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By R. G. Jensen** and J. A. Bassham

Photosynthesis in green plants requires not only photoelectron transport, oxygen evolution and photophosphorylation, but also the synthetic reactions whereby carbon dioxide is assimilated and reduced to a number of organic compounds via the photosynthetic carbon reduction cycle[1] and secondary biosynthetic pathways.[2] Complete photosynthesis with isolated chloroplasts has for many years been a goal of biochemists. Photosynthetic reactions could then be isolated from reactions of the cytoplasm, and the cell wall could be eliminated as a barrier to the assimilation of various added metabolites and chemicals. This achievement would greatly facilitate the study of the mechanisms of enzymic transformations and metabolic control in the synthetic reactions of photosynthesis.

The photochemical transfer of electrons from water to artificial electronic acceptors by subcellular particles from green plant cells was demonstrated many years ago by Hill and Scarisbrick.[3] Since that time, isolated chloroplasts and chloroplast particles have been found to be capable of photoelectron transport to NADP,[4-6] and of photophosphorylation.[7]

While very high rates, exceeding those required for in vivo photosynthesis, have been demonstrated for photoelectron transport to both natural and artificial electron acceptors and for photophosphorylation, rates of carbon reduction during photosynthesis by isolated chloroplasts have been disappointingly low. Fixation of ^{14}C-labeled carbon dioxide

into intermediate compounds of the photosynthetic carbon reduction cycle by isolated chloroplasts was reported by Allen et al. in 1955.[8] This ability to fix CO_2 was diminished with broken chloroplasts but could be restored by various cofactors,[9] and it was claimed that CO_2 fixation by chloroplasts could be accomplished in the dark if the cofactors ATP and NADPH were added.[10] A report from the same laboratory (in 1960) listed fixation rates of 4.5 to 6 μmoles CO_2/mg Chl/hr.[11] Others[12] found rates of about 4. It has long been assumed, and is verified by experiment in the present report, that spinach leaves should be capable of rates of CO_2 fixation exceeding 200 μmoles CO_2/mg Chl/hr.

Fixation of CO_2 has been stimulated by the addition of some intermediates of the carbon reduction cycle to broken chloroplasts,[9] or whole chloroplasts.[13] Walker[14] found a rate of 1.1 μmoles CO_2/mg Chl/hr. to be increased to 24.3 (and later,[15] 36.9) upon the addition of ribose-5-phosphate to isolated chloropalsts prepared according to his method. With the addition of 71.5 μmoles of ribose-5-phosphate per mg chlorophyll to the reaction mixture, Walker's accelerated CO_2 fixation rate resulted in a total fixation of 6.0 μmoles CO_2 per mg chlorophyll during the 20-min period of incubation. Thus the stimulated CO_2 fixation rate could result from the operation of only part of the carbon reduction cycle utilizing ATP from the photochemical reactions to convert ribose-5-phosphate to ribulose-1,5-diphosphate, the carboxylation reaction substrate. It would appear that Walker's preparation had a limited capacity for conversion of other sugar phosphates to pentose phosphates.

Another type of stimulation of CO_2 fixation by chloroplasts was achieved by Heber and Tyszkiewicz,[16] who added a concentrated solution

of soluble enzymes of the carbon reduction cycle to chloroplasts isolated in aqueous media and thereby obtained fixation rates of 4 μmoles CO_2/mg Chl/hr., which were further stimulated to 12 μmoles CO_2/mg Chl/hr. upon the addition of ATP and NADPH. Using chloroplasts from the marine chrysomonad Hymenomonas sp, Jeffrey et al.[17] measured a chloroplast fixation rate of 15.3 μmoles CO_2/mg Chl/hr., which was nearly 10% of the in vivo rate for that organism. Addition of chloroplast extract stimulated these chloroplasts to a rate of 34, or approximately 25%, of the fixation rate of whole cells.

In the studies reported below, efforts were made to obtain a well-defined, homogeneous preparation of chloroplasts with intact membranes, which would be capable of high rates of complete photosynthesis without the addition of either cofactors (other than inorganic ions) or enzymes. It was felt that such preparations would be more useful than reconstituted systems for studies of metabolic control and enzymic mechanisms.

Materials and Methods.--Seeds of Spinacia oleracea L. (var. Viroflay), obtained from the Ferry Morse Seed Co., were planted in 5 inches of vermiculite in flats, outdoors in direct sunlight. Upon germination, the plants were watered daily with Hoagland's solution. At the age of 4 to 5 weeks, leaves weighing approximately 1.2 to 1.5 gm were harvested and used immediately for experiments.

CO_2 fixation in intact leaves.--A freshly picked leaf was placed in a gas-tight transparent vessel with its stem in a vessel containing Hoagland's solution. The leaf chamber was attached by inlet and outlet tubes to the steady-state apparatus.[18] This apparatus contains a closed system of tubing and a pump for circulating gas through the leaf chamber

and then through an ionization chamber which monitors the level of ^{14}C when this tracer is present as $^{14}CO_2$.

After the leaf photosynthesized for 10 min with air, the gas handling system was closed and connected with a reservoir containing air and $^{14}CO_2$. The system then contained 200 μmoles of carbon dioxide, with a specific activity of 29.0 μc/μmole. The total CO_2 tension was 0.08%, or approximately twice that of air. The leaf was allowed to photosynthesize for 10 min with $^{14}CO_2$. We then quickly opened the chamber (less than 10 sec) and plunged the leaf into liquid nitrogen.

The frozen leaf material was ground in liquid nitrogen. We determined the chlorophyll content of the leaf,[19] with an aliquot sample of the frozen leaf material and found that the leaf contained 1.21 mg of chlorophyll. The remaining frozen powdered leaf material was denatured by immediate mixing with 80% methanol. An aliquot sample of this material was acidified and dried on a piece of filter paper, and the radioactivity was determined by counting between opposing thin-window G-M tubes. From the resulting count, known counter sensitivity, known specific activity of the $^{14}CO_2$, and the previously determined chlorophyll content, the photosynthetic assimilation rate of $^{14}CO_2$ into acid-stable compounds was calculated.

Rates of fixation by isolated chloroplasts.--On the same day, and from leaves taken from the plants used for the whole leaf fixation rate, isolated chloroplasts were prepared as described below. A 0.50 ml suspension of these chloroplasts containing 0.085 mg of chlorophyll was preilluminated for 3 min, after which radioactive bicarbonate was added and photosynthesis allowed to proceed for 6 min prior to killing in 80%

methanol. Determination of chlorophyll and of ^{14}C fixed (see above) gave the CO_2 fixation rate for isolated chloroplasts.

Preparation of chloroplasts.--The method described by Walker[14] has been considerably modified in our studies.

Each of three solutions used in the isolation and assay contain the following: 0.33 M sorbitol; 0.002 M $NaNO_3$; 0.002 M EDTA (dipotassium salt); 0.002 M Na isoascorbate; 0.001 M $MnCl_2$; 0.001 M $MgCl_2$; 0.0005 M K_2HPO_4.

In addition, the solution A contains 0.05 M MES[20] [2-(N-morpholino) ethanesulfonic acid)], adjusted with NaOH to pH 6.1; 0.02 M NaCl; the solution B contains 0.05 M HEPES[20] (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), adjusted with NaOH to pH 6.7; 0.02 M NaCl; solution C contains 0.05 M HEPES, adjusted with NaOH to pH 7.6; 0.005 M $Na_4P_2O_7 \cdot 10 H_2O$.

Ten gm of freshly cut leaves are washed, chilled, and the midribs are removed. The leaf strips and 30 ml chilled solution A are placed in a "semimicro" monel homogenizing vessel on a Waring Blendor and blended for only 5 sec at high speed. The slurry is poured and pressed through 6 layers of cheesecloth (42 threads per inch), and the resulting juice is centrifuged for 50 sec at 2000 x g. The resulting pellet may be stored at 0°C for up to 4 hr. Before the experiment the pellet is resuspended in the solution B at 0°C, to give a suspension which contains about 1.4 mg Chl/ml.

Photosynthesis with chloroplasts.--Photosynthesis is carried out in a round-bottom flask (diameter 3.3 cm) stoppered with a serum cap. The flask is mounted on a rack which holds 16 flasks and moves with a circular

motion in the horizontal plane (radius of motion 1/2 inch, frequency 200 cycles/min). This swirling motion distributes the suspension of chloroplasts around the round bottom of the flasks fairly uniformly without subjecting them to excessive agitation. Excessive shaking was found to cause loss of photosynthetic activity.

The flasks are held in a water bath at 20°C, and are illuminated through the transparent bottom of the bath by light from a bank of 20-watt Nu-Lite Ultralux fluorescent lamps which give an incident intensity at the flasks of 2400 foot-candles. The intensity was found to be sufficient for light saturation under the conditions used.

In each flask is placed 50 µl of chloroplast suspension (containing about 0.07 mg chlorophyll) and 430 µl of solution C. The shaking flasks are preilluminated for 3 min. Then 20 µl of $H^{14}CO_3^-$ solution is added to give a final volume of 0.5 ml, which is 0.006 M in HCO_3^- and contains 24 µc of ^{14}C. After 6 min of photosynthesis (unless otherwise specified) the chloroplast reactions are stopped by addition of 2 ml of methanol. Total radiocarbon fixed and chlorophyll content are then determined as described above.

Paper chromatography and radioautography.--Products of photosynthesis by spinach leaves and isolated chloroplasts were analyzed by two-dimensional chromatography in phenol-acetic acid-water and butanol-propionic acid-water, as described elsewhere.[21]

Test of pyrophosphate for metabolism.--In the course of investigations of possible buffers, it was found that pyrophosphate stimulated CO_2 fixation. Since pyrophosphate has been found to be formed from labeled orthophosphate in experiments with Chlorella, and its level is a function of light and dark,[22] and of the addition of inhibitors,[21] it was of interest to investigate its possible metabolism by the chloroplasts. Therefore, photosynthesis was carried out with ^{32}P-labeled pyrophosphate. After varying times up to 10 min photosynthesis, the chloroplasts were killed and analyzed. Very little labeled pyrophosphate was converted, probably via orthophosphate, which is formed slowly by hydrolysis of the pyrophosphate.

Because of its slow hydrolysis, sodium pyrophosphate is added to the photosynthesis medium (solution C) on the day of each experiment. Orthophosphate becomes gradually inhibitory above 0.0005 M.

Light saturation.--The effect of light intensity was tested under the conditions described above, except that illumination was supplied by two incandescent lamps (G.E. DXB Photospot) and the intensity varied by using neutral density screens, as shown in Results.

Effect of bicarbonate concentration.--The effect of bicarbonate concentration on the rate was tested under the conditions described above, except that the concentration of bicarbonate was varied as shown in Results. In order to minimize the effect of depletion of bicarbonate on its effective concentration during the measurement, the concentration of chloroplasts was reduced to give 0.02 mg of chlorophyll in the 500 µl reaction mixture.

Electron microscopy and optical microscopy.--An electron micrograph of chloroplasts prepared under the described conditions was made by Dr. Daniel Branton, using the freeze-etching technique[23] as adapted for this purpose by Branton and Park.[24]

Optical micrographs were prepared using the Zeiss photomicroscope under phase contrast.

Results and Discussion.--Intact and freshly harvested and healthy spinach leaves gave a CO_2 fixation rate of 245 μmoles CO_2/mg Chl/hr. Chloroplasts prepared the same day from leaves from the same plants gave a fixation rate of 155 μmoles CO_2/mg Chl/hr. This rate with isolated chloroplasts was obtained over a period of 6 min following 3 min preillumination. Thus the isolated chloroplasts, for a limited period of time, assimilated carbon dioxide at a rate that was 63% of the *in vivo* photosynthetic rate.

The total carbon dioxide fixation during various periods of time is shown in Figure 1. The rate of photosynthesis declines continuously with time of photosynthesis, and by 30 min is 40 μmoles CO_2/mg/Chl/hr.

The fixation rate becomes maximal with a bicarbonate concentration of about 3 mM (Figure 2). The bicarbonate level for 1/2 maximum rate is 6×10^{-4} M, compared to 1.1×10^{-2} M reported[25] for isolated ribulose diphosphate carboxylase (E.C. 4.1.1.39). Thus, the isolated chloroplasts used in our experiments resemble much more nearly *in vivo* photosynthesis in their capability for carbon dioxide fixation than does the isolated, purified enzyme which catalyzes the primary carboxylation reaction of photosynthesis.

The saturation of CO_2 fixation rate with light intensity is shown in Figure 3.

The appearance of the chloroplasts in the light microscope with phase optics is shown in Figure 4. It can be seen that approximately 75% of the chloroplasts are of the class I type (highly refractive without clear grana) as defined by Spencer and Unt.[26] It seems probable that the biochemically active chloroplasts in our experiments are the class I chloroplasts, which retain an intact membrane and have not lost soluble protein. A large proportion of class I chloroplasts is not in itself sufficient to produce high rates of CO_2 fixation, since Spencer and Unt reported a rate of 2.88 μmoles CO_2/mg Chl/hr.

Electron micrographs prepared using the freeze-etching technique with chloroplasts isolated as described in this paper are shown in Figures 5 and 6. They both show the close stacking and unswollen configuration of the thylakoids which are typical of chloroplasts in vivo.

The products of CO_2 fixation during 10 min in whole spinach leaves are shown in the radioautograph in Figure 7. The pool sizes of intermediates of the carbon reduction cycle are relatively small, and the radiocarbon passes quickly on into larger pools of various secondary products such as sucrose, alanine, aspartic acid and other amino acids.

In contrast, the pattern of labeling in isolated chloroplasts, shown in Figure 8, contains very high amounts of radiocarbon in intermediates of the carbon reduction cycle, particularly PGA, dihydroxyacetone phosphate, fructose and sedoheptulose diphosphates, and ribose-5-phosphate, as well as in glycolic acid. Work in progress indicates that these compounds are transported, or diffuse, rapidly from the isolated chloroplasts to the suspending medium. Hexose monophosphates and ribulose-1,5-diphosphate are mostly retained in the chloroplasts. The labeling of secondary products of low molecular weight is rather small.

Nonetheless, there is a significant amount of synthesis of macromolecules, as indicated by the large quantity of radiocarbon found at the origin.

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FIGURE CAPTIONS

Fig. 1. Time course of carbon dioxide fixation by isolated chloroplasts.

Fig. 2. Dependence of CO_2 fixation rate on bicarbonate concentration.

Fig. 3. Dependence of CO_2 fixation rate on light intensity. Light intensity given as incident intensity at surface of reaction flask. Light source was an incandescent lamp (see text) and was varied through the use of neutral density filters.

Fig. 4. Isolated chloroplasts under phase contrast in the light microscope. Class I chloroplasts appear bright and highly refractive with an outer, limiting membrane.

Fig. 5. Electron micrograph of isolated chloroplasts obtained by the freeze-etching technique.[24] These electron micrographs were prepared by Professor Daniel Branton of the Department of Botany, University of California, Berkeley, and reproduced by his courtesy. The chloroplast (C) with closely packed thylakoids is surrounded by an intact outer membrane (M). Another membrane face is left after the fracture process.

Fig. 6. Electron micrograph of isolated chloroplasts obtained by the freeze-etching technique. Part of an intact chloroplast is seen (bottom). At the top are seen remnants of a chloroplast without an outer membrane. The thylakoids (T) are still tightly packed. This micrograph is representative of a chloroplast preparation which gave low CO_2 fixation (20 μM/mg Chl/hr).

Fig. 7. Radioautograph of $^{14}CO_2$ photosynthetic products in whole spinach leaves. Detached spinach leaves were allowed to fix $^{14}CO_2$ for 10 min as described in text. After killing, the leaf material was analyzed by two-dimensional paper chromatography and radioautography. The

FIGURE CAPTIONS (p. 2)

numbers indicate the following compounds: 0 - origin; 1 - 3-phosphoglyceric acid; 2 - fructose-6-phosphate; 3 - sedoheptulose-7-phosphate and glucose-6-phosphate; 4 - ribulose-1,5-diphosphate, fructose-1,6-diphosphate, and sedoheptulose-1,7-diphosphate; 5 - ribose-5-phosphate and ribulose-5-phosphate; 6 - dihydroxyacetone phosphate; 8 - uridine diphosphoglucose; 9 - sucrose; 10 - malic acid; 11 - glyceric acid (?); 12 - citric acid; 13 - aspartic acid; 14 - serine; 15 - glycine; 16 - glutamic acid; 17 - threonine; 18 - alanine; 19 - glutamine.

Fig. 8. Radioautograph of $^{14}CO_2$ photosynthetic products in isolated spinach chloroplasts. Isolated chloroplasts were allowed to fix $^{14}CO_2$ for 6 min as described in text. After killing, the leaf material was analyzed by two-dimensional paper chromatography and radioautography. The numbers indicate the same compounds as for Fig. 7, plus: 7 - 3-phosphoglyceraldehyde; 20 - glycolic acid.

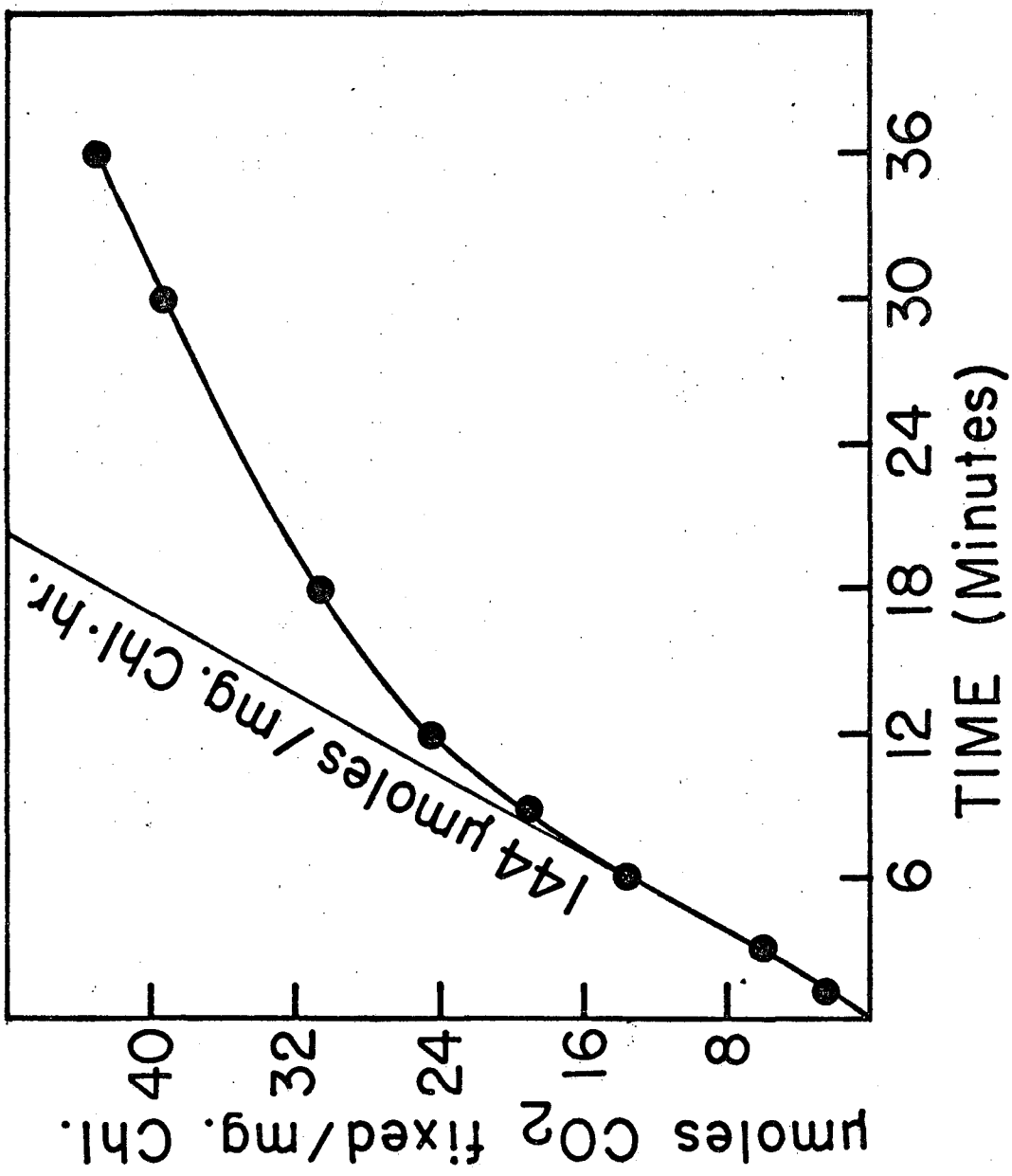
μmoles CO2 fixed/mg. Chl.
8
16
24
32
40
144 μmoles / mg. Chl. hr.
6
12
18
24
30
36
TIME (Minutes)

Fig. 1

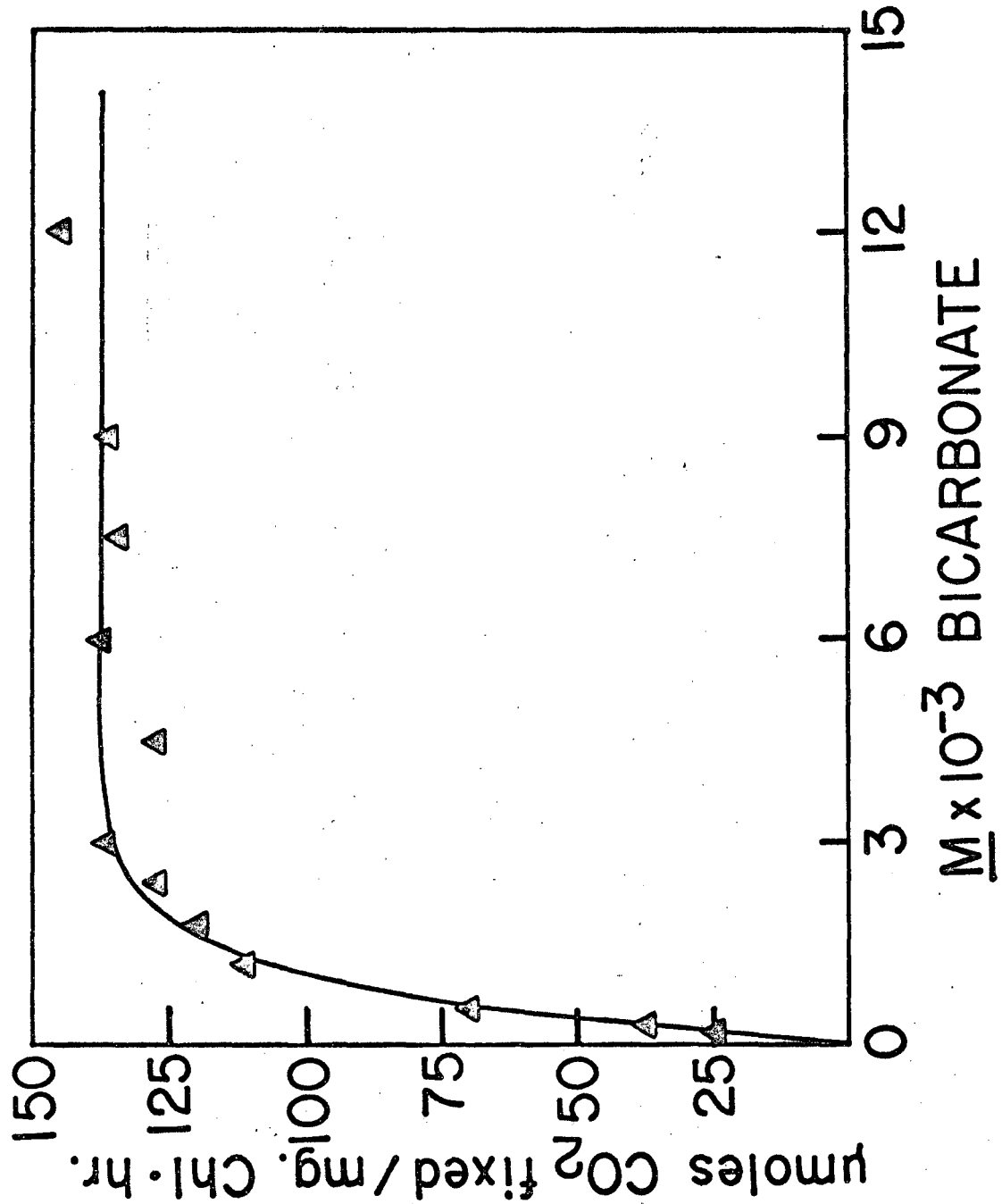
μmoles CO_2 fixed / mg. Chl. hr.
150
125
100
75
50
25
0
3
6
9
12
15
M x 10⁻³ BICARBONATE

Fig. 2

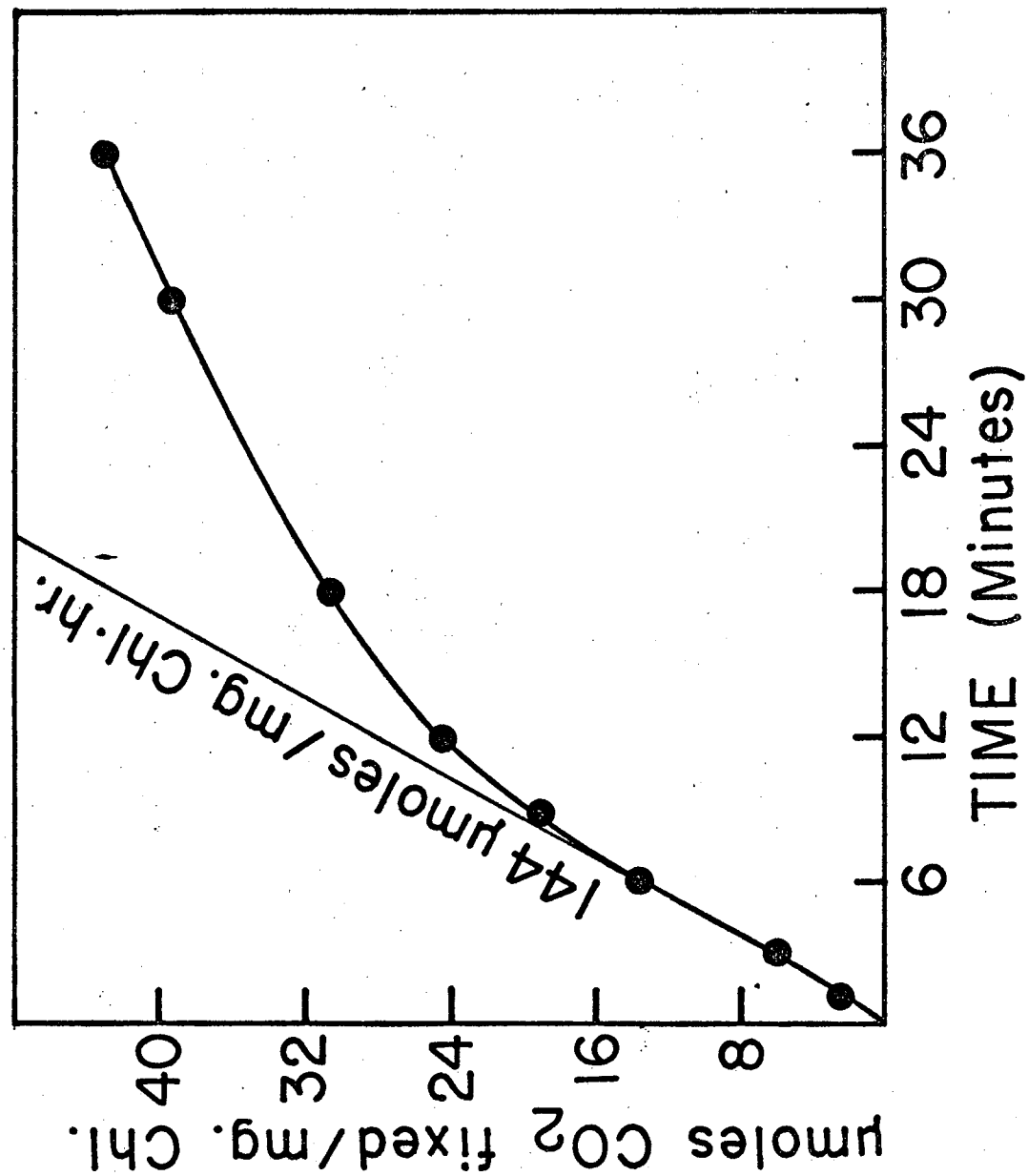
μmoles CO2 fixed/mg. Chl.
8
16
24
32
40
144 μmoles / mg. Chl. hr.
6
12
18
24
30
36
TIME (Minutes)

Fig. 1

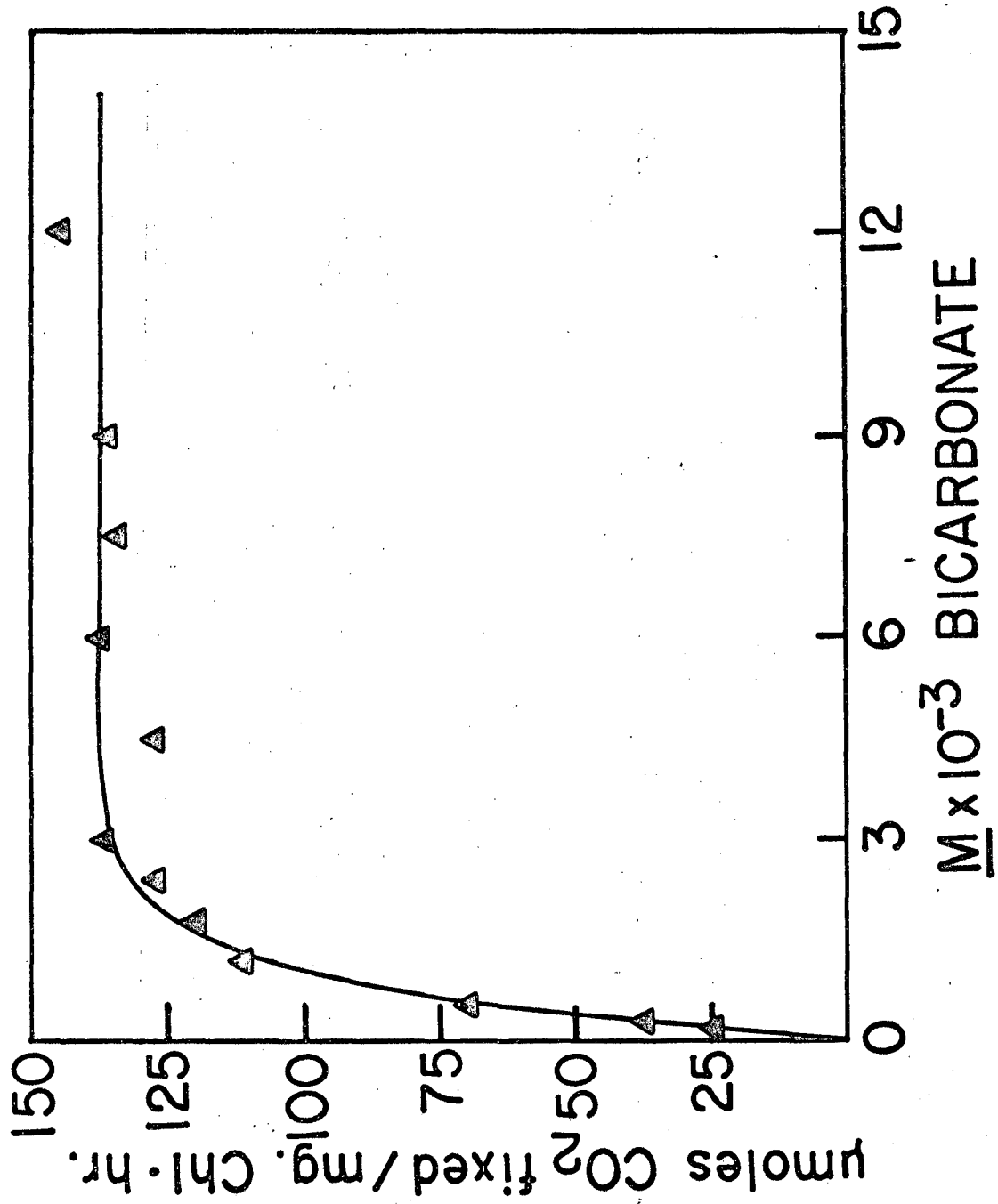
μmoles CO_2 fixed / mg. Chl. hr.
150
125
100
75
50
25
0
3
6
9
12
15
M x 10^{-3} BICARBONATE

Fig. 2

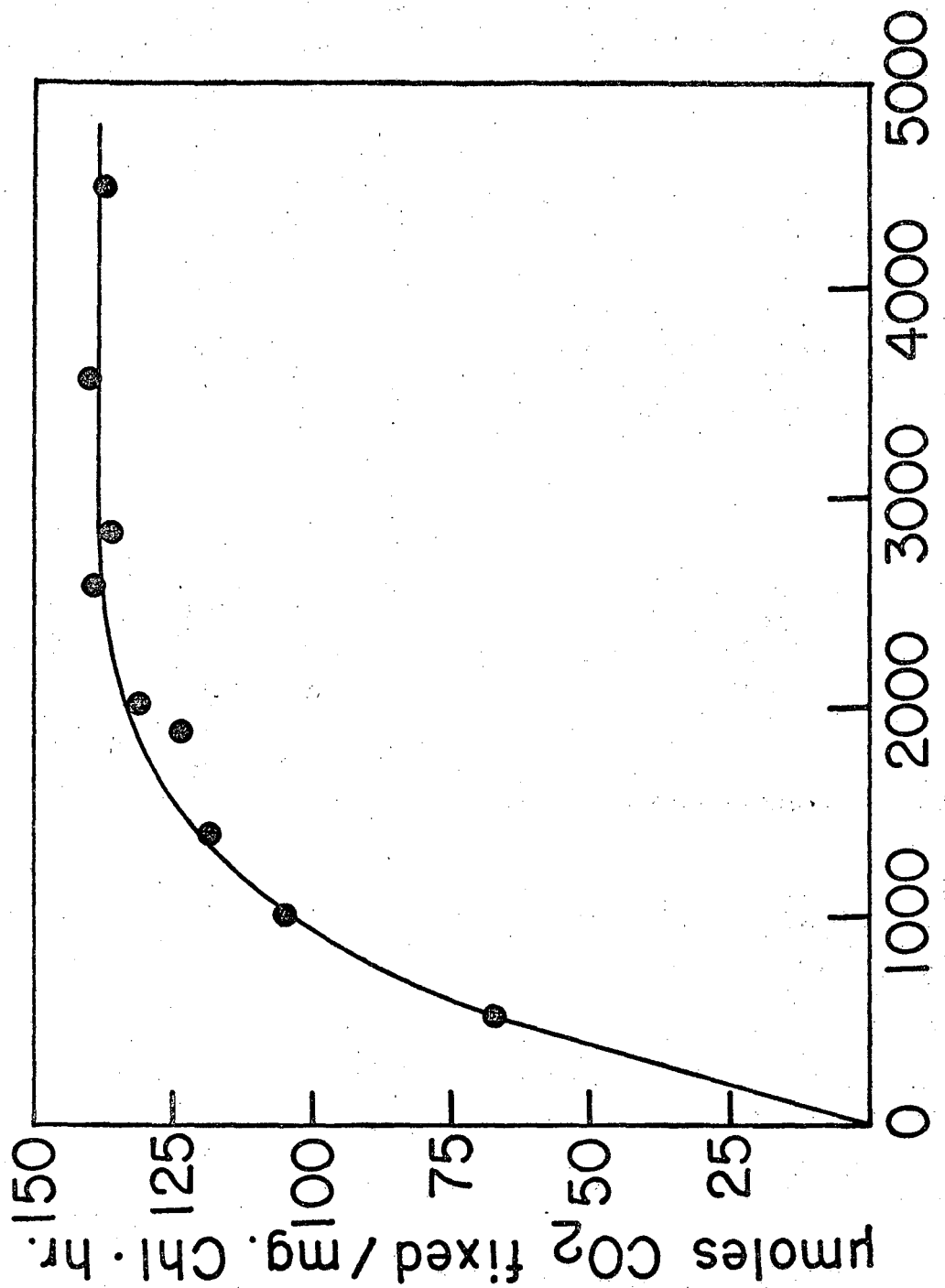
μmoles CO_2 fixed / mg. Chl · hr.
150
125
100
75
50
25
0
1000
2000
3000
4000
5000
LIGHT INTENSITY, ft.candles

Fig. 3

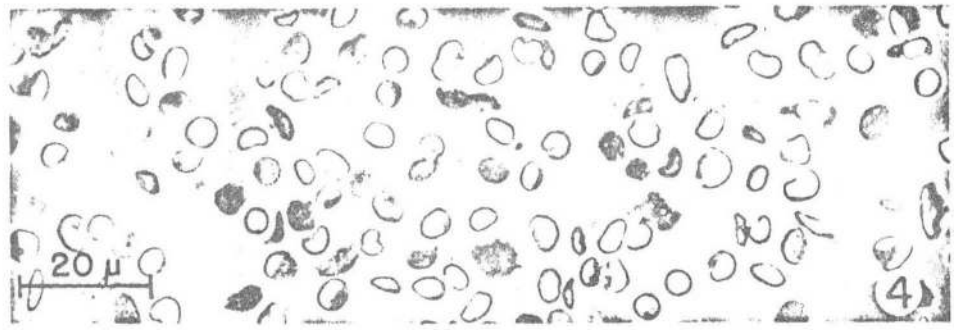

Fig. 4

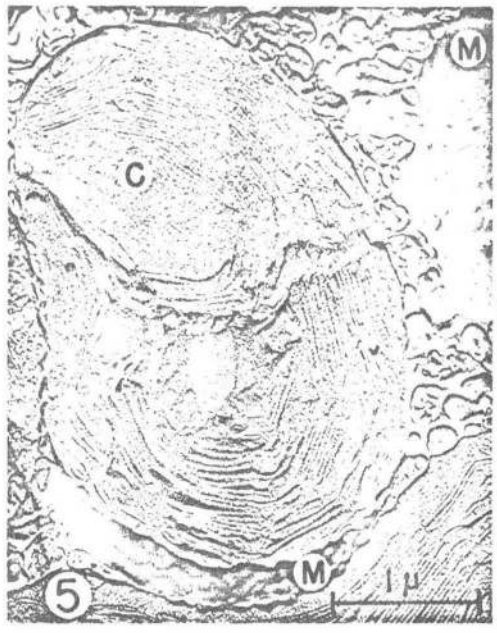

Fig. 5

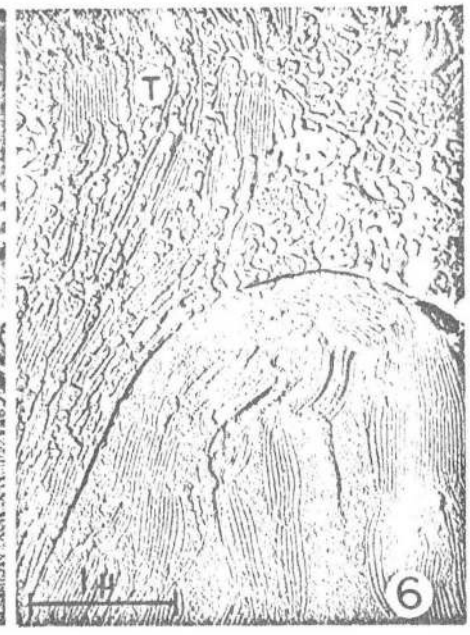

Fig. 6

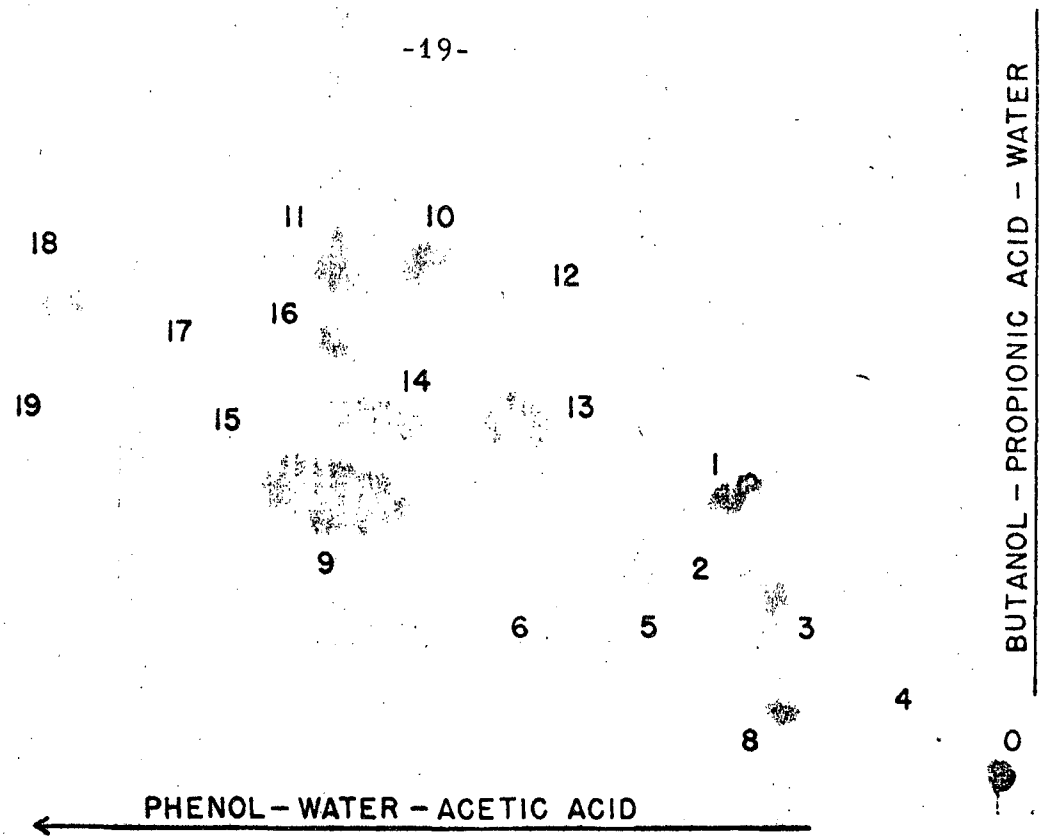
18
11
10
12
17
16
14
19
15
13
1
9
2
6
5
3
4
8
0
BUTANOL – PROPIONIC ACID – WATER
PHENOL – WATER – ACETIC ACID

Fig. 7

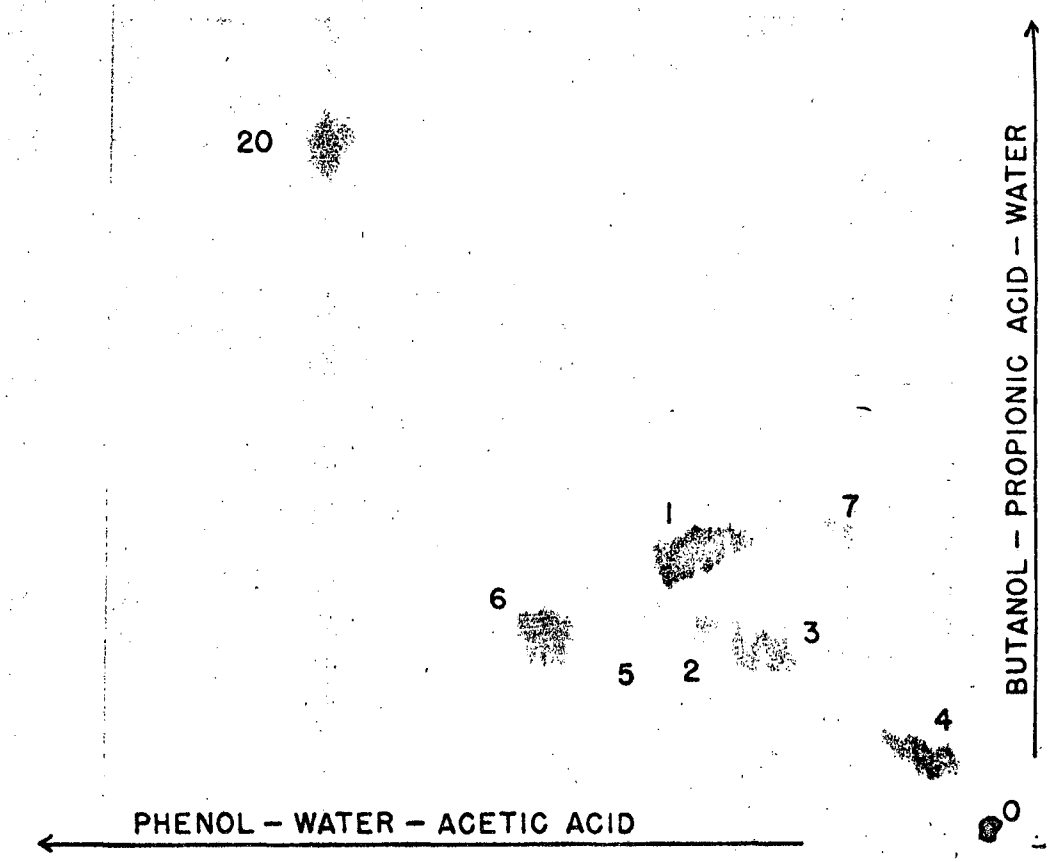
20
1
7
6
3
5
2
4
0
BUTANOL – PROPIONIC ACID – WATER
PHENOL – WATER – ACETIC ACID

Fig. 8