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J. A. Bassham, M. Kirk and M. Calvin

Radiation Laboratory and Department of Chemistry
University of California, Berkeley, California

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Abstract

Administration of hydroxylamine hydrochloride to algae, photosynthesizing with $C^{14}O_2$ for short periods has been found to result in two effects. If added before or during the fixation period, hydroxylamine hydrochloride often causes a stimulation of the rate of C^{14} incorporation. Whether added before, during or after the fixation period, it causes the appearance on a two-dimensional chromatogram of the algal extract of a new radioactive spot which on rechromatography is identified as phosphoenolpyruvate. These facts appear to negate the previously proposed hypothesis that addition of hydroxylamine hydrochloride results in a chromatographically stable derivative of some unstable intermediate in CO_2 fixation.

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Evidence for an unstable product or products formed in the early stages of carbon fixation during photosynthesis by algae has been presented by Metzner, et al.[1] In an attempt to stabilize, isolate, and characterize such unstable products, Metzner, et al.[2] added hydroxylamine to the algae just before, during or after a short period of photosynthesis with $C^{14}O_2$.

Analysis of the products of such fixation by the usual techniques of paper chromatography and radioautography led to observation of an apparently new radioactive compound on the chromatogram. This compound or "spot" usually appeared in the two-dimensional papers as a thin streak (its long dimension in the phenol direction) somewhere between aspartic and malic acids. Observations that the spot appeared to coincide with one of two fluorescent streaks (sometimes there was a radioactive compound coincidence with each of them), and that it might have an ultraviolet absorption peak of 238 mμ, led to the hypothesis that the new compound might be a complex newly-incorporated $C^{14}O_2$, possibly including a pterine.

We have attempted to identify this substance and to elucidate its role in photosynthesis. We have frequently obtained enhancement of C^{14} fixation by the addition of hydroxylamine hydrochloride to the photosynthesizing algae (both Chlorella and Scenedesmus) and have observed the fluorescent spots and a new radioactive spot (hereafter called F') which we believe to be the same as those previously reported at least in the two-dimensional chromatograms. Our results may be summarized as follows:

1) Addition of hydroxylamine hydrochloride to a concentration of 0.01 M in the suspension of photosynthesizing algae just before or during their fixation of $C^{14}O_2$ for five seconds in the light usually stimulates

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[1] H. Metzner, E. Simon and M. Calvin, Proc. Nat. Acad. Sci., 43, 892 (1957).

[2] H. Metzner, E. Metzner and M. Calvin, Proc. Nat. Acad. Sci., 44, (1958).

the incorporation of the C^{14} into compounds stable to acid treatment (paper chromatography, etc.). The increase in radioactivity is seen in phosphate esters, particularly phosphoglyceric acid.

2) A similar increase in fixation of C^{14} is seen if hydroxylamine hydrochloride is added to the algae suspension prior to or during dark fixation of $C^{14}O_2$ immediately following a period of illumination.

3) Addition of hydrochloric acid alone in an amount producing the same pH(4.8) in the algae suspension resulted in as much stimulation in one experiment as addition of hydroxylamine hydrochloride. In the same series of experiments addition of neutralized hydroxylamine caused an inhibition of $C^{14}O_2$ uptake.

4) Addition of hydroxylamine or hydroxylamine hydrochloride with the alcohol at the time of killing sometimes results in a slight increase in fixation and other times in no increase at all. However, if hydroxylamine hydrochloride is added for as short a time as one second before the algae are killed, there is sometimes a definite increase (around 10 percent higher than in the controls) in $C^{14}O_2$ fixation.

5) In cases 1 and 2 above there appears to be a decrease in C^{14} incorporated into malic acid.

6) In cases 1, 2 and 4 above the radioactive spot or streak (F') formed in the presence of hydroxylamine and running between aspartic acid and malic acid often but not always, coincides with one or the other of the fluorescent spots which run in the same area. Due to the fact that the radioactive compound and the fluorescent compound do not coincide in every case, it appears that they are not the same substance and that their frequent coincidence is a chromatographic accident, caused perhaps by their both being pushed along in the butanol-propionic acid direction by a third substance which may be hydroxylamine salt itself.

7) The radioactive spot, on rechromatography in the same solvents gives a spot which is phosphoenolpyruvic acid. This identity has been established by cochromatography with phosphoenolpyruvic acid (from California Foundation for Biochemical Research) followed by spraying with the Bayness-Isherwood phosphate spray. It was also established by cochromatography (with pyruvic acid) of the radioactive compound obtained after treating the original spot with phosphatase. The position of the pyruvic acid on the paper was located by treatment with silver nitrate in acetone followed by spraying with ethanolic sodium hydroxide.

8) When the radioactive spot, identified as phosphoenolpyruvic acid and obtained from a photosynthesis experiment with $C^{14}O_2$, is treated with 0.01 M hydroxylamine hydrochloride at room temperature for one hour, and the resulting solution is concentrated and chromatographed, a radioactive spot is obtained which has the same appearance and location on the chromatogram as the spot F'.

These results lead to the following conclusions:

1) The stimulation of fixation of $C^{14}O_2$ by hydroxylamine, which is most noticeable at very short exposures of the algae to C^{14}, is an actual

acceleration of one or more steps of the photosynthetic incorporation of CO_2 rather than the stabilization of an unstable intermediate. The entry of $C^{14}O_2$ into the cell seems a likely place at which stimulation might take place.

2) Hydroxylamine may form a compound or a stable salt with phosphoenolpyruvate, and this compound is stable only in the presence of excess hydroxylamine. Alternatively, hydroxylamine affects the chromatographic behavior of phosphoenolpyruvate in some manner to cause the observed movement on the chromatogram found experimentally. Once the excess hydroxylamine is removed (rechromatography) phosphoenolpyruvate runs to its usual position on the chromatogram.

3) The decrease in radioactivity found in malic acid may indicate that the hydroxylamine reacts with phosphoenolpyruvic acid in the algae during the period of photosynthesis and thereby inhibits C_1 to C_3 carboxylation.

4) The fluorescence which often coincides with radioactive spot is due to a different substance than the radioactive spot, and the coincidence is accidental.

While it thus appears that the effect of hydroxylamine is not a stabilization of unstable compounds, as was suspected earlier, the concept of a rather unstable CO_2 complex which could serve as a C_1 donor in the carboxylation reaction of the photosynthetic carbon reduction cycle is not ruled out by this fact. However, the principal instance in which there has appeared to be more total radiocarbon fixed during a short period of photosynthesis than is normally seen by the usual killing and analytical procedures (e.g., hot ethanol extraction and two-dimensional chromatography) was in the experiments in which hydroxylamine hydrochloride was added just prior to killing. Thus the hypothesis of an unstable CO_2 bearing intermediate (other than the β-keto acid carboxylation product from ribulose diphosphate) must be considered to be unsubstantiated by direct evidence at the present time.