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# PHOSPHATASE ACTION ON PHOSPHOGLYCOLIC, 3-PHOSPHOGLYCERIC, AND PHOSPHOENOL PYRUVIC ACIDS IN SPINACH CHLOROPLAST FRAGMENTS IN THE PRESENCE AND ABSENCE OF HIGH CONCENTRATIONS OF METHANOL

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## SUMMARY

Sonically broken spinach chloroplasts, before and after storage in the frozen state, were checked for their ability to phosphatase $^{14}C$-labelled phosphoglycolic, phosphoglyceric, and phosphoenol pyruvic acids, and a variety of sugar phosphates, in aqueous mixtures at room temperature, and in 50 % and 80 % aqueous methanol at 0°.

Only phosphoglycolic acid was found to be hydrolysed rapidly under the conditions examined, except in 80 % methanol. The other organic phosphates tested were metabolised in the absence of methanol to a number of other compounds usually built up during photosynthesis; in 50 % methanol they were hydrolysed either very slowly (phosphoglyceric and phosphoenol pyruvic acids) or not at all (sugar phosphates); in 80 % methanol they remained unchanged.

## INTRODUCTION

The detection of residual phosphatase activity in chloroplast fragments, algae, and several other biological materials suspended in alcohol as concentrated as 80 % (see ref. 1), led to the suspicion that this unexpectedly stable phosphatase might hydrolyse metabolic phosphates in experimental samples after the usual "killing" procedure of adding 4 volumes of cold alcohol. If this were so, the resulting label distribution obtained from the paper chromatographic analysis, would be invalid.

For this reason, a number of $^{14}C$-labelled phosphates appearing during photosynthetic $^{14}C$-fixation, and readily available by elution of the appropriate spots from two-dimensional chromatograms were checked. The main interest was in the hydrolysis of three phosphorylated hydroxy acids:

1. Phosphoglycolic acid, which, according to RICHARDSON AND TOLBERT[2], is rapidly split by phosphoglycolic acid phosphatase, and which is assumed by the same authors to be the only immediate precursor of free glycolic acid in photosynthesis.

2. 3-Phosphoglyceric acid which can be phosphatased to free glyceric acid;

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however, MORTIMER[3-5] considered this was also to account only for part of the free glyceric acid formed in certain higher plants *e.g.* soy beans. MORTIMER therefore deduced the existence of a different and not yet explored pathway of photosynthesis, involving glyceric acid derived from another precursor.

3. Phosphoenol pyruvic acid, which, as a high-energy phosphate, was supposed to be easily phosphatased even in mixtures with high alcohol content, because of its higher reactivity as compared to phosphoglycolic and phosphoglyceric acids.

## EXPERIMENTAL

### *Substrates*

Spots of $^{14}C$-labelled compounds, listed in Table I, were cut out from paper chromatograms obtained from usual photosynthesis experiments and run in the first dimension with either phenol–water (73:27) or "semistench"* for approx. 25 or 40 h respectively, and in the second dimension with butanol–propionic acid–water** for approx. 20 h. The spots were washed with diethyl ether to remove traces of the chromatographic solvents, and subsequently eluted with water. The activity of the substrate solutions varied within a wide range and depended mainly on the original activity of the eluted spots, the number of spots available for elution, and, to a minor extent, on the amount of water applied for the elution. When not immediately used, the substrate solutions were stored frozen.

TABLE I

SUBSTRATES USED FOR THE EXPERIMENTS

| | |
|---|---|
| Phosphoglycolic acid | Ribose 5-phosphate |
| 3-Phosphoglyceric acid | Glucose 6-phosphate |
| Phosphoenol pyruvic acid | Fructose 6-phosphate |
| Dihydroxyacetone-phosphate | Sedoheptulose 7-phosphate |
| 6-Phosphogluconic acid | Ribulose 1,5-diphosphate |
| Uridine diphosphoglucose | Fructose 1,6-diphosphate |

### *Chloroplast fragments*

Spinach chloroplasts were isolated by the aqueous procedure of PARK *et al.*[7], and, after resuspension in 0.025 M phosphate buffer (pH 7.5) sonicated for 90 sec in a Raytheon sonicator. The chlorophyll concentration of the suspension was adjusted to approx. 2.0 mg/ml. When not for immediate use, the chloroplast fragment suspension was stored in a deep freeze. This storage resulted in a considerable loss of photosynthetic activity, but had little effect on the phosphatase activity under investigation.

### *Incubation experiments*

The above substrate solutions were mixed with equal volumes of 0.2 M glycylglycine–NaOH buffer (pH 7.5). To samples (0.1 ml) of these mixtures, contained in

* Semistench[6]: 1.2 g EDTA + 100 ml 27% aq. $NH_3$ + 950 ml water + 350 ml *n*-propanol + 75 ml isopropanol + 75 ml *n*-butanol + 2.5 l isobutyric acid; aged for at least 24 h before use.

** Equal volumes of: a, 3.7 l *n*-butanol + 250 ml water; b, 1.8 l propionic acid + 2.2 l water, mixed immediately before use.

small screw cap vials, 0, 0.2 or 0.8 ml of methanol were added to make the alcohol concentration in the incubation mixtures 0, 50, or 80 % respectively. After adjusting to the desired temperature, 0.1 ml of chloroplast fragment suspension was added to each vial (zero time), and the samples were allowed to stand in weak daylight for the desired incubation times. The incubation was then stopped by heating the closed vials in boiling water for 3–4 min; methanol was added where necessary to bring the final concentration in each case to 80 % for this procedure. The total samples were later spotted onto the origins of one-dimensional paper chromatograms which were run in "semistench" for approx. 15 h to the edge of the paper (45–50 cm). The spots, localised by autoradiography on single-coated X-ray film, were subsequently counted for their relative activity by an automatic spot counting machine constructed in this laboratory[8]. The two tubes of this machine usually count 10.7 % of the total $^{14}C$-disintegrations occurring in spots on Whatman No. 4 paper, which was used for the described experiments.

## RESULTS

### *Sugar phosphates*

Incubation of the sugar phosphates listed in Table I with freshly prepared chloroplast fragments at room temperature without addition of methanol for 10–60 min resulted in the formation of a variety of other metabolic products, among them sucrose, other sugar phosphates, different from the starting material, amino acids, and glycolic acid. In no case could the dephosphorylated starting material be identified on one-dimensional chromatograms made from the samples. In the presence of 50 % or 80 % methanol there was no detectable reaction at all, either at 0° or at room temperature.

### *Phosphoglycolic acid*

Incubation of phosphoglycolic acid with freshly prepared chloroplast fragments at room temperature without addition of methanol led to rapid hydrolysis of the ester bond, as is shown in Table II. No other products appeared on the chromatograms, which were sprayed with saturated $NaHCO_3$ solution after drying, in order to prevent the glycolic acid from volatilising during the film exposure. The losses could be kept very small by this procedure.

Incubation of phosphoglycolic acid with frozen stored chloroplast fragments in a mixture containing 50 % methanol at 0° showed only a small decrease in the rate of cleavage (Table II). Performance of this experiment in a methanol concentration of 80 % yielded no free glycolic acid.

### *Phosphoglyceric acid*

The hydrolysis of phosphoglyceric acid was found to be under all circumstances much slower than that of phosphoglycolic acid. In the absence of methanol, fresh chloroplast fragments at room temperature formed a similar variety of compounds from phosphoglyceric acid as from sugar phosphates, so that quantitative measurements could not be made on one-dimensional chromatograms. The amount of free glyceric acid observed was small in any case.

Incubation with frozen stored chloroplast fragments at 0° in mixtures containing

TABLE II

CLEAVAGE OF PHOSPHOGLYCOLIC ACID

| Time (min) | In the absence of methanol at 22° (counts/min) | | | Glycolic acid (%) | In 50% methanol (by volume) at 0° (counts/min) | | | Glycolic acid (%) |
|---|---|---|---|---|---|---|---|---|
| | Glycolic acid | Phospho-glycolic acid | Σ | | Glycolic acid | Phospho-glycolic acid | Σ | |
| 0 | 0 | 3260 | 3260 | 0 | 15 | 1225 | 1240 | 1 |
| 0.5 | — | — | — | — | 180 | 1100 | 1280 | 14 |
| 1 | — | — | — | — | 470 | 790 | 1260 | 37 |
| 2 | 1620 | 1500 | 3120 | 52 | 520 | 700 | 1220 | 43 |
| 3.5 | — | — | — | — | 595 | 710 | 1305 | 46 |
| 5 | 2580 | 610 | 3190 | 81 | 825 | 450 | 1275 | 65 |
| 7.5 | — | — | — | — | 1030 | 210 | 1240 | 83 |
| 10 | 3020 | 360 | 3280 | 92 | 1210 | 80 | 1290 | 94 |
| 20 | 3140 | 70 | 3220 | 98 | 1230 | 20 | 1250 | 98 |
| 60 | 3150 | 20 | 3170 | 100 | 1270 | 30 | 1300 | 98 |

50% methanol resulted in a slow hydrolysis of phosphoglyceric acid (Table III) without the formation of any other compound. In 80% methanol, under otherwise the same conditions, no hydrolysis of phosphoglyceric acid could be detected.

*Phosphoenol pyruvic acid*

In the absence of methanol, phosphoenol pyruvic acid was metabolised by frozen stored chloroplast fragments at room temperature to several compounds (fewer than with sugar phosphates or phosphoglyceric acid as starting material; so they could be separated by one-dimensional chromatography) (Table IV). Among them were pyruvic acid and alanine, its transamination product, and an unidentified spot (Y). There was also an impurity always showing up in the same quantity (X).

In mixtures containing 50% methanol, the rate of hydrolysis of phosphoenol pyruvic acid at 0° was of the same order as that of phosphoglyceric acid under the same conditions (Table III). Even the samples immediately heated at zero time contained some free pyruvic acid, apparently formed by slow non-enzymic hydrolysis of the high-energy phosphate bond during the storage before chromatography. This effect was eliminated by keeping the sum of incubation and storage period constant

TABLE III

CLEAVAGE OF PHOSPHOGLYCERIC AND PHOSPHOENOL PYRUVIC ACID IN 50% METHANOL AT 0°

| Time | (Counts/min) | | | Glyceric acid (%) | (Counts/min) | | | | Pyruvic acid (%) |
|---|---|---|---|---|---|---|---|---|---|
| | Glyceric acid | 3-Phospho-glyceric acid | Σ | | Pyruvic acid | Phosphoenol pyruvic acid | X | Σ | |
| 0 | 65 | 5810 | 5875 | 1 | 180 | 1720 | 225 | 2125 | 8.5 |
| 10 min | 105 | 5350 | 5455 | 2 | 165 | 1660 | 200 | 2025 | 8.2 |
| 1 h | 190 | 5220 | 5410 | 4 | 260 | 1510 | 210 | 1980 | 13.1 |
| 5 h | 400 | 4160 | 4560 | 9 | 335 | 1380 | 230 | 1945 | 17.3 |
| 25 h | 1270 | 3670 | 4940 | 26 | 875 | 880 | 250 | 2005 | 43.7 |
| 50 h | — | — | — | — | 1380 | 420 | 245 | 2045 | 67.5 |
| 100 h | 3640 | 2030 | 5670 | 64 | — | — | — | — | — |

TABLE IV

CONVERSION OF PHOSPHOENOL PYRUVIC ACID AT 22° IN THE ABSENCE OF METHANOL

| Time (min) | (Counts/min) | | | | | | Alanine (%) | Pyruvic acid (%) | Alanine + pyruvic acid (%) |
|---|---|---|---|---|---|---|---|---|---|
| | Alanine | Y | Pyruvic acid | Phosphoenol pyruvic acid | X | Σ | | | |
| 0 | 0 | 0 | 155 | 1620 | 170 | 1945 | 0 | 8.0 | 8.0 |
| 2 | 45 | 40 | 215 | 1500 | 270 | 2070 | 2.2 | 10.4 | 12.6 |
| 5 | 90 | 50 | 335 | 1330 | 275 | 2080 | 4.3 | 16.1 | 20.4 |
| 10 | 180 | 80 | 470 | 1210 | 345 | 2285 | 7.9 | 20.7 | 28.6 |
| 25 | 435 | 155 | 475 | 450 | 350 | 1865 | 23.3 | 25.5 | 48.8 |
| 60 | 300 | 290 | 300 | 130 | 245 | 1675 | 42.4 | 17.9 | 60.3 |

for all the samples of the experiment. No enzymic formation of free pyruvic acid could be detected in samples treated in the same way, but containing 80% methanol.

## DISCUSSION

Of the phosphates examined, only phosphoglycolic acid is phosphatased with a high rate, at least in mixtures containing from 0 up to 50% methanol. It is quite likely that, if not destroyed immediately by heating, the crucial phosphatase often remains active for a while even in 80% methanol, especially when fresh chloroplasts or algae are used for the experiments instead of the frozen stored ones which have already been damaged to a certain extent by the freezing and thawing procedures. Thus a fraction of the glycolic acid found in photosynthesis experiments might be due to cleavage of phosphoglycolic acid after the still rather common "killing" procedure by simple dumping the samples, withdrawn from a lollipop or another incubation apparatus, into 4 volumes of cold methanol, storage for some time (a few minutes up to several hours) and subsequent heating which definitely kills all the enzymes. The rest is likely to be formed from phosphoglycolic acid by rapid enzymic hydrolysis during the incubation according to TOLBERTS's[2] theory that all glycolic acid derives from phosphoglycolic acid, which is formed first and then phosphatased during the incubation.

The phosphatase under investigation and the one responsible for the esterification of phosphate with added alcohols[1] might be identical with TOLBERT's recently isolated phosphoglycolic acid phosphatase[2] which has not yet been tested in the presence of high alcohol concentrations.

Phosphoglyceric acid, on the other hand, is phosphatased too slowly, even in the absence of methanol, so that the results of this work cannot be used, as far as isolated spinach chloroplasts are concerned, either for or against MORTIMER's proposal[3-5] of another pathway in photosynthesis to explain the appearance of appreciable quantities of free glyceric acid. But the results show that there is not much danger of getting additional free glyceric acid by the action of the surviving phosphatase after "killing" samples with cold methanol and heating them later up to several hours.

Although phosphoenol pyruvic acid is susceptible to very slow non-enzymic hydrolysis and, in the absence of methanol, is metabolised at a moderate rate to pyruvic acid and further to alanine, the enzymic hydrolysis of this substance in

alcoholic mixtures, like that of phosphoglyceric acid, was found to be too slow seriously to interfere with the results obtained by the mentioned "killing" procedure and subsequent paper chromatography. This is even more true for all the sugar phosphates tested.

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