STUDIES ON CARBON DIOXIDE FIXATION BY CHLOROPLASTS
THE EFFECT OF ARSENITE AND OTHER FACTORS

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SUMMARY

A comparison of the rate of photochemical reduction of CO_2 by intact chloroplasts and by fragmented chloroplast preparations was investigated. While arsenite, *p*-CMB, and iodoacetamide inhibited both types of preparations to the same extent, phosphate and arsenate did not.

The site of arsenite inhibition was investigated. The photochemical act was unaffected. None of the enzymes usually associated with the photosynthetic carbon cycle was inhibited.

INTRODUCTION

In a previous paper[1] the effect of pH, classical inhibitors, phosphate, arsenate and other factors on the light induced fixation of CO_2 by intact spinach chloroplasts was reported. In this paper some further results of our experiments on CO_2 fixation by intact and fragmented spinach chloroplasts are described. The most significant finding is the observation that the site of arsenite inhibition in photosynthesis is apparently in the carbon cycle. However, none of the enzymes generally considered to compose the photosynthetic carbon cycle was found inhibited by arsenite. A preliminary report on part of this investigation has appeared[2].

MATERIALS AND METHODS

Preparation of the intact chloroplasts and the reconstituted system

Intact chloroplasts were prepared by methods described in the preceding article[1]. The chlorophyll content of each preparation was determined by the method of ARNON[3] and aliquots containing 1 mg chlorophyll were used in each reaction vessel.

The reconstituted system which consisted of chloroplast extract and fragmented chloroplasts was prepared in the following way. Chloroplast extract was prepared by suspending once washed chloroplasts from 120 g of spinach leaf blades in 9 ml

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DPN, TPN, di- and triphosphopyridine nucleotide respectively; FMN, flavine mononucleotide; ADP, ATP, adenosine di- and triphosphate respectively; *p*-CMB, *p*-chloromercuribenzoate, BAL, 2,3-dimercaptopropanol; IAA, iodoacetamide.

of 0.02 *M* Tris buffer pH 7.5. After 30 min the supernatant fluid was obtained by centrifuging off the chloroplast fragments at 18,000 × *g* for 20 min.

Chloroplast fragments were prepared by suspending once washed intact chloroplasts from 40 g of leaves in 4 ml of 0.02 *M* Tris buffer pH 7.5. This suspension was used without further treatment.

Each reaction vessel received chloroplast extract equivalent to 2 mg chlorophyll and fragments equivalent to 1 mg chlorophyll.

Reagents

TPN, FMN, phenazine methosulfate, ADP, ATP, *p*-CMB and phosphoenolpyruvate were products of the Sigma Chemical Company. BAL was obtained from the K and K laboratories. IAA was purchased from the Nutritional Biochemical Company. Solutions of sodium arsenite were prepared from arsenious trioxide (Mallinckrodt Chemical Works) and sodium hydroxide.

Uptake of carbon dioxide

The method used in this investigation was similar to that employed previously[1,4].

Measurement of enzyme activity

Ribulose diphosphate carboxylase, transketolase, transaldolase, neutral and alkaline fructose 1,6-diphosphatases, phosphoriboisomerase, phosphoribulokinase, TPN and DPN associated glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglyceric acid kinase, photoreduction of TPN accompanied by O_2 evolution and photosynthetic phosphorylation activities were assayed by methods employed in previous studies[1,4].

The enzyme, phosphoenolpyruvate carboxylase was assayed by pipetting into a 12 ml centrifuge tube: 0.2 ml of chloroplast extract containing 40 μmoles Tris buffer, pH 7.5, 5 μmoles $MgCl_2$, 1 μmole phosphoenolpyruvate and 5 μmoles of $NaHCO_3$ containing ^{14}C. The total volume was 1.0 ml. After the reaction mixture was allowed to stand at room temperature for 10 min, it was placed in a boiling water bath for 2 min. For measurement of the total tracer fixed, 0.1-ml duplicate aliquots of the contents were pipetted onto aluminum planchets and evaporated to dryness under an infrared lamp. The dry samples were assayed with a gas-flow thin window counter.

For inhibition studies, vessels were kept at 0° until all components had been added. Then inhibitor was permitted to incubate at room temperature for 10 min before the introduction of substrate. When prevention of an inhibition was studied, inhibitor was added after 10 min of incubation, while for reversal studies inhibitor was added at zero time and the test compound was added 10 min later.

RESULTS

pH dependence of CO_2 fixation

Fig. 1 shows the effect of pH on CO_2 fixation by chloroplast fragments supplemented with chloroplast extract. The isotope incorporation rate proceeds at maximal velocity in the vicinity of pH 7.2 to 7.5. Below and above the values, the fixation rate falls off sharply. This result is somewhat similar to that observed with intact chloroplasts[1].

Action of iodoacetamide and p-*CMB on CO_2 fixation*

From the results outlined in Table I, it is evident that the alkylating agent, IAA and the mercaptide-forming compound, *p*-CMB, are both potent inhibitors of CO_2 fixation by the reconstituted system. Similar values were obtained previously with the intact chloroplast[1].

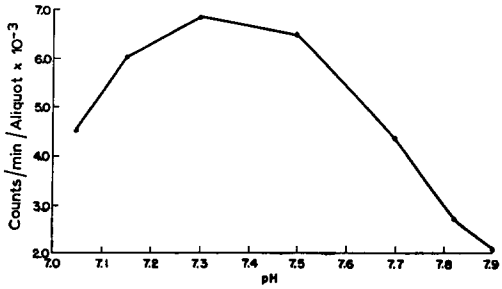

Fig. 1. The effect of pH on CO_2 fixation by the reconstituted system.

TABLE I

EFFECT OF IAA AND *p*-CMB ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM

Inhibitor concn. (M)	$^{14}CO_2$ *fixed counts/min/aliquot*	*% inhibition*
0	565	—
$5 \cdot 10^{-6}$ IAA	560	1
$1 \cdot 10^{-5}$ IAA	525	7
$5 \cdot 10^{-5}$ IAA	240	58
$5 \cdot 10^{-4}$ IAA	5	99
0	1920	—
$5 \cdot 10^{-6}$ *p*-CMB	1055	45
$1 \cdot 10^{-5}$ *p*-CMB	740	61
$5 \cdot 10^{-5}$ *p*-CMB	115	94

Effect of phosphate on CO_2 fixation

Increasing the concentration of phosphate from $5 \cdot 10^{-6}$ *M* to 10^{-2} *M* stimulates CO_2 fixation approx. 14-fold. Higher concentrations inhibit the fragmented chloroplast system. In contrast, the ability of the intact chloroplast to assimilate CO_2 was not stimulated until a concentration of 10^{-3} to $5 \cdot 10^{-4}$ *M* phosphate was reached. In addition, the intact system was strongly inhibited (60 %) by $2 \cdot 10^{-3}$ *M* phosphate[1].

A possible explanation for the effect of phosphate may be found in its action on the enzymes catalyzing the synthesis of ATP or on the enzymes of the reductive pentose phosphate cycle. The increased rate of CO_2 fixation is presumably caused by an accelerated synthesis of ATP (see ref. 5). In contrast, the ribulose diphosphate carboxylase was found to be inhibited by phosphate ions. Thus, in 0.01 *M*, 0.03 *M* and 0.05 *M* phosphate, the activity was only 90, 62 and 42 % of the control value, respectively. WEISSBACH *et al.*[6] have observed the same effect.

With respect to the concentration difference of phosphate in affecting CO_2 fixation in the intact chloroplast in contrast to the fragmented system, no final conclusion can be drawn. The results obtained here do suggest, however, that organization of the enzymes within the chloroplast must surely have a quantitative affect on CO_2 assimilation.

TABLE II

EFFECT OF PHOSPHATE AND ARSENATE ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM

Phosphate concn. (M)	*Arsenate concn. (M)*	*$^{14}CO_2$ fixed counts/min/aliquot*
0		60
$5 \cdot 10^{-6}$		60
$1 \cdot 10^{-5}$		90
$1 \cdot 10^{-4}$		145
$1 \cdot 10^{-3}$		155
$7 \cdot 10^{-3}$		743
$1 \cdot 10^{-2}$		860
$3 \cdot 10^{-2}$		570
$5 \cdot 10^{-2}$		325
$5 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	2225
$5 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	2115
$5 \cdot 10^{-3}$	$1 \cdot 10^{-2}$	1890
$5 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	315

Table II also reveals that the arsenate ion has an inhibitory effect on CO_2 fixation. This effect is additive to the one caused by phosphate. Like phosphate ions, arsenate ions were inhibitory toward the ribulose diphosphate carboxylase. Thus, 0.03 *M* and 0.05 *M* arsenate inhibited the enzyme 43 and 65 %, respectively. While 0.03 *M* phosphate inhibited the enzyme 38 %, the addition of 0.015 *M* arsenate to the mixture increased inhibition by 12 %.

The reduction of 3-phosphoglyceric acid to 3-phosphoglyceraldehyde catalyzed by 3-phosphoglyceric acid kinase and the TPN-dependent 3-phosphoglyceraldehyde dehydrogenase is also sensitive to arsenate. Thus in 0.02 *M* and 0.05 *M* arsenate ions, the reoxidation of TPNH was only 68 and 26 % of the control value, respectively. The arsenate ions apparently catalyzed an arsenolysis of 1,3-diphosphoglyceric acid.

Effect of arsenite on CO_2 fixation

Arsenite, under the conditions employed, inhibits the CO_2-fixing ability of chloroplast preparations (Table III, IV and V). However, a large variation was found. This variation was in part caused by endogenous material since one wash of

TABLE III

EFFECT OF ARSENITE ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM PREPARED FROM UNWASHED AND ONCE WASHED CHLOROPLASTS

Condition	*$^{14}CO_2$ fixed counts/min/aliquot*	*% inhibition*
Dark, washed*	80	—
Light, washed	2175	—
Light, washed	730	66
Dark, unwashed**	80	—
Light, unwashed	2460	—
Light, unwashed	1690	31
Arsenite concentration, $5 \cdot 10^{-3}$ *M*		

* Chloroplast fragments and extract were prepared as described in the MATERIALS AND METHODS section.

** Chloroplasts were not washed with 0.35 *M* NaCl before fragments and extract were prepared.

TABLE IV

EFFECT OF BAL ON ARSENITE INHIBITION OF $^{14}CO_2$ BY WASHED WHOLE CHLOROPLASTS

Expt.	*Compounds concn. (M)*	$^{14}CO_2$ *fixed counts/min/aliquot*	*% inhibition**	*% reactivation***
1	Complete	1415	—	—
	BAL $2 \cdot 10^{-4}$	1610	—	—
	BAL $2 \cdot 10^{-4}$, arsenite $7 \cdot 10^{-5}$***	780	52	0
	Arsenite $7 \cdot 10^{-5}$	665	53	—
2	Complete	600	—	—
	BAL $1 \cdot 10^{-3}$	845	—	—
	BAL $1 \cdot 10^{-3}$, arsenite $7 \cdot 10^{-5}$***	220	74	0
	Arsenite $7 \cdot 10^{-5}$	210	65	—

* Percent inhibition = [counts/min (complete) — counts/min (arsenite)]/counts/min (complete) × 100.

** Percent reactivation = [counts/min (after reactivation) — counts/min (arsenite)]/[counts/min (BAL) — counts/min (arsenite)] × 100.

*** Order of addition, BAL at zero time; arsenite 10 min later.

TABLE V

EFFECT OF ARSENITE AND BAL ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM

Expt.	*Compounds concn. (M)*	$^{14}CO_2$ *fixed counts/min/aliquot*	*% inhibition**	*% reactivation***
1	Complete	3880	—	—
	BAL $5 \cdot 10^{-4}$	1370	65	—
	BAL 10^{-4}	1495	61	—
	BAL $5 \cdot 10^{-4}$, arsenite 10^{-5}***	1035	—	64
	BAL 10^{-4}, arsenite 10^{-5}***	490	—	5
	Arsenite 10^{-5}	440	89	—
2	Complete	630	—	—
	BAL $5 \cdot 10^{-4}$	408	35	—
	BAL $5 \cdot 10^{-4}$, arsenite 10^{-5}***	314	—	75
	Arsenite 10^{-5}, BAL $5 \cdot 10^{-4}$§	330	—	77
	Arsenite 10^{-5}	35	94	—

* Percent inhibition = [counts/min (complete) — counts/min (arsenite)]/counts/min (complete) × 100.

** Percent reactivation = [counts/min (after reactivation) — counts/min (arsenite)]/[counts/min (BAL) — counts/min (arsenite)] × 100.

*** Order of addition, BAL at zero time; arsenite 10 min later.

§ Order of addition, arsenite at zero time, BAL 10 min later.

the intact chloroplasts resulted in a 2-fold increase in sensitivity to arsenite (Table III).

The data recorded in Table IV and V show that while BAL has little or no effect on the CO_2 fixing ability of the intact chloroplast, it can prevent as well as reverse the inhibition of CO_2 fixation by the reconstituted system caused by arsenite. It is also interesting to note that BAL itself is inhibitory. A similar effect has been noted for pyruvic acid oxidase which is also sensitive to arsenite[7].

Effect of arsenite on individual components of the CO_2-fixing system

Arsenite at 10^{-3} *M*, under the conditions employed, failed to inhibit the following enzymes or systems: ribulose diphosphate carboxylase, transketolase, transaldolase, ribose-5-phosphate isomerase, ribulose-5-phosphate kinase, TPN and DPN glyceral-

dehyde-3-phosphate dehydrogenase, 3-phosphoglyceric acid kinase, phosphoenolpyruvic acid carboxylase, photosynthetic phosphorylation using phenazine methosulfate, vitamin K_3 and riboflavin-5'-phosphate as electron acceptor and photoreduction of TPN accompanied by O_2 evolution and phosphorylation.

DISCUSSION

A comparison of the rate of CO_2 fixation by the intact chloroplast and the reconstituted chloroplast system brought out similarities as well as differences in their behaviour toward certain reagents. Thus, compounds like iodoacetamide, arsenite and *p*-CMB, which are considered to be non-competitive inhibitors affect both systems to the same extent. In contrast, while phosphate and arsenate ions presumably affect the same site in both systems, the concentration of each to attain this effect differs considerably. In addition, the concentrations of intermediates of the various cycles must surely differ in both systems and this may play a role in yielding variation. Clearly, the possibility must be considered that the structural organization plays an important role not only for the photochemical act of photosynthesis but also for subsequent carbon metabolism.

While at least one site of inhibition can be assigned to iodoacetamide, *p*-CMB, arsenate, and phosphate, the particular enzyme, or enzymes affected by arsenite is not clear. The data recorded here on the effect of arsenite on the photolysis of water, the photochemical formation of TPNH and ATP, are in agreement with the results obtained by SAN PIETRO AND LANG[8] and ARNON *et al.*[9] on the synthesis of "reducing power" in broken cell spinach preparations and by MCFADDEN AND ATKINSON[10] on the electron transfer from H_2 to O_2 in the intact, autotrophic bacterium, *Hydrogenomonas facilis*, and are thus consistent with the expectation that the site of arsenite inhibition is apparently the CO_2 reduction cycle.

Most of the enzymes which are usually considered to compose the photosynthetic carbon cycle were tested and found to be insensitive to arsenite.

The susceptibility of an enzyme catalyzed reaction to arsenite has generally been ascribed to the need for lipoic acid as a cofactor[11,12]. More recently, NIRENBERG AND JAKOBY[13] have found that various partially purified aldehyde dehydrogenases are inhibited by arsenite and that lipoic acid is not a participant. In this system, susceptibility is ascribed to the presence of two closely juxtaposed cysteine moieties. In both types, a dimercaptan is able to reverse the inhibition.

In a particulate system, another possibility exists; namely, that two sulfhydryl containing enzymes, individually unreactive toward arsenite, may be brought into proper position to produce a dithiol type structure. On fragmentation, the susceptibility toward arsenite would decrease. However, in the present study, this is apparently ruled out since the fragmented system as well as the intact system yielded similar inhibition values with arsenite.

Clearly, the results of this study does not settle the question of a site of arsenite inhibition in photosynthesis.

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UNBOUND SIALIC ACIDS IN FISH EGGS

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SUMMARY

Relatively large quantities of sialic acids occur in an unbound form in trout eggs.

Both N-glycolyl and N-acetylneuraminic acid are found within one cell, the trout egg.

Preliminary studies on the permeability of the egg to free sialic acids are reported.

INTRODUCTION

Sialic acids[1–3] are found in mammals[4,5], birds, amphibians[5], fish[6,7], bacteria[8], and in the eggs of the sea urchin[9]. Several forms of sialic acids (N-glycolyl, N-acetyl, N,O-diacetylneuraminic acid) have been described in the tissues of different animal species. Virtually all of the sialic acids found in tissues are bound either in large protein-carbohydrate complexes or in oligosaccharides such as neuramin-lactose of the mammary gland[10,11].

In this paper we wish to report some unusual characteristics of the sialic acids of trout eggs. Not only is much of the sialic acid free, but two forms of sialic acid are found in one egg.

MATERIALS AND METHODS

Eggs of the eastern brook trout (*Salzelinus fontinalis*) and rainbow trout (*Salmo gairdneri*) were kindly supplied by Dr. K. E. WOLF, Eastern Fish Disease Laboratory,