UNIVERSITY OF CALIFORNIA

Radiation Laboratory

BERKELEY, CALIFORNIA

UNIVERSITY OF CALIFORNIA
RADIATION LABORATORY

Cover Sheet
Do not remove

INDEX NO. UCRL-133
This document contains 14 pages
and — plates of figures.
This is copy 18 of 95. Series A
Issued to: Info Div.

UNCLASSIFIED

Classification

Each person who received this document must sign the cover sheet in the space below.

Route to	Noted by	Date	Route to	Noted by	Date
	Wilson	9/15/48			

UCRL-133

Chemistry-General

UNIVERSITY OF CALIFORNIA

Radiation Laboratory

Contract No. W-7405-eng-48

THE PATH OF CARBON IN PHOTOSYNTHESIS. III.

A. A. Benson and M. Calvin

Berkeley, California

Standard Distribution	Copy Nos.
Argonne National Laboratory	1-8
Armed Forces Special Weapons Project	9
Atomic Energy Commission, Washington	10-11
Battelle Memorial Institute	12
Brookhaven National Laboratory	13-22
Carbide & Carbon Chem. Corp. (K-25 Area)	23-26
Carbide & Carbon Chem. Corp. (Y-12 Area)	27-30
Columbia University (Failla)	31
General Electric Company	32-35
Hanford Directed Operations	36-42
Iowa State College	43
Kellex Corporation	44-45
Los Alamos	46-48
Massachusetts Institute of Technology	49
Monsanto Chemical Company, Dayton	50-51
National Bureau of Standards	52-53
Naval Radiological Defense Laboratory	54
NEPA	55
New York Directed Operations	56-57
Oak Ridge National Laboratory	58-69
Patent Advisor, Washington	70
Technical Information Division, ORDO	71-85
UCLA Medical Research Laboratory (Warren)	86
University of California Radiation Laboratory	87-91
University of Rochester	92-93
Western Reserve University (Friedell)	94
Chicago Directed Operations	95
Total	95

University of California
Radiation Laboratory
Berkeley, California

THE PATH OF CARBON IN PHOTOSYNTHESIS. III.

By

A. A. Benson and M. Calvin

Department of Chemistry and Radiation Laboratory

University of California, Berkeley, California*

Although the overall reaction of photosynthesis can be specified with some degree of certainty (CO_2 + H_2O + light ——> sugars + possibly other reduced substances), the intermediates through which the carbon passes during the course of this reduction have, until now, been largely a matter of conjecture. The availability of isotopic carbon, that is, a method of labeling the carbon dioxide, provides the possibility of some very direct experiments designed to recognize these intermediates and, perhaps, help to understand the complex sequence and interplay of reactions which must constitute the photochemical process itself.

The general design of such experiments is an obvious one, namely the exposure of the green plant to radioactive carbon dioxide and light under a variety of conditions and for continually decreasing lengths of time, followed by the identification of the compounds into which the radioactive carbon is incorporated under each condition and time period. From such data it is clear that in principle, at least, it should be possible to establish the sequence of compounds in time through which the carbon passes on its path from carbon dioxide to the final products. In the course of shortening the photosynthetic

---

* This paper is based on work performed under Contract #W-7405-Eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley, California.

times, one ultimately arrives at the condition of exposing the plants to the radioactive carbon dioxide with a zero illumination time, that is, in the dark. Actually, in our work the systematic order of events was reversed, and we have begun by studying first the dark fixation and then the shorter photosynthetic times.

The results of the beginnings of this sort of a systematic investigation are given in Table I which includes three sets of experiments, namely a dark fixation experiment and two photosynthetic experiments, one of 30 seconds duration and the other of 60 seconds duration.

TABLE I

CO_2 FIXATION BY SCENEDESMUS

	10 min. Preillumination 1 min. Dark Fixation	30 Second Photosynthesis	60 Second Photosynthesis
Total Fixed/cc. cells (c.p.m. 10^{-6})	0.97, 100%	6.2, 100%	12, 100%
Insoluble	0%	0%	5%
I. Ether extract at pH 1	12%	10%	3.7%
II. Amino Acids	39%	11%	3.7%
III. A. Sugar Phosphates	4.2%	44% (2% III A, 97% III B, 0.5% IV)	59% (29% III A, 64% III B, 15% IV)
III. B. Phosphoglycerate	42%	27%	20%
IV. Sugars	0.1%	4.7%	4.1%

The method that was developed for separating the various components of the cell are shown in diagrammatic form in Figure 1. Six fractions were obtained, and on the basis of the method of fractionation, certain general properties of the compounds contained in each of these fractions can be specified.

The insoluble fraction, which contained practically none of the very quickly formed radioactive products, consists of the high molecular weight substances such as the proteins, cellulose and starch together with the very water-insoluble low molecular weight materials such as the fats and pigments. Fraction I will contain those materials which can be ether extracted from an acid aqueous medium by a continuous operation extending over a period of fifteen hours. These consist of the fatty acids and the di-and tricarboxylic acids as well as the lower hydroxylated carboxylic acids such as malic acid, lactic acid, glyceric acid, and citric acid. The higher polyhydroxy acids such as gluconic acid and the phosphate esters and anhydrides would not be extracted under these conditions. Fraction II will contain those substances which are or can be cationic. This is limited to the nitrogenous bases, amino acids, and oxonium compounds. Fraction III consists of those anionic materials which could not be ether extracted, namely the organic phosphates and highly hydroxylated carboxylic and enolic acids. Fraction IV consists of the non-ether extractable neutral molecules, that is, the simpler carbohydrates.

Fraction III was further separated into two parts. Part A consists of that group of anionic substances elutable off an anionic exchange resin by ammonia. Part B consists of those anionic materials which are not elutable with ammonia but which can be removed from the resin with sodium hydroxide. So far, the only substance which

we have found to display the behavior of Fraction III-B on the anion resin is phosphoglyceric acid.

A relatively limited group of compounds are known to exhibit the behavior corresponding to Fraction III-A. These consist of the sugar phosphates, both hexose and triose, together with substances like gluconic and mucic acids. An additional characteristic of Fraction III-A makes possible its further breakdown into three more groups of radioactive components.

If the original Fraction III-A ammonia eluate is concentrated by vacuum evaporation and then passed again through the anion column, one finds that all of it is no longer absorbable on the resin, and, furthermore, of that which is absorbed, a certain fraction has become non-ammonia elutable and has been identified with the original III-B fraction, namely phosphoglyceric acid. That part of Fraction III-A which is very readily converted to phosphoglyceric acid we believe to be triose phosphate (1). That part of III-A which becomes non-absorbable we believe to be easily hydrolyzable hexose phosphates such as glucose-1-phosphate, while the remainder of Fraction III-A which is apparently unchanged by contact with ammonia and evaporation in all probability is the more difficultly hydrolyzable hexose phosphate such as glucose-6-phosphate, although it might also contain substances such as gluconic or mucic acids.

The specific identification of most of the radioactive components in these fractions has already been described (1) (2). A

---

(1) Calvin, M., and Benson, A. A., Science, 107, 476 (1948).

(2) Calvin, M., Benson, A.A., Aronoff, S., Haas, V., Hall, A. G., Bassham, J. A., and Weigl, J., "C^{14} in Photosynthesis", monograph, in press, 1948.

more detailed identification of the amino acid fraction by means of radio autographs of paper chromatograms has also been described (3). Therein, the presence of radioactive aspartic acid, alanine, asparagine, β-alanine, serine, and phenylalanine was demonstrated. Although large quantities of glutamic acid were always present, it was never found to be radioactive.

An examination of the three experiments given in Table I reveals smooth trends between all of the three conditions in each of the fractions and in the general nature of the distribution. There is no sharp discontinuity between the photosynthetic experiments and the dark fixation experiment. This indicates a close relationship between the dark fixation and photosynthesis. However, some doubt existed as to the significance of this dark fixation in photosynthesis. This arose from the demonstrated reversal of certain decarboxylation reactions found in non-photosynthetic organisms. At the present writing, these consist of only the two following reactions:

1. CO_2 + pyruvic acid ——— oxalacetic acid (4)

2. CO_2 + ketoglutaric acid ——— oxalsuccinic acid (5)

In view of the absence of radioactivity in any of the components of the tricarboxylic acid cycle having more than four carbon atoms under any circumstances in the early products of photosynthesis, we

---

(3) Stepka, W., Calvin, M., and Benson, A. A., Science, 1948, in press.

(4) Evans, E. A., Vennesland, B., and Slotin, L., J. Biol. Chem., 147, 771 (1943).

(5) Ochoa, S., J. Biol. Chem., 174, 145 (1948).

need only consider Reaction 1. That the dark fixation following preillumination is not due to the simple reversibility of respiratory or fermentative reactions has already been demonstrated (1) and is readily apparent from the mere fact of its great dependence upon preillumination of the cells in the absence of carbon dioxide. The suggestion purporting to account for the increased dark fixation following preillumination depends upon a net mass action reversal of the decarboxylation reaction and those leading up to it by the increased carbon dioxide concentration which obtains upon the addition of radioactive carbon dioxide. The way in which this might be brought about is illustrated by the following reaction schemes:

$$CO_2 \longrightarrow A \xrightarrow{h\nu} B \longrightarrow C \longrightarrow \cdots \longrightarrow \text{Products}$$

$$\uparrow$$

$$CH_3COCO_2^-$$

1.

$$^-O_2C\text{-}CH_2\text{-}CO\text{-}CO_2^- + R \longrightarrow {}^-O_2C\text{-}CH_2\text{-}CHOH\text{-}CO_2^- + Ox. \quad 1a$$

During the preillumination in the absence of carbon dioxide, the residual carbon dioxide concentration (of necessity intra-cellular) is reduced by the normal photosynthetic mechanism through a series of intermediates, A, B, C, etc., presumed unknown. This by hypothesis is the only effect of the preillumination in the absence of carbon dioxide. Upon the addition of the radioactive carbon dioxide, the carbon dioxide concentration is increased producing a mass action reversal of a sequence of reactions to and through the decarboxylation reaction. For very small amounts of carbon dioxide fixed with respect

to the total fixing capacity, that is, the saturation value, the amount fixed should be simply proportional to the increase of carbon dioxide concentration and independent of the total carbon dioxide concentration.

Thus, if a plot is made of the amount fixed against the carbon dioxide added, the initial slope of this curve should be independent of the total amount of carbon dioxide present. Such a plot is shown in Figure 2. The initial slope for the preilluminated algae is over one hundred times that for the non-preilluminated algae. The ordinates on this figure are calculated in terms of the initial specific activity of the carbon fed for both curves. If the difference between the two curves is due to a higher initial non-radioactive carbon dioxide concentration in the predark cells, the added radioactive carbon dioxide would have been more dilute (lowered specific radioactivity) in these cells than in the preilluminated cells by the ratio of the initial non-radioactive carbon dioxide concentrations. In order to make the slope the same, the ratio of residual carbon dioxide concentrations in the predark cells to that in the preilluminated cells must have been greater than one hundred, that is, the preillumination must have reduced the residual carbon dioxide concentration in the cells by a factor of over one hundred.

Since the cells were being continually swept with carbon dioxide-free helium at a rate of about 500 cc. a minute throughout the entire experiment which lasted about an hour, of which the first half hour consisted merely of sweeping the cell suspension in the dark, the

residual carbon dioxide concentration within the cells could not have been greater than that corresponding to a partial pressure of the order of 0.1 mm. Therefore, the preillumination would have had to reduce the carbon dioxide concentration to a value corresponding to a partial pressure of less than 0.001 mm. This we know the light cannot do.

The rate of photosynthesis in a wide variety of green plants begins to fall off in the vicinity of 1.0 mm. partial pressure of carbon dioxide. In a medium containing one-fifth atmosphere of oxygen, the steady state carbon dioxide partial pressure cannot be reduced below approximately 0.1 mm. This is, of course, due to a balance between the photosynthetic rate of removal of carbon dioxide and the production of carbon dioxide by respiratory and fermentative mechanisms. While it is true that in our case (anaerobic), carbon dioxide is presumably produced only by a fermentative path, this would also produce a corresponding lower maximum possible residual carbon dioxide concentration in the predark algae and the lower limit for the steady state carbon dioxide partial pressure would be reduced in the same ratio. It is, perhaps, significant that the dependence of the dark fixation on carbon dioxide partial pressure shown in Figure 2 resembles very much the dependence of photosynthetic rates on carbon dioxide concentration.

The curves in Figure 2 can very readily be understood if the function of the preillumination is to increase the concentration of the reducing agent (R in Reaction 1a) and of the carbon dioxide

acceptor (s), such as the pyruvate, through some cyclic path. Such a cycle has already been presented (1) (2), and is reproduced here although the various experimental data leading up to it will not be reviewed again in this paper. The two reactions labeled with a (?) are introduced as possible routes to account for the appearance of a considerable amount of radioactivity in the carboxyl groups of succinic acid and alanine when carboxyl-labeled acetate is fed to the algae.

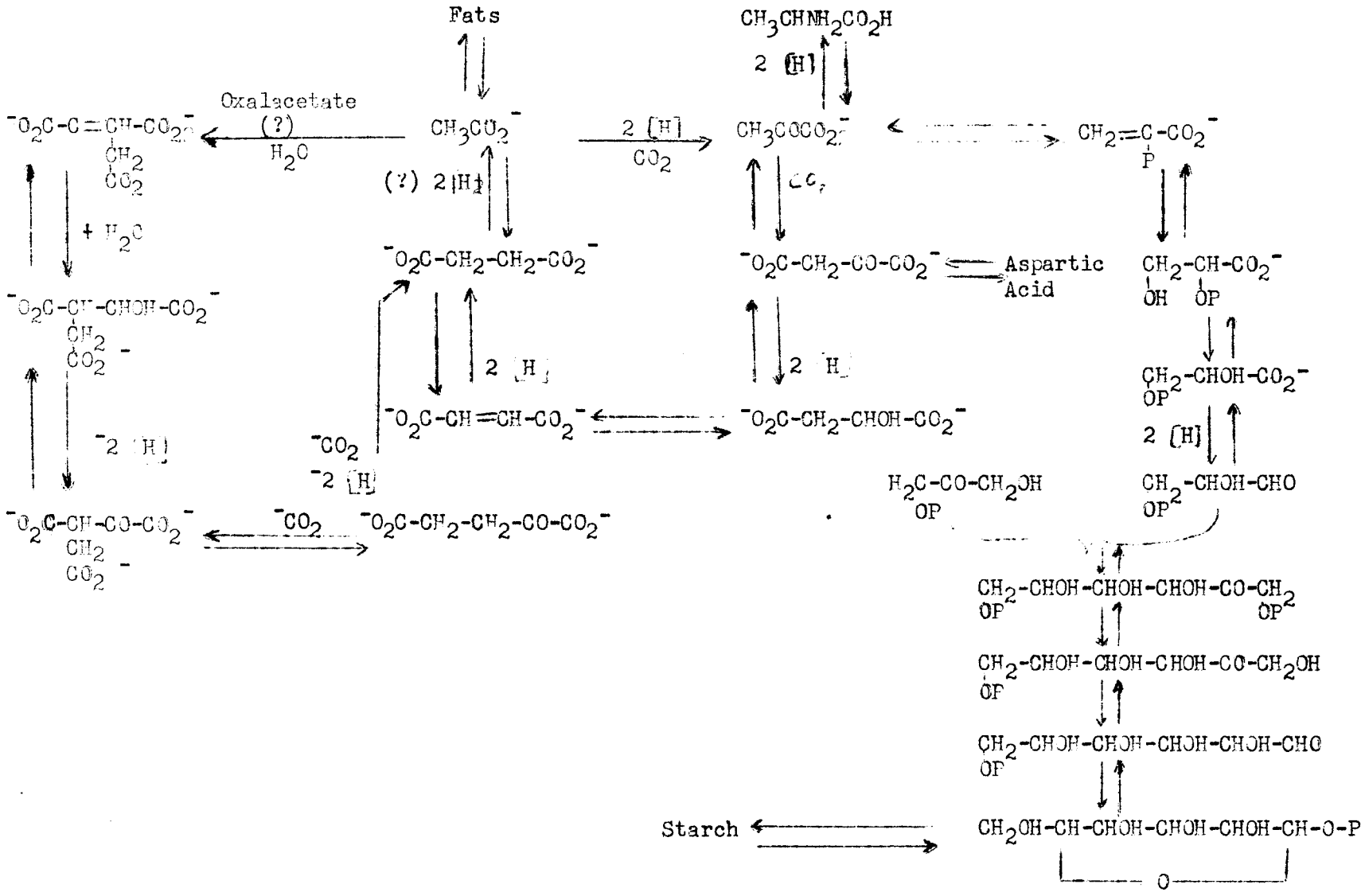

Fats
Oxalacetate
(?)
H_2O
$CH_3CO_2^-$
2 [H]
CO_2
$CH_3CHNH_2CO_2H$
2 [H]
$CH_3COCO_2^-$
CO_2
$CH_2=C-CO_2^-$
P
$^-O_2C-C=CH-CO_2^-$
CH_2
CO_2^-
+ H_2O
(?) 2 [H]
$^-O_2C-CH_2-CH_2-CO_2^-$
$^-O_2C-CH_2-CO-CO_2^-$
Aspartic Acid
$CH_2-CH-CO_2^-$
OH OP
$^-O_2C-CH-CHOH-CO_2^-$
CH_2
CO_2^-
2 [H]
2 [H]
$CH_2-CHOH-CO_2^-$
OP
$^-O_2C-CH=CH-CO_2^-$
$^-O_2C-CH_2-CHOH-CO_2^-$
$^-CO_2$
$^-2$ [H]
2 [H]
$H_2C-CO-CH_2OH$
OP
$CH_2-CHOH-CHO$
OP
$^-O_2C-CH-CO-CO_2^-$
CH_2
CO_2^-
$^-CO_2$
$^-O_2C-CH_2-CH_2-CO-CO_2^-$
$CH_2-CHOH-CHOH-CHOH-CO-CH_2$
OP OP
$CH_2-CHOH-CHOH-CHOH-CO-CH_2OH$
OP
$CH_2-CHOH-CHOH-CHOH-CHOH-CHO$
OP
Starch
$CH_2OH-CH-CHOH-CHOH-CHOH-CH-O-P$
O

FIGURE 1

FRACTIONATION OF RADIOACTIVE PRODUCTS OF PHOTOSYNTHESIS BY ALGAE

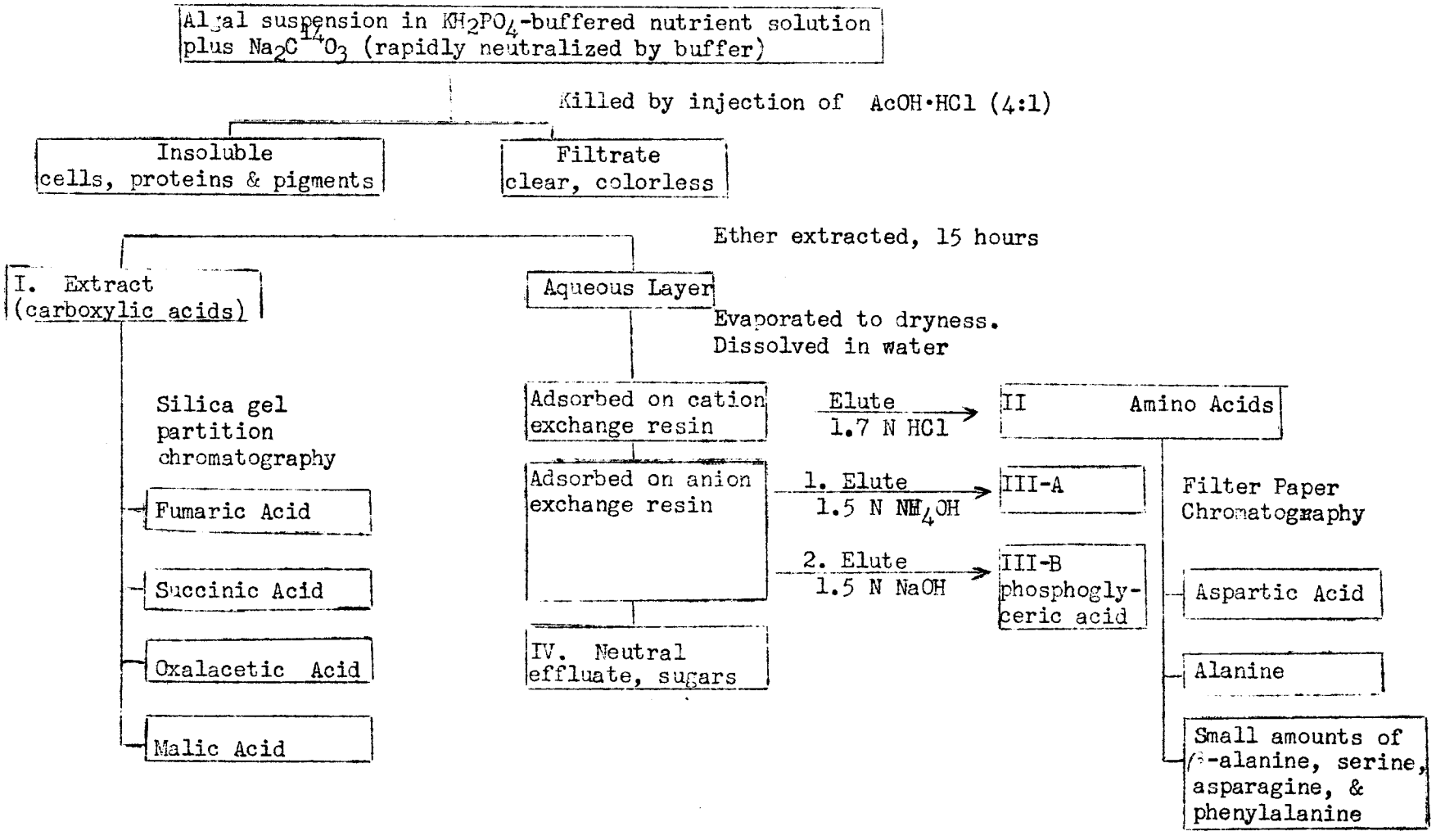
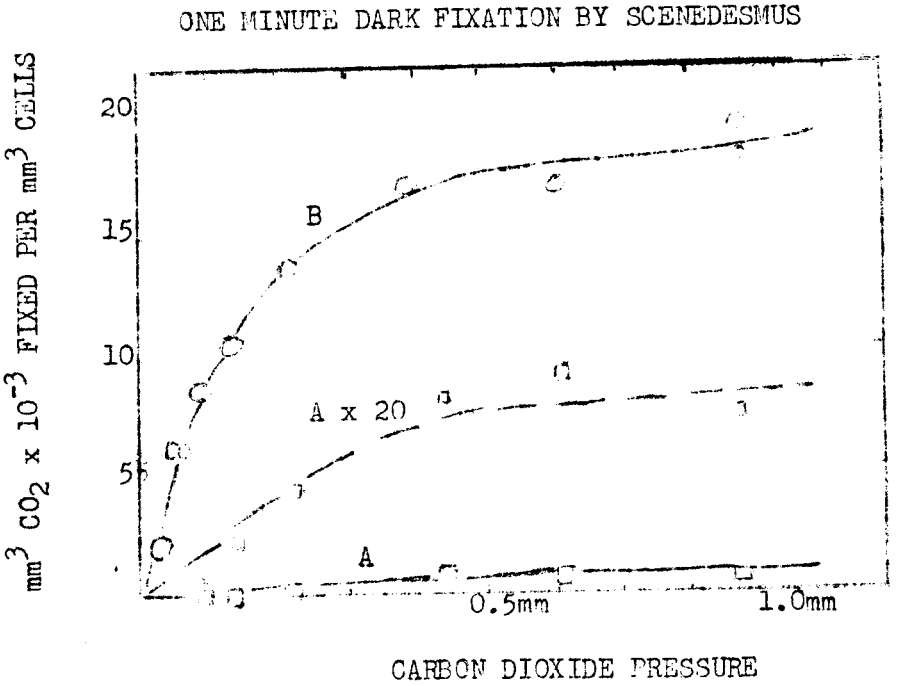


FIGURE 2


ONE MINUTE DARK FIXATION BY SCENEDESMUS
mm3 CO2 x 10-3 FIXED PER mm3 CELLS
20
15
10
5
B
A x 20
A
0.5mm
1.0mm
CARBON DIOXIDE PRESSURE