT CHLOROPLAST PREPARATIONS TO AMMONIA AS CONTROL

The value of c_{50} is referable to $+62\%$.

Base	pK_b	*Preparation*	*—log [unionized base]*												
			6.17	*5.57*	*5.22*	*4.75*	*4.25*	*3.92*	*3.67*	*3.25*	*2.95*	*2.40*	*2.00*	*1.50*	*1.17*
Piperidine	2.80	a	33	88	107	108	64	28	0						
Pyrrolidine	2.89	b	14	43	90	116									
Diethylamine	2.90	a	18	67	85	95	79	63	47						
Cyclo-hexylamine	3.36	b	18	51	99	103	98								
Piperazine	4.19, 8.43	b			7	15	32	44	54	87					
Benzylamine	4.62	a			4	28	64	96	107						
Ammonia	4.75	a			10	43	92	117	124	88	60	6			
Morpholine	5.70	b					10	27	47	102	122	128	110		
β-Bromoethyl-amine	6.20	c						38	46	87	130	211	192		
Imidazole	6.92	d								41	70	128	85	—50	
γ-Picoline	7.96	f								11	24	72	110	127	109
Pyridine	8.81	e							17	36	48	71	98	126	122
Aniline	9.42	f	(% below basal rate)			—6	—5	—5	—11	—13	—11	—11	—14	—27	—34

The results are shown in Table III. The failure of aniline to increase the rate of ferricyanide reduction was confirmed[3] but all other bases used were in some degree active. Piperidine is especially so. For example, only 7 µM free piperidine is needed to give complete uncoupling and 0.6 µM produces a 30 % increase over control. The efficiency is thus some 30 times greater than that of NH_3 in terms of free base. It should be pointed out, however, that with respect to the concentration of cation required to give maximum electron flow at the same pH, piperidine is less active by a factor of two. The similarity in shape between the concentration curves of these same bases suggests that the pathway blocked by "excess" NH_3 (Table I), is also correspondingly more sensitive to piperidine. Further, the compensation point between the opposing influences of added base does not seem to vary greatly with the base used, so that all active bases uncouple to ± 10 % of the same optimal electron flow rate, with the notable exception of β-bromoethylamine.

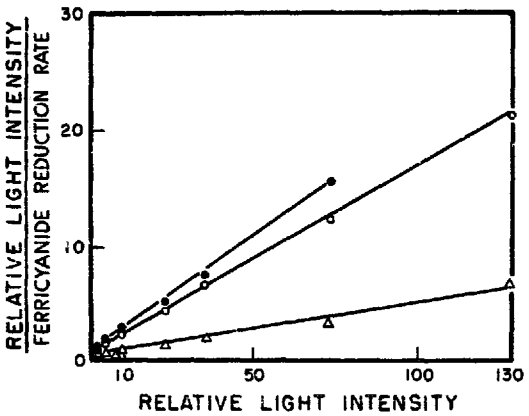

Fig. 4. Effect of NH_3 on rate-limiting reactions. Basal ferricyanide reduction at pH 8.0 with addition of: ○—○, nil; △—△, 0.1 mM NH_3; ●—●, 5.6 mM NH_3.

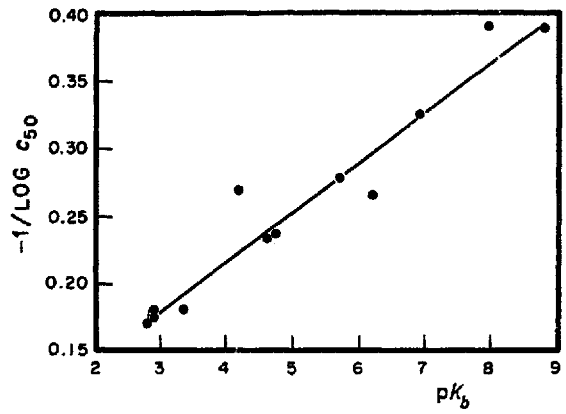

Fig. 5. Relation of c_{50} to pK_b. Points correspond to the sequence of bases given in Table III omitting aniline.

During this study we found that strong bases generally were the more efficient uncoupling agents in terms of free base concentration. In order to test this generalization, a further series of compounds covering a range of pK values was selected. The free base concentration needed to give an enhancement equal to 50 % of the maximum rate increase obtainable by addition of NH_3 to the same preparation, was designated c_{50}.

Fig. 5 plots the relation

$$pK_b = -n \frac{1}{\log c_{50}} \tag{4}$$

for the bases shown in Table III. Irrespective of structure, the uncoupling activity was approximately related as above to the pK_b.

The failure of aniline to uncouple cannot be explained simply on the basis of Fig. 5. The value of c_{50} corresponding to $pK_b = 9.3$ would be 3.6 mM, yet there is no rate enhancement at any point up to 0.125 M—the highest studied. Either this amine is inactive by reason of some structural or physical characteristic (unlikely in view of the activity of pyridine and benzylamine), or the slope of Fig. 5 must rise rapidly to infinity above $pK_b = 8.8$ (pyridine). The latter seems a preferable interpretation.

Piperazine, with two dissociable basic groups, is thought to be the first known instance of a polyfunctional uncoupler. At pH 8.0 very little exists as the uncharged species yet it is a function of this form which correlates most closely with the slope of Fig. 5, the values being: $pK_b = 4.19$, $-1/\log c_{50} = 0.27$. If the singly positive ion were also active, the value of this second term would rise to 0.53.

Since there is much similarity between this base and 1,5-diaminopentane we were concerned at the reported inactivity of the latter[3]. It was possible, however, to calculate from the data of GOOD's experiment the actual concentrations of the three molecular species making up a total of 2.0 mM at pH 7.5. The free base proved to be only 0.5 μM. From Fig. 5 one can predict that for a base with $pK_b{}^1 = 3.0$, the value of c_{50} is about 1.25 μM and so, assuming inactivity on the part of the cations (as for piperazine), it seemed possible that higher concentrations of diaminopentane might have induced uncoupling. This proved to be the case as shown by Table IV, in which optimum uncoupling requires at least 0.01 M total base at pH 7.5. The value of $-1/\log c_{50}$ from these figures is 0.165 which gives a reasonable fit with $pK_b = 3.0$ on the slope of Fig. 5.

TABLE IV

UNCOUPLING BY 1,5-DIAMINOPENTANE

	Undissociated diaminopentane (μM)					
	0.0	*0.3*	*0.8*	*1.6*	*2.6*	*7.8*
Ferricyanide reduction*	161	173	216	242	278	237
Percentage above basal	0	7	34	50	73	47

* μmoles ferricyanide/h/mg chlorophyll. Basal reaction conditions at pH 7.5. Free base concentration calculated using $pK_b{}^1 = 3.0$, $pK_b{}^2 = 4.3$ (see ref. 9).

One may conclude that substances with two basic functions can also uncouple but that activity is restricted to the totally unionized form of the molecule. Thus a nitrogenous base will uncouple provided (1) that the pK_b is below about 9.0, and (2) that the molecule carries no charge.

DISCUSSION

Uncoupling induced by nitrogenous bases appears to be reversible and dependent on the concentration of unionized molecules. Inhibition of O_2 evolution by excess base had the same characteristics, and can likewise be shown to affect a limiting dark reaction. It is clear from other work, however, that two distinct stages are concerned in these analogous patterns of behaviour. Reduced indophenol dye can for instance be made to supply electrons to the chloroplast system at a point prior to that of coupled phosphorylation, which is sensitive to uncoupling by low NH_3 concentrations[10], yet unresponsive to the presence of inhibitory levels of free base[4]. The reduction of dye is contrariwise refractory to uncoupling[6], but most sensitive to inhibitors of O_2 evolution. This latter process is also curtailed by a variety of nitrogenous reagents which have no obvious affinity for the coupling reaction. Thus NH_3 and the other bases mentioned herein display two types of inhibitory activity.

The changes in pH optimum and reaction rate for the reduction of ferricyanide which are observed on uncoupling chloroplasts are most readily ascribed to modification of an existing electron-transport pathway respecting the turnover rate of a limiting intermediate. The altered mechanism would be relatively efficient only at low pH.

WHITTINGHAM AND BISHOP[11] have studied the thermal processes between the two light reactions and concluded that basal and coupled ferricyanide reduction have a common dark process which is not involved in dye or uncoupled ferricyanide reduction. Since their pH conditions were such (pH 7.4) as to favour increased electron flow on uncoupling these results are consistent with the above hypothesis. The thermal process in question would be that characterized by a pH-8.0 optimum, in the absence of which a somewhat more acid value might be predicted to yield the shortest optimal flash separation interval.

GOOD has suggested that penetration of the unionized form of a nitrogenous base into the lipid fraction of the chloroplast is responsible for uncoupling. This accounts for the inactivity of compounds having hydrophilic groups such as $-OH$, $-COO^-$ and of course $-NR_1R_2R_3H^+$.

While our results are in substantial agreement with this hypothesis we have a slightly different view as to the actual mechanism of uncoupling. The relation of activity to pK_b suggests that the bases behave as nucleophilic agents, and in a non-aqueous system we would prefer to think of their involvement with an electrophilic group rather than a proton. There are doubtless many components of the chloroplast lipid with which reaction could occur, including the possibility of interaction with the electrophilic unsubstituted ring position of plastoquinone. There is evidence that amines react with electron-deficient sites of quinones by first forming a loose complex[12]. This might so modify a quinone as to prevent fulfilment of its normal role in phosphorylation. The energy loss on formation of such complexes is probably not more than 5 kcal/mole, hence reversibility on washing uncoupled chloroplasts is not a point of objection. Full evaluation of these ideas must await a clearer understanding of the function of quinones in photophosphorylation.

Finally a warning should be issued against the use of imidazole buffers in the study of chloroplast reactions. This base is clearly an efficient uncoupling agent at millimolar concentrations.

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