BBA 45636

CHLOROTRI-*n*-BUTYLTIN AN INHIBITOR OF PHOTOPHOSPHORYLATION IN ISOLATED CHLOROPLASTS*

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(Received August 14th, 1967)

SUMMARY

1. Chlorotri-*n*-butyltin was found to be a specific inhibitor of photophosphorylation in isolated chloroplasts. Only very small amounts were required for complete inhibition, and the reaction of the inhibitor with the chloroplasts appeared to be stoichiometric, requiring 1 mole of chlorotributyltin to 60–120 moles chlorophyll.

2. No direct effect of chlorotributyltin on electron transport could be observed, although it inhibited ferricyanide and $NADP^+$ reduction indirectly in coupled chloroplasts. This inhibition could be completely reversed by the addition of an uncoupler such as NH_4Cl.

3. Photophosphorylation as well as the light-dependent "pH rise" were markedly stimulated by low concentrations of chlorotributyltin in chloroplasts which were deficient in coupling factor. It was postulated that the chlorotributyltin binds to the chloroplasts close to or at the site of binding of the coupling factor and thus prevents the loss by hydrolysis of a high-energy intermediate.

INTRODUCTION

The demonstration by LEE AND ERNSTER[1] that oligomycin at low concentrations will stimulate oxidative phosphorylation in partially uncoupled mitochondrial fragments had led to a re-examination of the role of of coupling factors in stimulating the rate of ATP formation. It was suggested that the binding of oligomycin to the particles prevents the loss of a high-energy intermediate by hydrolysis, and that the binding of the coupling factor acts in a similar fashion. However, the presence of enzymatically active coupling factor was demonstrated to be obligatory for the oligomycin-stimulated phosphorylation[2]. In addition, the stimulation of phosphorylation in uncoupled beef heart mitochondrial particles by coupling factor from yeast mitochondria depended on the presence of residual amounts of beef heart coupling factor[3].

Abbreviations: DCC, *N,N'*-dicyclohexyl carbodiimide; DCIP, 2,6-dichlorophenolindophenol.

* Contribution from the Biochemistry Department, School of Agriculture and Life Sciences, and School of Physical Sciences and Applied Mathematics. Paper No. 2429 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N.C.

A coupling factor has been isolated from chloroplasts of spinach[4] and *Euglena gracilis*[5]. McCARTY AND RACKER[6] have recently reported that *N,N'*-dicyclohexyl carbodiimide (DCC), while inhibiting photophosphorylation and electron transport in coupled chloroplasts, will stimulate phosphorylation in uncoupled chloroplasts in the presence of added coupling factor. The inhibition of electron transport by DCC could, however, be reversed only partially by ammonia and atebrin, thus making the determination of its site of action somewhat difficult, although it is probably very close to the site of uncoupling by ammonia. McCARTY, GUILLORY AND RACKER[7] have also reported on another inhibitor of photophosphorylation, Dio-9, which has been postulated to inhibit photophosphorylation at a terminal step. Its inhibition of electron transfer is maximal only in the presence of ADP and phosphate, and it also inhibits the ATPase activity of the isolated coupling factor.

Recently, IZAWA, WINGET AND GOOD[8] have shown that phlorizin will also serve as an inhibitor of photophosphorylation, requiring, however, very high concentrations ($> 1 \cdot 10^{-3}$ M) for optimal effect.

Oligomycin does not have any effect on photophosphorylation. We have consequently investigated other inhibitors which affect mitochondria in a way similar to oligomycin to see whether they will inhibit photophosphorylation.

This report will describe the inhibition of photophosphorylation by chlorotri-*n*-butyltin, which has been reported to have an oligomycin-like effect on oxidative phosphorylation[9]. It will be shown that chlorotributyltin reacts stoichiometrically with the chloroplasts, inhibiting photophosphorylation, but having no direct effect whatsoever on electron transport. Also, in contrast to the effect of oligomycin on corn shoot mitochondria[10], ADP and phosphate are not required to obtain maximal indirect inhibition by chlorotributyltin of electron transport. Moreover, it will be shown that low concentrations of chlorotributyltin will greatly stimulate photophosphorylation in uncoupled Euglena, but only slightly in uncoupled spinach chloroplasts.

MATERIALS AND METHODS

Chloroplasts were prepared from *Euglena gracilis* as previously described[11] and from spinach by the method of JAGENDORF AND AVRON[12]. The chloroplasts were uncoupled by lysis in the presence of $1 \cdot 10^{-3}$ M EDTA as described by AVRON[13].

The reaction mixtures for assay of photophosphorylation are described in each table. The samples were illuminated for 2 min at 17°, with 20000 lux of red light (610–780 nm), and ATP formation measured with the aid of $^{32}P_i$ by a modification of the method of LINDBERG AND ERNSTER[14]. The reduction of $NADP^+$ and 2,6-dichlorophenolindophenol (DCIP) was followed spectrophotometrically at 340 and 610 nm, respectively, and ferrocyanide formation by *o*-phenanthroline as described by AVRON AND SHAVIT[15]. For assay of ATPase activity the samples were incubated for 10 min at 36°. Chlorophyll was measured by the method of ARNON[16]. Chlorotri-*n*-butyltin was purchased from the Aldrich Chemical Company, and stock solutions were made up in 50 % methanol.

RESULTS

Inhibition of photophosphorylation

Chlorotributyltin inhibited both cyclic and non-cyclic phosphorylation at extremely low concentrations (Tables I and II), Euglena chloroplasts being somewhat

more sensitive than spinach chloroplasts. The low concentration required for complete inhibition prompted us to examine the possibility that the ratio of chloroplasts to chlorotributyltin determines the degree of inhibition. We consequently varied the chloroplast concentrations in the reaction mixture and measured their inhibition by varying concentrations of chlorotributyltin. It appeared that, especially for Euglena chloroplasts, the ratio of chlorophyll to chlorotributyltin was the major determinant of the degree of inhibition rather than the chlorotributyltin concentration, with the inhibition for a given concentration of chlorotributyltin being inversely proportional to the amount of chloroplasts in the reaction mixture. For Euglena, about 1 mole chlorotributyltin to 120 moles chlorophyll gave complete inhibition, while for spinach about 1 to 60 was required. This indication of tight binding between chlorotributyltin and the phosphorylation sites was corroborated by preincubating chloroplasts in a

TABLE I

THE INHIBITION BY CHLOROTRI-*n*-BUTYLTIN OF PHOTOPHOSPHORYLATION BY EUGLENA CHLOROPLASTS

The reaction mixture, in a final volume of 1.5 ml, contained: 10 μmoles Tris buffer, pH 7.8, 35 μmoles NaCl, 1 μmole ADP, 4 μmoles phosphate buffer containing 400000 counts/min $^{32}P_i$, 15 μmoles glucose, 5 μmoles $MgCl_2$, 1 μg (0.15 unit) crystalline hexokinase, 0.5 mg crystalline bovine serum albumin, 300 μmoles mannitol, chloroplasts containing 20 μg chlorophyll and cofactor as described in the table.

Chlorotributyltin ($M \times 10^8$)	*ATP formation* (*μmoles/mg chlorophyll per h*)		
	Pyocyanin (*0.05 μmole*)	*DCIP* (*0.15 μmole*)	*$Fe(CN)_6^{3-}$* (*0.75 μmole*)
0	36.9	9.9	16.1
0.5	34.7	—	—
1	30.7	9.1	14.8
2	22.8	8.4	13.5
4	10.9	5.2	5.9
8	2.7	1.8	1.4

TABLE II

THE INHIBITION BY CHLOROTRI-*n*-BUTYLTIN OF PHOTOPHOSPHORYLATION BY SPINACH CHLOROPLASTS

Reaction mixture as in Table I.

Chlorotributyltin ($M \times 10^8$)	*ATP formation* (*μmoles/mg chlorophyll per h*)			
	Pyocyanin (*0.05 μmole*)	*DCIP* (*0.15 μmole*)	*$Fe(CN)_6^{3-}$* (*0.75 μmole*)	*$NADP^+$* (*0.5 μmole*)
0	1008	167	131	114
0.5	954	—	—	—
1	892	162	125	111
2	825	138	115	106
4	672	—	—	—
5	—	109	106	83
8	506	—	—	—
10	—	46	66	67
12	283	—	—	—

reaction mixture with chlorotributyltin, then separating them by centrifugation. The chloroplasts were then assayed for photophosphorylation with fresh reaction mixture, and to the supernatant fresh chloroplasts were added and assayed too. This experiment showed (Table III) that Euglena chloroplasts bound and removed most of the chlorotributyltin from the reaction mixture, while spinach chloroplasts removed about half. The binding of chlorotributyltin was unaffected by the presence of NH_4Cl or atebrin during the preincubation.

TABLE III

THE BINDING OF CHLOROTRI-*n*-BUTYLTIN TO CHLOROPLASTS

Chloroplasts in a reaction mixture as described in Table I were treated with or without $8 \cdot 10^{-8}$ M chlorotributyltin for Euglena and $1.2 \cdot 10^{-7}$ M chlorotributyltin for spinach, incubated in an ice bath in the dark for 15 min and then separated by centrifugation. The chloroplasts were taken up in fresh reaction mixture without chlorotributyltin, and fresh, untreated chloroplasts were added to the supernatant. The numbers in parentheses represent the percentage of control.

	ATP formed (μmoles/mg chlorophyll per h)	
	Euglena	*Spinach*
Control		
Supernatant + fresh chloroplasts	18.8 —	556 —
Chloroplasts + fresh mixture	12.3 —	496 —
Chlorotributyltin-treated		
Supernatant + fresh chloroplasts	14.8 (79)	345 (62)
Chloroplasts + fresh mixture	4.6 (37)	323 (65)
Control + $1 \cdot 10^{-3}$ M NH_4Cl		
Chloroplasts + fresh mixture	12.0 —	
Chlorotributyltin-treated + $1 \cdot 10^{-3}$ M NH_4Cl		
Chloroplasts + fresh mixture	4.9 (41)	

TABLE IV

THE EFFECT OF CHLOROTRI-*n*-BUTYLTIN AND NH_4Cl ON THE RATE OF FERRICYANIDE REDUCTION BY EUGLENA AND SPINACH CHLOROPLASTS

Reaction mixture, in a final volume of 2 ml, contained: 40 μmoles Tris buffer, pH 7.8, 18 μmoles NaCl, 3 μmoles $MgCl_2$, 0.75 μmole $K_3Fe(CN)_6$, and chloroplasts containing 10 μg chlorophyll for spinach, 20 μg for Euglena.

	Ferricyanide reduction (μmoles/mg protein per min)		
	Reaction mixture	*+ 5 μmoles P_i*	*+ 5 μmoles P_i and + 1 μmole ADP*
I. Euglena			
Control	48.7	35.8	41.9
+ $5 \cdot 10^{-4}$ M NH_4Cl	52.7	43.7	37.8
+ $5 \cdot 10^{-7}$ M chlorotributyltin	36.2	25.9	32.1
+ NH_4Cl and chlorotributyltin	51.6	47.8	45.5
II. Spinach			
Control	245	221	322
+ $1 \cdot 10^{-3}$ M NH_4Cl	281	302	265
+ $5 \cdot 10^{-7}$ M chlorotributyltin	167	158	185
+ NH_4Cl and chlorotributyltin	288	379	323

Effect of chlorotributyltin on ferricyanide reduction

The rates of ferricyanide reduction of chloroplasts which were uncoupled by the addition of NH_4Cl or atebrin were not affected by chlorotributyltin even at concentrations 100 × those required for inhibition of phosphorylation ($5 \cdot 10^{-6}$ M). The same held true for the reduction of DCIP and $NADP^+$ (see also Table V). In coupled chloroplasts, however, the rate of ferricyanide reduction was markedly inhibited by chlorotributyltin, and this inhibition was completely reversible by NH_4Cl (Table IV). The inhibition and its reversal were, however, independent of the presence of ADP and P_i. The same effect could be shown on $NADP^+$ reduction in Euglena but not in spinach chloroplasts (Table V).

Enhancement of photophosphorylation in EDTA-uncoupled chloroplasts

Chloroplasts from which the coupling factor was removed by lysis in dilute EDTA[13] showed an enhancement of their residual rate of photophosphorylation by

TABLE V

THE EFFECT OF CHLOROTRI-*n*-BUTYLTIN AND NH_4Cl ON THE RATE OF $NADP^+$ REDUCTION BY EUGLENA AND SPINACH CHLOROPLASTS

The reaction mixture, in a final volume of 3 ml, contained: 6 μmoles Tris buffer, pH 7.8, 35 μmoles NaCl, 300 μmoles mannitol, 0.5 μmole $NADP^+$, 2 × saturation spinach ferredoxin, and chloroplasts, containing 30 μg chlorophyll.

	$NADP^+$ reduction (μmoles/mg chlorophyll per h)	
	Euglena	*Spinach*
Control	7.2	98.9
+ $5 \cdot 10^{-7}$ M chlorotributyltin	0.6	99.9
+ $1 \cdot 10^{-3}$ M NH_4Cl	7.0	96.6
+ chlorotributyltrin + NH_4Cl	6.8	103.2

TABLE VI

EFFECT OF CHLOROTRI-*n*-BUTYLTIN ON PHOTOPHOSPHORYLATION BY UNCOUPLED CHLOROPLASTS

Reaction mixture as in Table I with pyocyanin as cofactor. Chloroplasts were uncoupled by suspending them in 10^{-3} M EDTA. In Expt. I they were suspended at a final concentration of 400 μg chlorophyll per ml, and in Expt. II, at a final concentration of 100 μg/ml. They were then reisolated by centrifugation for 20 min at 36000 × *g*. 0.2 ml of the EDTA supernatant of the Euglena was used as the source of coupling factor (CFE).

Chloro-tributyltin ($M \times 10^8$)	*ATP formation (μmoles/mg chlorophyll per h)*				
	Euglena			*Spinach*	
	Expt. I	*Expt. II*		*Expt. I*	*Expt. II*
		−CFE	*+CFE*		
0	7.8	0.70	1.17	209	10.2
0.5	9.8	0.93	1.23	217	9.9
1	16.6	1.17	1.69	236	9.1
2	7.5	2.14	1.06	197	9.7
4	5.1	1.98	1.02	189	8.1
8	1.9	0.97	0.84	121	5.1
12	—	—	—	62	2.7

low concentrations ($1 \cdot 10^{-8}$ M) of chlorotributyltin, although higher concentrations ($8 \cdot 10^{-8}$ M) were as inhibitory as they were to coupled chloroplasts (Table VI). The addition of coupling factor decreased the enhancement obtained by the addition of chlorotributyltin.

Restoration of the light-induced "pH rise" in uncoupled chloroplasts

Coupled chloroplasts showed no effect on their light-induced "pH rise" by the addition of chlorotributyltin, as is the case with other inhibitors of photophosphorylation[6,17]. In chloroplasts uncoupled by EDTA, however, the residual low "pH rise" was greatly stimulated by chlorotributyltin (Table VII), being restored to the value of the coupled chloroplasts.

TABLE VII

EFFECT OF CHLOROTRI-*n*-BUTYLTIN ON THE LIGHT-INDUCED "pH RISE" IN COUPLED AND UNCOUPLED CHLOROPLASTS

Isolated chloroplasts were suspended either in 0.01 M NaCl + 0.2 M mannitol or in $1 \cdot 10^{-3}$ M EDTA to a chloroplast concentration of 0.25 mg/ml, reisolated by centrifugation and resuspended in the NaCl–mannitol medium. The reaction mixture for the assay contained 300 μmoles mannitol, 0.05 μmole pyocyanin and chloroplasts containing 400 μg chlorophyll for Euglena, 100 μg for spinach, in a final volume of 1.5 ml. The reaction was run at 5°, and each value represents the mean of duplicate runs, with 3 light–dark cycles for each. Starting pH was 6.15. Light intensity was 80000 lux.

	"pH rise" (ΔpH)	
	Euglena	*Spinach*
Coupled chloroplasts	+0.044	+0.176
+ $5 \cdot 10^{-7}$ M chlorotributyltin	+0.039	+0.133
Uncoupled chloroplasts	+0.018	+0.036
+ $5 \cdot 10^{-7}$ M chlorotributyltin	+0.041	+0.088

TABLE VIII

INHIBITION BY CHLOROTRI-*n*-BUTYLTIN OF THE Ca^{2+}-DEPENDENT ATPASE ACITIVITY OF THE EUGLENA COUPLING FACTOR

The coupling factor was isolated as described by us[5]. The reaction mixture, in a final volume of 3 ml, contained 150 μmoles Tris buffer, pH 8.0, 7.5 μmoles ATP, 15 μmoles $CaCl_2$ and enzyme as described in the table.

Chlorotributyltin (M)	*ATP hydrolysis (μmoles/mg protein per min)*	
	Chloroplast fragments (approx. 300 μg protein)	*Purified enzyme (4 μg protein)*
None	0.037	9.36
$1 \cdot 10^{-7}$	0.038	—
$5 \cdot 10^{-7}$	0.033	8.82
$1 \cdot 10^{-6}$	0.033	7.52
$2 \cdot 10^{-6}$	—	4.56
$5 \cdot 10^{-6}$	0.030	2.96
$1 \cdot 10^{-5}$	0.024	1.76
$2 \cdot 10^{-5}$	—	0.88
$5 \cdot 10^{-5}$	0.003	—

Inhibition of the Ca^{2+}-dependent ATPase activity of the coupling factor from Euglena chloroplasts

The ATPase activity of the isolated coupling factor was inhibited by chlorotributyltin, although the concentration required was much higher than that required to inhibit photophosphorylation (Table VIII). In spite of the high concentration of chlorotributyltin required, the inhibition could well be a specific reaction and not a non-specific interaction with protein, since the same concentration of chlorotributyltin was required to obtain complete inhibition in crude as compared to purified enzyme preparations.

DISCUSSION

The results presented show that chlorotributyltin at low concentrations is a specific inhibitor of photophosphorylation. Comparing its effect to that of other inhibitors and uncouplers of photophosphorylation, chlorotributyltin lacks the direct effect on electron transport which DCC has even in the presence of NH_4Cl (ref. 6), and, on the other hand, it does not require ADP and P_i for maximal inhibition of electron transport in coupled chloroplasts as do Dio-9[7] and phlorizin[8]. It can thus tentatively be concluded that the site of binding of chlorotributyltin lies between that of DCC and Dio-9.

We have no explanation to date for the inhibition by chlorotributyltin of $NADP^+$ reduction in Euglena chloroplasts which was not observed in spinach chloroplasts. This inhibition, which is completely reversible by NH_4Cl, is particularly curious since we could show no stimulation whatsoever of $NADP^+$ reduction by the addition of ADP and phosphate.

The "pH rise" is currently considered as a manifestation of a high-energy intermediate in ATP formation. Since chlorotributyltin reverses the loss of the light-induced pH rise in chloroplasts deficient in coupling factor, it probably prevents the hydrolysis of a non-phosphorylated high-energy intermediate ($X \sim I$) prior to the coupling factor. This can also be concluded from the enhancement of phosphorylation in uncoupled chloroplasts by low concentrations of chlorotributyltin. In fact, since the enhancement of phosphorylation by chlorotributyltin was decreased by added coupling factor, it is possible that chlorotributyltin binds to the same site as the coupling factor.

The inhibition of the Ca^{2+}-dependent ATPase by higher concentrations of chlorotributyltin may represent a second specific action of chlorotributyltin on the coupling factor, inhibiting the binding of inorganic phosphate to form the phosphorylated intermediate ($X \sim P$). It could well be that such a dual action by chlorotributyltin on chloroplasts is similar to the one recently suggested by HINKLE, PENEFSKY AND RACKER[18] for the dual action of oligomycin on mitochondria, acting both on the non-phosphorylated, as well as the phosphorylated high-energy intermediate in ATP formation.

The very small amount of chlorotributyltin required for complete inhibition of photophosphorylation puts an upper limit to the ratio of chlorophyll to ATP-forming site of about 60 for spinach and 120 for Euglena chloroplasts. This will hold true provided that each molecule of chlorotributyltin inhibits only one site, and not a number of sites, as has recently been suggested for the uncoupling of oxidative

phosphorylation by carbonylcyanide phenylhydrazone derivatives[19]. In light of the tight binding of chlorotributyltin by the chloroplasts, such a multi-site inhibition appears improbable.

ACKNOWLEDGEMENTS

The able technical assistance of Mrs. THEA SCHOSTAG and Mr. RICHARD THACKER is gratefully acknowledged.

This work was supported in part by Public Health Service Grant No. GM-09710-05 from the National Institutes of Health, Division of General Medical Sciences.

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