Title

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Authors

Metzner, Helmut
Metzner, Barbara
Calvin, Melvin

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ABSTRACT

Some chemical, physical, and chromatographic properties of the hydroxylamine-stabilized early products of CO_2 fixation in photosynthesis are described. Although no definitive structural information is yet available, these properties, together with the biochemical context in which the material appears, make possible some likely suggestions about the nature of the substances.

EARLY UNSTABLE CO_2-FIXATION PRODUCTS IN PHOTOSYNTHESIS

Helmut Metzner, *Barbara Metzner, † and Melvin Calvin

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

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A comparison of the influence of different killing procedures on CO_2 fixation in short periods of photosynthesis (5 to 10 seconds) suggested that a part of the CO_2 is unstably bound.[1] Cold solvents extract some of this labile complex but it apparently decomposes during the following fractionation and counting procedure. If the algae are poured into boiling ethanol, however, less CO_2 appears to be fixed, presumably because the unstable compound(s) is (are) at least partly decomposed to give back CO_2.[2] The addition of hydroxylamine (to a concentration of 0.01 M) to the algal suspension immediately before the killing process gives a significantly higher CO_2 fixation than occurs in the absence of hydroxylamine. The additional fixation product(s) is (are) relatively stable during the succeeding procedure.

To study this phenomenon in greater detail Scenedesmus suspensions were allowed to assimilate $C^{14}O_2$ for periods between 5 seconds and 5 minutes. They were then killed by acetone or ethanol at about -30°C. After addition of acetic acid (to remove excess radioactive bicarbonate) an aliquot was sealed (after rapid evacuation at -45°C) into a glass bomb containing a copper–copper oxide mixture together with some glucose as a carrier substance.[3] Most of the manipulation and storage of these bombs was carried out in a liquid oxygen bath and the temperature was not allowed to rise above -20°C at any time before the combustion vessel was sealed. The organic material was burned (at about 650°C) and the activity of the resulting CO_2 measured in an ionization chamber with a vibrating-reed electrometer. By this means a value for the "total fixation" was obtained. The difference between this amount and that obtained by the standard procedure of plating the suspension on aluminum planchets and counting with a Geiger-Mueller tube gives the amount of "unstable fixation." It was found that after 5 seconds of photosynthesis more than half the activity is bound in a labile complex which is lost during the normal procedure. At least some of this loss can be prevented by the addition of hydroxylamine to the suspension either before, together with, or (if the temperature is low) after addition of the solvent used for the killing of the algae. Thus the experiments with the gas counting method confirmed the results of our earlier experiments, in which the stabilizing effect of the hydroxylamine was indicated.[2] Table I gives some data obtained by the usual

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*Rockefeller Fellow, 1956-1957. Permanent address: Plant Physiological Institute of the University of Göttingen, Germany.

†Guest Research Worker, 1956-1957.

[1] Metzner, Metzner, and Calvin: Arch. Biochem. Biophys. (in press).

[2] H. Metzner and B. Metzner, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 4; Metzner, Simon, Metzner, and Calvin, Proc. Natl. Acad. Sci. U.S. 43, 892 (1957).

[3] H. Metzner, H. Simon, and B. Metzner, in Chemistry Division Quarterly Report, UCRL-3950, Sept. 1957, p. 5.

plate counting procedure which illustrate the effect of both alcohol temperature and hydroxylamine on the amount of CO_2 fixed. The influence of the hydroxylamine upon the fraction of the activity that is soluble in ethanol is not very pronounced. In all acetone experiments, however, a marked effect of hydroxylamine on the distribution of activity between soluble and insoluble components is apparent (Table II). At least a part of the increased fraction of the activity appearing in the acetone-soluble components is accounted for by the increased amount of stable phosphate esters apparently brought into solution by the hydroxylamine. It appears as if the hydroxylamine liberated the phosphate from either a salt- or anhydride-type linkage with the enzyme, thus allowing the phosphate to dissolve. The application of hydroxylamine may very well lead to the detection of new phosphorylated compounds, which during the normal extraction procedure are retained by the insoluble fraction.

Table I

Influence of NH_2OH on the stable CO_2 fixation*

(5 sec photosynthesis after 90 sec flushing with N_2 in the light)

Killing Procedure	Without NH_2OH	With NH_2OH
Boiling ethanol	3098	5892
Ethanol (-35°C)	5506	7390

*Values are in thousands of counts/min/cc wet packed cells, determined as total plate counts of the original algal suspension in ethanol.

Table II

Stable CO_2 fixation and activity of the acetone-soluble fraction*

(5 sec photosynthesis after 90 sec flushing with N_2 in the light)

Killing procedure	Stable fixation in total original suspension	Activity of the acetone-soluble fraction†	Relative amount of phosphorous esters in acetone-soluble fraction (%)
Acetone (-36°C)	3099	650	44.6
NH_2OH followed by acetone (-35°C)	5517	4387	88.6
Acetone (-35°C) followed by NH_2OH	3490	3320	83.5

*Values in thousands of counts/min/cc wet packed cells.

†Practically all the radioactivity that does not appear in the acetone is extracted by H_2O immediately following the acetone extraction.

The discovery of the hydroxylamine effect encouraged us to develop a method for the study of the initially unstable complex, or its more stable derivative(s), by paper chromatography. Preliminary experiments were performed to see whether the labile complex survives contact with the solvent systems we were using. Aliquots of two Scenedesmus suspensions killed by subzero acetone, both with and without previous addition of hydroxylamine, were pipetted onto a sheet of chromatography paper. One spot of each series was cut out and burned in a glass bomb. The remaining spots were wet either with phenol or with the butanol–propionic acid mixture used in the normal chromatographic separation. After the paper was dried in a stream of cold air (total contact time with solvent 5 to 10 minutes) these spots were cut out and burned in a similar way. Whereas the aliquots taken of extracts with alcohol or acetone upon contact with phenol suffered a loss of about 30% of the activity compared with the spot that had no solvent contact, nothing was lost from those extracts involving the hydroxylamine. The butanol–propionic acid mixture, on the other hand, leads to practically the same loss (about 10%) from both types of extracts. This suggested one-dimensional chromatography using only phenol. To improve the separation the paper was cut as described elsewhere.[4]

Two series of experiments were performed using Scenedesmus cells that had been photosynthesizing in $C^{14}O_2$ for 5 seconds. In one, the cells were killed and extracted with alcohol (Fig. 1) and in the other with acetone (Fig. 2). In each series the organic insoluble residue was extracted with water. The two extracts (organic solvent and water extracts) were chromatographed separately in one dimension with phenol. All the chromatograms of material to which hydroxylamine was added either before, during, or after the killing process showed two fluorescing spots which coincided with two blackened bands on the exposed x-ray films. The faster-moving spot (F) runs just behind malic acid and is not in general well separated from it even after an 18-hour run. The other fluorescing compound (F') lies between malic and aspartic acids. In the control experiments (without hydroxylamine) with cold and hot ethanol as well as with cold acetone there is only a trace of a lower band that might correspond to F'. From phenol chromatograms of the alcohol-soluble components (Fig. 1, left) it was possible to estimate the amounts of F and F' (in Experiment 6) at about 9% of the total extracted into alcohol. Since this extract contains about half of the total C^{14} fixed, the amount of F and F' that survives the chromatography constitutes about 5% of the total.

Two-dimensional chromatograms do not seem to show the upper band (F) (Fig. 3); it appears to have been destroyed by the butanol–propionic acid system. In the hydroxylamine experiments, however, some of the lower spot (F') remains. It often separates into two bands (F' and F'', with the latter running close to aspartic acid). Its F' activity decreases within two weeks to about one-third of its original value. Another fluorescing compound moving faster than F is also present but is not radioactive. No fluorescent area was seen on any of the two-dimensional chromatograms of the control series (without hydroxylamine). It thus appears that the compound F is more stable to phenol than to acidified butanol, whereas F', at least to some extent, can survive the butanol–propionic acid solvent, particularly if hydroxylamine is added. However, because traces of what appears to be F' are to be seen in the phenol chromatograms even after killing

[4] H. Metzner, Planta 45, 493 (1955).

with boiling ethanol in the absence of hydroxylamine (Fig. 1, Column 1), it is probably not the original labile material.

The fluorescing areas clearly show ultraviolet absorption when examined between a fluorescent screen and its activating light source. An attempt was made to determine the ultraviolet absorption spectrum of the unstable fluorescent material F, by eluting it with 0.1 N NaOH from a one-dimensional phenol chromatogram as soon as possible after the chromatographic separation and development of the exposed film. We tried to balance out the paper contamination by eluting an equal weight of paper which had no plant extract on it to use as a blank. The spectrum showed two peaks, one at 255 and another at about 285 mμ, sometimes with a shoulder at longer wave lengths (about 340 mμ). The uncertainty of the balance, however, makes the value of these measurements doubtful as yet.

The time of appearance, chromatographic behavior, lability, and particularly the fluorescent behavior suggest a complex carrier of a one-carbon compound as a likely possibility for this "active CO_2." The spectrum, if real, would suggest the presence of a pterine moeity.[5] The limited information available does not eliminate the possibility of a nucleotide[6] as well, and the variety of fragments into which it breaks upon manipulation may be taken as a reflection not only of its reactivity but of its complexity as well.

If such an "active CO_2" were the natural substrate for the carboxylation reaction[7] of carboxydismutase[8] it would account for the apparently too-small turnover rate of this enzyme system with CO_2 as substrate.[9] Further-more, a

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[5] Van Baalen, Forrest, and Myers, Proc. Natl. Acad. Sci. U.S. 43, 701 (1957).

[6] Bachhawat, Woessner, and Coon, Federation Proc. 15, 214 (1956).

[7] C. R. Greenberg, Federation Proc. 13, 221 (1954).

[8] Quayle, Fuller, Benson, and Calvin, J. Am. Chem. Soc. 76, 3610 (1954).

M. Calvin and P. Massini, Experientia 8, 445 (1952).

Weissbach, Horecker, and Hurwitz, J. Biol. Chem. 218, 795 (1956).

E. Racker, Nature 175, 249 (1955).

[9] E. Racker, The Reductive Pentose Phosphate Cycle. I. Phosphoribulokinase and Ribulose Diphosphate Carboxylase, J. Biol. Chem. (in press);

Ning G. Pon, Studies in the Carboxydismutase System and Related Materials (Thesis), University of California, Berkeley (1958).

particular sensitivity to fluoride ion[10] of such an "active CO_2" complex could also account for the fluoride-liberated CO_2 observed by Warburg et al.[11]

In any case, the stabilizing effect of hydroxylamine seems best interpreted in terms of an extremely rapid and facile reaction, probably involving a carbonic-phosphoric anhydride, thiocarbonate, carbamate, or some combination or reactive derivative of these.

This work was done under the auspices of the U. S. Atomic Energy Commission.

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[10] Flavin, Castro-Mendoza, and Ochoa, Biochim. et Biophys. Acta 20, 591 (1956).

[11] O. Warburg and J. Kripphal, Z. Naturforsch. 11b, 718 (1956).

Figure Captions

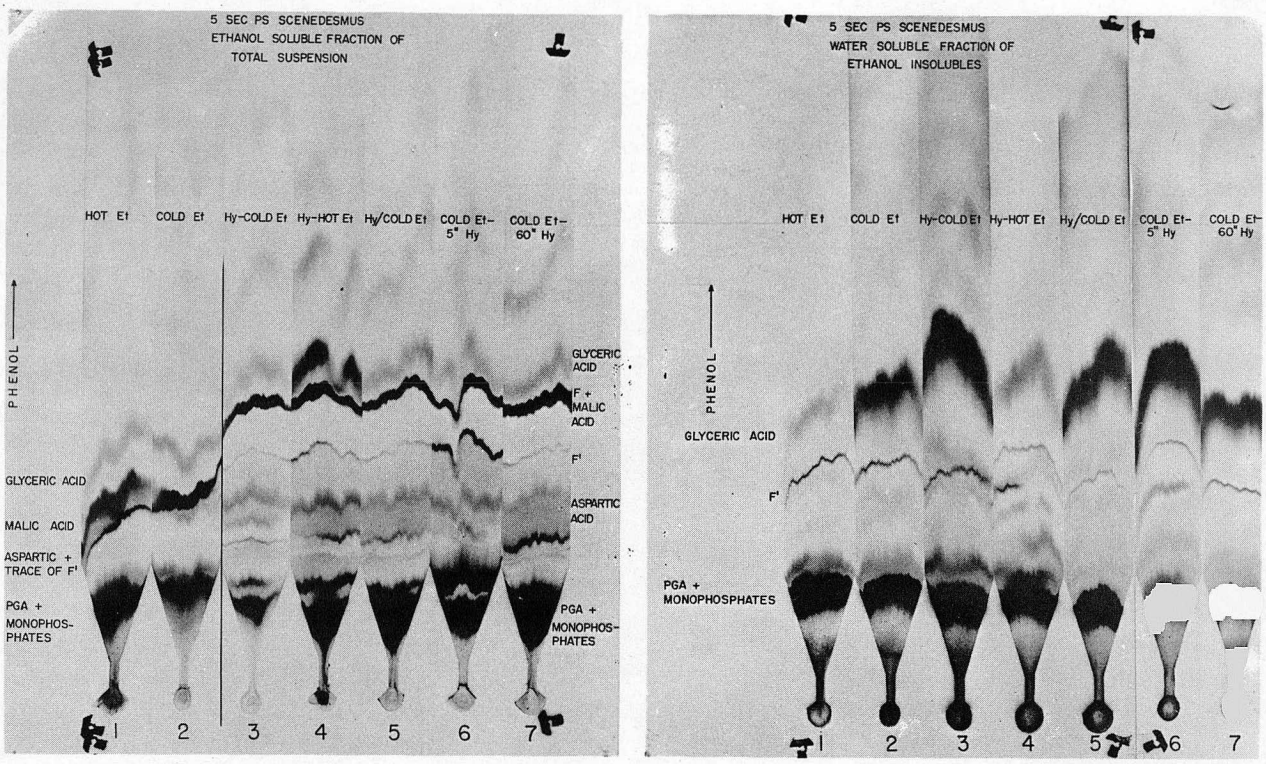
Fig. 1. Radioautograph of one-dimensional phenol chromatograms of various alcohol and water extracts of Scenedesmus cells that have been photosynthesizing in $C^{14}O_2$ for 5 sec and then are killed in a variety of ways. (Left, the ethanol extracts; right, the water extracts of the alcohol-insoluble residue.)

Key: (1) hot alcohol; (2) cold alcohol (-35°C); (3) hydroxylamine added to cell suspension to make it 0.01 M in hydroxylamine and then immediately killed in cold alcohol (-35°C); (4) hydroxylamine added to cell suspension to make it 0.01 M in hydroxylamine and then immediately killed in hot alcohol; (5) algae killed by dropping into a 0.01 M solution of hydroxylamine in cold ethanol (-35°C); (6) cold alcohol (-35°C) followed in 5 sec by the addition of hydroxylamine; (7) cold alcohol (-35°C) followed in 60 sec by the addition of hydroxylamine.

Fig. 2. Radioautograph of a one-dimensional phenol chromatogram of various acetone and water extracts of Scenedesmus cells that have been photosynthesizing in $C^{14}O_2$ for 5 sec, and then have been killed in a variety of ways. Left, the acetone extracts; right, the water extracts of the acetone-insoluble residue.

Key: (8) killed by cold acetone (-40° C); (9) killed in cold acetone (-40° C) immediately after addition of hydroxylamine to cell suspension to make it 0.01 M in hydroxylamine, (10) killed in cold acetone (-40° C) followed in 5 sec by the addition of hydroxylamine.

Fig. 3. Radioautographs of chromatograms of 5-sec photosynthesis experiments using Scenedesmus: light turned off and hydroxylamine added; after 1 min ethanol (-36° C) added. Top, ethanol extract; bottom, water extract of alcohol-insoluble material.


5 SEC PS SCENEDESMUS
ETHANOL SOLUBLE FRACTION OF
TOTAL SUSPENSION
HOT Et
COLD Et
Hy-COLD Et
Hy-HOT Et
Hy/COLD Et
COLD Et-
5" Hy
COLD Et-
60" Hy
PHENOL
GLYCERIC ACID
MALIC ACID
ASPARTIC +
TRACE OF F'
PGA +
MONOPHOS-
PHATES
GLYCERIC
ACID
F +
MALIC
ACID
F'
ASPARTIC
ACID
PGA +
MONOPHOS-
PHATES
1
2
3
4
5
6
7
5 SEC PS SCENEDESMUS
WATER SOLUBLE FRACTION OF
ETHANOL INSOLUBLES
HOT Et
COLD Et
Hy-COLD Et
Hy-HOT Et
Hy/COLD Et
COLD Et-
5" Hy
COLD Et-
60" Hy
PHENOL
GLYCERIC ACID
F'
PGA +
MONOPHOSPHATES
1
2
3
4
5
6
7

ZN-1860

Fig. 1

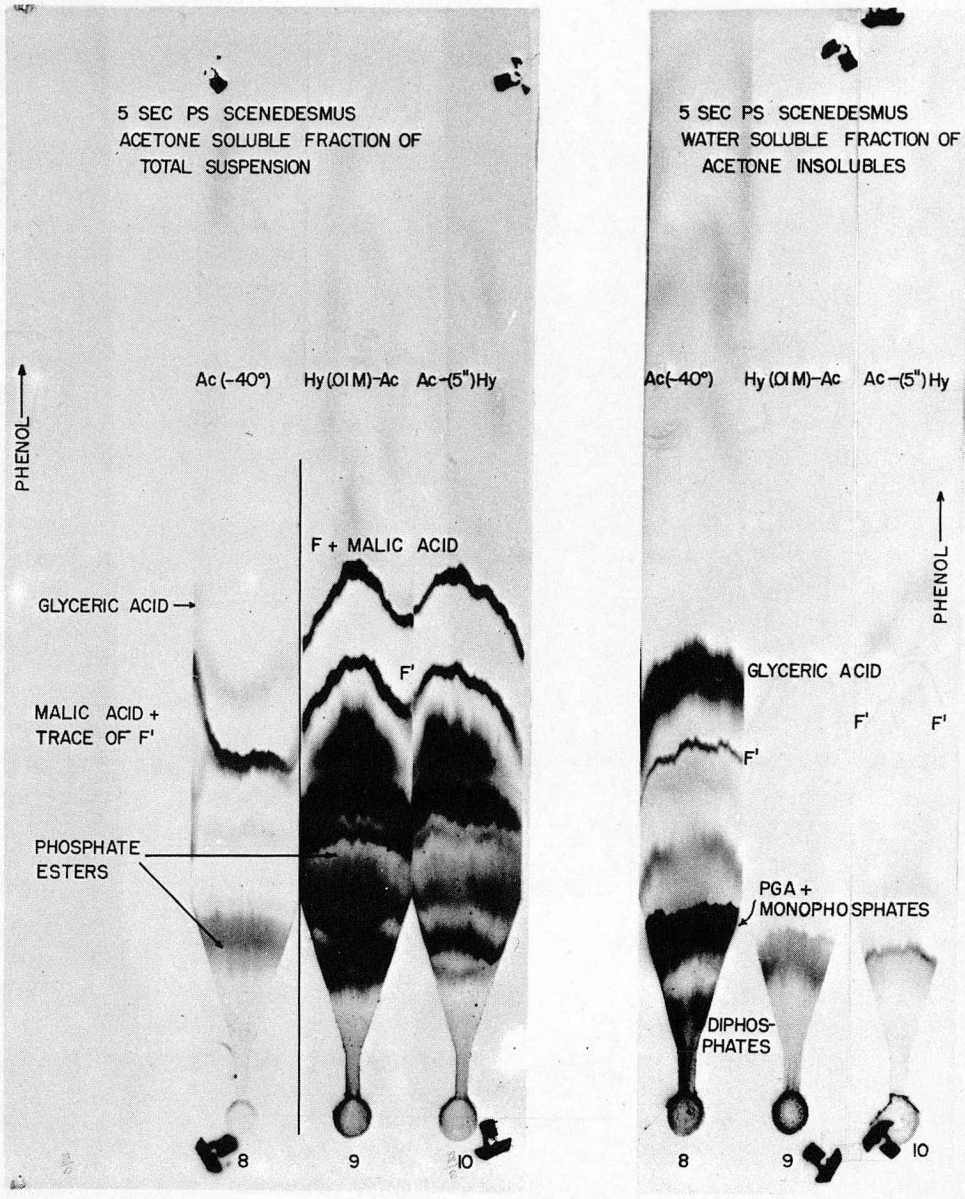
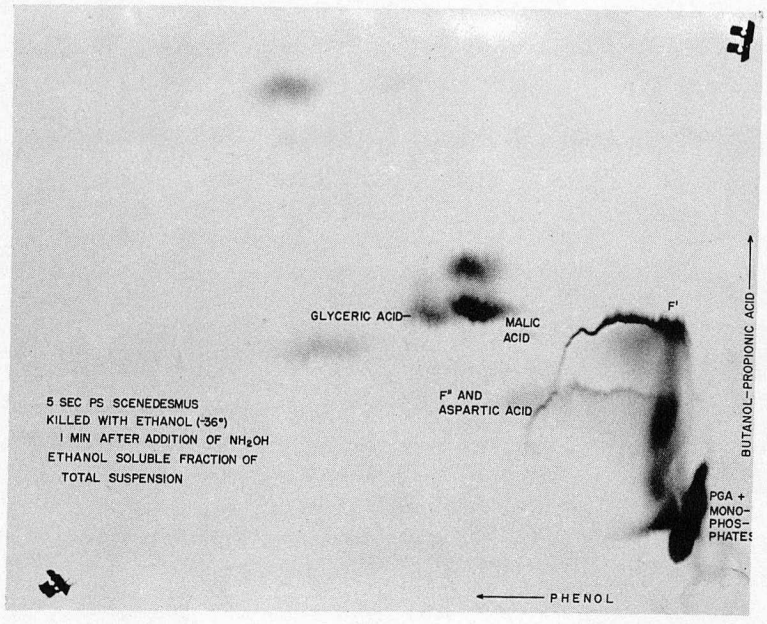
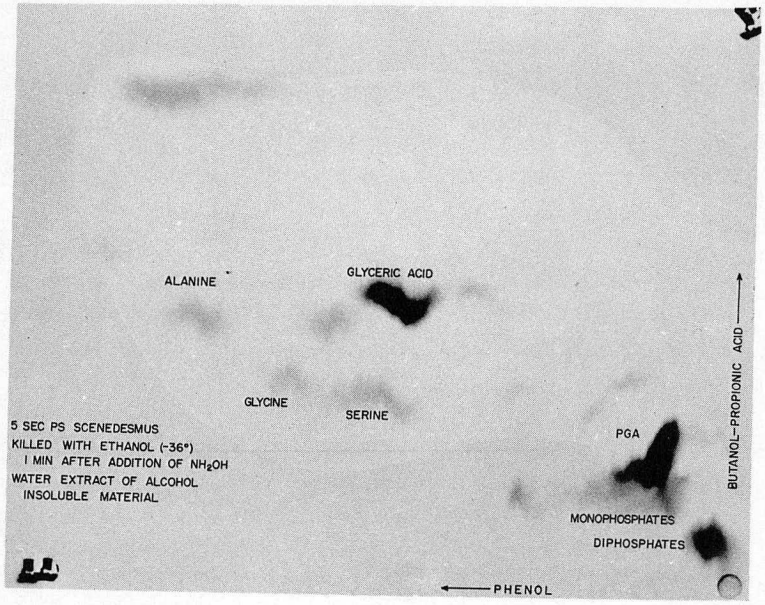

5 SEC PS SCENEDESMUS
ACETONE SOLUBLE FRACTION OF
TOTAL SUSPENSION
Ac (-40°)
Hy (.01 M)-Ac
Ac-(5")Hy
PHENOL
F + MALIC ACID
GLYCERIC ACID
F'
MALIC ACID +
TRACE OF F'
PHOSPHATE
ESTERS
8
9
10
5 SEC PS SCENEDESMUS
WATER SOLUBLE FRACTION OF
ACETONE INSOLUBLES
Ac(-40°)
Hy (.01 M)-Ac
Ac-(5")Hy
PHENOL
GLYCERIC ACID
F'
F'
F'
PGA +
MONOPHOSPHATES
DIPHOS-
PHATES
8
9
10
ZN-1861

Fig. 2.


GLYCERIC ACID
MALIC ACID
F'
F² AND ASPARTIC ACID
5 SEC PS SCENEDESMUS
KILLED WITH ETHANOL (-36°)
1 MIN AFTER ADDITION OF NH₂OH
ETHANOL SOLUBLE FRACTION OF
TOTAL SUSPENSION
BUTANOL-PROPIONIC ACID
PGA + MONO-PHOS-PHATES
PHENOL


ALANINE
GLYCERIC ACID
GLYCINE
SERINE
5 SEC PS SCENEDESMUS
KILLED WITH ETHANOL (-36°)
1 MIN AFTER ADDITION OF NH₂OH
WATER EXTRACT OF ALCOHOL
INSOLUBLE MATERIAL
PGA
MONOPHOSPHATES
DIPHOSPHATES
BUTANOL-PROPIONIC ACID
PHENOL

ZN-1859

Fig. 3.