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## STUDIES ON THE CARBOXYLATION REACTION OF PHOTOSYNTHESIS

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## ABSTRACT

Studies have been made on the carboxylation of ribulose-1,5-diphosphate by cell-free extracts of Chlorella; this reaction has been shown to be independent of light. The action of various cofactors and inhibitors has been studied, and a scheme of reactions catalyzed by the extract, which is consistent with the data, is presented.

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## INTRODUCTION

Previous work in this laboratory with intact photosynthetic organisms[1] and cell-free preparations of Chlorella[2] indicated that ribulose-1,5-diphosphate (RuDP) acts as the primary carbon dioxide acceptor of photosynthesis, the RuDP being carboxylated to yield phosphoglyceric acid as the first and major reaction product.

The work reported here was undertaken as a study of this carboxylation reaction in cell-free systems, with particular reference to the effect of various metabolic inhibitors thereon. Although the reactions occurring in such systems are many and varied, it was possible, by this approach, to obtain information on the stability of the carboxylating enzyme or enzymes, and to shed some light on the sequence of transformations of carbon compounds in photosynthesis.

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active areas was determined by direct counting of $C^{14}$ on the paper.

$CO_2$-Fixation Experiments

In experiments where $C^{14}$-RuDP was used, the source of carbon dioxide was 40 µl of 0.025 M unlabeled sodium bicarbonate adjusted to pH 7.6.

Purification of Charcoal

Before it was used to remove pyridine nucleotides from solution, "Nuchar 00" charcoal was suspended in boiling HCl (10%) for one hour, filtered, washed to neutrality with distilled water, and dried at 100° C.

## RESULTS

The Stability of the Carboxylation Enzyme System

Storage of the cell-free extract at 3° for periods up to 24 hours did not adversely affect the power of the extract to catalyze the carboxylation of RuDP; on the contrary, the total quantity of phosphoglycerate (PGA) formed increased threefold after 24 hours' storage (Table I). Similarly, the total radioactivity appearing as aspartate increased sevenfold during this time. This increase in the amounts of $C^{14}O_2$ fixed into PGA is probably due to a stabilization of the RuDP, i.e., a progressive deterioration of enzyme systems tending to remove RuDP by routes other than the carboxylation reaction. The increased appearance of the $C^{14}$ in aspartate similarly argues for a preservation of the enzyme systems carboxylating pyruvate and forming aspartate from oxalacetate or malate, while other systems--e.g., those enzymes involved in the oxidation of pyruvate--deteriorate. This latter effect is similar to the results noted after treatment of the cell-free extracts with iodoacetate (see later).

It is thus apparent that the enzyme systems involved in the carboxylation reaction are stable over the time tested, and that extracts aged up to 24 hours could be safely employed as a source of carboxylating enzymes.

The Effect of Illumination on the Carboxylation Reaction

In the preliminary studies of the carboxylation reaction, it had been noted that when sonically disrupted Chlorella suspensions were centrifuged at low speeds (3,000 rpm), and the supernatant solutions were used as the carboxylating system, the extracts appeared to fix thrice as much $C^{14}O_2$ into PGA in the light as in the dark (Table II, Tubes 1 and 3). If the supernatant solutions were centrifuged at 40,000 rpm for 15 minutes in a "Spinco" Centrifuge (No. 4 head), a quantity of green solid was removed, the remaining solutions were very pale green in color, and -- in the dark -- fixed the same quantity of $C^{14}O_2$ as did the slower-spun extract under the same conditions. In the light the fixation was increased by a factor of about 50% Table II, (Tubes 2 and 4).

Table II

The effect of illumination and temperature on the carboxylation reaction

Tubes 1 to 4, 5 to 8, and 9 to 11 contained, respectively, extracts from three different batches of Chlorella. Each tube contained 100 µl extract in 0.02 M phosphate, pH 6.8; 50 µl RuDP Solution, 40 µl 0.025 M $NaHC^{14}O_3$, pH 7.6 (400 µc/ml), and water to 270 µl. Incubation time = 12 min.

| Tube No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Chlorella extract used | 3,000 | 40,000 | 3,000 | 40,000 | 3,000 | 40,000 | 3,000 | 40,000 | 40,000 | 40,000 | 40,000 |
| RuDP used | 1 | 1 | 1 | 1 | 2a | 2a | 2a | 2a | 2a | 2a | 2a |
| Illumination | dark | dark | light | light | dark | dark | light | light | dark | dark | dark |
| Temperature (°C) | 25 | 25 | ? | ? | 22.5 | 22.5 | 22.5 | 22.5 | 0 | 25 | 37 |
| PGA formed (counts/min) | 630 | 640 | 1720 | 930 | 980 | 940 | 880 | 970 | 100 | 2,000 | 2,440 |
| Aspartic Acid formed(counts/min) | 190 | 120 | 490 | 170 | <50 | <50 | <50 | <50 | ... | 570 | 1,650 |

# THE EFFECT OF INHIBITORS ON THE CARBOXYLATION REACTION SYSTEM

## A. Studies with $C^{14}O_2$ and unlabeled RuDP

### 1. The effect of iodoacetate (IAA)

When *Chlorella* extracts were incubated with RuDP and $C^{14}$-sodium bicarbonate, in the presence of iodoacetate, it was noticed that no inhibition of the carboxylation reaction occurred but that, on the contrary, there was a considerable increase in the total radioactivity incorporated, as measured by counting the "origin" spot of the chromatograms prior to development (Table III). This increased radioactivity was not, however, due to an increase in the amount of PGA formed, but was due to a greater incorporation of $C^{14}$ into malate and aspartate. In the presence of $10^{-3}$ M iodoacetate, this incorporation of $C^{14}$ into malate and aspartate was slight (less than 20% of the labeled PGA formed); in the presence of $10^{-2}$M iodoacetate, however, the labeled malate and aspartate formed contained together over 50% of the total radioactivity fixed into PGA by the extract. It was therefore apparent that the action of iodoacetate on the reaction mixture was to leave the carboxylation reaction quite unaffected and to facilitate the fixation of $C^{14}O_2$ into malate and aspartate. Since it was known that the oxidation of pyruvate via the Krebs cycle is inhibited by iodoacetate, it was conceivable that this increased $C^{14}O_2$ incorporation into malate and aspartate was a symptom of the preservation of pyruvate (probably arising by glycolysis) in the extract, thus allowing the fixation of $CO_2$ by pyruvate (to yield first oxalacetate or malic and, secondly, by transamination, to give aspartate) to play a greater role than in the absence of the inhibitor.

The result of an experiment to test this point is given in Table IV. The normally observed incorporation of $C^{14}$ into PGA, with only slight formation of aspartate and malate, was shown by this extract when incubated with RuDP and $NaHC^{14}O_3$ (Tube 2). When RuDP was left out, no PGA and only a trace of malate appeared (Tube 1). In the presence of iodoacetate ($10^{-2}$M) but in the absence of RuDP, there was no formation of labeled PGA, but labeled malate and aspartate were formed in quantities similar to those found in the experiments recorded in Table III (Tube 3). When pyruvate ($10^{-2}$M) was added to the extract in the absence of RuDP and IAA (Tube 4), there was again no formation of labeled PGA, but amounts of labeled malate and aspartate in excess of those found in Tube 1 appeared. Similarly, in the presence of pyruvate and iodoacetate, but in the absence of RuDP, considerable quantities of labeled malate and aspartate were found (Tube 5), although here a small amount of PGA also appeared. With boiled *Chlorella* extract (Tubes 6 and 7), no labeled materials appeared on the chromatograms.

These results show that the fixation of $C^{14}O_2$ by the *Chlorella* extracts in the presence of IAA proceeds via at least two independent paths: (a) the fixation of $C^{14}O_2$ by RuDP to form labeled PGA, unaffected by the presence of IAA, and (b) the fixation of $C^{14}O_2$ by (probably) pyruvate to form oxalacetate or malate and, by transamination, aspartate. This aspect is further discussed with the experiments performed with cyanide as inhibitor.

Table IV

The incorporation of $C^{14}O_2$ into aspartate in the absence of RuDP

Each tube contained the reaction mixture (RM) of 100 µl cell-free extract of Chlorella (40,000) + 40 µl $C^{14}$-$NaHCO_3$ solution, other substances being added as marked in the table. Total volume: 210 µl. Incubation time: 10 minutes. Temperature: 25°.

| | | $C^{14}O_2$ Incorporated into | | |
|---|---|---|---|---|
| Tube | Contents | PGA | Aspartate | Malate |
| 1 | RM | ... | ... | <50 |
| 2 | RM + RuDP[2] | 1110 | 70 | 50 |
| 3 | RM + NaIAA($10^{-2}$M) | ... | 760 | 550 |
| 4 | RM + Na pyruvate($10^{-2}$M) | ... | 100 | 130 |
| 5 | RM + Na pyruvate($10^{-2}$M)+ NaIAA($10^{-2}$M) | 100 | 500 | 500 |
| 6 | Boiled extract + $C^{14}$-$NaHCO_3$ | ... | ... | ... |
| 7 | Boiled extract + $C^{14}$-$NaHCO_3$ NaIAA ($10^{-2}$M) | ... | ... | ... |

Table V

The effect of sodium fluoride on the incorporation of $C^{14}O_2$

Each tube contained 100 µl Chlorella extract + 50 µl RuDP solution + 40 µl $NaHC^{14}O_3$ (RM). The Chlorella extract used was spun at 40,000 rpm in all experiments except No. 24, in which it was spun at 3,000 rpm.

| Experiment No. | Contents | Total Volume (µl) | Incubation Time (min) | Temperature (°C) | $C^{14}$ Incorporated into PGA | PEP | Asp. | Mal. |
|---|---|---|---|---|---|---|---|---|
| 20 | RM[1] | 210 | 10 | 24 | 1200 | <50 | <50 | <50 |
| | RM + $10^{-3}$M-NaF | 210 | 10 | 24 | 1400 | ... | <50 | <50 |
| 21 | RM[1] | 210 | 10 | 22 | 1050 | <50 | <50 | ... |
| | RM + $10^{-2}$M-NaF | 210 | 10 | 22 | 1500 | ... | ... | ... |
| 24 | RM[2a] | 210 | 10 | 25 | 2160 | 70 | 50 | <50 |
| | RM + $10^{-2}$M-NaF | 210 | 10 | 25 | 3400 | ... | 70 | <50 |
| | RM[2a] | 210 | 10 | 40? | 5200 | 60 | 170 | 60 |
| | RM + $10^{-2}$M-NaF | 210 | 10 | 40? | 6200 | ... | 100 | ... |
| 25 | RM[2a] | 270 | 12 | 22 | 970 | ... | 170 | ... |
| | RM + $10^{-2}$M-IAA ± $10^{-2}$M-NaF | 270 | 12 | 22 | 1570 | ... | 540 | 140 |

The addition of ATP to the carboxylation system caused no stimulation of labeled PGA formation (Experiment 23); indeed, the amount of $C^{14}O_2$ incorporated into PGA in the presence of ATP was less than 40% of that fixed in the absence of ATP.

For investigation of the effect of $TPN^+$ on the carboxylation, it was desirable to remove any of the nucleotide already existing in the extract; preliminary tests showed that $TPN^+$ added to M/50 phosphate buffer, pH 6.8, to a final concentration of $10^{-3}$M, could be quantitatively removed by treating the extract (4 ml) with washed charcoal (20 mg) at $0^o$ for 3 minutes and filtering through sintered glass. Such a procedure has been used for removal of $DPN^+$ from glyceraldehyde-3-phosphate dehydrogenase preparations by Taylor et al.[5] Chlorella extract, centrifuged at 40,000 rpm and similarly treated with charcoal, was still capable of carboxylating RuDP with only a 20% reduction of activity (Experiment 21). Addition of $TPN^+$ to the charcoal-treated extract caused a small stimulation of $C^{14}O_2$ fixation (22%) into PGA; addition of $TPN^+$ to extract that had not been treated with charcoal caused an apparent stimulation of 40% of PGA formation. These results suggest that $TPN^+$ does not participate directly in the carboxylation reaction as, even if some protein-bound $TPN^+$ had not been removed by the charcoal treatment, the magnitude of the $TPN^+$ effect is too small for a direct involvement of the nucleotide in the $CO_2$-fixation process. Subsequent studies on the partially purified carboxylation system from spinach have indeed shown that $TPN^+$ is not involved in the fixation of $CO_2$ by RuDP to form PGA.[6,7,8]

## The Effect of Cyanide

Potassium cyanide was found to exert an inhibitory effect on the carboxylation of RuDP ranging from 98% inhibition at $10^{-2}$ M concentration to ~2% at $10^{-4}$ M (Table VII). The action of this inhibitor could be due either to cyanhydrin formation with the RuDP, or to metal complex formation should the carboxylation require the presence of a metal either as ion or metalloprotein. It was not possible, at the time of writing, to distinguish between these possibilities.

It is interesting to note that at the high concentration of $10^{-2}$ M KCN, which should have removed any pyruvic acid as cyanhydrin, the incorporation of $C^{14}O_2$ into malic and aspartic acids still proceeded to approximately the same extent. This indicates that the formation of malic and aspartic acids may proceed via a system other than the "malic" enzyme or the Wood-Werkman reaction, e.g., the carboxylation of PEP to give oxalacetate, as reported by Bandurski and Greiner.[9] If this system is mainly responsible, then the proposed explanation for the enhanced fixation in the presence of iodoacetate, as previously discussed, applies equally well, as any stabilization of the pyruvate pool would lead to an increased concentration of PEP.

## The Effect of o-phenathroline, K-chloro-Hg-benzoate and glutathione

The effect of these reagents on the carboxylation of RuDP is summarized in Table VIII. o-Phenathroline at a concentration of $10^{-3}$M had very little effect on the incorporation of $C^{14}O_2$ into PGA; raising the concentration to $10^{-2}$ M caused the PGA count to drop to 37% of its value in the uninhibited system. It seems unlikely from this result that the presence of iron is necessary for the carboxylation reaction.

Table VIII

The effect of o-phenanthroline, K chloro-Hg-benzoate, and glutathione on the incorporation of $C^{14}O_2$

Each tube contained 100 µl *Chlorella* extract, centrifuged at 40,000 rpm, + 50 µl RuDP solution + 40 µl $C^{14}$-$NaHCO_3$ + water to 190 µl; this mixture is referred to as RM, other substances added as marked. Incubation time: 10 minutes.

| Contents of tubes | Total $C^{14}O_2$ fixed on origin of paper | $C^{14}$ incorporated into | | | |
|---|---|---|---|---|---|
| | | PGA | PEP | Asp. | Malate |
| RM | 5120 | 3670 | 700 | 90 | <50 |
| RM + o-phenanthroline ($10^{-3}$ M) | 6000 | 4180 | 810 | <50 | <50 |
| RM + K chloro-Hg-benzoate ($10^{-3}$ M) | 600 | 340 | ... | ... | ... |
| RM + glutathione ($10^{-3}$ M) | 4280 | 3420 | 560 | <50 | <50 |

Table IX

Studies with $CO_2$ and $C^{14}$-RuDP

A. Test of carboxylating activity of extract.

Both tubes contained the reaction mixture (RM) of 100 µl Chlorella extract (40, 000) + 40 µl $NaHC^{14}C_3$, unlabeled RuDP being added as marked. Total volume = 250 µl. Incubation time: 10 minutes. Temperature: 25°.

| Tube No. | Contents | $C^{14}O_2$ incorporated into PGA |
|---|---|---|
| 1 | RM | ... |
| 2 | RM + RuDP[3] | 1190 |

B. Purity of $C^{14}$-RuDP

| Tube No. | Contents | Total counts used | Ribulose | Hexose |
|---|---|---|---|---|
| 3 | $C^{14}$-RuDP + phosphatase | 12, 600 | 8, 440 | 780 |

C. Action of extract on $C^{14}$-RuDP, with and without inhibitors present.

Each tube contained the reaction mixture (RM) of 100µl Chlorella extract (40, 000) + 40 µl $NaHCO_3$ solution, other substances being added as marked in the table. Total volume = 250 µl. Incubation time = 12 minutes. Temperature = 25°.

| Tube No. | Contents | Total counts put on paper | Remaining on origin of chromatogram | Unchanged RuDP | PGA | P-glycolic acid | Hexose + heptose-P | Pentose-P | Triose-P | Recovery of counts | % Recovery |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | RM*+$C^{14}$-RuDP | 23, 300 | 2540 | 13, 580 | 340 | 760 | 250 | 800 | 220 | 18, 490 | 79 |
| | | | | | | | Radioactivity in these regions, compounds not identified | | | | |
| 5 | RM+$C^{14}$-RuDP | 23, 300 | 1200 | 12, 020 | 665 | 625 | 1590 | 1480 | 800 | 18, 380 | 79 |
| 6 | Repeat of (5) | 22, 300 | 1240 | 9, 870 | 800 | 600 | 1530 | 1130 | 540 | 15, 710 | 70 |
| 7 | RM+$C^{14}$-RuDP+ IAA ($10^{-2}$M) | 26, 200 | 1070 | 12, 450 | 1100 | 980 | 1620 | 1410 | 720 | 19, 350 | 74 |
| 8 | RM+$C^{14}$-RuDP+ NaF($10^{-2}$M) | 25, 200 | 1250 | 12, 610 | 980 | 480 | 1580 | 1050 | 750 | 18, 700 | 74 |
| 9 | RM+$C^{14}$-RuDP+ 2:4DNP($10^{-3}$M) | 25, 900 | 1280 | 10, 080 | 1000 | 660 | 1380 | 1190 | 510 | 16, 100 | 62 |
| 10 | RM+$C^{14}$-RuDP+ IAA($10^{-2}$M) +NaF($10^{-2}$M)+ 2:4DNP($10^{-3}$M) | 23, 800 | ← 13, 350 → | | 1350 | 2750 | ← | 2530 | → | 19, 980 | 84 |

(*) Boiled extract used

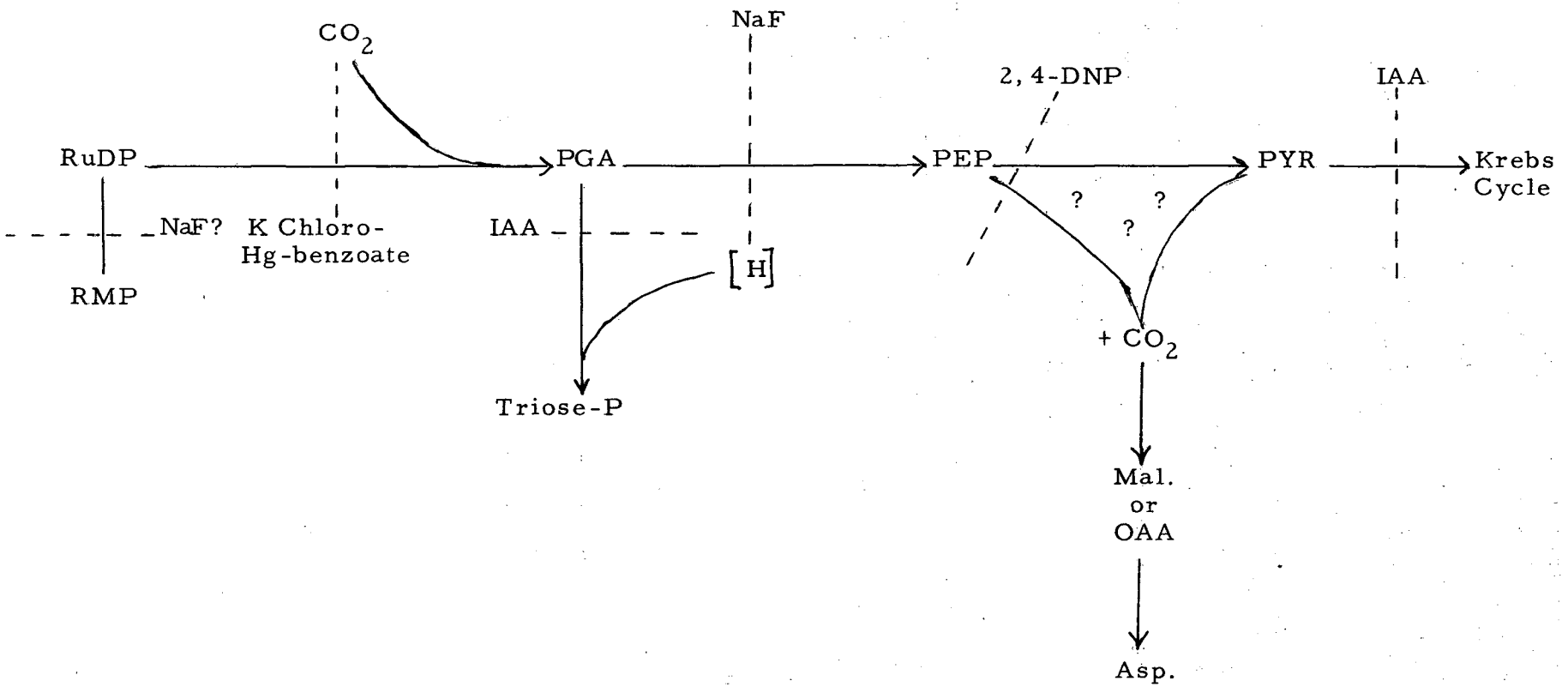


Fig. 1. Postulated scheme of reactions catalyzed by cell-free Chlorella extracts.

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