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Lawrence Radiation Laboratory
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Contract No. W-7405-eng-48

PART I. SEARCH FOR UNSTABLE INTERMEDIATES IN THE CARBOXYLATION REACTION OF PHOTOSYNTHESIS
PART II. SOME REACTIONS OF DIAZOMETHANE IN AQUEOUS SOLUTION

George Agapios Akoyunoglou

August 1962

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Printed in USA. Price $2.50. Available from the
Office of Technical Services
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Washington 25, D.C.

ABSTRACT

PART I. SEARCH FOR UNSTABLE INTERMEDIATES IN THE CARBOXYLATION REACTION OF PHOTOSYNTHESIS

Carboxydismutase (extracted from chloroplasts of Spinacea oleracea and fractionated with ammonium sulphate) was carboxylated with $H^{14}CO_3^-$ in the presence of Mg^{++} ions, and the complex was isolated in about 45% yield by passage through a Dowex 1 column. $^{14}CO_2$ was quantitatively transferred from $^{14}CO_2$~enzyme to ribulose-1,5-diphosphate (RuDP) yielding ^{14}C-labeled phosphoglyceric acid (PGA).

The carboxylation of carboxydismutase was Mg^{++}-dependent. The rate of $^{14}CO_2$~enzyme formation was dependent on the concentration of enzyme, Mg^{++}, and bicarbonate. It was also temperature dependent. The formation of the complex was not inhibited by avidin or ATP and it was not ATP-dependent.

Preincubation studies with the substrates and inactivation of the enzyme with cold methanol before incubation showed that Mg^{++} with bicarbonate stabilized the enzyme against methanol while RuDP did not. When the enzyme plus Mg^{++} is preincubated with bicarbonate, then treated with methanol followed by incubation with RuDP, some PGA is obtained, but when the enzyme is preincubated with RuDP, no PGA is formed.

All efforts failed to produce any evidence for the existence of the postulated diphosphate ester of a beta-keto acid intermediate in the carboxylation reaction of RuDP. This can be explained by any of the following: the amount present is very small; the life time very short; or the free beta-keto acid does not occur at all, and the reaction goes through an enzyme~CO_2~RuDP complex. These

efforts included treatment of the products with liquid ammonia and then separation by low temperature paper chromatography, and treatment of the photosynthetic material or the carboxydismutase system with diazomethane.

The results of this study provided conclusive evidence that the overall carboxydismutase reaction proceeds in two or probably in three steps, with the enzyme-bound CO_2 as an intermediate.

PART II. SOME REACTIONS OF DIAZOMETHANE IN AQUEOUS SOLUTION

Carbon dioxide was allowed to react with diazomethane prepared by alkaline hydrolysis of N-methyl-N-nitroso-N'-nitroguanidine. The different products were separated, and identified as dimethyl carbonate, methyl carbamate, N-methyl methyl carbamate, and N-dimethyl methyl carbamate.

The presence of ammonia was necessary for the formation of methyl carbamate, N-methyl methyl carbamate, and N-dimethyl methyl carbamate when carbon dioxide was allowed to react with diazomethane prepared by alkaline hydrolysis of N-methyl-N-nitroso-p-toluenesulfonamide.

Ammonia was found to be present in the ethereal solution of diazomethane prepared by alkaline hydrolysis of N-methyl-N-nitroso-N'-nitroguanidine.

The products of the reaction between ammonia, carbon dioxide and diazomethane in aqueous solution are dimethyl carbonate, methyl carbamate, N-methyl methyl carbamate, and N-dimethyl methyl carbamate. A mechanism for their formation is proposed.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to all of the members, both past and present, of the Lawrence Radiation Laboratory's Bio-Organic Chemistry Group, for their many suggestions and their interest. It is with particular gratitude that he acknowledges the advice of Drs. J.A. Bassham and N.G. Pon.

I am deeply indebted to Professor Melvin Calvin, under whose stimulating directions and encouragement this research was accomplished.

I particularly want to express my gratitude to the Greek State Scholarship Foundation for the award of a scholarship which made it possible for me to start graduate studies at the University of California.

Last, but by no means least, I want to express my appreciation to my wife. Without her encouragement and patience, this work could not have been completed.

This work was done under the auspices of the United States Atomic Energy Commission and the Greek State Scholarship Foundation.

PART I. SEARCH FOR UNSTABLE INTERMEDIATES IN THE CARBOXYLATION REACTION OF PHOTOSYNTHESIS

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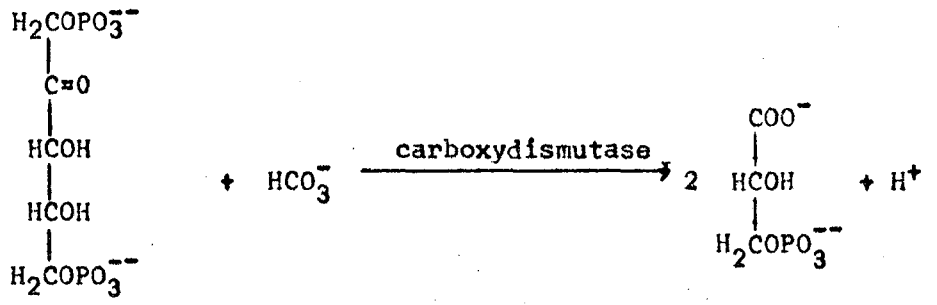
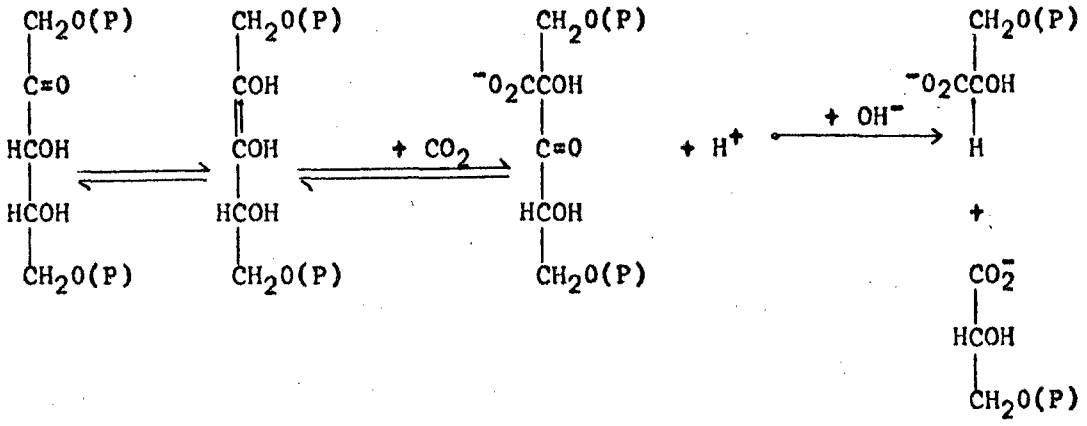
I. INTRODUCTION

It has been well established that ribulose diphosphate is the carbon dioxide acceptor in the carboxylation reaction of photosynthesis in green plants, and that phosphoglyceric acid is the stable product of the reaction[1-7]. This reaction has the following stoichiometry[3,7,8]:



ribulose-1,5-diphosphate 3-phosphoglycerate

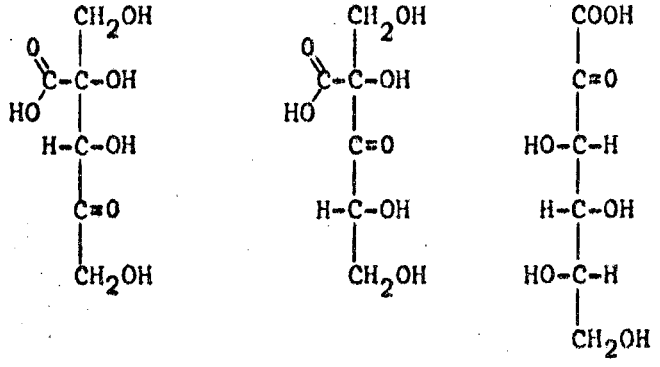
Calvin[9,10] has suggested that the reaction proceeds via an intermediate, a six-carbon sugar beta-keto acid diphosphate (2-carboxy-3-ketopentitol-1,5-diphosphate), an unstable compound, which is hydrolyzed easily to form two molecules of PGA. The proposed mechanism of the carboxylation reaction is the following[11]:



An electrophilic attack of the carbon dioxide on the enolic

form of RuDP occurs, to form the beta-keto acid, which in turn is hydrolyzed to form two molecules of PGA.

Many attempts have been made to demonstrate the existence of the beta-keto acid or of any other unstable intermediate, which is probably formed in the carboxylation reaction of photosynthesis. Moses and Calvin[12] were able to isolate two radioactive substances from Chlorella pyrenoidosa after 3 min of photosynthesis with ^{14}C-bicarbonate. They characterized these substances as belonging to a carboxyketopentitol diphosphate type. They had been tentatively proposed as the diphosphates of 2-carboxy-4-ketopentitol (I) and 2-carboxy-3-ketopentitol (II), a gamma-keto acid and beta-keto acid respectively.



(I) (II) (III)

Both of them are isomers, and it was suggested that the gamma-keto acid was formed from the beta-keto acid, the proposed unstable product of the carboxylation reaction of RuDP. Recently[13], it was found in this laboratory that the gamma-keto acid was really the diphosphate of the 2-keto-aldonic acid (III), and it was not an intermediate in the carbon reduction cycle of photosynthesis, but was formed from glucose. It is not known if the compound once

proposed as beta-keto acid is really the compound (II), because its identification was based on the identification of compound (III) as gamma-keto acid. Shkolnik and Doman[14] used liquid nitrogen to stop the photosynthetic reactions in leaves, liquid ammonia to extract and stabilize some unstable acids in the ground leaves, and low temperature chromatography (-30°) to separate the different radioactive products. When this technique was used on geranium leaf, after one second exposure to labeled CO_2, radioactivity was observed on chromatograms at the beginning of the diphosphate zone. If the same material were treated less carefully this radioactivity was absent. They were not able to reproduce these experiments on all plant species tested, but only on geranium and, to a lesser extent, on beans. As far as it is known this radioactive compound observed by Doman has not yet been identified. Pon[7] made many attempts to demonstrate the existence of this beta-keto acid in the isolated enzyme system. He used many methods to slow down the reaction, to stop it or stabilize and separate the products. Despite these extensive studies, he failed to produce any evidence for the existence of the beta-keto acid.

It is well known that the RuDP carboxylation reaction is an enzymatic reaction. A cell-free extract of algae capable of yielding phosphoglyceric acid from ribulose diphosphate and $^{14}CO_2$ was obtained in this laboratory in 1954[2]. About the same time, Weissback et al. obtained a soluble spinach extract capable of forming phosphoglyceric acid from $^{14}CO_2$ and ribose-5-phosphate in the presence of adenosine triphosphate and triphosphopyridine

nucleotide[3]. Many studies of the ribulose diphosphate carboxylase (or carboxydismutase as it is called) that was derived from different sources followed, and the requirement of a metal ion (Mg^{++}) as cofactor was established[3,7].

Pon[7] studying the carboxydismutase system found that preincubation of the enzyme with Mg^{++} ions and bicarbonate increases its activity. He suggested two possible interpretations of this phenomenon: (1) the enzyme combines with the metal ion and bicarbonate to form a complex (active CO_2), and then reacts with ribulose diphosphate; or (2) the metal ion or bicarbonate activates the enzyme by reaction at some site other than the active site. A comformation change of the enzyme is induced accompanying the activation process[6,15].

Various forms of (active CO_2) have been suggested as intermediates in different enzymically catalyzed carboxylation reactions, such as the carboxylation of (1) propionyl CoA to form methyl-malonyl CoA[16], (2) beta-methylcrotonyl CoA to form beta-methyl-glutaryl CoA[17], etc. Lynen et al.[17] were able to prepare a CO_2~biotin unstable complex by adding biotin as substrate in place of methylcrotonyl CoA in the methylcrotonyl CoA carboxylase system. This complex was stabilized by conversion to the dimethyl ester with diazomethane. It was also found that only the d-biotin reacted and formed the complex, the l-enantiomorph being inactive. Lynen et al. explained their results with the assumption that the methylcrotonyl CoA carboxylase can bind d-biotin in a dissociable fashion, and that the "CO_2" combined

with biotin covalently bound to the enzyme can be tranferred to the dissociably bound biotin to form the $CO_2\sim$ biotin complex. Recently Ochoa et al.[18,19] prepared and separated a $CO_2\sim$ enzyme complex (propionyl CoA carboxylase), using Dowex 1-Cl (X8, 200 to 400 mesh) ion exchange column which was eluted with 0.02 M-Tris-HCl buffer, pH 8.0, containing 0.001 M-GSH. This complex was relatively stable near 0° and very unstable at higher temperatures. All the above enzyme systems require ATP. Lynen's $CO_2\sim$ biotin and Ochoa's $CO_2\sim$ enzyme complexes are not formed in the absence of ATP.

It is possible that a $CO_2\sim$ enzyme complex is also formed as an intermediate in the carboxylation reaction of ribulose diphosphate, which subsequently reacts with RuDP to form phosphoglyceric acid, but no evidence is hitherto reported.

The purpose of this study was:

1) To search for the proposed beta-keto acid or any other unstable intermediate formed in the carboxylation reaction of ribulose diphosphate.

2) To search for a $CO_2\sim$ enzyme complex of the carboxydismutase system.

3) To prepare, isolate and study the $CO_2\sim$ enzyme complex of the carboxydismutase system.

4) Finally, to study the effects of various additions (e.g. activators and inhibitors) on the carboxydismutase system related with the $CO_2\sim$ enzyme complex formation.

II. EXPERIMENTAL PROCEDURE

Abbreviations. The following abbreviations are henceforth used throughout the first part of this text.

cpm	: counts per minute	ppt	: precipitate
dpm	: disintegrations/min.	rpm	: revolutions/min.
G-M	: Geiger-Mueller	soln	: solution
g	: gram, or acceleration due to gravity	hr	: hour
uC	: microcurie(s)	temp	: temperature
mC	: millicurie(s)	sec	: second
l	: liter(s)	EtOH	: ethanol
ml	: milliliter(s)	MeOH	: methanol
ul	: microliter(s)	GSH	: glutathione
uM	: micromole(s)	CoA	: coenzyme A
mg	: milligram(s)	PGA	: phosphoglyceric acid
mM	: millimole(s)	PS	: photosynthesis
M	: molar	PEPA	: phosphoenolpyruvic acid
N	: normal	SMP	: sugar monophosphates
min	: minute(s)	SDP	: sugar diphosphates
EDTA	: ethylenediaminetetraacetic acid	TRP	: triose phosphate
ATP	: adenosine-5'-triphosphate	suprn	: supernatant liquid

PPO : 2,5-diphenyloxazole (primary scintillator "fluor)

POPOP : 1,4-bis-(2-(5-phenyloxazole))benzene (secondary scintillator "fluor")

tris : tris(hydroxymethyl)aminomethane

R_f : ratio of distance travelled by spot to distance travelled by solvent front - distance is measured from the origin to the densest portion of the spot.

Apparatus

Homogenizer: A Waring blender operated at high speed was used for the rupture of spinach leaves.

Centrifuge: For preparative purposes the following centrifuges were used: 1) International Clinical Centrifuge (up to 2,500 X g); 2) International Portable Refrigerated Centrifuge, Model PR-2 (up to 2,500 X g); and 3) Spinco Ultracentrifuge, Model L (from 3,000 to 145,000 X g). All g-values were maximum values.

Radioactive counters: The radioactivity of samples was measured either with a flow counter equipped with a thin window G-M tube or with a scintillation counter. (1) G-M counters: An automatic counter, equipped with a G-M tube with a Micromil* window, was used for precise counting. The automatic counter consisted of a Nuclear-Chicago scaling unit (model 183), an automatic sample changer (model C-110A) and a printing timer (model C-111B). The quenching gas (Q gas) flowing through the system was composed of 99.05% He and 0.95% isobutane. For counting individual samples or radioactive spots on chromatographic paper a G-M scaler (Nuclear Chicago, model 181A), equipped with a modified G-M tube (Scott) covered with a Mylar** end-window[20] and flushed continuously with Q gas was used.

The samples were spread out on thin aluminum planchets. The planchets were first cleaned by rinsing with alcohol, and then the sample, acidified with acetic acid, was transferred to the planchet on a rotating table[21] with the aid of a micropipette. One or two drops of detergent (Aquet***-H_2O, 1:500 w/v) were added in the sample in

* Supplied directly by the Nuclear Chicago Corp.

** Mylar is DuPont's registered trademark for its polyester film.

*** From the Emil Greiner Co., 22 N. Moore St., New York.

order to facilitate the spreading of material on the planchet. The counters were corrected for coinicdence, and an "infinitely thin" $Ba^{14}CO_3$ plate covered with a film of shellac was used as a stable reference standard.

(2) Scintillation counter: The liquid scintillation counter used in this study was a coincidence anticoincidence counter, "Tri-Carb"*, described earlier[22]. Two scintillation solutions were used, No. 2 for aqueous samples, and No. 6 for nonpolar samples. The composition of No. 2 was: Toluene (2.0 l), p-dioxane (2.0 l), absolute EtOH (1.2 l), naphthalene (260 g), PPO (26 g), and POPOP (0.5 g). The composition of No. 6 was: Toluene (1.0 l), PPO (4.5 g), and POPOP (0.1 g).

For the determination of the radioactivity, aliquots of radioactive material to be counted (not exceeding 0.5 ml) were pipetted accurately with a micropipette into 20 ml glass vials with aluminum-lined screw tops, which contained 10 ml of the scintillation solution No. 2 or No. 6. Each sample was counted in the scintillation counter a sufficient number of times to obtain the desired statistical accuracy. The variability of the composition of the sample was controlled by the use of an internal standard. The internal standard used was toluene-1-^{14}C, which had been distilled and diluted to an appropriate concentration. 0.2 ml of toluene-^{14}C (1.23×10^5 dpm/ml) was pipetted into the counted sample and the vial was recounted. The first count, minus the background, is termed C_1; the second count minus the background is termed C_2.

* "Tri-Carb" liquid scintillation spectrometer, model 214, Packard Instrument Co., Inc., LaGrange, Illinois.

$$\text{Efficiency of counting} = \frac{C_2 - C_1}{\text{std. activ. added}} = \frac{C_2 - C_1}{2.46 \times 10^4}$$

$$\text{dpm of sample} = \frac{2.46 \times 10^4 \times C_1}{C_2 - C_1} = \frac{C_1}{\text{efficiency}}$$

The glass vials were used only once, in order that their contamination might be prevented. To prevent also inaccurate measurements due to the variability of the composition of the sample and the solvent, and the"colour quenching"* of some coloured samples, internal standard was added to every sample that was counted.

Dialyzer: Dialysis of the enzymic solution against a very dilute buffer was necessary in order to remove salts and low molecular weight material present in it. The sample to be dialyzed was placed within a dialysis bag made of Visking cellulose acetate tape, and both ends were knotted. For complete dialysis, the external buffer solution was changed 5 to 6 times, once every two hours.

Rotating evaporator: Solutions of relatively large volume were concentrated under reduced pressure using a Rinco rotating evaporator (Rinco Instrument Co., Greenville, Ill.) in conjunction with dry ice and liquid nitrogen traps.

Low temperature chromatography box: A special chromatography box-freezer combination made by Labline Inc. (Chicago, Ill.) was used for low temperature chromatography. The dimensions of the box were 78 x 54 x 70 cm. The instrument was capable of holding 8-10 papers each time, for one or two-dimension chromatography. The temperature of the box could range from room temperature to

* "Colour quenching": Strongly coloured samples tend to absorb some of the light emitted by the scintillator fluor, and subsequently lower the sensitivity of the scintillation counter.

-40° C, and it could be kept constant (± 1° C) during the run and the drying of the chromatograms. A preliminary drying of the chromatograms was done under a forced current of dry cold air at the temperature of the run, and then they were removed to another box at room temperature for complete drying.

Analyses.

Paper chromatography: After a reaction or a photosynthetic experiment was completed, the usual method used to separate the products was paper chromatography. Whatman No. 4 papers were used after being washed with oxalic acid according to Benson[23]. Descending chromatograms were made in the boxes usually used in this laboratory[11]. The material was spotted on an origin 3 inches from the top and the side of the paper. Water saturated phenol and butanol-propionic acid-water were used as developing solvents[11,23]. The solvents were run for eight to twelve hr in each dimension, and then the papers were dried under a forced current of air at room temp. The temp during the development of the chromatogram was 20 to 25° C. The butanol-propionic acid-water solvent was prepared just before it was used, by mixing equal volumes of butanol-water (1,246:84 v/v) and propionic acid-water (620:790 v/v).

For the separation of the phosphorylated esters another set of solvents was also used: Butanol-propionic acid-water in the first dimension and isobutyric acid-water-concentrated ammonia-EDTA (2520:1450:100 v/v:1.175 g)[24] in the second dimension.

Butanol-acetic acid-water (4:1:5 v/v) and isobutyric acid-water-concentrated ammonia-EDTA were also used for the separation of the methylated products. The methylation was carried out with diazomethane.

Many solvents were tried for the separation of the products at low temp and two of them which gave the best results were used: methanol-water-liquid ammonia (80:20:20 v/v) and acetone-methanol-water-liquid ammonia (30:28:12:130 v/v). The temp during the development of the chromatograms was -30° to -35° C.

Radioactive compounds on the chromatogram were located by exposing the paper to single coated Kodak x-ray film. The identity of the chromatographically separated compound was established by its R_f value, and confirmed by cochromatography of the eluted radioactive compound with an authentic marker compound. The identity of phosphate esters was further confirmed by treating the eluates with acid phosphatase purified from Polidase-S (Schwartz Laboratories, Inc.). The enzymic hydrolysate was cochromatographed with authentic marker compounds. In each case, exact coincidence of the radioactivity with the marker compound was taken as criterion of identity.

For the detection of the authentic marker compounds on the chromatogram, either the paper was sprayed with an acidified ammonium molybdate reagent and then exposed to sunlight[25] (detection of organic phosphates), or the paper was dipped into an acetonic solution of silver nitrate and then sprayed with ethanolic NaOH[26] (detection of sugars and sugar acids).

Anion exchange column: The separation of the $CO_2 \sim$ enzyme complex was accomplished by passing the reaction mixture through a Dowex 1 column, as described by Ochoa *et al.*[18]. Dowex 1-Cl (X8, 200 to 400 mesh) was washed and equilibrated with 0.02 *M* Tris-HCl buffer, pH 8.0, and then packed into a 0.8 x 4.0 cm column. The column was washed

with tris-HCl buffer pH 8.0, 0.02 M, and immediately before use with 3 ml of 0.02 M tris-HCl buffer, pH 8.0, containing 0.001 M GSH. The column was loaded with the reaction mixture (usually 0.5 ml) ans subsequently washed with 0.6 ml 0.02 M tris-HCl-GSH buffer. 0.02 M tris-HCl-GSH buffer (1.8 ml), pH 8.0, was used for the elution of the column. The eluate contained 45% (w/w) of the initial protein. An aliquot sample of the eluate (0.2 ml) was placed in a liquid scintillation counter for determination of ^{14}C content. Preliminary experiments showed that all free bicarbonate ion from a reaction mixture which contained all the components except the enzyme stayed in the column after elutionwith 8.0 ml of 0.02 M tris-HCl-GSH buffer, pH 8.0. In the presence of enzyme the 1.8 ml eluate contained all the enzyme which could be eluted, the effluent, wash and further eluate being free of enzyme. All the operations were carried out in the cold room (0 to 2° C).

Plant Material

The plant material used in our experiments were (1) unicellular green algae, *Chlorella pyrenoidosa*; (2) leaves of horse beans, *Vicia taba*; (3) spinach leaves, *Spinacea oleracea*.

Green algae (Chlorella pyrenoidosa): *Chlorella* is being continually cultured in this laboratory in Myer's medium[27] and the methods used were those described earlier[11,28]. With these methods of culture the conditions of growth are the same from day to day. The harvested algal suspension was centrifuged at about 1600 x g, then suspended in a buffer solution, and centrifuged again. The algae was then resuspended in the same buffer, to a concentration of

from 1% to 4%, depending on the nature of the experiment. The buffer used was 2.5×10^{-5} M K_2HPO_4-KH_2PO_4 (3:2). The algae were washed with the buffer solution because the initial nutrient solution contained a quantity of salts which often interferred with the analysis of biochemical compounds formed following exposure to the tracer element. The above particular buffer was used because the small amount of added phosphate improves the subsequent chromatography. The resuspended algae were aerated at room temp in weak light until the time they were used. A small amount of the final suspension of algae (2 to 6 ml) was used in each photosynthetic experiment.

Leaves of horse beans (Vicia taba): Small leaves were taken about 40 days after germination of the plant, washed with cold tap water, drained and then used in the photosynthetic experiment. Leaves of this particular plant were used because they were easily extractable, they had a high photosynthetic rate, and they were more suitable for our experiments.

Spinach leaves (Spinacea oleracea): Fresh spinach leaves obtained from the market were used for preparation of chloroplasts, and for the isolation of the ribulose diphosphate carboxylase (carboxydismutase) from the chloroplast extract. The chloroplasts were first isolated and then lysed osmotically to yield the chloroplast extract. This extract was fractionated with ammonium sulfate.

Preparation of Carboxydismutase

The following methods for preparing spinach chloroplasts and carboxydismutase were used[7,29]. Fresh spinach leaves with petioles and some of the midribs removed were washed in cold tap water twice,

drained dry by tumbling in a wire basket, and placed in a cold room for several hours to allow the leaves to become turgid. All subsequent operations were carried out at or near 0° C. All glassware and centrifugation apparatus were precooled to about 0° C.

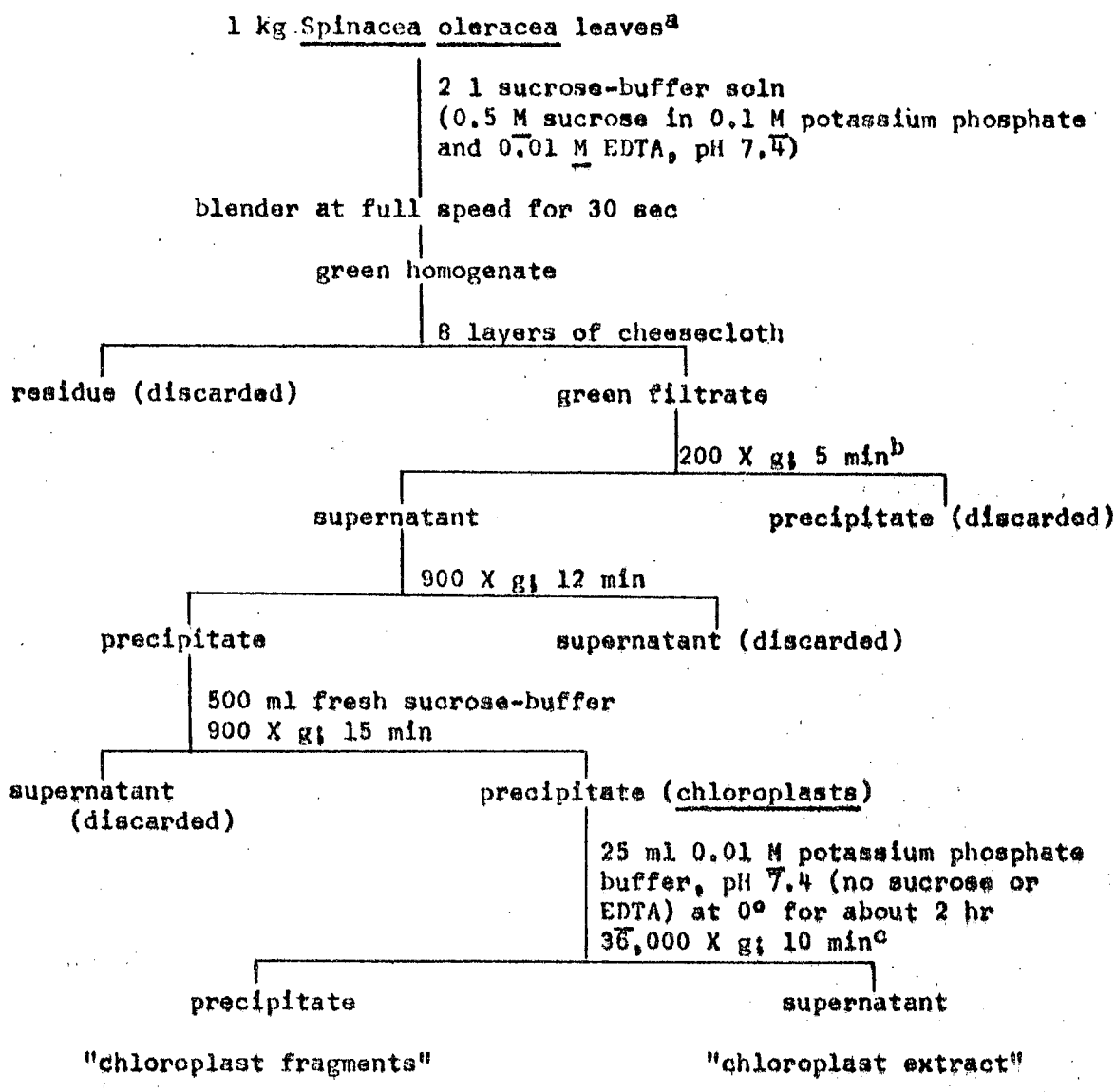
One kilogram of leaves was used for each experiment. The leaves were cut into strips 0.5 to 1 cm wide and were divided into eight equal portions. Each portion (125 g) was ground in a blender at full speed for 30 sec, together with 250 ml of sucrose-buffer solution. (Sucrose-buffer: 0.5 M sucrose in 0.1 M potassium phosphate and 0.01 M EDTA, pH 7.4.) The steps for the preparation are given in Fig. 1 and Fig. 2.

Preparation of Ribulose Diphosphate

Ribulose diphosphate (RuDP) solution was prepared from the barium salt of RuDP*. In a 10 ml centrifuge tube containing 0.60 ml water and 0.60 ml of 0.10 M HCl, 30 mg of Ba-RuDP were suspended. The larger particles were crushed with the aid of a small glass rod. The mixture was cooled in an ice bath and 1.20 ml of 0.10 M Na_2SO_4 plus 0.60 ml of 0.10 M NaOH were added. The mixture was allowed to stand for 10 to 15 min before centrifuging the precipitate. The supernatant liquid containing the RuDP was stored in a frozen state.

RuDP-^{14}C was prepared by extraction from algae after being allowed to undergo photosynthesis in the presence of $NaH^{14}CO_3$ for 2 to 3 min[4,7]. The radioactive products extracted from the algae were separated by paper chromatography, and the separated RuDP-^{14}C was eluted from the paper.

* Kindly supplied by Dr. N.G. Pon.

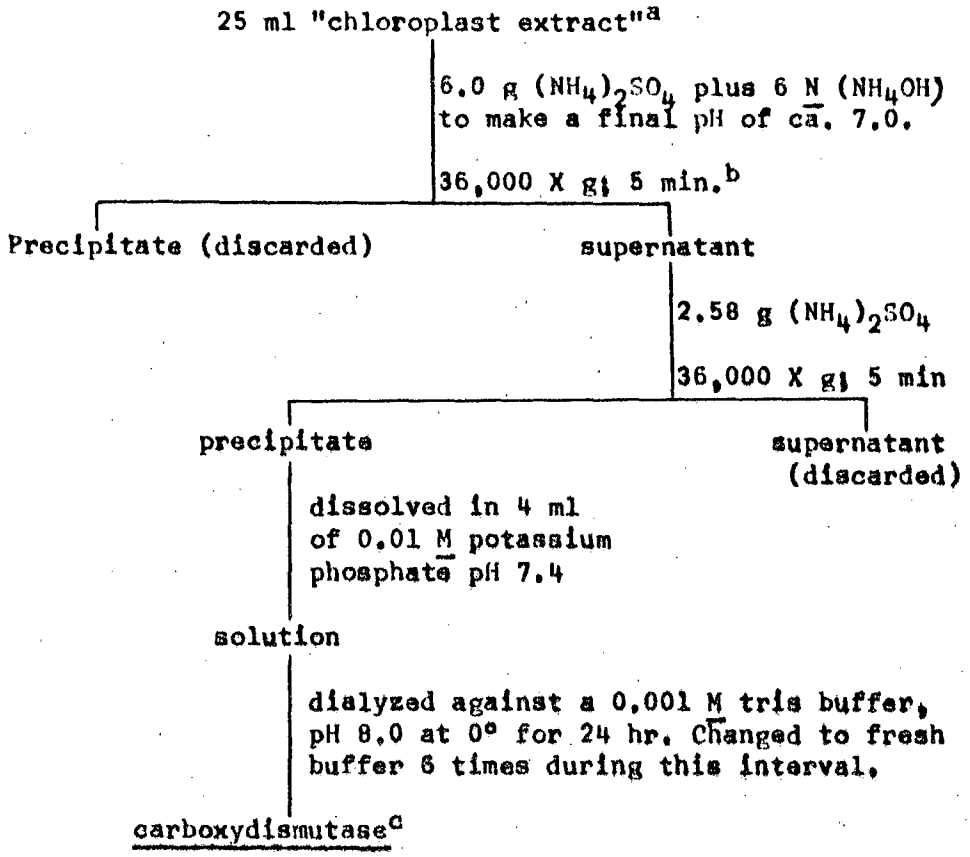


a) All operations were carried out at, or near, 0° C.

b) International Portable Refrigerated Centrifuge, model PR-2.

c) Spinco Ultracentrifuge, model L, rotor number 40.

Fig. 1. The preparation of the chloroplast extract.



a) All operations were carried out at, or near, 0° C.

b) Spinco Ultracentrifuge, model L, rotor number 40; all accelerations due to gravity (g) are maximum values.

c) The enzyme stored at 0° C.

Fig. 2. Ammonium sulfate fractionation of the chloroplast extract.

Preparation of Diazomethane

Diazomethane was prepared by alkaline saponification of N-methyl-N-nitroso-N'-nitroguanidine, as is described in the second part of this thesis. The ethereal solution of diazomethane was either used immediately or was stored for a short time in the deep freeze (-16°) before use.

Photosynthesis Experiments with Algae

The harvested Chlorella cells were washed and resuspended in the phosphate buffer to give a 2 to 4% solution (in ml of wet packed algae/vol. of suspension). 1 to 10 ml of the cell suspension were pipetted either into the vessels illustrated in Fig. 3 or into the vessels illustrated in Figs. 4 and 5. The vessel in Fig. 3 is a modified "lollipop" designed to allow sufficient light to illuminate a small volume of algae. An additional advantage of the modified "lollipop" is that it allows the rapid killing of the algae by means of cold (-60°) MeOH poured into the extra volume. Illumination was provided by reflector spotlights, which gave a light intensity of about 7,000 foot candles on either side of the vessel. Excessive heat was avoided by the use of infrared, water-cooled filters placed between the reflector spotlights and the photosynthesis vessel (Fig. 6).

The algae were preilluminated for 30 min in the light, while a continuous stream of 1.5% CO_2 in air (v/v) was bubbled through the algae suspension ("pre-adaptation" period), then swept with nitrogen gas for 1 min. Afterwards a solution of ^{14}C-labeled bicarbonate was added and the mixture was allowed to stand for 1 to 5 sec.

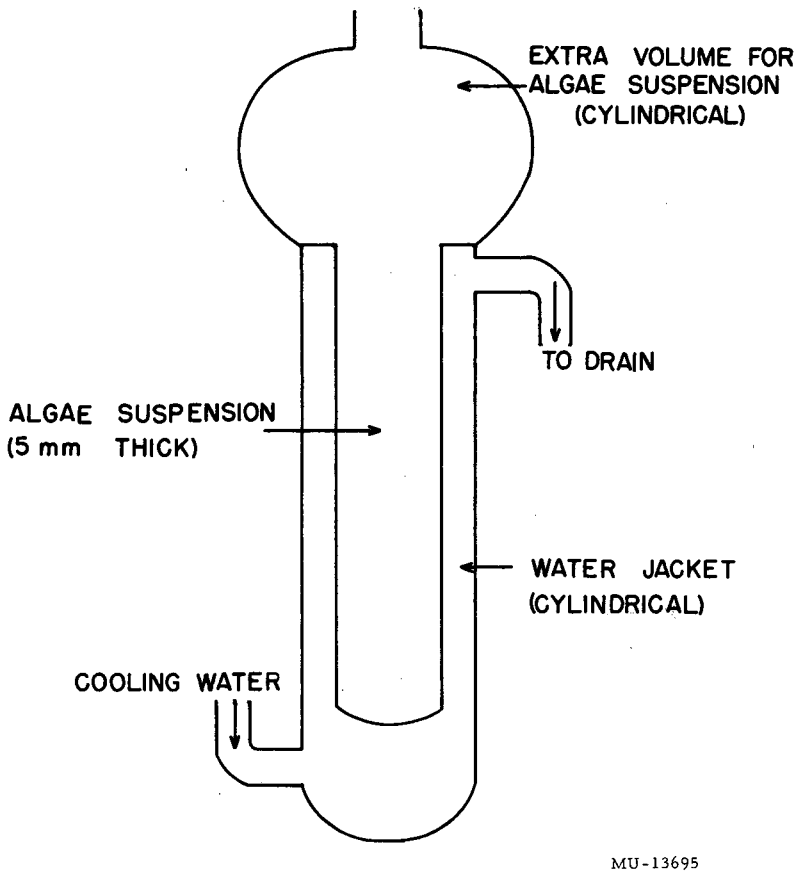


Fig. 3. Modified "lollipop" for short time photosynthesis experiments with algae.

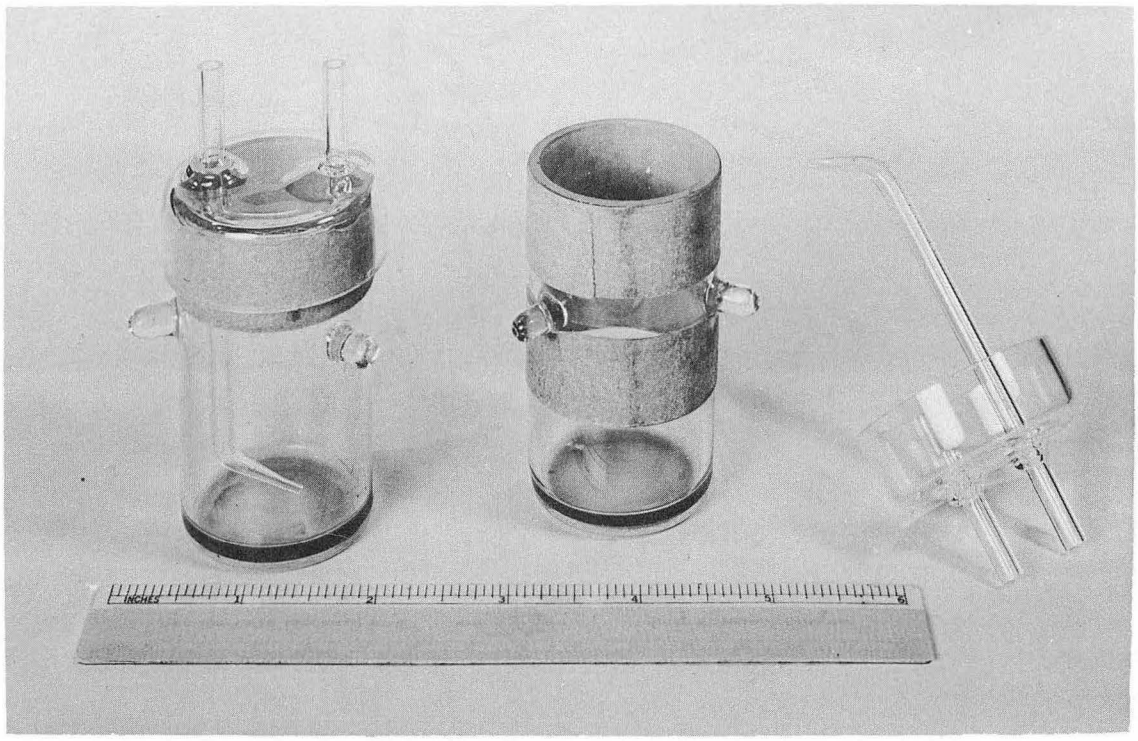


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Fig. 4. Illumination vessel for exposing algal suspensions to $H^{14}CO_3^-$ under several conditions.

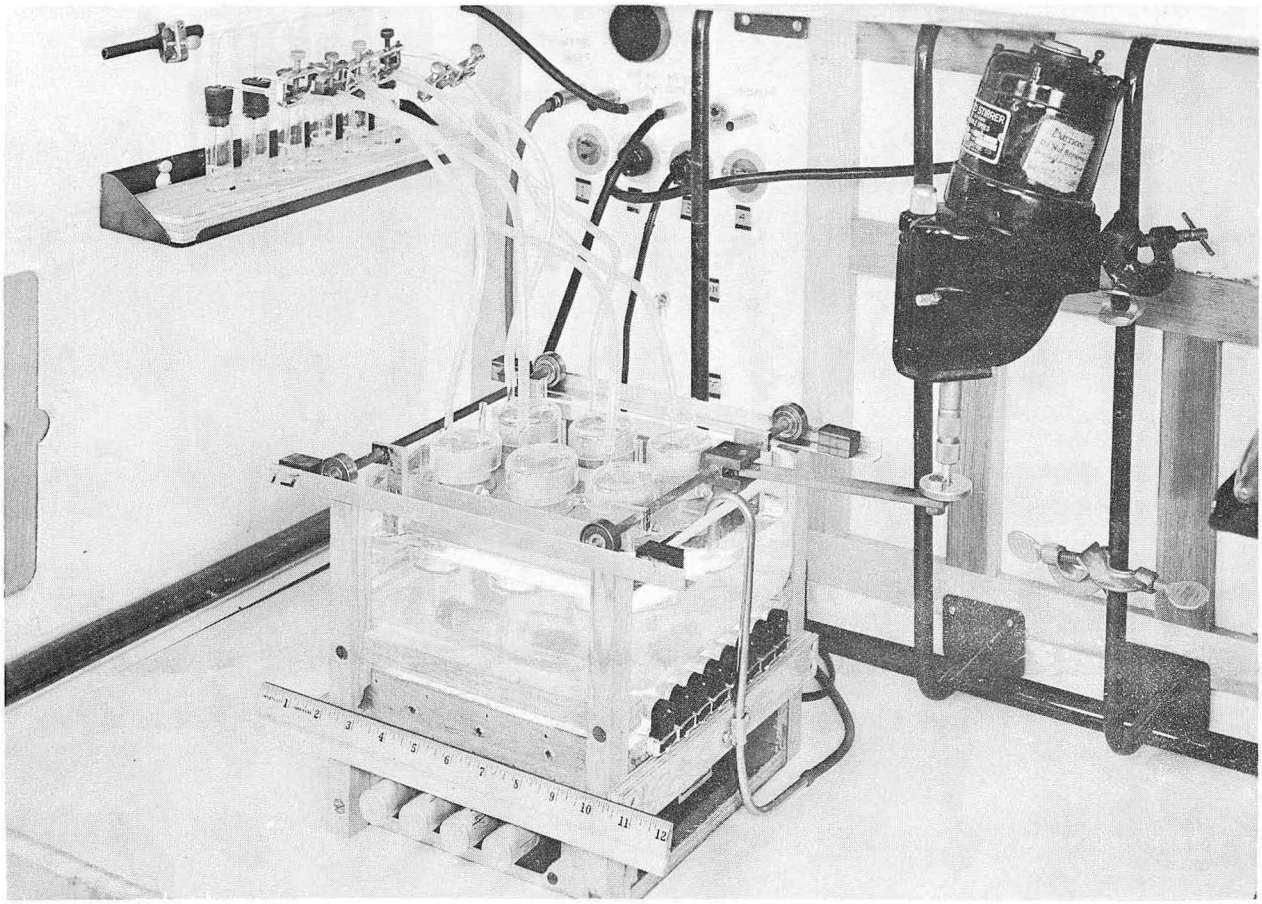

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Fig. 5. Apparatus for exposing algal suspensions to $H^{14}CO_3^-$ under several conditions.

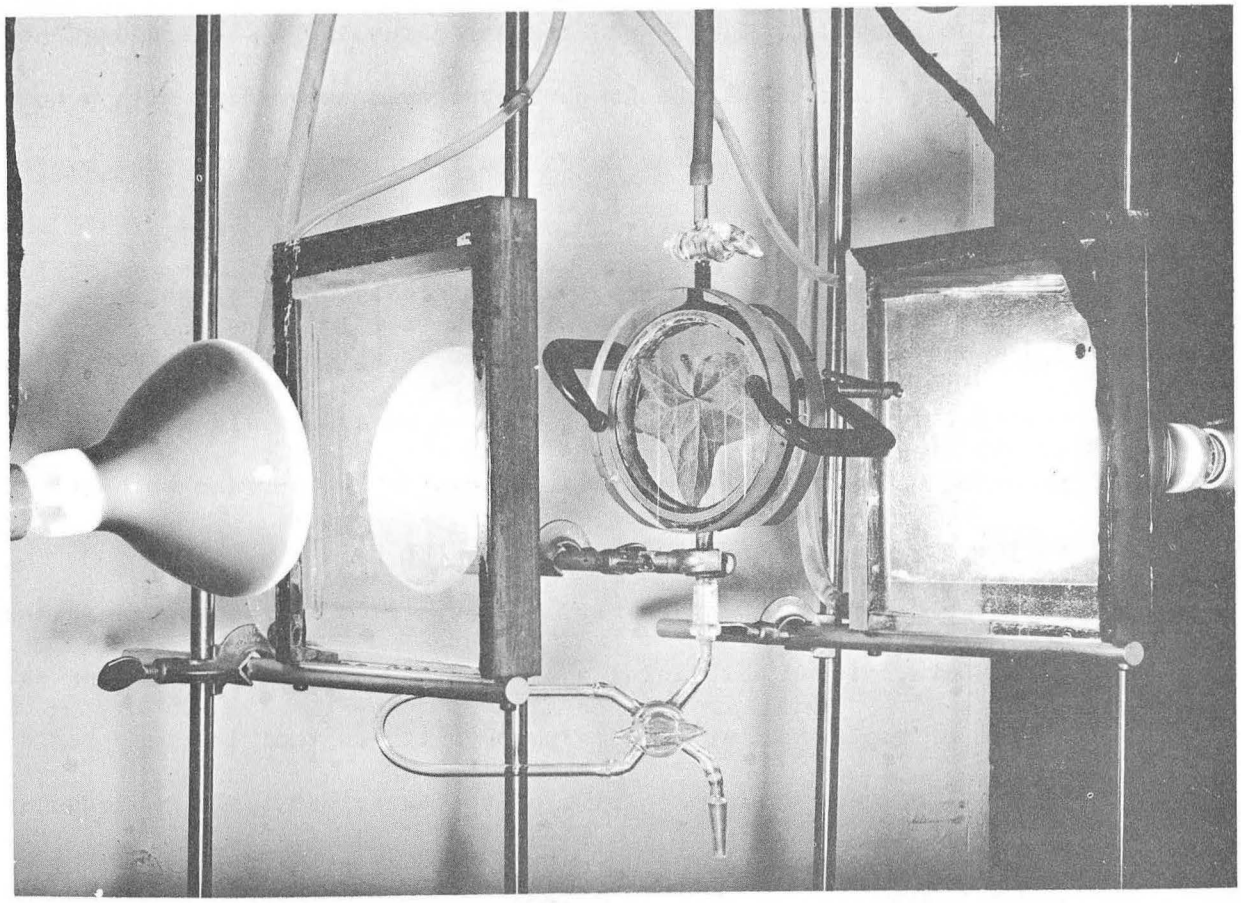

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Fig. 6. Apparatus for exposing leaf to $^{14}CO_2$.

The algae were killed by injection of a sufficient amount of cold MeOH (-60°) to give a final solution of 80% in MeOH. During the time of photosynthesis with $H^{14}CO_3^-$ the cell suspension was stirred by bubbling nitrogen gas through it. The killed algae were either extracted or reacted first with diazomethane and then extracted.

The vessels in Fig. 4 and the apparatus in Fig. 5 are designed[30] to provide for a simultaneous photosynthesis experiment with a number of samples under conditions which are identical save for one systematically varied factor.

The vessels were constructed from glass tubing of about 3.5 cm internal diameter and 7 cm long, to which a flat glass disc was rigidly attached to form a bottom. A removable lid was provided with an outlet and inlet tube; the latter reaches nearly to the bottom of the vessel.

The apparatus shown in Fig. 5 consisted of a rectangular glass water bath, fitted with inlet and outlet tubes to permit a constant stream of cooling water to pass through it, so that the temp could be kept constant during the time of the experiment. The rectangular glass water bath was arranged over a bank of eight 6-watt fluorescent lights. At the top of the water bath two rails were fitted, along which ran a small carriage consisting essentially of four wheels fixed to a plate having six holes punched in it. Six of the glass incubation vessels were suspended from this plate so that the bottoms of the vessels nearly reached the bottom of the water bath. The vessels were prevented from falling through the plate by three glass "ears" on each vessel arranged near its top.

The whole carriage was made to run back and forth along its rails by means of a connecting rod attached to an eccentric on the drive shaft of a stirrer motor. The carriage oscillated about 3 cm at approximately 250 cyc/min. The light intensity at the bottom of the glass was about 2000 foot candles. While illumination to each vessel was fairly constant, there was some variation between different positions on the shaker. For this reason the samples were supplied with radioactive bicarbonate one at a time in a serial manner, each flask being moved to one particular position on the shaker for the period of incubation with labeled bicarbonate.

During the "pre-adaptation" period before the experiment, in which the cells were allowed to come to a steady metabolic rate, the cell suspension (1.5 ml of 4% algal suspension) was shaken in the light for 30 min, while air containing 1.5% (v/v) CO_2 was blown over the surface of the cell suspension. The gas mixture was pre-wetted by bubbling through water to minimize evaporation from the cell suspension and to serve as a control measure for the rate of aeration. After the "pre-adaptation" period, the algal suspension was swept with a stream of nitrogen for 1 min, and then a solution of ^{14}C-labeled bicarbonate was added. The algae were allowed to incubate for 1 to 5 sec, before being killed by adding 4 ml cold (-70°) MeOH. The shaker was kept in motion while both additions were made to ensure rapid mixing. The killed algae were either extracted or reacted first with diazomethane and then extracted. In the case when low temp chromatography was used for the separation of the radioactive compounds formed in the short time of the

incubation of the algae with $H^{14}CO_3^-$, the algae were killed by pouring the algal suspension into a flask containing liquid nitrogen.

Photosynthesis Experiments with Leaves of Bean Plant

A single excised leaf from a "horse bean" plant, about 750 mg/leaf, was used in each experiment. The apparatus used is shown in Fig. 6[1]. The leaf was placed in the circular flat illumination chamber with a detachable face, with the stem of the leaf immersed in water. The chamber was equipped with two tubes, the one at the top leading through a two-way stopcock to a loop containing $^{14}CO_2$, and to a tank containing 1.5% CO_2 in air. Illumination was provided by reflector spotlights, which gave a light intensity of about 7,000 foot candles on either side of the leaf. Excessive heat was avoided by the use of infrared, water-cooled filters placed between the reflector spotlights and the illumination chamber. The leaf was illuminated for 5 min, while a continuous stream of 1.5% CO_2 in air (v/v) was passed through the illumination chamber. Then the $^{14}CO_2$ was swept into the chamber by the CO_2-air mixture. The leaves were exposed to $^{14}CO_2$ for 1 to 5 sec, and then plunged into liquid nitrogen.

Carboxydismutase Assay

There were two steps in the carboxydismutase assay: 1) the preliminary incubation (preincubation) step and 2) the incubation step[7]. The constituents of the incubation mixture were: Enzyme solution; $MgCl_2$ (0.2 M); ^{14}C-labeled bicarbonate, and RuDP. In some cases 1 M tris-buffer, pH 8.3 was added. In the preincubation

step all but one or two of the components of the assay system were mixed and allowed to stand either at 0° or 25° C for a predetermined time. At the end of this period the rest of the component(s) was added (the incubation step) and the complete system was incubated for a fixed time at 25° C. (In some cases where two components were preincubated with the enzyme, the first component was allowed to be in contact with the enzyme for a time "a", followed by preliminary incubation with the second component for a time "b". The first component would then be preincubated with the enzyme for a total time (a + b).) The reaction was stopped either by adding cold (-60° C) MeOH to give a final volume of 80% in MeOH, or by adding acetic acid, or liquid ammonia.

Carboxylation of Enzyme

Each reaction mixture contained tris-buffer (pH 8.3), enzyme solution, $MgCl_2$ and ^{14}C-bicarbonate. There were two steps in the reaction of carboxylation of the enzyme. In the first step all the components but bicarbonate were mixed and allowed to stand at 0° C for a predetermined time. At the end of this period the $H^{14}CO_3^-$ was added and the complete system stayed for a fixed time at 0° C (or at another fixed temp). Afterwards, the $CO_2 \sim$ enzyme complex was separated by anion exchange column chromatography.

Reaction of Algae, Leaves or Products of Enzyme Assay with CH_2N_2

a) Treatment of killed algae with diazomethane: The cold, 80% MeOH suspension of the killed algae was divided into two equal portions. The one of them was evaporated under reduced pressure

almost to dryness at a bath temp of -10° C. To the residue a cold ethereal solution of diazomethane was added until the yellow color persisted, and the reaction was allowed to proceed for 24 hr at 0° C, and 2 hr at room temp (23° C). The ethereal solution was separated from the residue and it was concentrated at room temp to a small volume. An aliquot was put on chromatograms for chromatographic analysis of the radioactive compounds. The residue was extracted first with pure MeOH, then with a solution of 80% MeOH, again with a solution of 20% MeOH and finally with water at 60 to 70° C, for 30 min each time. The extracts were pooled and the, residue discarded. The extracts were concentrated to a small volume *in vacuo* below 30° C, and the radioactive compounds were separated by paper chromatography.

To the second portion of the killed algae suspension which served as a control, 1 ml of 10% acetic acid solution was added and the mixture was evaporated almost to dryness *in vacuo* at bath temp of 40°. The rest of the procedure was the same as above.

b) Treatment of leaves with diazomethane: The leaves that were killed by liquid N_2 after having been exposed to labeled CO_2 for 1 to 5 sec, were carefully ground in a ceramic mortar under liquid nitrogen and the powder thus obtained was divided into two equal portions. To one of them was added 2 ml of cold (-40°) MeOH, followed by 15 ml of an ethereal solution of diazomethane. To the other, which served as a control, 4 ml of 10% acetic acid in MeOH were added, and the mixture was left at 50° C for 10 min. Afterwards, the mixture was evaporated to dryness *in vacuo* at a bath temp of 40° C. To the residue was added 2 ml of MeOH, followed by 15 ml of an

ethereal solution of diazomethane. Both samples were left at 0° for 24 hr and at room temp for 2 hr. The samples were centrifuged and the ethereal solutions were separated from the residues. The residues were extracted first with pure MeOH, then with a soln of 80% MeOH, again with a soln of 20% MeOH and finally with water, at 60 to 70° C for 30 min each time. The ethereal soln and the extracts of each sample were pooled and concentrated to a small volume *in vacuo* at room temp. The radioactive compounds of each sample were separated by paper chromatography.

c) Treatment of the enzyme assay system with diazomethane: The enzyme suspension, after incubation with ^{14}C-labeled bicarbonate in the presence of $MgCl_2$ and RuDP for 1 to 10 min, and inactivation with cold (-60°) MeOH, was divided into two equal portions. To one of them 15 ml of an ethereal soln of diazomethane was added and the mixture left at 0° C for 24 hr and at room temp for 2 hr. The residue was separated from the ethereal soln and extracted first with a soln of 80% MeOH, then with a soln of 20% MeOH and finally with water at 60° to 70° for 30 min each time. The extracts and the ethereal soln were pooled and concentrated to a small volume *in vacuo* at room temp. The other portion, which served as a control, was centrifuged and the residue extracted with a soln of 80% MeOH, then with a soln of 20% MeOH and finally with water at 60 to 70° for 30 min each time. The extracts and the supernatant were pooled and the residue discarded. To the extracts 1 ml of a 10% acetic acid soln was added and the mixture was concentrated to a small volume *in vacuo* at 40°. To the concentrated extracts 15 ml of an ethereal soln of diazomethane was added. The mixture was left at 0° C for 24 hr and at room temp for 2 hr,

and then it was concentrated to a small volume at room temp. The radioactive components of both samples were separated by paper chromatography.

Separation of the Radioactive Products by Low Temp Paper Chromatography

In the case when low temp paper chromatography was used for the separation of the radioactive compounds which were formed after a short time photosynthesis in the presence of ^{14}C-carbon dioxide, the algae or leaves were first killed by liquid nitrogen, and then they were carefully ground in a ceramic mortar under liquid nitrogen. The powder thus obtained was divided into two portions. To one of them was added 8 ml of liquid ammonia and after 10 min the residue was separated from the supernatant liquid. A part of the residue was evenly applied on the origin of a chromatographic paper, and the chromatogram was run in the low temp box, using non-freezing solvents. The supernatant liquid was concentrated to approximately 1 ml, and the radioactive components were separated by low temp paper chromatography. During this procedure the temp did not rise above -30° C. The chromatographic paper was kept in the cold chromatographic box for 2 hr before it was used. The temp during the development of the chromatogram was -35 to -30° C. The solvents were run for fourteen to sixteen hr. This procedure is approximately the same with the one described by Doman[14].

The same procedure was also followed for the other portion, which served as control, except that the powdered algae or leaves were heated to 75° C for 1 hr and then cooled to -30° C before the addition of liquid ammonia.

Low temp chromatography was also used for the separation of the radioactive products of the carboxydismutase assay system. Enzyme soln, $MgCl_2$ and ^{14}C-bicarbonate were added into two centrifuge tubes, and after 5 min preincubation at 0° C RuDP was added. The concentration of enzyme, $MgCl_2$, bicarbonate and RuDP was the same in both tubes. After 5 min incubation, the reaction mixture of one of the tubes was immersed into liquid ammonia (4 ml), and the radioactive components were separated by low temp (-30°) paper chromatography. To the mixture of the other tube, which served as a control, two drops of acetic acid were added, and the mixture was allowed to stand at 60° C for 30 min. Afterwards, the mixture was cooled to 0° C and then 4 ml liquid ammonia were added. The radioactive components were also separated by low temp (-30°) paper chromatography.

III. RESULTS AND DISCUSSION

Search for the Unstable Beta-keto Acid.

As stated in the introduction section of the first part of this thesis, one of the objectives of this research was to search for the unstable beta-keto acid, postulated by Calvin as an intermediate in the carboxylation reaction of RuDP. Beta-keto acids are generally unstable compounds, and the beta-keto acid formed from RuDP and CO_2 with two phosphoric groups present, is expected to be even more unstable. Therefore, special methods are needed for its detection.

Many attempts were made to demonstrate the existence of this unstable compound in algae (Chlorella pyrenoidosa), in leaves of "horse bean" plant, and in the isolated enzyme system (carboxydismutase). Algae had the disadvantage that it was accompanied by a large amount of free ^{14}C-bicarbonate which interferred with the separation of the small amount of radioactive products formed after a short time photosynthesis. The use of the isolated enzyme system had the advantage that even with a crude extract of the spinach chloroplasts the only stable radioactive product formed after a short time of incubation (1 to 15 min) was PGA. This made it easier to look for and distinguish any other radioactivity present. In each experiment the killed material was divided into two equal portions, the one serving as a control was treated less carefully. Conditions for the photosynthetic experiment and incubation varied. For example, in one case a high concentration of ^{14}C-bicarbonate in the algae suspension and a very short time of exposure were applied. In another case use of high concentration of enzyme

(2 to 3 mg) was made. In the last case the enzyme was preincubated with $H^{14}CO_3^-$ at 0° C for a long time (2 to 3 hr) and then incubated with RuDP at 25° C for a short time (1 to 2 min). It was hoped that in each case the amount of the keto acid intermediate would increase. Two analytical methods were used in this search: 1) Treatment of the material at low temp, and separation of the radioactive products present by low temp paper chromatography. The material was also treated with liquid ammonia before the run of the chromatogram, so that the more stable salts of unstable acids should be formed. It was hoped that under these conditions the rate of dissociation of any unstable compound present would be very slow and would make its detection easier, 2) Treatment of the material killed with cold (-60°) MeOH with an ethereal solution of diazomethane, so that the stable methyl esters of unstable acids should be formed, and separation of the radioactive products by paper chromatography.

Despite a great number of experiments and the mild conditions used, no evidence was produced for the existence of the postulated beta-keto acid intermediate. Either the amount of this acid is too small to be detected even with tracer techniques, or the acid is not present as a free acid but as a complex combined with the enzyme, the complex being hydrolyzed to give PGA and free enzyme. Another possibility is that the beta-keto acid does not exist at all, i.e. the formation of PGA from RuDP, CO_2 and H_2O proceeds through another mechanism.

It was mentioned earlier that Doman and Shkolnik[14] observed a small amount of radioactivity at the beginning of the diphosphate

area on a chromatogram of geranium leaves which was run at low temp. The leaves were previously treated with liquid ammonia under mild conditions. This radioactivity was not present if the material was treated less carefully. They were not able to reproduce this experiment with other plants, except with geranium and to a lesser extent with beans. As far as is known this compound has not yet been identified. We were not able to reproduce the above experiment with algae, "horse bean" leaves or the isolated enzyme from spinach chloroplasts. Before making any speculations on the above experiment, the radioactive compound has to be identified, or at least some of its properties and its relation with the carbon reduction cycle of photosynthesis be studied.

It is interesting to note that when algae or leaves were treated at low temp, and low temp paper chromatography was used for the separation of the radioactive compounds, a difference in the relative radioactivity of the stable compounds was observed, compared to radioactive compounds of the control system. That was probably due to the difference in the equilibrium of the reactions at different temps.

In the experiments with diazomethane, algae was used initially as the photosynthetic material, and later the isolated enzyme system was chosen as being more convenient. Some experiments with leaves of "horse bean" were also conducted. In the first experiments with diazomethane and algae it was observed that a great number of radioactive compounds were formed when algae was exposed to ^{14}C-bicarbonate for 1 to 5 sec and then treated with diazomethane. The total stable

radioactivity was many times higher than in the control sample. This is shown in Figs.7 and 8. The amount of radioactivity which was stabilized with diazomethane depended on the concentration of bicaronbate present in the algae suspension, as is shown by comparing Figs. 9 and 10 with Fig. 7. Fig. 11 is a radioautograph of the chromatogram of the control sample of the experiment described in the legend to Fig. 9.

In order to show if the unstable complexes formed from bicarbonate and algae were related to the carbon reduction cycle of photosynthesis, a number of experiments were done with dead algae. In these experiments an algal suspension was preilluminated for 30 min with 1.5% CO_2, swept 1 min with N_2 gas and then killed with hot or cold MeOH. After 30 min ^{14}C-bicarbonate was added and the mixture was concentrated at low temp before the addition of diazomethane, as described in the experimental section. Figs. 12 and 13 are radioautographs of two chromatograms (extract of the residue, and ethereal supernatant) showing the results of one of these experiments. A great number of radioactive compounds are present on both chromatograms. Needless to say, no radioactivity was found on the chromatogram of the control of the above experiment with dead algae. This showed that unstable radioactive compounds stabilized with diazomethane were not related to the carbon reduction cycle of photosynthesis, but were carbon dioxide addition products. These are usually present in a solution of the precursors of the above products and bicarbonate.

Amines, amino acids, peptones, proteins etc. are typical compounds which form addition products with carbon dioxide of the carbamate

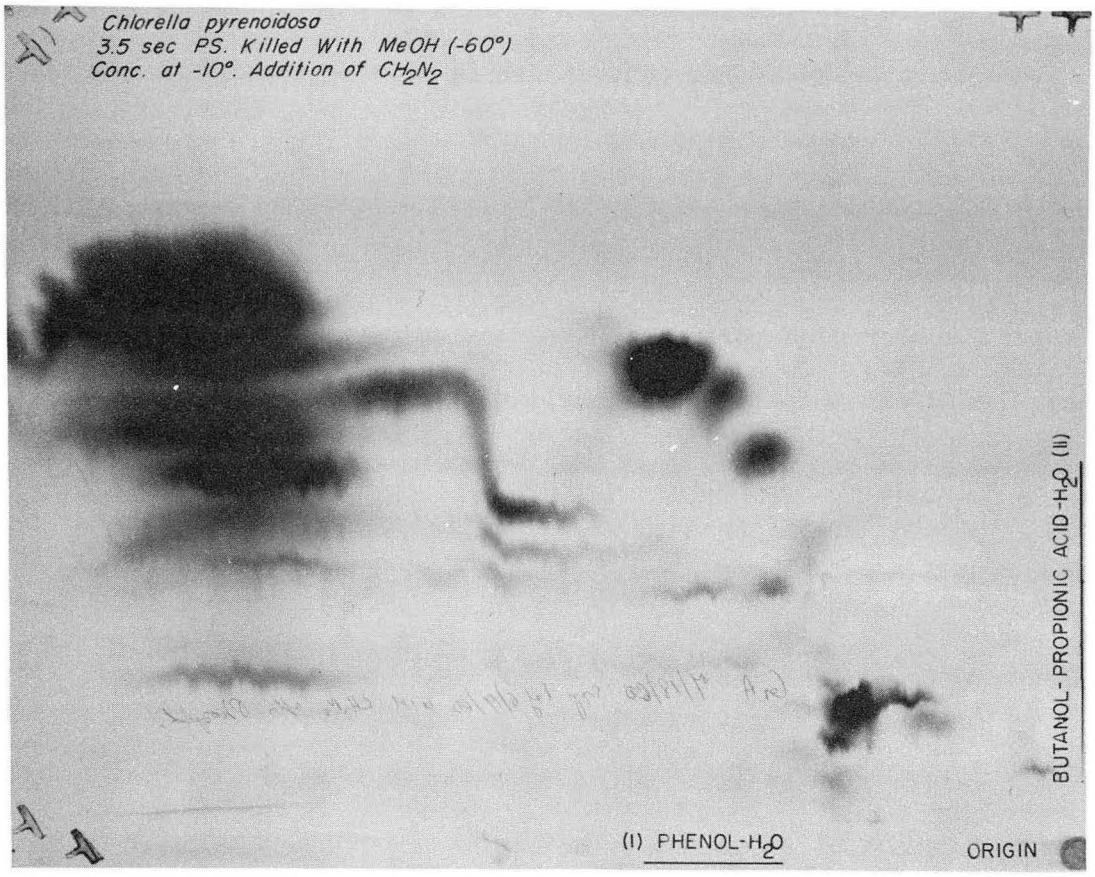

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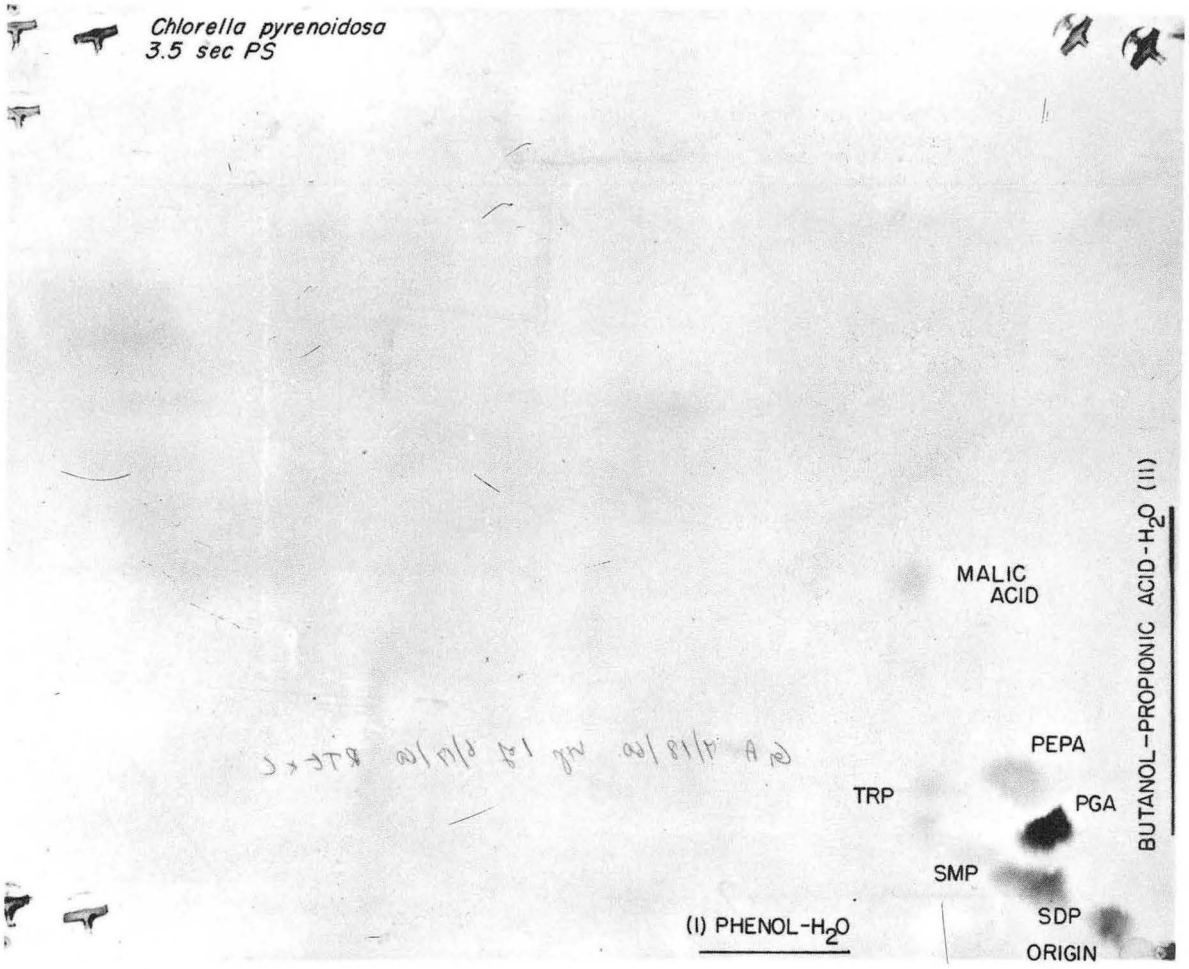
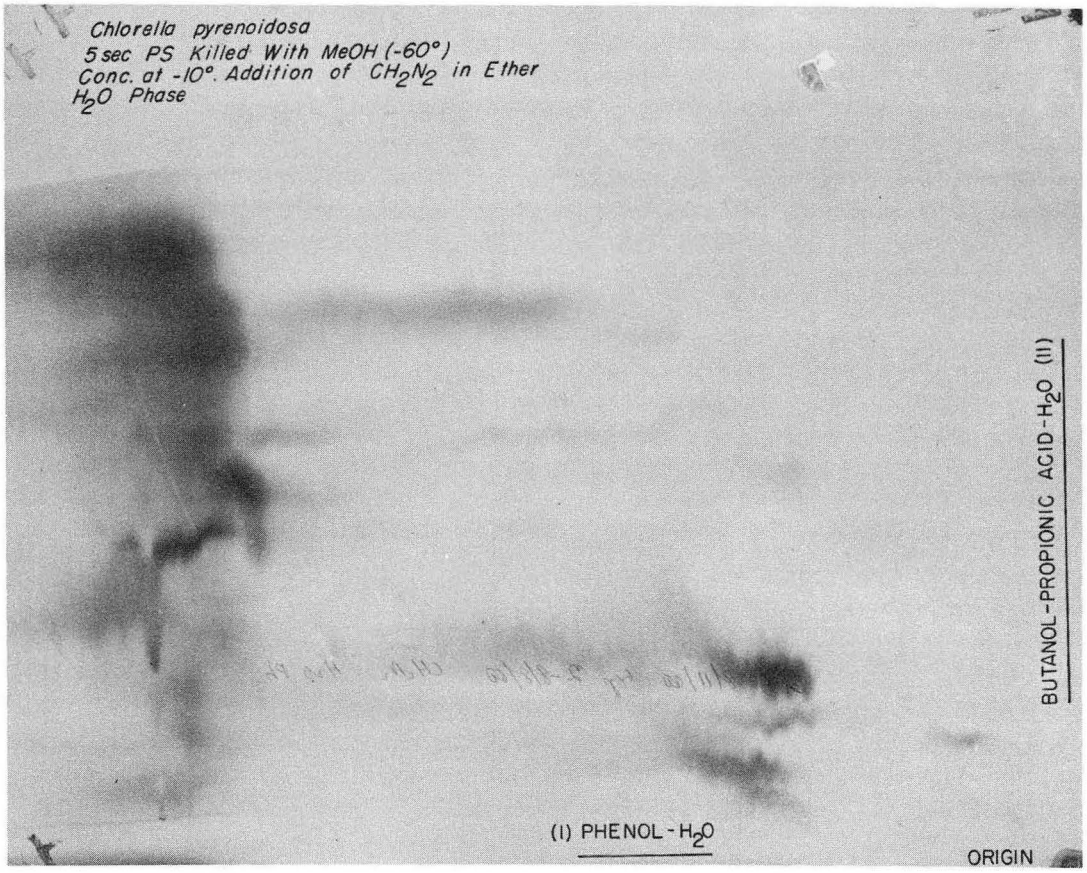
Fig. 7. Radioautograph of the chromatogram of the extracts of algae which had been treated with diazomethane. Experimental conditions: 2 ml of 4% algal suspension were preilluminated 30 min with CO_2, swept 1 min with N_2, 3.5 sec PS with 0.4 ml $H^{14}CO_3^-$ solution (2 mc/ml, 0.20 M), killed with 8 ml cold (-60°) MeOH, conc. at -10°. Addition of CH_2N_2. Extraction of residue with a soln of 80% MeOH, again with a soln of 20% MeOH and finally with H_2O at 65°C. Conc. of extracts and supernatant to a small volume.



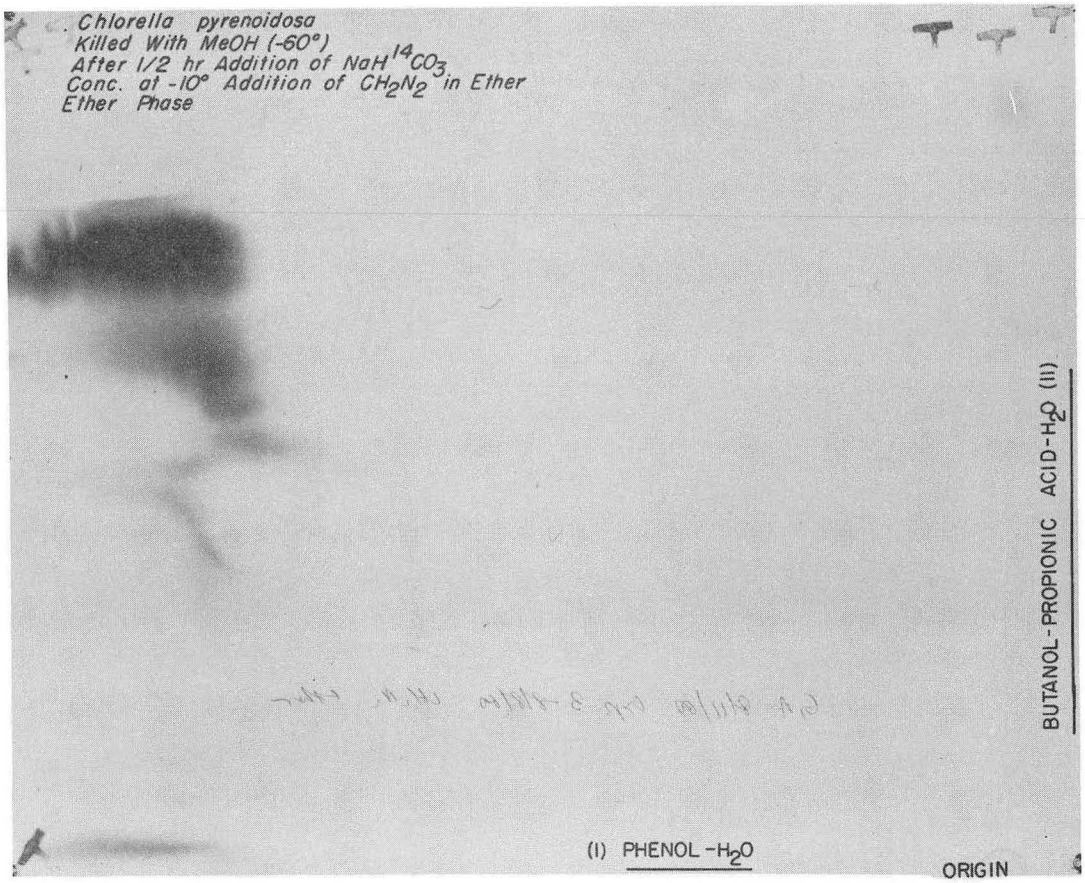
ZN-3177

Fig. 8. Radioautograph of a chromatogram of algae extract after 3.5 sec PS. Control of experiment in Fig. 7.



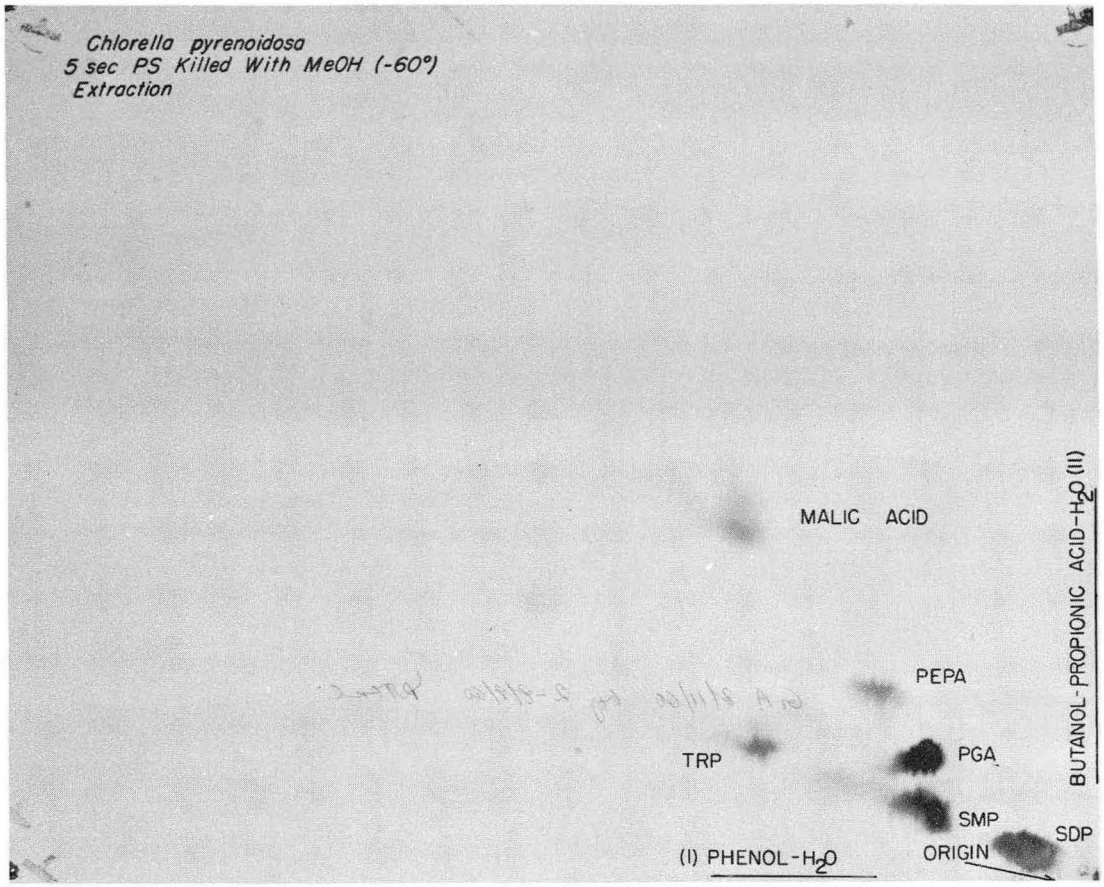
ZN-3179

Fig. 9. Radioautograph of the chromatogram of the extracts of algae which had been treated with diazomethane. Experimental conditions: 2 ml of 4% algal suspension were preilluminated 30 min with CO_2, swept 1 min with N_2, 5 sec PS with 0.25 ml $H^{14}CO_3^-$ soln (2 mc/ml, 0.06 M), killed with 8 ml cold (-60°) MeOH, conc. at -10°. Addition of CH_2N_2, separation of supernatant. Extraction of residue with 80% MeOH, 20% MeOH, water, at 65°. Conc. of extracts to a small volume.



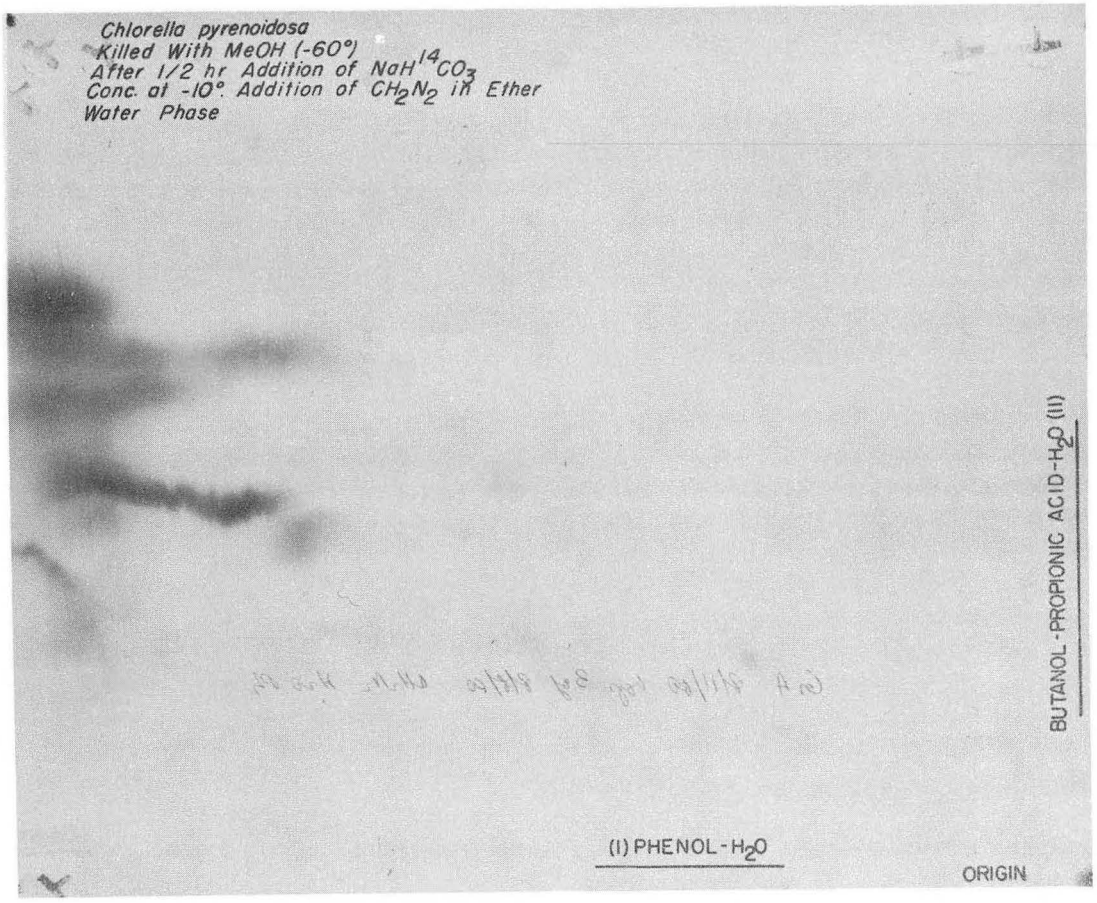
ZN-3178

Fig. 10. Radioautograph of the chromatogram of the supernatant of experiment in Fig. 9.



ZN-3170

Fig. 11. Radioautograph of a chromatogram of algae extract after 5 sec PS. Control of experiment in Fig. 9.



ZN-3172

Fig. 12. Radioautograph of a chromatogram of extracts of dead algae, after being treated with $H^{14}CO_3^-$ and CH_2N_2. Experimental conditions: 2 ml of 4% algal suspension were preilluminated 30 min with CO_2, swept 1 min with N_2, killed with 8 ml MeOH. After 30 min conc. to initial volume and 0.5 ml $H^{14}CO_3^-$ soln (2 mc/ml, 0.06 M) were added, conc. at -10°. Addition of CH_2N_2, separation of supernatant. Extraction of residue with 80% MeOH, 20% MeOH and water at 65°C. Conc. of extracts to a small volume.

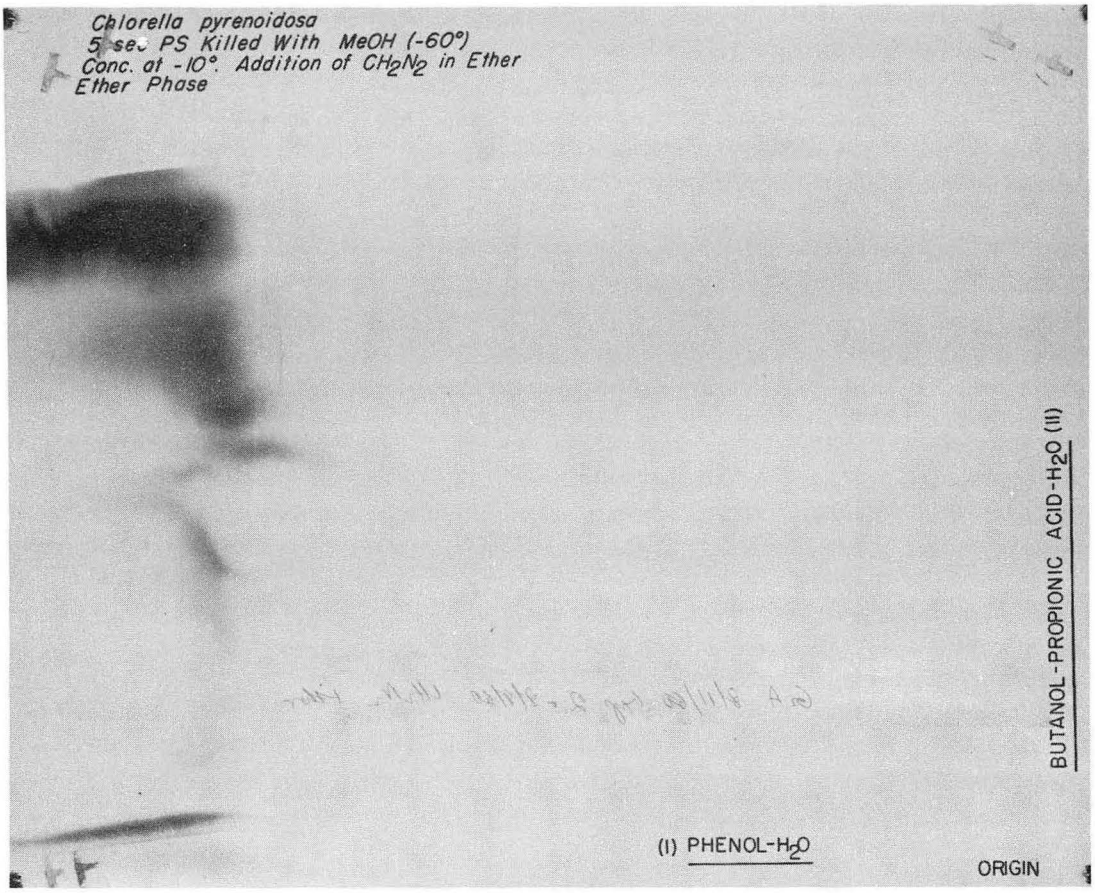


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Fig. 13. Radioautograph of the chromatogram of the supernatant of experiment in Fig. 12.

form[31,32]. Experiments with a soln of crystalline bovine albumin or with solns of seven different amino acids confirmed the formation of carbon dioxide addition products. These products were stabilized with diazomethane and separated by paper chromatography. The amino acids used were: D,L-aspartic acid, L-asparagine, L-glutamic acid, glycine, L-cystine, D,L-leusine and D,L-proline. In the amino acids or protein solns ^{14}C-bicarbonate was added and the mixture was treated with an ethereal soln of diazomethane. After concentration the radioactive products were separated by paper chromatography. Two radioactive spots were observed on many of the chromatograms of the amino acids, probably due to the mono- and dimethyl esters of the carbamates. Radioactive spots were also observed on the chromatogram of the protein which was partially hydrolyzed with HCl.

This carbamination reaction is of particular importance for the transport of carbon dioxide by the blood[33,34] but it is not known whether it is of great importance in photosynthesis as suggested by Siegfried[35]. Probably the formation of carbon dioxide addition products in algae or other plants helps in the increase of carbon dioxide concentration and consequently in an increase in the rate of photosynthesis.

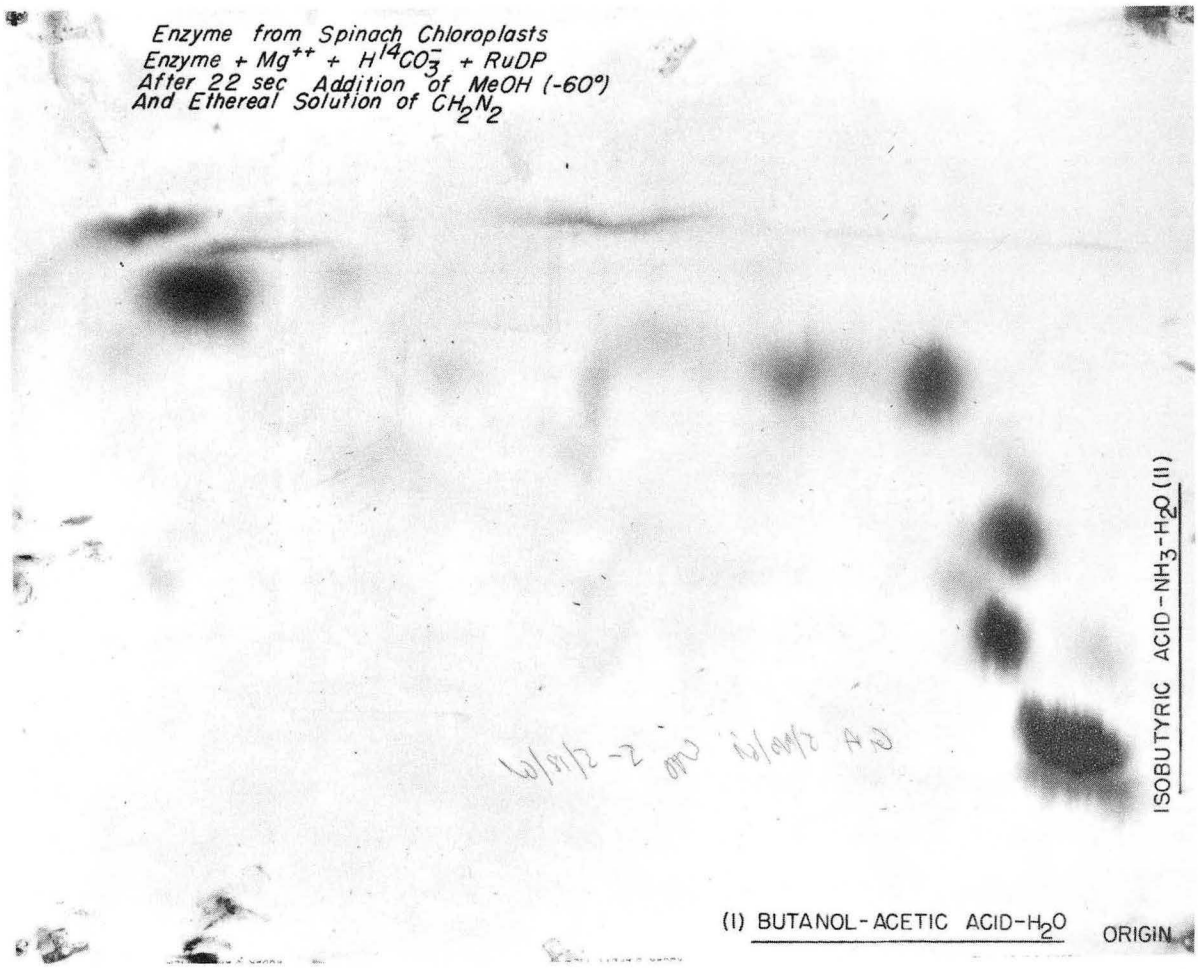
It is of interest to note that one of the radioactive spots on the chromatogram of the ethereal supernatant of the algae treated with diazomethane (Figs. 10 and 13) was green, and a coincidence of the radioactivity and color was observed, which may be due to a carbon dioxide addition to chlorophyll compound. It is known that an affinity of chlorophyll for carbon dioxide

exists[31], but the question of the observed green radioactive compound being a CO_2-chlorophyll complex requires further experimentation.

The isolated enzyme system was used more extensively in the search for the unstable keto acid, using diazomethane as a stabilizing reagent. These experiments also failed to produce any evidence for the existence of the keto acid intermediate. There were many radioactive spots on the chromatogram of the extracts of the enzyme system which had been treated with diazomethane, as is shown in Fig. 14. Exactly the same radioactive compounds were obtained with the enzyme system which was inactivated with cold (-60°) or hot (65° C) MeOH and treated with diazomethane, or with PGA eluted from paper and treated with diazomethane. This means that the different radioactive compounds are derivatives of PGA, formed by the action of diazomethane on it. Fig. 15 is a radioautograph of a chromatogram of the control of the experiments with the isolated enzyme system.

Search for a $CO_2 \sim$ enzyme Complex

The carboxydismutase system is a complicated one because of the fact that there are present two substrates (CO_2 and RuDP) and one cofactor (Mg^{++}). An analysis of the carboxylation reaction of RuDP presents many difficulties. In any enzymically catalyzed reaction two steps have to be taken into account, the activation and the reaction step. The activation of the enzyme involves, in its simplest form, the formation of the enzyme-substrate complex, whereas in the more complicated systems, the sequence of combination of the various constituents of the enzyme system is involved.



ZN-3181

Fig. 14. Radioautograph of the chromatogram of the extracts of the isolated enzyme system after being treated with diazomethane. Experimental conditions: enzyme (carboxydismutase), 2 mg; $MgCl_2$, 4 uM; $NaH^{14}CO_3^-$, 10 uM (2 mc/ml, 0.06M); RuDP, 0.8 uM; final volume, 400 ul. Preincubation of the enzyme with Mg^{++} and $H^{14}CO_3^-$ at 0° for 1 hr; the $H^{14}CO_3^-$ was added 5 min after the addition of Mg^{++}. Incubation with RuDP at 25° for 22 sec. Addition of cold MeOH (1.6 ml, -60°), then 5 ml of an ethereal soln of CH_2N_2. Extraction of the residue (protein ppt) with 80% MeOH, 20% MeOH and water at 65°. Conc. of extracts and supernatant to a small volume.

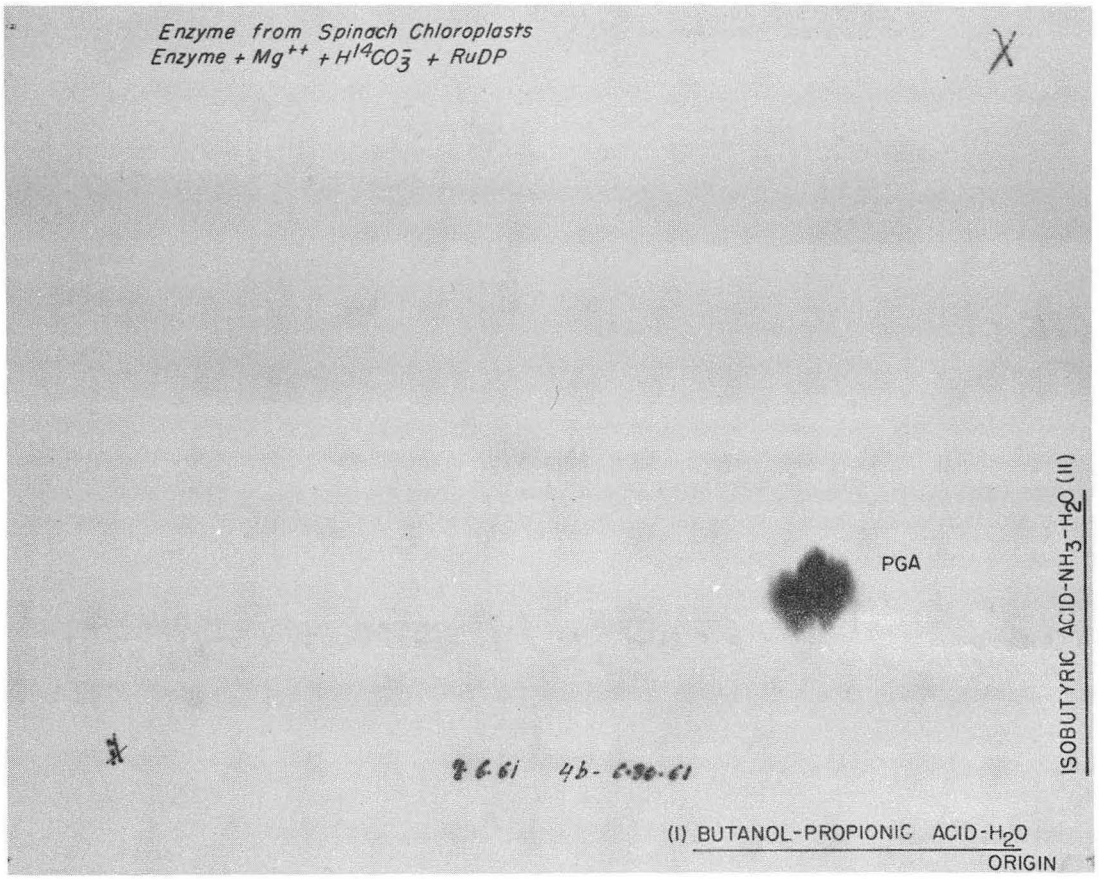


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Fig. 15. Radioautograph of the chromatogram of the extracts of the enzyme system after 5 min incubation at 25°. Experimental conditions: enzyme, 2 mg; $MgCl_2$, 4 uM; $NaH^{14}CO_3$, 10 uM (2 mc/ml, 0.06 M); RuDP, 0.8 uM; final volume, 400 ul. Preincubation at 0° for 1 hr. Incubation at 25° for 5 min. Addition of 1.6 ml MeOH. Extraction of the residue (protein ppt) with 80% MeOH, 20% MeOH, and water at 65°. Conc. of extracts to a small volume.

A tertiary enzyme system containing a metal ion cofactor, M, a substrate, S, and an enzyme, E, may have at least two different sequences of combination[7]:

Mechanism I	Mechanism II
1) $E + M \rightleftharpoons EM$	1) $M + S \rightleftharpoons MS$
2) $EM + S \rightleftharpoons EMS$	2) $MS + E \rightleftharpoons EMS$
3) $EMS \longrightarrow EM + P$	3) $EMS \longrightarrow E + P + M$

where P, EM and EMS are product, enzyme-metal ion complex and enzyme-metal ion-substrate complex, respectively. The mathematical treatment of these two sequences gives the same results, namely:[7,36,37,38]

$$v = k\,(E)_o / \left(1 + K_m/(S) + K_m/K_{x,m}(M)\,(S)\right)$$

where v is the velocity of the reaction; k is the specific rate constant of the decomposition of EMS into EM or E and P; K_m is the Michaelis constant; $K_{x,m}$ is the association constant for the formation of either EM or MS; $(E)_o$, (M) and (S) are the concentrations of enzyme, metal ion, and substrate, respectively.

There is also a third mechanism for the formation of the enzyme-substrate-metal ion complex. This can be written as follows:

$$E + S \rightleftharpoons ES; \quad ES + M \rightleftharpoons EMS; \quad EMS \longrightarrow E + P + M$$

where ES is the enzyme-substrate complex and the rest are the same as above.

Analysis of the kinetic data or the measurement of the rate of the reaction in both the forward and reverse directions so that the value of $K_{x,m}$ would be obtained kinetically, have been applied for the study of many enzyme systems.

Pon[7], in his study of the carboxydismutase system, used another approach for the study of the mechanism of the activation of the enzyme.

He observed a time effect in the activation of the enzyme (preincubation effect), and the time required for the activation could be extended over a measureable time interval by lowering the preincubation temperature. He performed a series of experiments in which carboxydismutase was preincubated with Mg^{++}, RuDP, and $H^{14}CO_3^-$, in various sequences and combinations at 0° C and for different intervals of time, and then incubated for 5 min at 25° C. The results are shown in Table I and Fig. 16. It can be seen clearly that the enzyme is more active when it is preincubated with either metal ion or bicarbonate first than when it is preincubated with RuDP first (Table I). Moreover, in the presence of metal ion, the activity of the enzyme increases with increasing time of preincubation with bicarbonate, whereas the activity decreases with increasing time of preincubation with RuDP (see Fig. 16).

As was mentioned in the introduction section of this thesis, Pon[7] suggested two interpretations of this phenomenon, one of which is that the enzyme combines first with the metal and bicarbonate and then reacts with RuDP. A proof of the above interpretation would be the isolation of a "$CO_2 \sim$ enzyme" complex which would subsequently react with RuDP. Two approaches were used for the study of this problem: first, inactivation of the enzyme with MeOH after being preincubated with Mg^{++} and $H^{14}CO_3^-$, followed by addition of RuDP to the mixture; and second, the isolation of a $^{14}CO_2 \sim$ enzyme complex using anion exchange column, similar to that used by Ochoa *et al.*[18] for the separation of the $CO_2 \sim$ enzyme complex of the propionyl CoA carboxylase, and subsequently reaction with RuDP to form PGA.

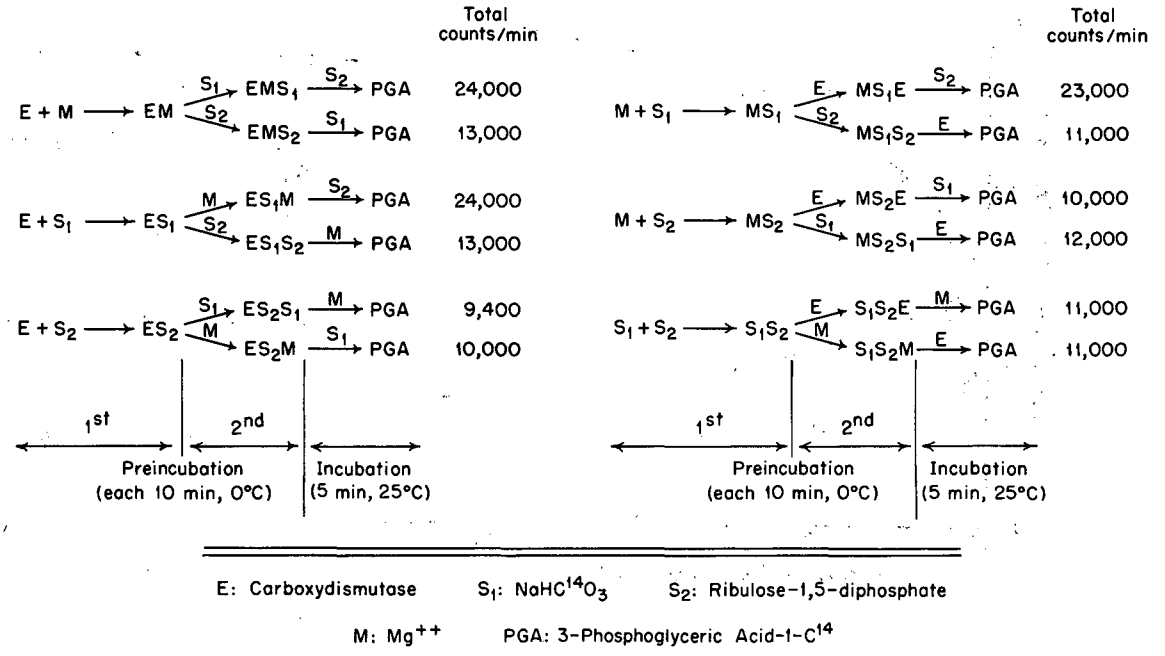

Table I. The Preincubation of Carboxydismutase with Substractes (S_1, S_2) and cofactor (M). (From N. G. Pon (7), p. 159)

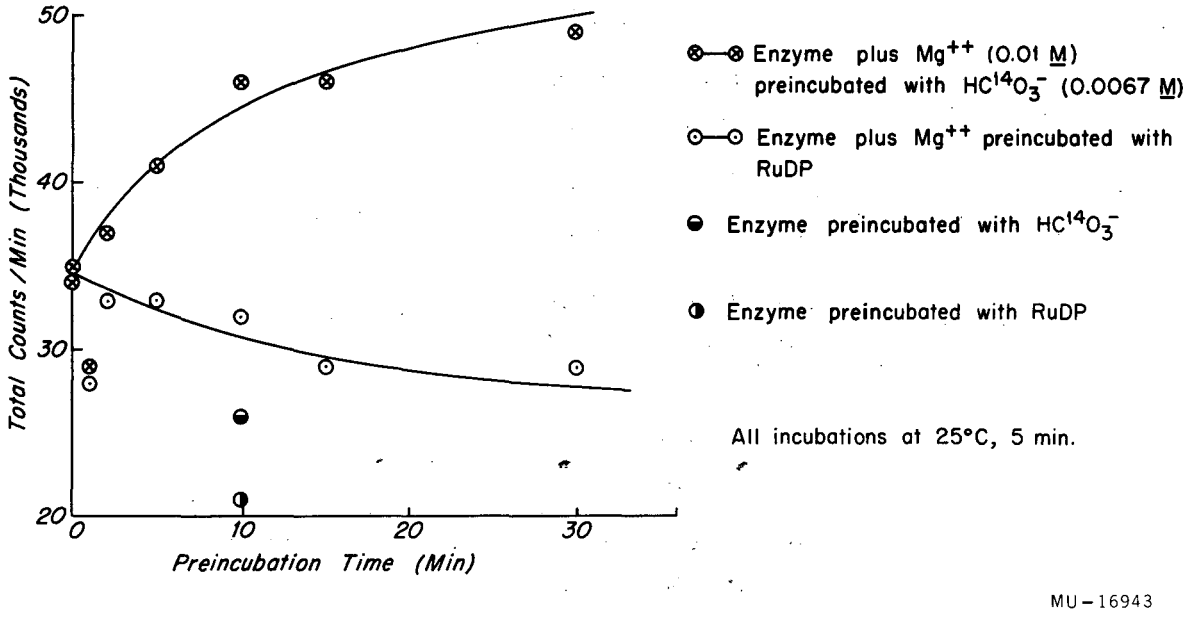

Fig. 16. The preincubation of carboxydismutase with substrates at 0°. (From N. G. Pon (7), p. 160). Preincubation conditions: temp, 0° C. Enzyme plus Mg^{++} case: enzyme preincubated 1 min at room temp with $MgCl_2$ and tris, then preincubated with one of the substrates; reaction started by addition of the second substrate. Enzyme case: enzyme preincubated with one substrate shown above; reaction started by addition of the other substrate plus $MgCl_2$. Incubation conditions: 5 min. at 25°C.

The results of the first method used are shown in Table II. It can be seen clearly that some PGA is formed when the enzyme is preincubated with Mg^{++} and $H^{14}CO_3^-$ first, then inactivated with MeOH, and afterwards incubated with RuDP. No PGA is present when the enzyme is preincubated with Mg^{++} or with Mg^{++} and RuDP first and the $H^{14}CO_3^-$ is added after the inactivation of the enzyme (Table II).

One interpretation of this phenomenon is that the enzyme combines with bicarbonate and Mg^{++} to form a complex which is stable at low temps even in the presence of 80% MeOH, and which can react with RuDP to form PGA. Once the CO_2 has been transferred from the complex to the RuDP, the free enzyme molecule is not capable of combining with another molecule of CO_2 and the reaction stops.

Another interpretation is that the presence of bicarbonate increases the resistance of the enzyme against MeOH, and the reaction goes on for a short time until all the molecules of the enzyme present in the solution are inactivated.

The result of the B-c experiment does not favor the second interpretation. (See Table II.) After the preincubation of the enzyme with Mg^{++} and bicarbonate, and addition of MeOH, the mixture was centrifuged and the supernatant was discarded. The residue was resuspended in tris buffer and then ^{14}C-bicarbonate and RuDP were added. There is an increase in the amount of radioactivity fixed compared with the results of the other experiments (B-a and B-b). However, this cannot be explained by assuming that some of the enzyme molecules were still active after separation from the MeOH soln, because in that case one should expect even higher radioactivity than was observed.

Table II. Preincubation of Carboxydismutase with Substrates and Cofactor and Inactivation of the Enzyme with MeOH Before Incubation.

		total dpm/mg protein
A:	(a) $E + M + S_1 \longrightarrow EMS_1 \xrightarrow[(-40°)]{MeOH} EMS_1 + MeOH \xrightarrow{S_2} PGA$	20,000
	(b) $E + M + S_1 \longrightarrow EMS_1 \xrightarrow[(-40°)]{MeOH} EMS_1 + MeOH \xrightarrow{S_2} PGA$	35,500
	(c) $E + M + S_1 \longrightarrow EMS_1 \xrightarrow[(-40°)]{MeOH} EMS_1 + MeOH \xrightarrow{S_2} PGA$	36,000
	(d) $E + M + S_2 \longrightarrow EMS_2 \xrightarrow[(-40°)]{MeOH} EMS_2 + MeOH \xrightarrow{S_1} PGA$	-----
	(e) $E + M \longrightarrow EM \xrightarrow[(-40°)]{MeOH} EM + MeOH \xrightarrow{S_1 + S_2} PGA$	-----
B:	(a) $E + M + S_1 \longrightarrow EMS_1 \xrightarrow[(-40°)]{MeOH} EMS_1 + MeOH \xrightarrow{S_2} PGA$	28,000
	(b) $E + M + S_2 \longrightarrow EMS_2 \xrightarrow[(-40°)]{MeOH} EMS_2 + MeOH \xrightarrow{S_1} PGA$	600
	(c) $E + M + S_1 \longrightarrow EMS_1 \xrightarrow[(-40°)]{MeOH} EMS_1 + MeOH \xrightarrow[- \text{suprn}]{+(\text{tris} + S_1 + S_2)} PGA$	52,000
	1st Preincubation / 2nd Inactivation / 3rd Incubation	

E: Carboxydismutase, S_1: $NaH^{14}CO_3$, S_2: Ribulose-1,5-diphosphate, M: Mg^{++}, PGA: 3-Phosphoglyceric acid-1-^{14}C

Experimental Conditions: A: Each reaction mixture contained (umoles): Enzyme, 3.4 mg (dry weight); Mg^{++}, 5; $NaH^{14}CO_3$, 4 (specific activity 31 uC/uM); RuDP, 0.8; MeOH, to make a final conc. 80% in MeOH. Final volume of the preincubation mixture, 0.2 ml. Preincubation (1st) 2 min at room temp. Inactivation with cold MeOH (-40°) stayed 30 min at -10° (2nd). Incubation at room temp for 1 hr (a), or 2 hr (b, d and e) or 16 hr (c). Extraction of the residue

Table II (cont.).

(protein ppt) with 80% MeOH, 20% MeOH, water at 60°, for 30 min each time. Separation of the PGA formed by paper chromatography.

B: Each reaction mixture contained (umoles): Mg^{++}, 5; $NaH^{14}CO_3$, 9 (21 uC/uM); RuDP, 0.8; enzyme, 3.6 mg (dry weight); MeOH, to make a final conc. of 80% in MeOH. Final volume of the preincubation mixture 0.25 ml. Preincubation at 0° C for 10 min (b) and for 2 hr (a and c). Inactivation 10 min at 0°. Incubation 18 hr at room temp. The acid stable radioactivity was counted on planchets with a G-M counter tube. In the experiment B-c the inactivated mixture was centrifuged, supernatant discarded and in the residue 200 uM of tris buffer, pH 8.1, S_1 (3 uM) and S_2 were added. Final volume 1 ml. Incubation 18 hr at room temp.

Using a Dowex 1-Cl (X 8, 200 to 400 mesh) anion exchange column we were able to separate a $^{14}CO_2 \sim$ enzyme complex from a reaction mixture containing enzyme, Mg^{++}, $H^{14}CO_3^-$ and tris buffer (pH 8.3), which subsequently reacted with RuDP and formed ^{14}C-PGA. Preliminary experiments showed that the above column retained all the radioactivity from a reaction mixture containing all the components of the carboxylation system except the enzyme, and in the same concentration as the complete system. The carboxylation of the enzyme was carried out as described in the experimental section and in the legends to the tables and figures (Tables III, IV and V; Figs. 18, 19, 21, 22). The isolation of the $^{14}CO_2 \sim$ enzyme complex from the reaction mixture by passage through a Dowex 1 anion exchange column is described in the experimental section. Tris buffer (pH 8.3) was used in the carboxylation system because, as was observed by Pon[7] (see Fig. 17), the activity of carboxydismutase depends on the pH, and there is a maximum of its activity around pH 8.

The $^{14}CO_2 \sim$ enzyme complex is relatively stable at 0° C, and upon incubation of the complex with RuDP at room temp for 10 or more min, approximately all radioactivity was found in the PGA formed. In some experiments the $^{14}CO_2 \sim$ enzyme complex was incubated with RuDP and a large amount of non-labeled bicarbonate at room temp for 5 to 10 min. In this case approximately 35% of the radioactivity was found as acid-stable, i.e. in PGA. The acid-stable radioactivity was dropped in the latter case because two competing reactions were taking place in the incubation mixture, the carboxylation of RuDP, and the exchange of $^{14}CO_2$ in the complex with $^{12}CO_2$ from the soln.

Table III. Carboxylation of Carboxydismutase (Carboxylation by Mg^{++} + $H^{14}CO_3^-$).

Enzyme + Mg^{++} + "$^{14}CO_2$" $\longrightarrow$ (Enzyme-Mg^{++})-$^{14}CO_2$

System 1	Radioactivity of enzyme, dpm/mg	System 2*	Radioactivity of enzyme, dpm/mg
Complete	352,000	Complete	460,000
No Mg^{++}	19,600	No Mg^{++}	37,400

Experimental conditions: System 1: Each reaction mixture contained (umoles): Mg^{++}, 5; tris buffer (pH 8.3) 150; $NaH^{14}CO_3$ (specific activity 21 uC/uM), 12; enzyme, 2 mg (dry weight). Final volume 0.5 ml. Preincubation (without bicarbonate) 5 min at 0°. Incubation 2 hr at 0°. Separation of the $^{14}CO_2$~enzyme complex by anion exchange column at 0°.

System 2: Each reaction mixture contained (umoles): tris buffer (pH 8.3), 200; Mg^{++}, 10; $NaH^{14}CO_3$ (specific activity 21 uC/uM), 9; enzyme, 2.4 mg (dry weight). Final volume 0.5 ml. Preincubation (without bicarbonate) 5 min at 0°. Incubation 3 hr at 0°. Separation of the $^{14}CO_2$~enzyme complex by anion exchange column at 0°.

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* The enzyme of system 2 is from a different preparation.

Table IV. Dependence of the Amount of $^{14}CO_2$~ enzyme Formed on Mg^{++} Concentration.

Concentration of Mg^{++}	Radioactivity of $^{14}CO_2$~ enzyme (dpm/mg)
No Mg^{++}	19,600
1×10^{-4} M	32,500
1×10^{-3} M	104,000
2×10^{-3} M	148,500
1×10^{-2} M	352,000
5×10^{-2} M	425,000
1×10^{-1} M	480,000

Experimental conditions: Each reaction mixture contained (umoles): tris buffer (pH 8.3), 150; Mg^{++}, see table; $NaH^{14}CO_3$ (specific activity 21 uC/uM), 12; enzyme, 2 mg (dry weight). Final volume 0.5 ml. Preincubation (without bicarbonate) 5 min at 0°. Incubation 2 hr at 0°. Separation of the $^{14}CO_2$~ enzyme complex by anion exchange column at 0°.

Table V. Carboxylation of Carboxydismutase in the Presence of Avidin or ATP.

System 1		System 2	
Complete system: enzyme, Avidin, Mg^{++}, $NaH^{14}CO_3$, tris buffer (pH 8.1)		Complete system: enzyme, ATP, Mg^{++} $NaH^{14}CO_3$, tris buffer (pH 8.1)	
	Radioactivity of $^{14}CO_2$~ enzyme, dpm/mg		Radioactivity of $^{14}CO_2$~ enzyme, dpm/mg
Complete	360,000	Complete	430,000
No Avidin	430,000	No ATP	430,000
No Enzyme	70,000		
Complete + Biotin	420,000		

Experimental conditions: System 1: Each reaction mixture contained (umoles): tris buffer (pH 8.1), 150; Mg^{++}, 10; $NaH^{14}CO_3$ (specific activity 21 uC/uM), 12; enzyme (when present), 2 mg; Avidin (when present) 6 units; biotin (when present), 6 ug. Final volume 0.5 ml. Before preincubation the mixture of enzyme, Avidin, tris buffer and biotin (or without biotin) stayed 30 min at 0°. Preincubation 5 min at 0°. Incubation 2 hr 30 min at 0°. Separation of the $^{14}CO_2$~ enzyme complex by anion exchange column at 0°.

System 2: Each reaction mixture contained (umoles): tris buffer (pH 8.1), 150; Mg^{++}, 10; $NaH^{14}CO_3$ (specific activity 21 uC/uM), 12; enzyme, 2 mg (dry weight); ATP (when present), 0.25 mg. Final volume 0.5 ml. Preincubation 5 min at 0°. Incubation 2 hr 30 min at 0°. Separation of the $^{14}CO_2$~ enzyme complex by anion exchange column at 0°.

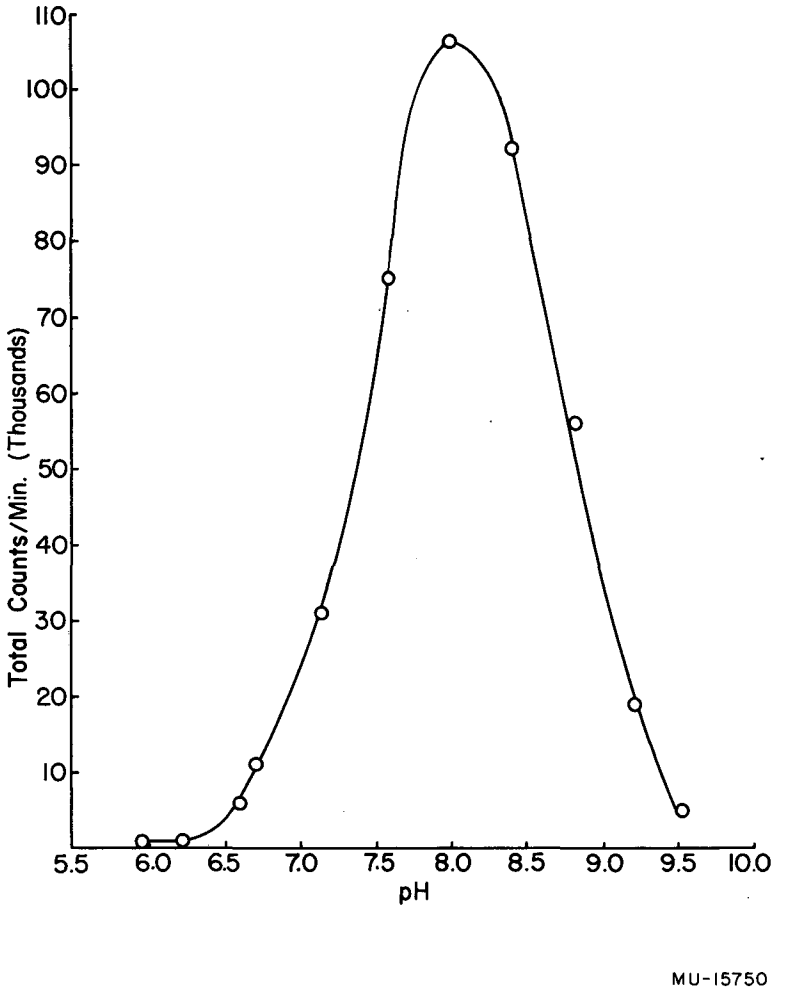

Fig. 17. The effect of pH on the activity of carboxydismutase. (From N. G. Pon (7) p. 139.)

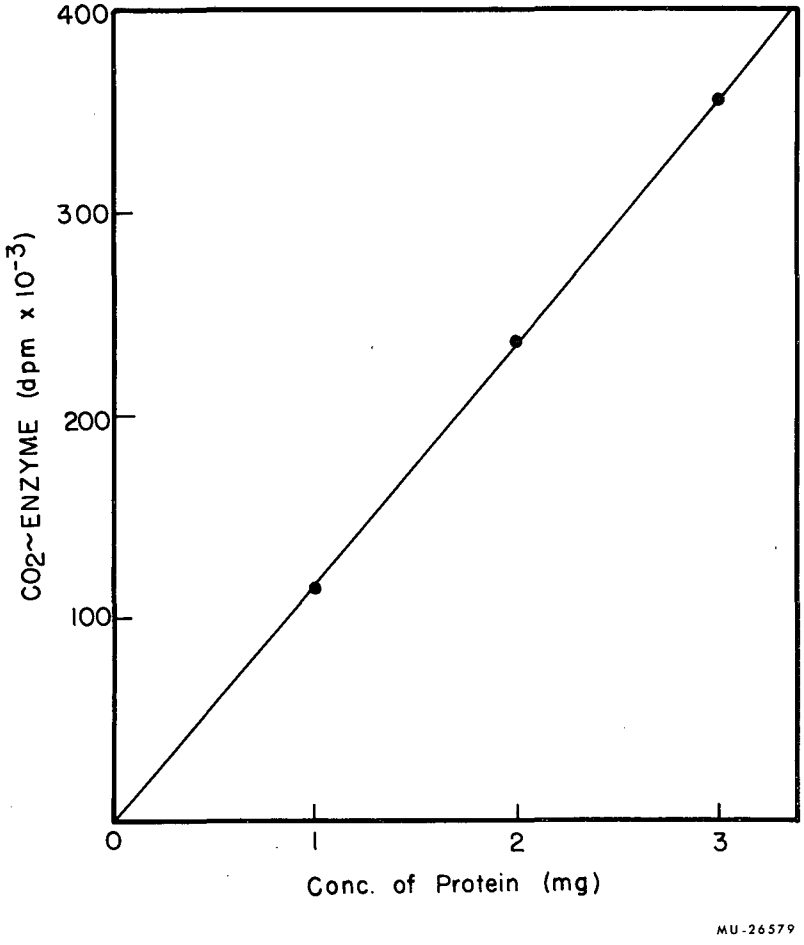

Fig. 18. The dependence of the amount of $^{14}CO_2$~ enzyme formed on enzyme concentration. Carboxylation of enzyme: Each reaction mixture contained (umoles): tris buffer (pH 8.3), 125; $MgCl_2$, 5; $NaH^{14}CO_3$ (specific activity 21 uC/uM), 7.5; enzyme, see curve. Final volume 0.4 ml. Preincubation (without bicarbonate) 1 min at 25°. Incubation 10 min at 0°. Separation of the $^{14}CO_2$~ enzyme complex by anion exchange column.

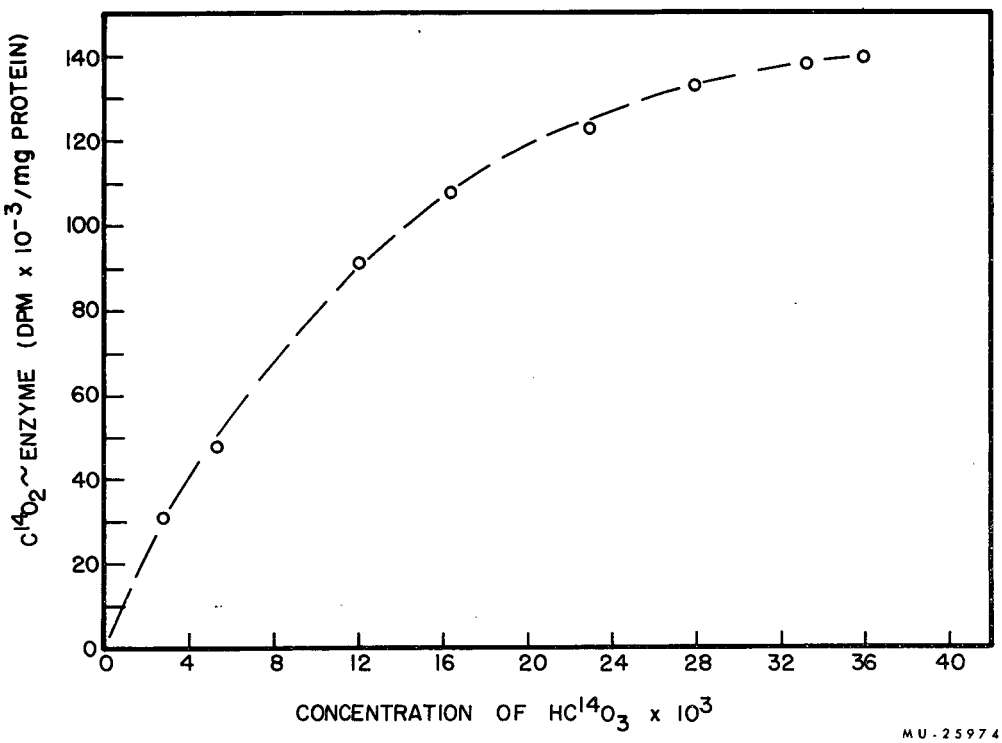

Fig. 19. The dependence of the amount of $^{14}CO_2$~ enzyme formed on bicarbonate concentration. Carboxylation of enzyme: Each reaction mixture contained (umoles): tris buffer (pH 8.3), 125; $MgCl_2$, 5; $NaH^{14}CO_3^-$, see curve (specific activity 21 uC/uM); enzyme (extracted from chloroplasts of Spinaces oleracea and fractionated with ammonium sulphate), 2 mg (dry weight). Final volume, 0.5 ml. Preincubation (without bicarbonate) 5 min at 0°. Incubation 10 min at 0°. The $^{14}CO_2$~ enzyme complex was isolated by anion exchange column.

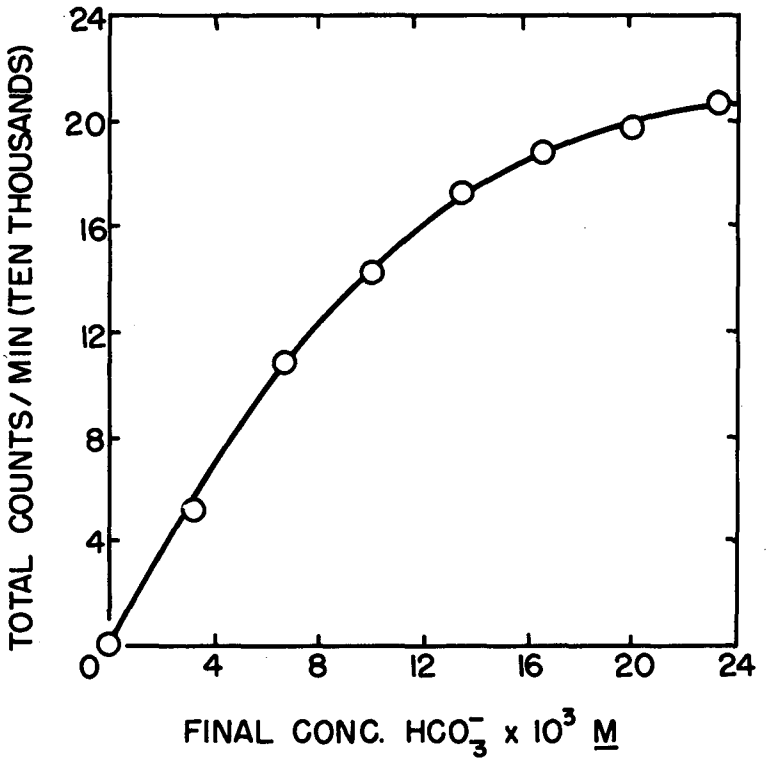

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Fig. 20. The dependence of enzyme activity on bicarbonate concentration. (From N. G. Pon (7), p. 135.)

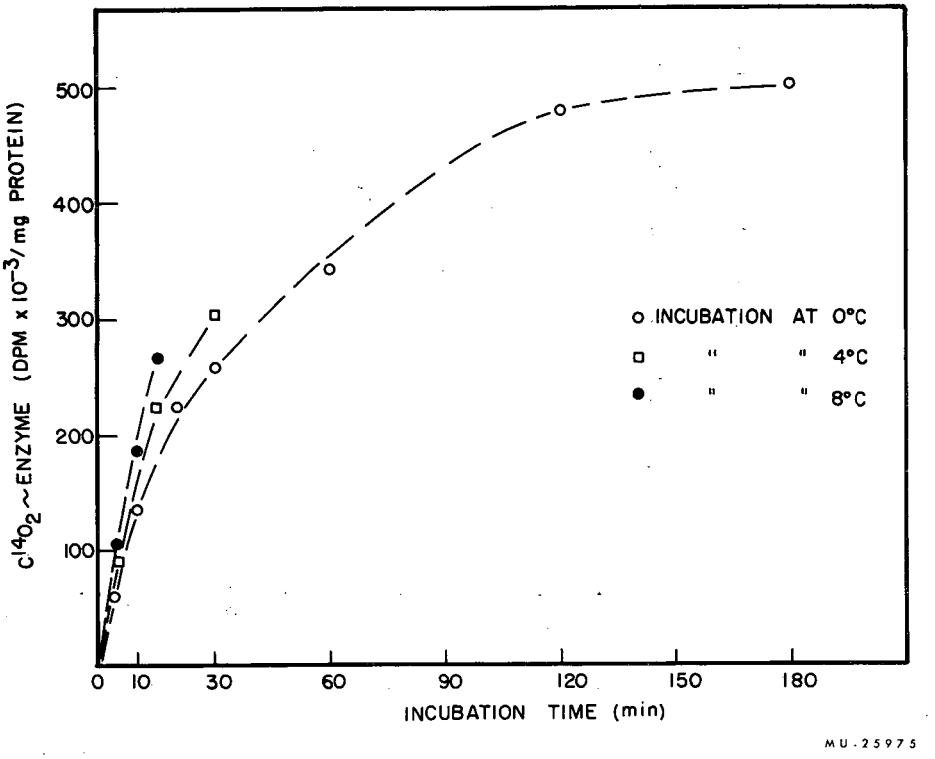

Fig. 21. Rate of $^{14}CO_2$~ enzyme formation. Carboxylation of enzyme: Each reaction mixture contained (umoles): tris buffer (pH 8.3), 125; $MgCl_2$, 5; $NaH^{14}CO_3$, 18 (specific activity 21 uC/uM), final concentration of bibarbonate 36×10^{-3} M; enzyme (extracted from chloroplasts of Spinacea oleracea and then fractionated with ammonium sulphate), 2 mg (dry weight). Final volume, 0.5 ml. Preincubation (without bicarbonate), 5 min at 0°. Incubation time and temp, see curve. The $^{14}CO_2$~enzyme complex was isolated by anion exchange column.

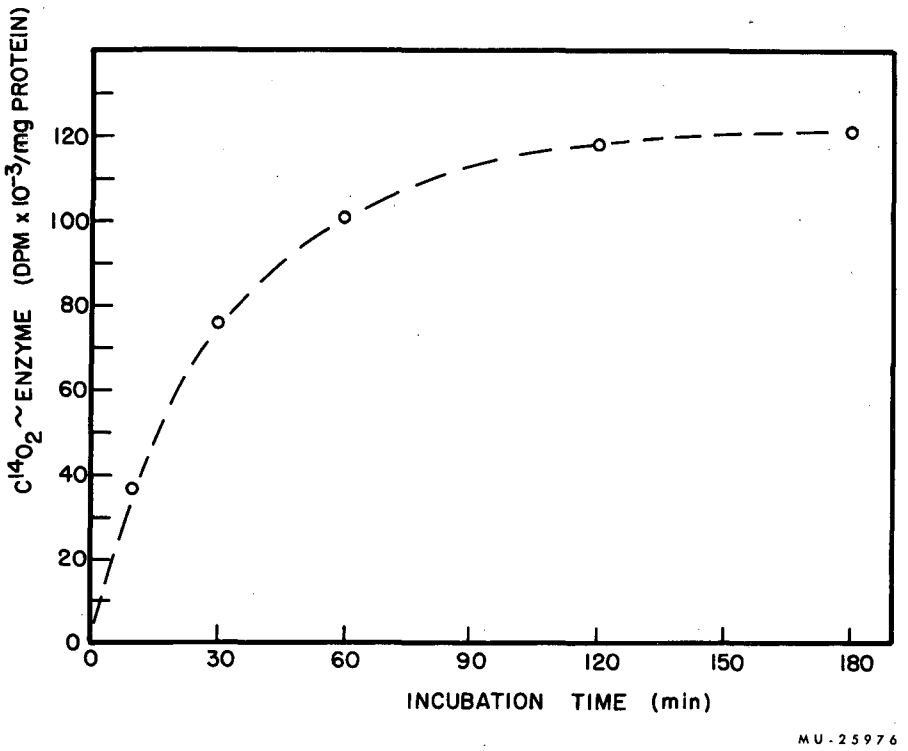

Fig. 22. Rate of $^{14}CO_2$ enzyme formation at 0°. Carboxylation of enzyme: Each reaction mixture contained (umoles): tris buffer (pH 8.0), 36; $MgCl_2$, 7; $NaH^{14}CO_3$, 1.8 (specific activity 21 uC/uM), final concentration of bicarbonate 3.6×10^{-3} M; enzyme (extracted from chloroplasts of Spinacea oleracea and fractionated with ammonium sulphate), 2 mg (dry weight). Final volume, 0.5 ml. Preincubation (without bicarbonate) 5 min at 0°. Incubation time, see curve. Incubation temp 0°. The $^{14}CO_2$ ~ enzyme complex was isolated by anion exchange column.

Dependence of $^{14}CO_2 \sim$ enzyme Formed on Mg^{++} Concentration

It is known[7] that Mg^{++} ions are necessary for the activation of carboxydismutase, and furthermore that the activation depends on the concentration of Mg^{++} ions. In order to find out whether these Mg^{++} ions are necessary for the carboxylation of the enzyme, or for the transcarboxylation from the $CO_2 \sim$ enzyme to the RuDP, experiments were performed in the presence and absence of Mg^{++}. The results are shown in Table III. Thus carboxylation of the enzyme is dependent on the presence of Mg^{++} ions. The small radioactivity of the enzyme incubated in the absence of Mg^{++} ions may be due to the presence of a small amount of metal ions in the enzyme preparation, which were not possible to separate even after 24 hr of dialysis, and to the formation of carbamino compounds.

Table IV shows the dependence of the complex formed on the concentration of Mg^{++} ions. There is an increase in the complex formed as the concentration of Mg^{++} ions increases.

Many other metal ions besides Mg^{++} were tested in the carboxylation of the enzyme. These are the following: Ni^{++}, Mn^{++}, Hg^{++}, Fe^{++}, Fe^{+++}, Cu^{++}, Zn^{++}, Cr^{+++} and Co^{++}. All of these ions were tested at a final concentration of 0.01 M. The carboxylation system was preincubated (without bicarbonate) at 0° for 5 min and incubated 2 hr at 0° C. With each metal ion a control experiment was run; a reaction mixture containing all the components of the carboxylation system except the enzyme and in the same concentration as in the complete system, was passed through the column and the eluate tested for radioactivity. Hg^{++}, Cu^{++} and Zn^{++} gave no increase in the radioactivity of the enzyme compared to the radioactivity present when the enzyme was incubated in the absence of any metal ion. All of the other cations except Ni^{++} gave a great increase in the radioactivity present in the eluate. However, the same amount of

radioactivity was present in the eluate whether the enzyme was there or not. Ni^{++} gave the following results: complete system, 275,000 dpm/mg protein; no enzyme, a total of 75,000 dpm. ^{14}C-labeled PGA was formed when the eluate of the experiment with enzyme, Ni^{++} and $H^{14}CO_3^-$ was incubated with RuDP.

The high radioactivity present in the eluate in the experiments with Mn^{++}, Fe^{+++}, Fe^{++}, Cr^{+++} and Co^{++} in the presence or absence of enzyme is probably due to the formation of different chelate compounds. The results of the experiments with Ni^{++} are in agreement with the results of Pon[7], who found a substantial fixation of radioactivity when carboxydismutase was incubated with Ni^{++}, $H^{14}CO_3^-$, RuDP and tris buffer.

Dependence of $^{14}CO_2 \sim$ enzyme Formed on Enzyme and Bicarbonate Concentrations

The rate of $^{14}CO_2 \sim$ enzyme formation follows a linear dependence with respect to the enzyme concentration, Fig. 18, which is in agreement with the results found by Pon[7]. Pon studied the rate of CO_2 fixation by the carboxydismutase system vs enzyme concentration.

The effect of bicarbonate concentration on the rate of $^{14}CO_2 \sim$ enzyme formation is shown in Fig. 19. The reaction was carried out at 0° C for 10 min, and as the rate of the reaction at that temp was slow, it may be considered the initial rate. The rate increased as the concentration of bicarbonate was increased up to 34×10^{-3} M. At greater concentrations the rate was approximately constant. The value of Michaelis constant for bicarbonate (the bicarbonate concentration at which the carboxylation of the carboxydismutase rate is one half the maximum rate) is 0.009 ± 0.001 M. This value compares well with those quoted by other investigators, viz., 0.01 to 0.02M[3,5], or greater than 0.006 M[7].

A similar curve was obtained by Pon[7] for the dependence of the enzyme activity on bicarbonate concentration (see Fig. 20). Pon measured the $^{14}CO_2$ fixation by the carboxydismutase system vs bicarbonate concentration.

By using a high concentration of bicarbonate and leaving the mixture to react for a long time (see Fig. 21), we would expect that all the active sites of the enzyme would be virtually saturated with carbon dioxide. It was found that 1 mg of enzyme protein had bound approximately 11.5×10^{-3} uM of bicarbonate. Assuming that the molecular weight of the enzyme is 400,000, 1 mg of protein contains about 2.5×10^{-3} uM, which corresponds to 5 molecules of CO_2 per molecule of protein. The number is probably higher because of the impurity of the enzyme preparation and the fact that the amount of complex formed depends also on the concentration of Mg^{++} ions.

Rate of $^{14}CO_2 \sim$ enzyme Formation

Fig. 21 shows the rate of $^{14}CO_2 \sim$ enzyme formation at 0° C, 4° C and 8° C. The reaction is complete after two to three hr at 0° C and goes much faster as the temperature increases.

Fig. 22 shows again the rate of the reaction at 0° C but at smaller bicarbonate concentration.

A time effect on the activity of carboxydismutase was observed by Pon[7], as was mentioned earlier. Pon observed an increase in the activity of the enzyme when it was preincubated with bicarbonate in the presence of Mg^{++} at 0° C. The activity of the enzyme increased as the time of preincubation increased (Fig. 16). This is in accord with our observations on the rate of complex formation at 0° C.

Carboxylation of Carboxydismutase in the Presence of Avidin or ATP

Experiments were performed (carboxylation of carboxydismutase) in the presence of Avidin, or Avidin plus biotin, or ATP. Biotin is a cofactor for many carboxylases[17,39]. Avidin is an inhibitor for biotin dependent enzyme systems[17,40]. ATP is a cofactor for ATP-dependent biotin containing enzymes such as (1) propionyl carboxylase[41,42], (2) beta-methylcrotonyl carboxylase[17,43], acetyl carboxylase[44,45,46], and ATP-dependent pyruvic carboxylase[47]. With beta-methylcrotonyl carboxylase Lynen et al., prepared a CO_2~ biotin complex, and Ochoa et al. isolated a CO_2~ enzyme complex of propionyl CoA carboxylase.

The results of our experiments are shown in Table V. There is no decrease or increase in the amount of complex formed in the presence of or absence of ATP. This is in accord with the observation that the carboxydismutase is not an ATP-dependent enzyme. Furthermore, Pon[7] observed an inhibition inthe enzyme activity of carboxydismutase system by ATP. A similar inhibition in the enzyme activity was also observed with other phosphorylated esters, such as thiamine pyrophosphate, 3-phosphoglyceric acid, threose-2,4-diphosphate, etc. The inhibition of the above phosphorylated esters may be due to a phosphate effect, and probably they inhibit the transcarboxylation of RuDP from the CO_2~ enzyme complex and not the carboxylation of the enzyme.

There is a decrease in the amount of CO_2~ enzyme formed in the presence of Avidin, but the decrease is not as high as one should expect it to be if carboxydismutase was a biotin-dependent enzyme. The small decrease is probably due to the decrease in the concentration of Mg^{++} ions because of the presence of avidin. This is in agreement with the results obtained by Pon[7], who found that avidin had no effect on the carboxydismutase activity.

It is worthwhile to mention here that carboxydismutase may have -SH functional groups because, as was observed by Mayaudon *et al.*[4], carboxydismutase was inhibited by p-chloromercuribenzoate, and the reaction was reversed by addition of cysteine. An inhibition by p-chloromercuribenzoate was also observed by Fager[48] on enzymic extracts of *Chlorella*.

Mechanism of the Carboxydismutase Reaction

Indirect evidence obtained by Pon[7] in accord with evidence from the present work indicates that the carboxydismutase reaction involves two or perhaps more separate steps. The first step is the formation of the CO_2~ enzyme complex. The rate of this reaction depends on the concentration of enzyme, bicarbonate, Mg^{++} ions and the temp. The second step is the transcarboxylation from the CO_2~ enzyme complex to the RuDP. The mechanism is the following:

$$\text{enzyme} + \text{"}CO_2\text{"}^{*} + Mg^{++} \rightleftharpoons (\text{enzyme-}Mg^{++})\text{-}CO_2$$

$$(\text{enzyme-}Mg^{++})\text{-}CO_2 + \text{RuDP} \xrightarrow{H_2O} \text{enzyme} + Mg^{++} + 2\ \text{PGA}$$

$$\text{sum: "}CO_2\text{"} + \text{enzyme} + \text{RuDP} + H_2O \xrightarrow{Mg^{++}} \text{enzyme} + 2\ \text{PGA}$$

whether the metal ion (Mg^{++}) forms a complex with the enzyme before bicarbonate, or *vice versa*, is uncertain.

The proposed mechanism for the carboxylation reaction of RuDP, via an unstable beta-keto acid, is described in the experimental section. There is no evidence so far for the existence of this postulated keto acid intermediate. Either the amount present is very small or the life time very short and so very difficult to detect,

---

* "CO_2" denotes the reacting species of bicarbonate in the carboxydismutase system.

or it does not exist at all. There is also another possibility, as was mentioned earlier, that the beta-keto acid is not formed as a free intermediate but as a complex combined with the enzyme, which is hydrolyzed to yield PGA, enzyme and Mg^{++} ions.

According to the above hypothesis the mechanism of the carboxydismutase reaction can be indicated as follows:

$$\text{enzyme} + Mg^{++} + \text{"}CO_2\text{"} \rightleftharpoons (\text{enzyme-}Mg^{++})\text{-}CO_2$$

$$(\text{enzyme-}Mg^{++})\text{-}CO_2 + \text{RuDP} \rightleftharpoons ((\text{enzyme-}Mg^{++})\text{-}CO_2)\text{-RuDP}$$

$$((\text{enzyme-}Mg^{++})\text{-}CO_2)\text{-RuDP} \xrightarrow{H_2O} \text{enzyme} + Mg^{++} + 2\ \text{PGA}$$

Experiments with enzyme, Mg^{++} and ^{14}C-labeled RuDP in the absence of bicarbonate showed either that a RuDP-enzyme complex is not formed, or if it is formed, the bond between RuDP and enzyme is not stronger than the bond between RuDP and the ion exchange resin. Experiments with enzyme, Mg^{++}, ^{14}C-labeled RuDP and unlabeled bicarbonate also showed no evidence for an enzyme-CO_2-RuDP complex, either because the bond between RuDP and the enzyme-CO_2 is not stronger than the bond between RuDP and the ion exchange column or the rate of hydrolysis of the complex is faster than the rate of elution of the column.

It is worthwhile to mention here that in the experiments with the carboxydismutase system and diazomethane it was observed that when the enzyme was inactivated with cold MeOH, then treated with diazomethane and the residue (protein) extracted first with a soln of 80% MeOH, then with a soln of 20% MeOH and finally with water at 65° C, some radioactivity remained in the residue, which was not susceptible to extraction. When the residue was hydrolyzed with HCl and the hydrolysate chromatographed, some PGA was observed

on the paper in addition to other radioactive compounds, probably carbamino compounds. The amount of PGA was much less when the carboxydismutase was inactivated with hot MeOH and then treated with diazomethane.

One interpretation of this phenomenon is that PGA forms a complex with the enzyme which is stabilized by diazomethane. The other interpretation is that an enzyme-CO_2-RuDP complex is formed which is stabilized by diazomethane and which after hydrolysis with HCl gives PGA.

SUMMARY

All efforts failed to produce any evidence for the existence of the postulated diphosphate ester of a beta-keto acid intermediate in the carboxylation reaction of RuDP. This can be explained by any of the following: the amount present is very small; the life time is very short; the beta-keto acid does not occur at all; or the reaction goes through an enzyme-CO_2-RuDP complex. These efforts included treatment of the products with liquid ammonia and then separation by low temp paper chromatography, and treatment of the photosynthetic material or the carboxydismutase system with diazomethane.

Carboxydismutase (extracted from chloroplasts of Spinacea oleracea and fractionated with ammonium sulphate) was carboxylated with ^{14}C-labeled bicarbonate in the presence of Mg^{++} ions, and the complex was isolated in about 45% yield by passage through a Dowex 1 anion exchange column. Approximately 5 moles of $^{14}CO_2$ were taken up per mole of enzyme. $^{14}CO_2$ was quantitatively transferred from $^{14}CO_2$~ enzyme to RuDP yielding ^{14}C-labeled PGA.

The carboxylation of carboxydismutase was Mg^{++}-dependent and the amount of complex formed increased as the concentration of Mg^{++} ions increased. The rate of $^{14}CO_2$enzyme formation was linearly dependent on enzyme concentration. Bicarbonate also exhibited a similar behavior at low substrate concentration, but at high concentrations, a typical saturation curve was obtained. The rate of the complex formation was also temperature dependent.

A value of 0.009 M bicarbonate was calculated for the Michaelis constant, in accord with the values obtained by other investigators with the complete carboxydismutase system.

The formation of the $^{14}CO_2$~ enzyme complex is not inhibited by avidin or ATP and it is not ATP-dependent.

Preincubation studies with the substrates and inactivation of the enzyme with cold MeOH before incubation showed that Mg^{++} with bicarbonate stabilized the enzyme against MeOH, while RuDP did not. When the enzyme plus Mg^{++} is preincubated with ^{14}C-bicarbonate, then treated with MeOH followed by incubation with RuDP, some ^{14}C-labeled PGA is obtained, but when the enzyme is preincubated with RuDP, no PGA is formed.

The results of this study provided conclusive evidence that the overall carboxydismutase reaction proceeds in two or probably three steps, with the enzyme-bound CO_2 as an intermediate.

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PART II. SOME REACTIONS OF DIAZOMETHANE IN AQUEOUS SOLUTIONS

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I. INTRODUCTION

Diazomethane is a well known reagent, which is very often employed for the preparation of different organic compounds or their derivatives. It methylates compounds containing acidic hydrogen in a neutral medium and at room temperature; it reacts with unsaturated compounds as well as with aldehydes and ketones to form a variety of products; it can be used for the preparation of the derivatives of different naturally occurring organic compounds present in very small amounts; it can also be used for the stabilization of some unstable compounds, e.g., beta-keto acids, by forming their stable derivatives. Diazomethane reacts under very mild conditions, and the by-products of its reaction are generally volatile compounds.

Diazomethane has been used in the search for unstable intermediates in the carboxylation reaction of photosynthesis, i.e., the carboxylation of ribulose -1,5-diphosphate to give phosphoglyceric acid, the first stable product in the carbon reduction cycle of photosynthesis. In all the experiments with diazomethane, C^{14}-bicarbonate was present in the reaction mixture. It is known that dimethyl carbonate is the product of the reaction of bicarbonate and diazomethane. However, by the use of paper and gas chromatography for the separation of the products of the above reaction, three other compounds were found in addition to dimethyl carbonate.

The purpose of this study was:

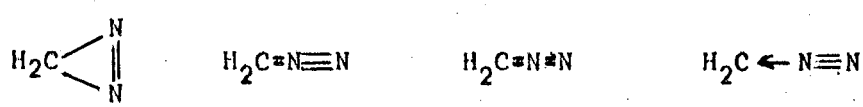
1) To separate and identify the products of the reaction of diazomethane (prepared by alkaline hydrolysis of N-methyl-N-nitroso-N'-nitroguanidine) with bicarbonate or carbon dioxide.

2) To study the mechanisms of the reaction.

II. CHEMISTRY OF DIAZOMETHANE

Constitution of Diazomethane

Diazomethane like all the aliphatic diazo compounds is one of the few organic compounds for which no one formula can be written. According to classical theory two formulas were suggested for it, the ring (I) by Curtius[2], and the linear one (II) by Angeli and Thiele[3].



(I) (II) (III) (IV)

Langmuir[4,5] proposed the structure (III), as the Angeli-Thiele formula containing a pentacovalent nitrogen atom could not be correct, and later the structure (IV) was considered. In the (III) the middle nitrogen donates two electrons to the other nitrogen atom, and in the (IV) the two electrons are donated to the carbon atom.

Electron diffraction[6], and infrared spectrophotometry studies have established conclusively a linear relationship for the carbon and nitrogen atom in diazomethane with the CN and the NN bond length of 1.34 $\pm$ 0.05 and 1.13 $\pm$ 0.04 Å, respectively. These values require that the CN bond has about 42% double-bond character, and the molecule

of diazomethane must be represented as a resonance hybrid of several charge-separated[8-11] forms (V, VI, VII), none of which can be regarded as a satisfactory approximation.

$$^{+}CH_2-N=N^{-} \longleftrightarrow CH_2=N^{+}=N^{-} \longleftrightarrow {}^{-}CH_2-N^{+}\equiv N$$

(V) (VI) (VII)

Forms (V,VI,VII) come from the initial proposed formulas (I,III,IV) after the shifting of an electron pair. The fact that diazomethane is represented as a mesomeric system with the extreme formulas (V, VI, VII) can explain its reaction with different groups of organic compounds, and its reactivity.

Preparation of Diazomethane

V. Pechmann[1] prepared diazomethane first by treating different nitrosoderivatives of methylamine, such as N-methyl-N-nitroso--benzamide, methylnitrosourethan, etc., with alkali. Since then many methods have been developed for the preparation of gaseous or solutions of diazomethane. Diazomethane solutions are normally ethereal.

The following methods are common ones for the preparation of diazomethane, and they can be arranged into two groups:

<u>Preparation of gaseous diazomethane:</u> from chloroform and hydrazine[12] according to the reaction:

$$CHCl_3 + H_2NNH_2 + 3\ KOH \longrightarrow CH_2N_2 + 3\ KCl + 3\ H_2O.$$

Preparation of diazomethane solution: By the alkaline saponification of compounds having the general formula[10] $R\text{-}N(CH_3)NO$, where R represents groups such as $CONH_2$, $CO_2C_2H_5$, COC_6H_5, $C(NH)NHNO_2$, $SO_2C_7H_7$, etc.

a) From nitrosomethylurethan[13] according to the reaction:

$$CH_3N(NO)CO_2C_2H_5 + KOH \longrightarrow CH_2N_2 + K_2CO_3 + C_2H_5OH + H_2O$$

b) From nitrosomethylurea[13] according to the reaction:

$$CH_3N(NO)CONH_2 + KOH \longrightarrow CH_2N_2 + KOCN + 2\ H_2O$$

c) From N-methyl-N-nitroso-N'-nitroguanidine[14,15]:

$$CH_3N(NO)C(NH)NHNO_2 + KOH \longrightarrow CH_2N_2 + K(NCNNO) + H_2O$$

d) From N-methyl-N-nitroso-p-toluenesulfonamide[16]:

$$CH_3N(NO)SO_2C_7H_7 + C_2H_5O^- \longrightarrow CH_2N_2 + C_7H_7SO_2C_2H_5 + H_2O.$$

Reactions of Diazomethane

As was mentioned earlier, diazomethane is a very reactive compound. It reacts with a variety of organic and inorganic compounds forming different products. Because of its reactivity and the fact that it reacts under very mild conditions, it is used very often in synthetic and analytical organic chemistry. The use of diazomethane increased recently, because new reactions have been studied which are useful in obtaining important compounds easily and inexpensively.

Of all the reactions of diazomethane the most common three series will be very briefly considered here.

Reaction with acidic hydrogen: Acidic hydrogen is the hydrogen which is able to leave a compound as a positively charged proton, the rest of the molecule becoming a negatively charged ion. Diazomethane reacts with compounds which have an acidic hydrogen and forms their methyl derivatives. The general reaction is

$$X - H + CH_2N_2 \longrightarrow XCH_3 + N_2$$

$$XH_2 + CH_2N_2 \longrightarrow X(CH_3)_2 + N_2$$

The reaction is irreversible and uses up all the diazomethane. The rate of the reaction depends on the acidity of the compound. Eister[10] suggested the following mechanism for the above reaction: The mobile proton of the acidic compound (XH) attaches to the lone pair of electrons on the carbon atom in the extreme formula (VII) of diazomethane (nucleophilic state) and forms a hydrogen bond. If the compound is acidic enough, the hydrogen migrates to the carbon atom of diazomethane, and a methyldiazonium cation and the anion X^- are formed. The methyldiazonium cation loses nitrogen and the remaining CH_3^+ group (carbenium cation) combines with the anion X^- to form the methylester derivative of the acid $X\text{-}CH_3$.

$$X\text{-}H + :^-CH_2\text{-}^+N{\equiv}N \longrightarrow X\text{-}H\text{-}CH_2{\ddagger}N{\equiv}N: \longrightarrow X^- + CH_3\text{-}^+N{\equiv}N:$$

$$CH_3\text{-}^+N{\equiv}N: \longrightarrow CH_3^+ + N_2, \text{ and } CH_3^+ + X^- \longrightarrow X\text{-}CH_3$$

Kuhn and Reulius[18] accept this theory for the reaction of diazomethane with water or with aqueous solutions of acidic compounds. Arndt[17] finds the above theory unsatisfactory for the reactions of diazomethane with solutions of acidic compounds in nonpolar solvents, i.e., solvents in which the acidic compound (XH) reacts as it is, because (1) the diazonium cation will give azo compounds with enols and phenols and not methyl derivatives, and (2) the methyl cation (CH_3^+) which is formed as an intermediate in the reaction would be "free to choose" a site of the anion with which to react.

Arndt[17] proposed the <u>continuous adiabatic process mechanism</u>: In this theory, too, the first step is the formation of a hydrogen bridge between the acid molecule (XH) and the carbon atom of diazomethane (extreme formula (VII) of diazomethane), the more acidic XH is the more the bridge proton is attracted to the carbon atom of dizomethane, and greater is the electronic shift within the diazo group toward the distribution N N. This is not stable and splits off as nitrogen. The remaining group $X\text{-}H\text{-}CH_2$, after the loss of nitrogen, is rearranged in a "continuous adiabatic process" due to hyperconjugation effects into $X\text{-}CH_3$.

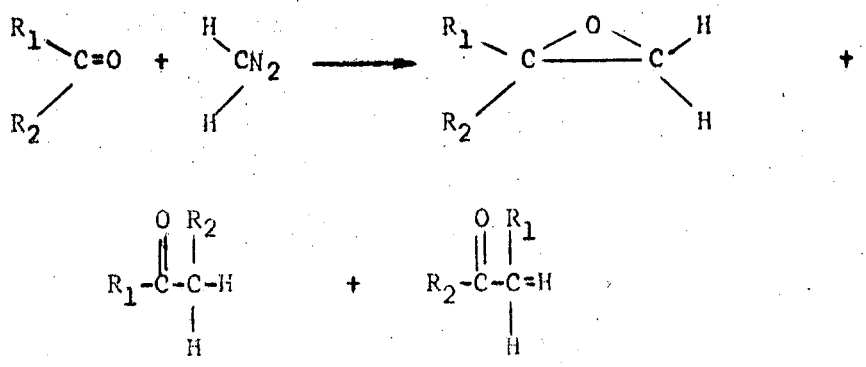
$$X\text{-}H + :^{-}CH_2\text{-}^{+}N{\equiv}N: \longrightarrow X\text{-}H\text{-}CH_2\text{-}^{+}N{\equiv}N: \longrightarrow N_2 + X\text{-}H\text{-}CH_2$$

and $$X\text{-}H\text{-}CH_2 \longrightarrow X\text{-}CH_3$$

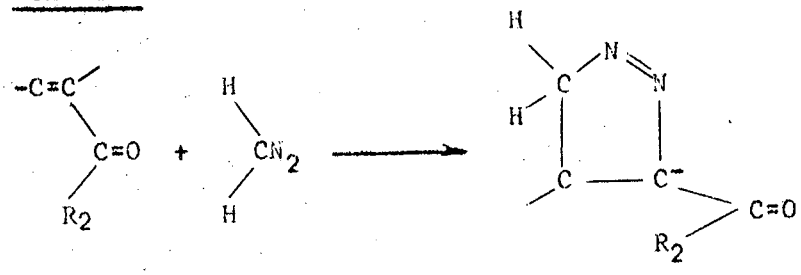
This theory is now accepted by Distert[19] for the case of the reaction of diazomethane with acidic compounds in nonpolar solvents, but it is not possible to decide which mechanism applies in the case of aqueous solutions.

Reaction with carbonyl compounds: Diazomethane reacts with aldehydes and ketones and forms a variety of products, depending on the nature of the carbonyl compound. The reaction follows scheme A, B, or C[8], but for the most of the aldehydes and ketones, scheme A is followed:

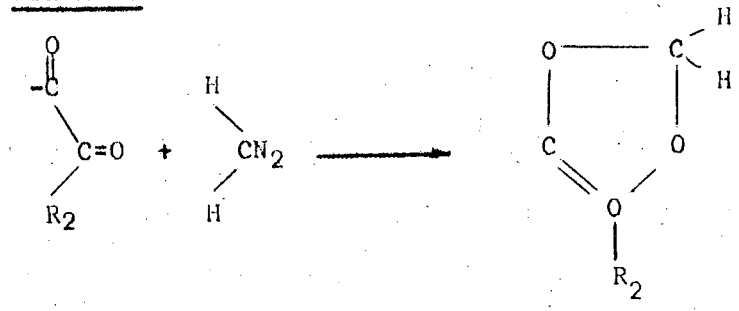
Scheme A



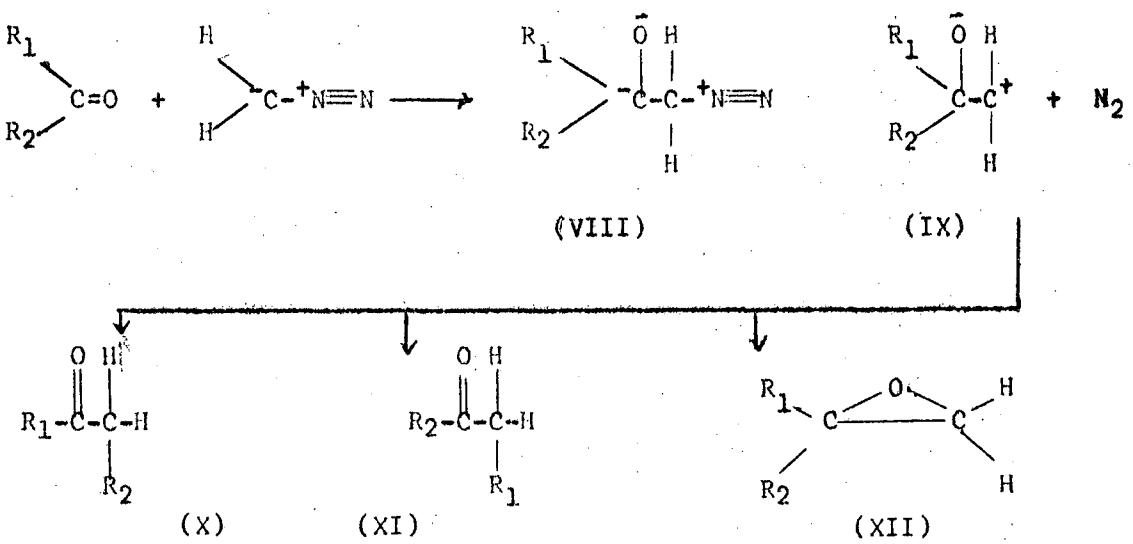
Scheme B



Scheme C



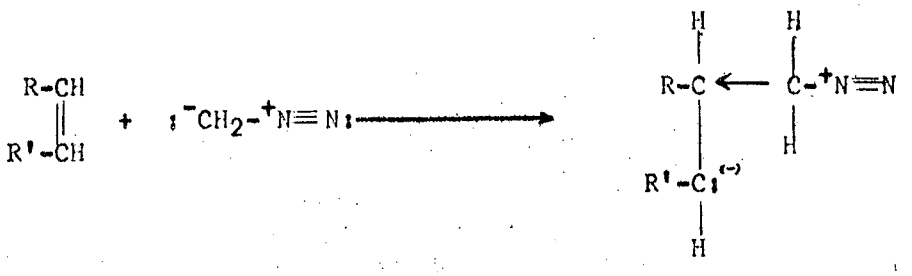
The extreme formula (VII) of diazomethane is the effective one for the reaction according to scheme A. In this case nucleophilic attack of the diazomethane (extreme formula VII) occurs on the carbonyl group to yield an addition compound, currently expressed as the diazonium betaine (VIII). The betaine (VIII) is decomposed and forms any or all of the three products (X,XI,XII) possibly through a labile intermediate (IX) which is stabilized by any or all of the three paths to yield, X, XI and/or XII.[20,21,8]



Reaction with unsaturated carbon bonds: Diazomethane reacts with unsaturated carbon bonds and forms pyrazolines, pyrazoles and various other products[10]

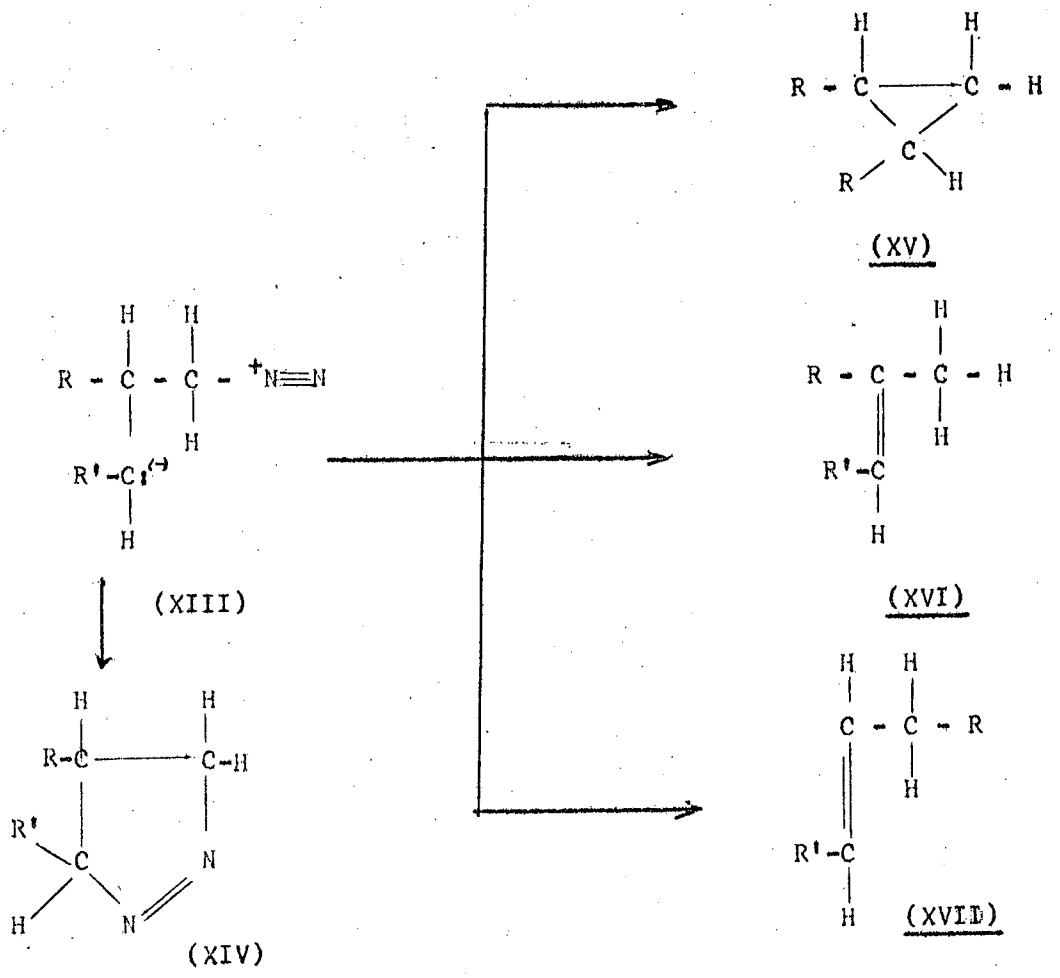
Although these reactions have received little systematic investigation, it appears that the reaction of the diazomethane with the carbon-carbon double bond is similar to that with carbonyl group. A nucleophilic attack of the diazomethane (extreme formula VII) on the double bond forms a diazonium betaine (XIII). This intermediate

can be stabilized either by migration of a proton from the methylene group of diazomethane with the formation of a pyrazoline ring (XIV), or by splitting of nitrogen, and the remaining fragment forming compounds XV, XVI, and/or XVII[10].



(XIII)

and



(XV)

(XVI)

(XVII)

(XIII)

(XIV)

Evidence for the existence of the addition product intermediate (XIII) has been established by von Pechmann and Seel[22].

II. EXPERIMENTAL PROCEDURE

Abbreviations: The following abbreviations are henceforth used throughout the second part of this text.

cm	:centimeter
cpm	:counts/min
dpm	:disintegrations/min
eq(s)	:equation(s)
IR	:infrared
mC	:millicurie(s)
uC	:microcurie(s)
ug	:microgram
ml	:milliliter
ul	:microliter
ma	:milliamperes
mM	:millimole(s)
min	:minute
Mol.W.	:molecular weight
M	:molar
N	:normal
sc.c.	:scintillation counter
sec.	:second(s)
soln	:solution
hr	:hour
v.	:volt(s)
temp	:temperature
DMC	:Dimethyl carbonate
MCR	:Methyl carbamate
NMMCR	:N-methyl-methyl-carbamate
NDMMCR	:N-dimethyl-methylcarbamate
MeOH	:Methanol
EtOH	:Ethanol
EDTA	:Ethylenediamine-tetraacetic acid
VPC	:Vapor Phase Chromatography
EGS	:Ethyleneglycol polyester of succinic acid
MNNG	:N-methyl-N-nitroso-N'-nitroguanidine
R_f	Ratio of distance travelled by solvent front. Distance is measured from the origin to the densest position of the spot

III. GENERAL METHODS

Paper Chromatography

For the separation of the products of the reaction of diazomethane with bicarbonate and the hydroxamate derivatives of them, Whatman No. 4 oxalic acid washed papers were used, with 95% EtOH-p-dioxane-water-acetic acid (60:26:9:5 v/v)[23] and butanol-acetic acid-water (4:1:5 v/v)[24] as developing solvents. Isobutyric acid-water-concentrated ammonia-EDTA (2520:1450:100 v/v:1.175 gr)[25] and butanol-acetic acid-water were also used as developing solvents. The temperature during the development of the chromatogram was 20°C to 25°C. The papers were dried under a forced current of air at room temperature. Radioactive compounds on the chromatogram were located by exposing the paper to single coated Kodak x-ray film. For the detectionof the nonradioactive hydroxamates, the chromatogram was sprayed after its development with a solution of 1% (w/v) ferric chloride in 95% EtOH, containing 0.1% HCl[26]. Hydroxamates form an intense red purple complex with ferric chloride on a yellow background.

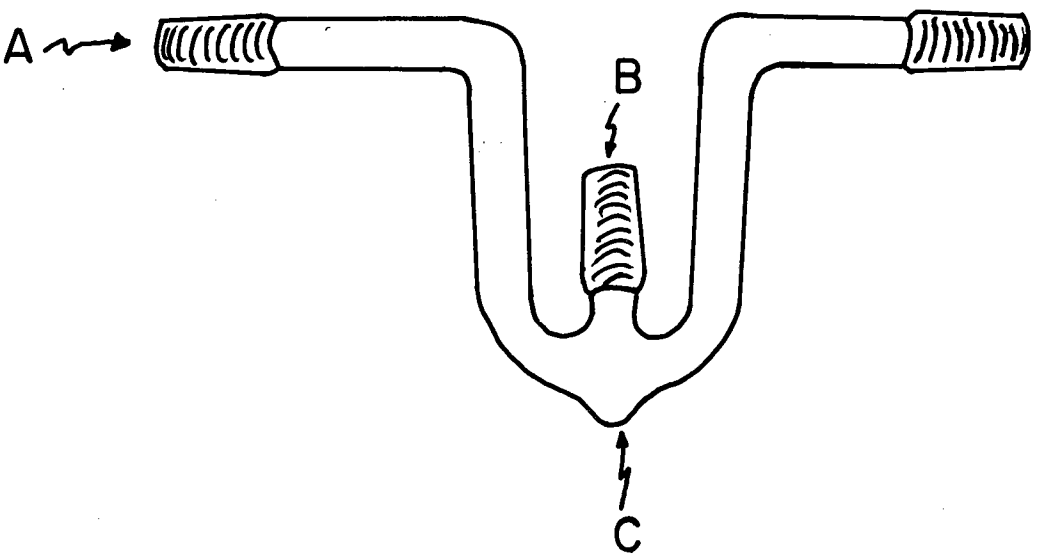
The identities of the chromatographically separated hydroxamates were established by their R_f values. The radioactive compound was then eluted and cochromatographed with authentic marker compound (ca. 100 ug of marker) for confirmation. Exact coincidence of the radioactivity with the marker compound was taken as criterion of identity.

Vapor Phase Chromatography

As the products of the bicarbonate-diazomethane reaction were relatively volatile, Wilkens standard gas chromatograph Model A90, connected with a Brown recorder (Brown Potentiometer, Brown Intrument Division, Philadelphia, Pennsylvania) and a 10 foot EGS column were used for the separation and identification of them. The collector shown in Fig. 23 was used in conjunction with the chromatography unit to collect the different compounds separately. The collector was cooled by a dry ice-acetone bath or by liquid nitrogen. The analytical conditions were the following: Injection port and column temperature, 150°C; detector voltage, 12 v; filament current in ma, 140; helium flow rate, 58 ml/min; chart speed, 1 cm/min; sample size, 0.001 to 0.050 ml.

Vapor Phase Chromatography in Conjunction with Proportional Counter

For the separation and detection of the radioactive products of the C^{14} bicarbonate-diazomethane reaction, a gas chromatograph in conjunction with proportional counter was used. Wolfgang and Rowland[27] and Wolfgang and Mackay[28] have described proportional counters for use in conjunction with gas chromatography to detect radioactive effluent materials. Whittemore[29] has described the one which is used in our laboratory, and which is similar to that described in

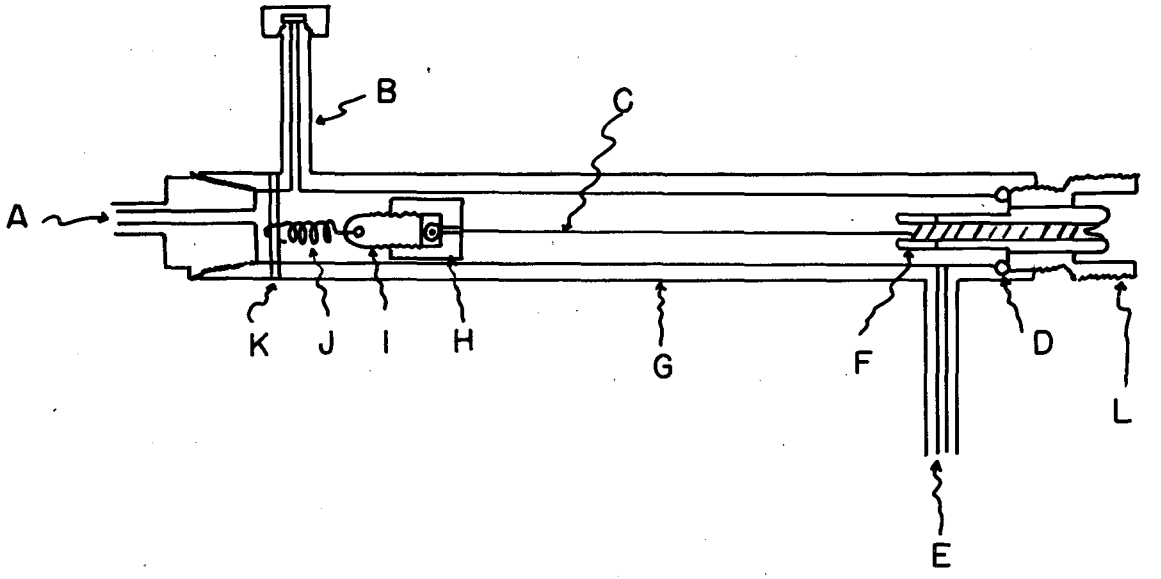


MU-26031

Fig. 23. Collector used in gas chromatography
A. Input from gas chromatography column
B. Outlet to facilitate removal of trapping compounds
C. Reservoir for the collection of separated compounds.

Refs. 27 and 28 with certain modifications. Fig. 24 is a diagram of the proportional counting tube, and Fig. 25 is a block diagram of the apparatus.

The proportional counter enables one to determine total activities in various components of radioactive mixtures that can be separated by gas chromatography. It can detect the various radioactive components of a mixture even if they are present in very small amounts which are not detectable by the mass detector Combination of the mass and radioactivity measurements enables one to calculate specific activities of components without the necessity of trapping them. The chromatography unit is a Wilkens Model A90 or Wilkens linear programmed Model A350 (Wilkens Instrument and Research, Inc., Walnut Creek, California). The proportional counting tube is home-made and it is described in the legend to Fig. 24. The printing register is a Hewlett-Packard Model 560A digital recorder (Hewlett-Packard Company, Palo Alto, California). The printer is set to print out the number of register pulses recorded on the scaler in certain intervals (e.g., each 20 sec.). Thus every 20 sec a record of the counting rate of the tube is printed in a quantitative fashion. This eliminates the necessity of integrating the area under the activity peaks, and, in addition, enables one to detect small peaks with greater reliability than can be achieved through sole dependence on the trace derived from the rate meter output.

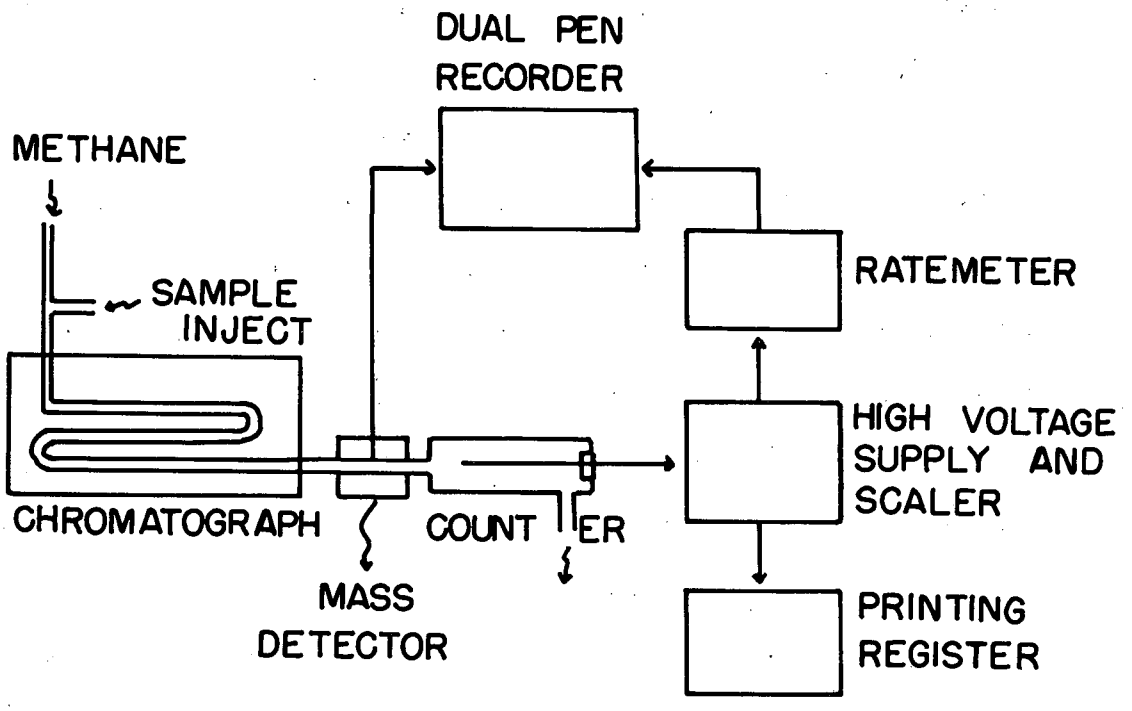


MU-21334-A

Fig. 24. Proportional counting tube used in gas chromatography*

A. Connection to chromatography unit.
B. Inlet for auxiliary counting gas
C. 2 mil stainless steel wire soldered to receptable at one end and to washer at the other
D. Teflon gasket
E. Same connection as outlet of chromatography unit
F. Teflon sleeve
G. Brass counter tube
H. Grooved teflon
I. Nylon
J. Weak spring
K. Pin
L. UG-496/U receptacle (modified)

*From Whittemore[20]



MU-21335

Fig. 25. Apparatus used for detection of radioactive components in gas chromatography.*

*From Whittemore[20]

With the Wilkens Model A90 chromatography unit the analytical conditions were the same as with the VPC without proportional counter, except that methane was used as a carrier and counting gas. With the Wilkens Model A350 linear programmed chromatography unit, the analytical conditions were the following: Injector port temp, 195°C; detector temp, 215°C; column temp, 150°C; collector temp, 175°C; detector voltage, 12 v; filament current in ma, 140; methane flow rate, 60 ml/min; chart speed, 1 cm/min; sample size, 0.001 to 0.008 ml; high voltage of scaler, 2800 v.

The identities of the chromatographically separated compounds were established by cochromatography with authentic marker compounds (ca. 100 ug of marker). Exact coincidence of the radioactivity peak with the mass peak of the marker compound was taken as criterion of identity.

Infrared Spectroscopy

Infrared spectrum analyses of the different compounds, from 2.5 to 15 microns, were carried out using an Infra-Red Beckman Spectrophotometer Model IR7 (Beckman Instruments, Inc., Fullerton, California), equipped with NaCl liquid cells 0.1 cm path. The absorption cells were exactly matched for transmissivity. Carbon tetrachloride was used as solvent and reference. Exact coincidence of the infrared spectrum of an unknown compound with that of an authentic marker was taken as criterion of identity.

Mass Spectrometry

Mass spectrometric analyses of the unknown compounds were carried out using a Consolidated Electrodynamics Corporation 21-130 mass spectrometer. The highest mass value of the spectrum was taken as the molecular weight of the compounds. It was then compared with the value found from osmotic pressure differences.

Melting Point

The melting point determinationof one of the unknown compounds which was solid at room temperature was carried out using a Fisher micro melting point apparatus (Fisher Scientific Co., Eimer and Amend, New York). No significant change of the melting point of an unknown compound in a mixture of an authentic marker was taken as a criterion of identity.

Chemical Analysis for C, H, N and Determination of Molecular Weight

The determination of the molecular weight using osmotic pressure differences, and the analyses for carbon, nitrogen and hydrogen were carried out at the Analytical Chemistry Laboratory of the Chemistry Department of the University of California. The oxygen content was determined by subtracting the sum of C, H and N content from one hundred.

Preparation of Diazomethane

Diazomethane was prepared by alkaline saponification of N-methyl-N-nitroso-N'-nitroguanidine, or N-methyl-N-nitroso-p-toluenesulfonamide (Aldrich Chemical Company, Inc.).

From N-methyl-N-nitroso-N'-nitroguanidine[14,15]: Crystals of N-methyl-N-nitroso-N'-nitroguanidine were added slowly to a 50% solution of sodium hydroxide covered with ether in a 250 ml distilling flask fitted with an efficient condenser, set downward for distillation. The condenser was connected to a receiving flask which contained 70-100 ml ether. The inlet tube of the receiver dipped below the surface of the ether. Both flasks were cooled to 0°C. When the reaction was terminated, the ethereal solution of diazomethane was either used immediately or it was stored for a short time in the deep freeze (-16°C) before use.

From N-methyl-N-nitroso-p-toluenesulfonamide (DIAZALD)[16]: 100 ml of 95% EtOH was added to 40 ml of 60% potassium hydroxide solution in a 250 ml distilling flask fitted with dropping funnel and an efficient condenser set downward for distillation. The condenser was connected to two receiving flasks in series, the second of which contained 70-100 ml ether. The inlet tube of the second receiver dipped below the surface of the ether, and both receivers were cooled to 0°C. The flask containing the alkaline solution was heated in a water bath to 65°C, and a solution of 86 gr. "Diazald" in 400 ml of ether was added through the dropping funnel, slowly. The rate of addition was equal to the rate of distillation.

When the dropping funnel was empty, another 50 ml of ether was added and the distillation was continued until the distilling ether was colorless. The ethereal solutions of diazomethane were combined and used immediately. The solution of diazomethane prepared this way always contained some EtOH.

Reaction of Diazomethane with Carbon Dioxide

In the reaction of diazomethane with carbon dioxide, either a solution of bicarbonate-C^{14} or gaseous carbon dioxide-C^{12} were used.

Reaction of $HC^{14}O_3^-$ with Diazomethane

0.50 ml of $HC^{14}O_3^-$ solution (1.98 mC/ml; 0.06 M) were mixed with an ethereal solution of diazomethane until the yellow color persisted. The reaction mixture was left for a few hours at room temp, and overnight in the deep freeze (-16°C). In some experiments a small amount of MeOH, HCl, or ammonia was added. The solution was concentrated under a current of air at 5°C and the different radioactive components of the mixture were separated by gas chromatography in conjunction with a proportional counter.

Reaction of Carbon Dioxide with Diazomethane

Gaseous carbon dioxide (100%) was bubbled through an inlet tube into the ethereal solution of diazomethane, in the receiving flask of the diazomethane preparation apparatus, at 5°C until the solution became colorless. The flask contained also a small amount of water (2.5 ml). The inlet tube was dipped below the surface of the water layer. In some experiments, small amounts of MeOH, ammonia, methylamine, or dimethylamine were present. The solution was concentrated at 5°C under a current of air and the different components of the reaction mixture were separated by vapor phase chromatography and collected separately.

Preparation of Methyl Carbamate (MCR)

Methyl carbamate (methyl urethan) was prepared from methyl chlorocarbonate and methylamine as described by Hartman and Brether[30] with the exception of using smaller amounts of reagents. Into a 250 ml flask fitted with a mechanical stirrer, a thermometer, two dropping funnels, and cooled by an ice-salt mixture, 30 ml of ether and 18.6 gr of a 33% aqueous methylamine solution was placed. When the stirred mixture had cooled to 5°C, 18.9 gr of methyl chlorocarbonate was added slowly, keeping the temperature to below 5°C. When almost half of the methyl chlorocarbonate had been added, a cold solution of 8 gr of pure sodium hydroxide in 12 ml of water, was added gradually together with the rest of the methyl chlorocarbonate while stirring continuously at such a

rate that the last of the two solutions were added simultaneously. After standing for 15 min, the ether layer was separated and the aqueous solution was extracted with 15 ml of ether. The combined ether solutions were rapidly dried by shaking for a short time with about 1.0 gr of potassium carbonate which was added in two portions. The ether was removed and the residue was distilled under reduced pressure. A small portion of the methyl carbamate was further purified by gas chromatography.

The methyl chlorocarbonate (carbomethoxychloride) was prepared by the procedure described by Bergmann and Zervas for the preparation of carbobenzoxychloride[31,32]. The phosgene, necessary for the preparation of the methyl chlorocarbonate, was prepared by the procedure described by Vogel[33].

Analysis	%C	%H	%N	%O (Calcd)
Calcd for $C_2H_5O_2N$	32.00	6.66	18.66	42.66
Found for MCR	31.79	6.58	17.95	43.50

Preparation of N-Methyl-methyl Carbamate (NMMCR)

NMMCR was prepared from dimethyl carbonate and methylamine, as described by Delepine and Schving[34]. A mixture of 3.1 gr of methylamine in 100 ml benzene and 9 gr of dimethyl carbonate were kept in the cold for four to five hours. The NMMCR was precipitated with 20 ml water and the aqueous solution was extracted with 30 ml of ether. The ether was removed and the residue was distilled

under reduced pressure. The N-methyl-methyl carbamate was further purified by gas chromatography.

Analysis	%C	%H	%N	%O (Calcd)
Calcd for $C_3H_7O_2N$	40.45	7.86	15.73	35.95
Found for NMMCR	40.57	7.94	15.60	35.89

Preparation of N-Dimethyl-methyl Carbamate (NDMMCR)

The NDMMCR was prepared from dimethyl carbonate and dimethylamine as described by Delepine and Schving[34]. Dimethylamine (4.5 gr) in 100 ml of benzene and 9 gr of dimethyl carbonate were refluxed for twelve hours. The products were precipitated with 15 ml of water and the aqueous solution extracted with 30 ml of ether. The ether was then removed and the residue was distilled. The NDMMCR was further purified by gas chromatography.

Analysis	%C	%H	%N	%O (Calcd)
Calcd for $C_4H_9O_2N$	46.60	8.74	13.59	31.07
Found for NDMMCR	46.86	8.52	13.17	31.46

Preparation of Hydroxamates[23]

Two ml of a freshly mixed alkaline solution of hydroxylamine were added to: (1) an alcoholic solution of the radioactive products of the reaction of diazomethane with C^{14}-bicarbonate; or (2) 1 mg of the methyl ester of an acid in MeOH. Sodium hydroxide (2.25 N in

absolute MeOH) and hydroxylamine hydrochloride (1.67 N in absolute MeOH) = 1:1 v/v was cooled to 0°C and the NaCl formed was removed by centrifugation. The salt was washed once with methanol, and the washing was added to the supernatant to restore the original volume of the hydroxylamine solution. The mixture of the methyl esters and the alkaline solution of hydroxylamine was maintained at room temperature for one hour. The solution of the hydroxamates was concentrated over steam and separated by paper chromatography.

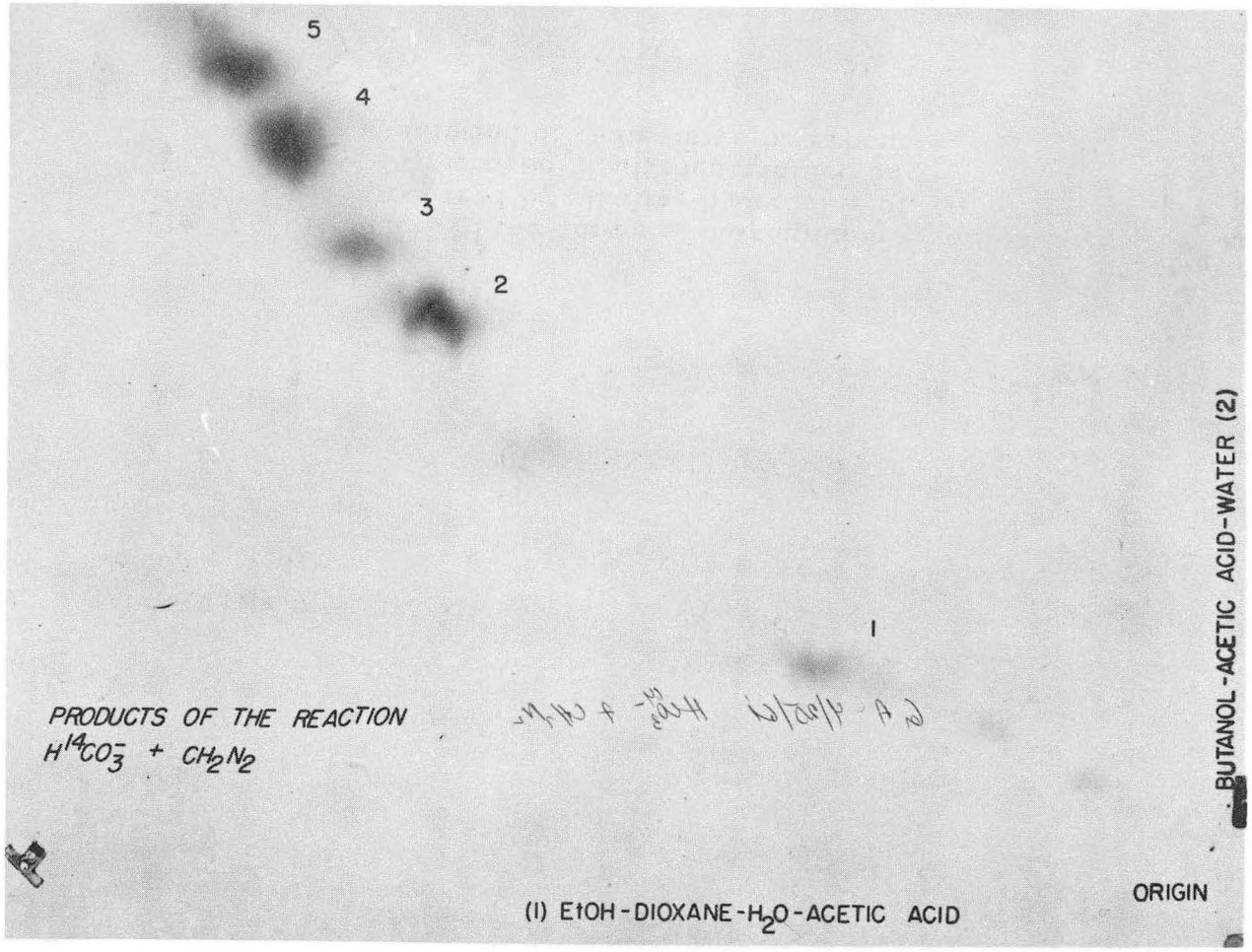
IV. RESULTS

Separation of the Products of the CO_2, CH_2N_2 Reaction

Paper chromatography was initially used for the separation of the products of the reaction between C^{14}-bicarbonate and diazomethane. The fact that after radioautography of the developed chromatogram, several radioactive spots were found instead of only one or none (as the dimethyl carbonate is a volatile compound) was the reason for this study.

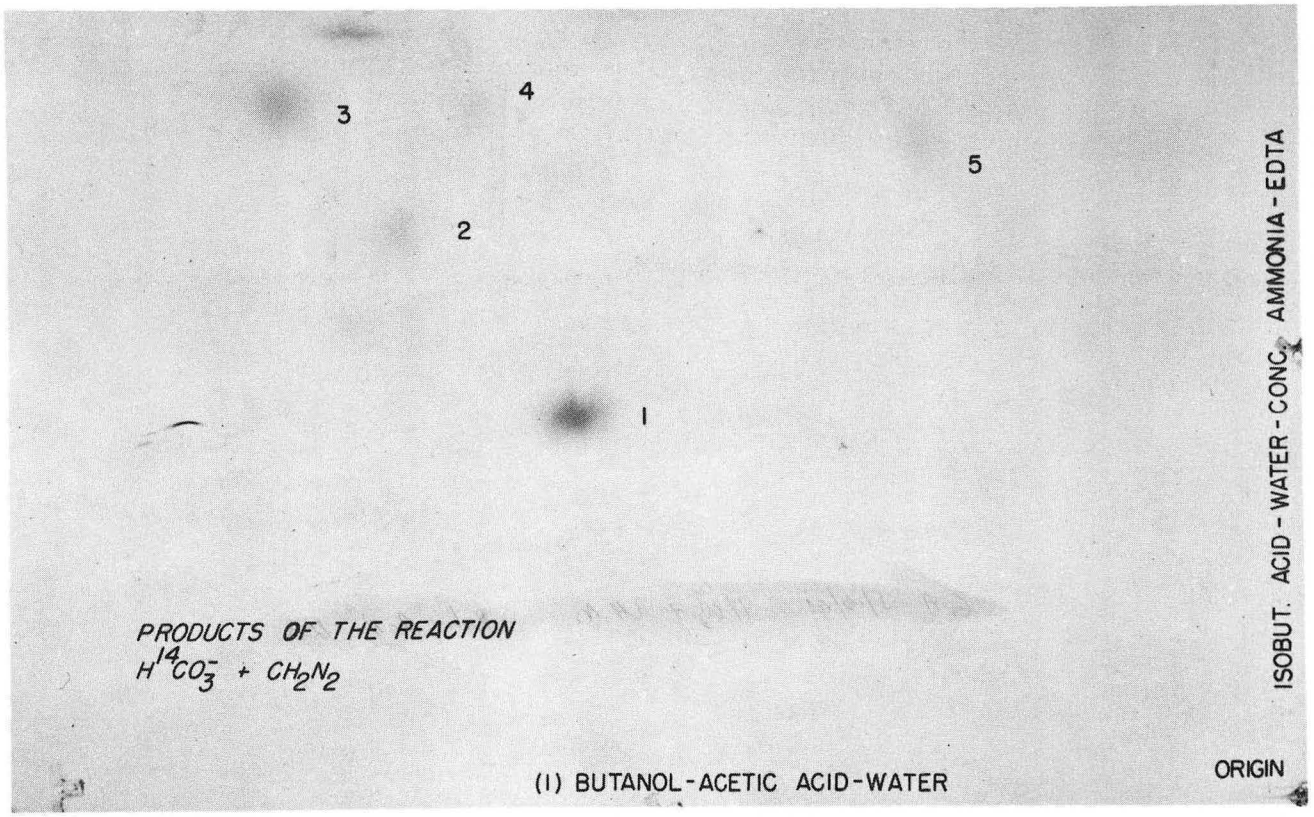
Figs. 26 and 27 are radioautographs of paper chromatograms of the products of the above reaction which have been developed with two different sets of solvents.

By counting the radioactivity on the chromatogram before and after its development, it became evident that some or all of the products were relatively volatile. The total activity that remained on the paper after the development of the chromatogram was only a small fraction of the initial radioactivity that was applied on the origin. This became more evident when the products were treated with an alkaline solution of hydroxylamine, such that the nonvolatile hydroxamates of the different acids would be formed and then chromatographed. Fig. 28 is a radioautograph of the chromatogram of the products after treatment with hydroxylamine. Approximately 75% of the initial radioactivity remained on the chromatogram after its development.



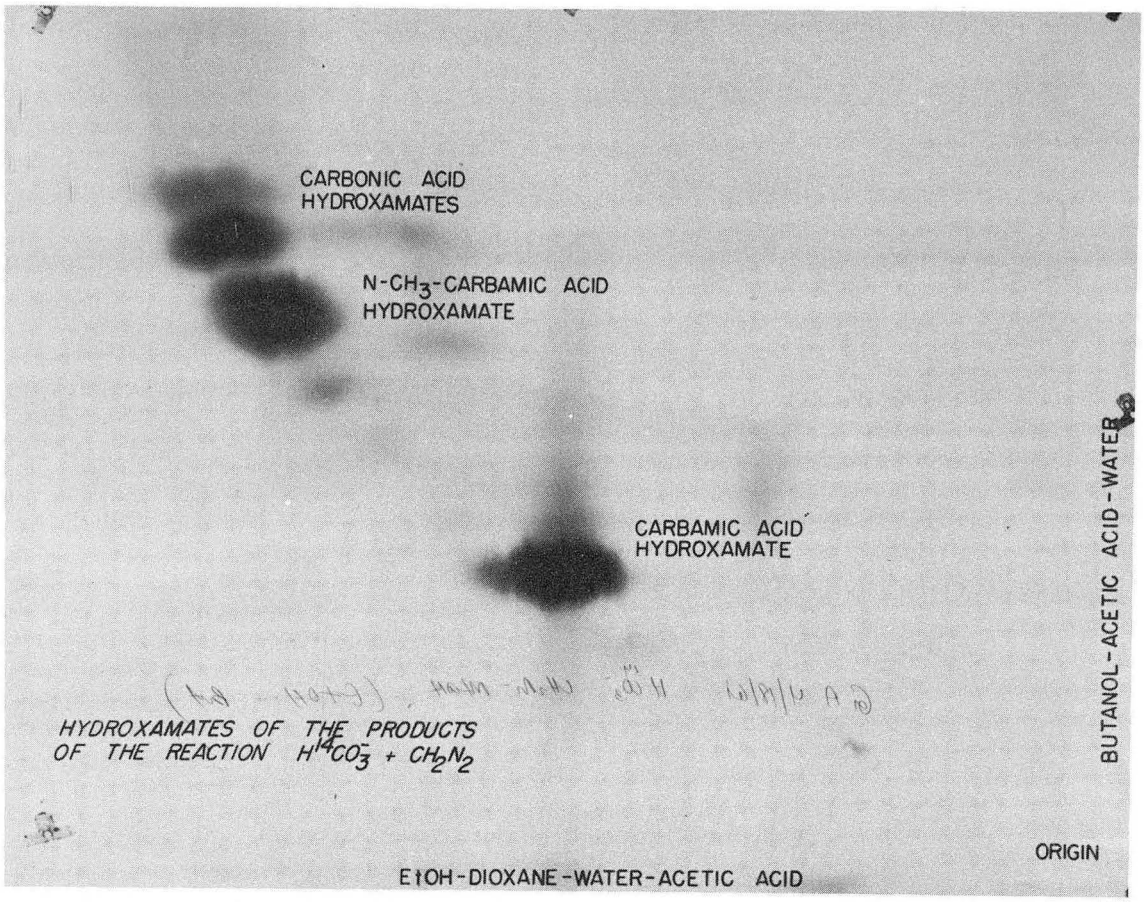
ZN-3174

Fig. 26. Products of the action of diazomethane which it was prepared by alkaline hydrolysis of N-methyl-N-nitrose-N'-nitroguanidine, on aqueous solution of radioactive bicarbonate.



ZN-3175

Fig. 27. Products of the action of diazomethane, which it was prepared by alkaline hydrolysis of N-methyl-N-nitroseo-N'nitroguanidine, on aqueous solution of radioactive bicarbonate.



ZN-3180

Fig. 28. Products of the action of diazomethane, which it was prepared by alkaline hydrolysis of N-methyl-N-nitroso-N' nitroguanidine, on aqueous solution of radioactive bicarbonate, after they have been treated with an alkaline solution of hydroxylamine.

The use of vapor phase chromatography in conjunction with a proportional counter was found to be a more suitable method for the separation of the different components of the radioactive mixture. It was found that the mixture consisted of five radioactive compounds, all of them using ^{14}C-bicarbonate as precursor, as shown in Fig. 29 where five radioactive peaks are present. Compounds I, II, III, IV and V were named depending on their emergence time from the VPC. The same products were always formed (compounds I, II, III, IV and V) by the action of diazomethane, prepared from MNNG, on aqueous solution of ^{14}C-bicarbonate.

Gas and paper chromatography of the untreated aqueous solution of ^{14}C-bicarbonate showed that there were no radioactive impurities present, and that the bicarbonate was the precursor of all five compounds which were formed by the action of diazomethane on $NaH^{14}CO_3$.

Cochromatography (VPC) of the radioactive mixture with different authentic marker compounds, such as dimethyl carbonate, carbon dioxide, methyl acetate, methyl glyoxylate, methyl glycolate, methyl pyruvate, etc., assisted the preliminary identification of two out of the total of five compounds. Compound I was identified as carbon dioxide and compound II as dimethyl carbonate. DMC is the product that one should expect to be formed from the reaction of bicarbonate with diazomethane, and CO_2 is one of the reagents. However, it was impossible to identify any of the other unknown compounds (III,IV,V).

Treatment of each compound, after separation and collection, with an ethereal solution of diazomethane showed that compounds II, III, IV and V remained unchanged, but compound I yielded a mixture of all five. That experiment showed that none of the compounds

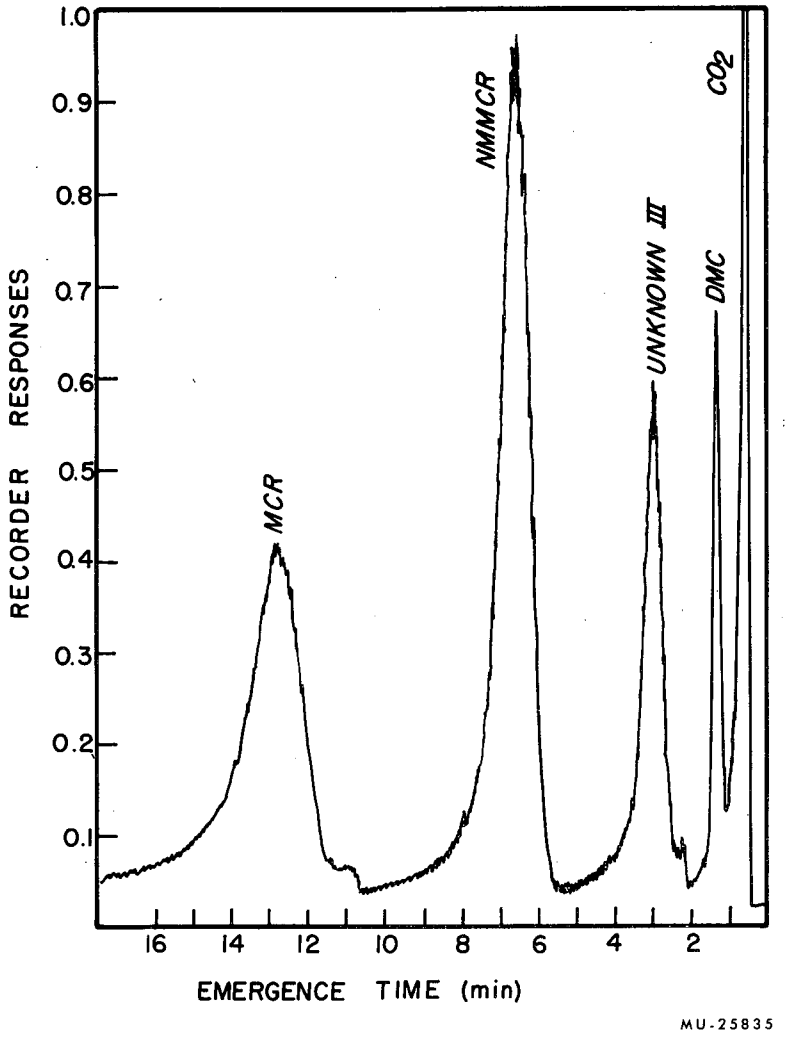


Fig. 29. Gas chromatogram of the radioactive mixture of the products of the reaction of $NaHC^{14}O_3$ with diazomethane prepared from MNNG. Unknown compound III was identified as N-dimethylmethyl carbamate.

II, III, IV or V were precursors of any of the others, and that compound I yielded a mixture of all of them with diazomethane, because it was carbon dioxide. They were hydrolyzed relatively easily with sodium hydroxide or hydrochloric acid, and they yielded CO_2 as the only radioactive product.

For the identification of the other three unknown compounds, relatively higher amounts of them (nonradioactive) were prepared from gasoue carbon dioxide (100%) and diazomethane, as described in the experimental section. The components of the reaction mixture were separated by gas chromatography, and collected separately in the collector shown in Fig. 23. Fig. 30 is a gas chromatogram of the products of the CO_2-CH_2N_2 reaction (CH_2N_2 was prepared from MNNG), and the peaks shown are the mass peaks of each component of the mixture. Fig. 31 is a gas cochromatogram of the radioactive mixture, prepared from $NaHC^{14}O_3$ and CH_2N_2 and the nonradioactive products prepared from $C^{12}O_2$ and CH_2N_2. Coincidence of the radioactive and mass peaks shows that in either case the products of the reaction are the same. Compounds II, III and IV are liquid at room temperature and compound V is solid.

Using chemical and physical methods, such as molecular weight determination, carbon-nitrogen-hydrogen-oxygen analysis, infrared spectrophotometry and mass spectrometry, melting point determination and cochromatography with authentic marker compounds, it was possible to identify all of them. Compound I is carbon

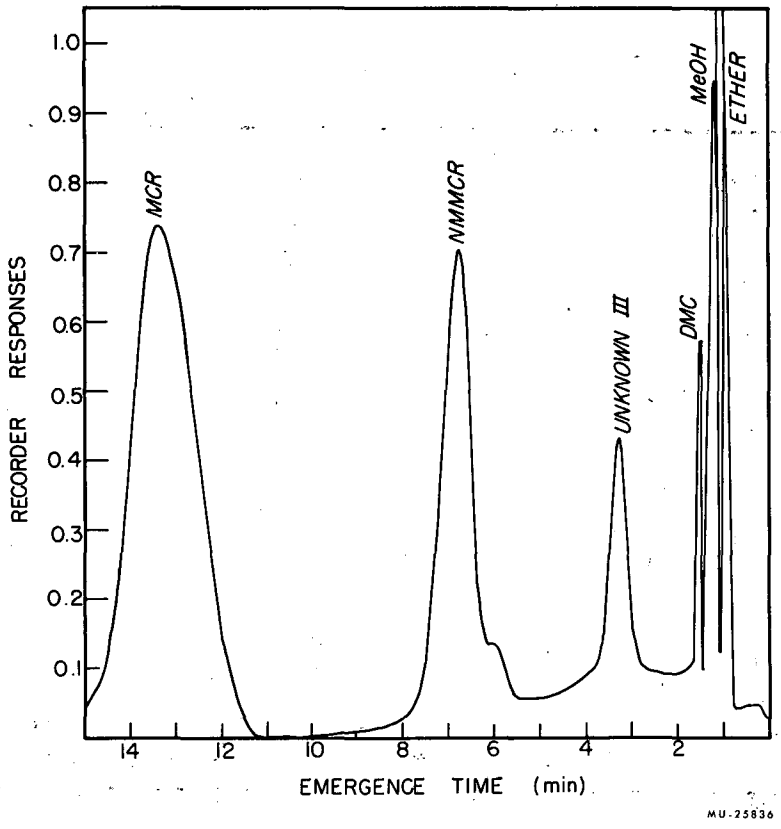


Fig. 30. Gas chromatogram of the nonradioactive mixture of the products of the reaction of carbon dioxide with diazomethane prepared from MNNG. Unknown Compound III was identified as N-dimethylmethyl carbamate.

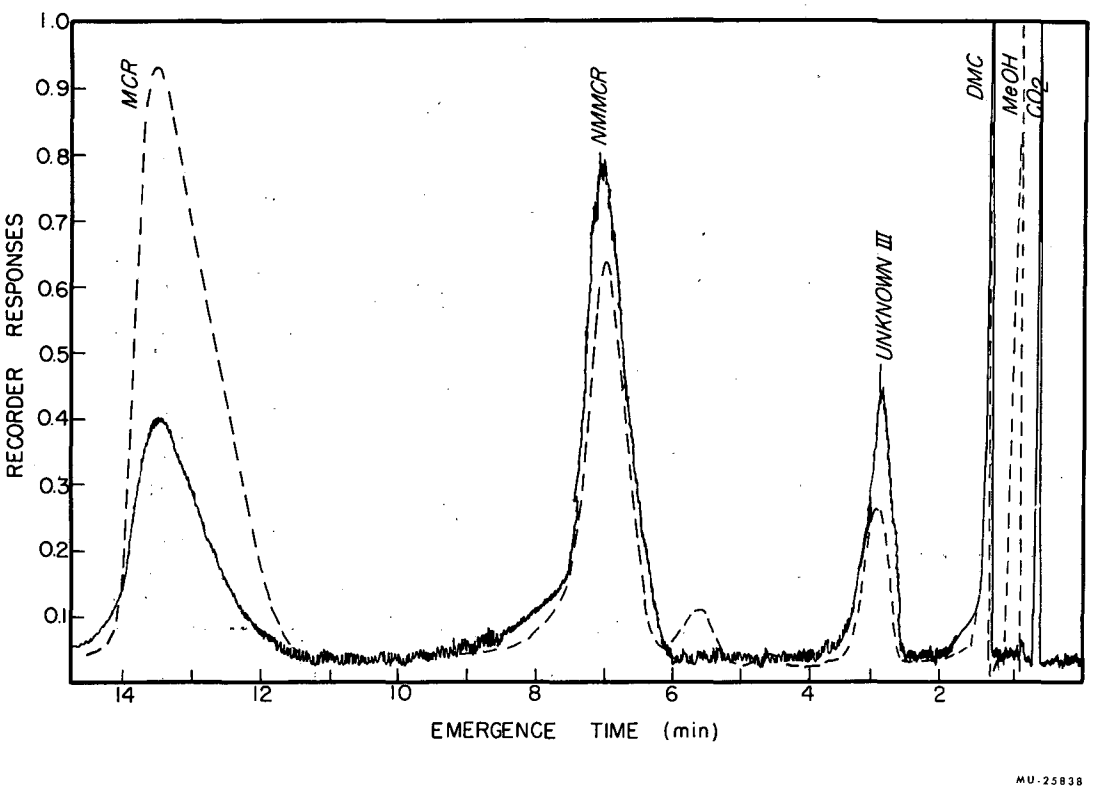


Fig. 31. Gas cochromatogram of the radioactive mixture of the products of the reaction $NaHC^{14}O_3 + CH_2N_2$, and the nonradioactive products of the reaction $CO_2 + CH_2N_2$. Diazomethane was prepared from MNNG. Solid line is the radioactivity recording; dotted line is the mass recording.

dioxide; compound II is dimethyl carbonate (DMC); compound III is N-dimethyl-methyl carbamate (NDMMCR); compound IV is N-methyl-methyl carbamate (NMMCR); and compound V is methyl carbamate (MCR).

Identification of Compounds II,III,IV and V

The identification of the unknown compounds II,III,IV and V was based on: (1) the analysis for C,H,N,O and the determination of molecular weight; (2) the study of the mass and infrared spectra, and their comparison with the mass and infrared spectra of authentic marker compounds; and (3) the cochromatography of the unknown compounds, or their hydroxamate derivatives, with authentic marker compounds.

Gas cochromatography of the separately collected radioactive compounds II,III,IV and V with authentic dimethyl carbonate, NDMMCR, NMMCR and MCR, respectively, showed in each case an exact coincidence of the radioactivity with the mass peaks. There was also an exact coincidence of the radioactivity with the marker compound, in the case of paper cochromatography of the (1) hydroxamate of C^{14}-II with the hydroxamate of carbonic acid; (2) hydroxamate of C^{14}-III with the hydroxamate of N-dimethyl carbamic acid; (3) hydroxamate of C^{14}-IV with the hydroxamate of N-methyl carbamic acid; and (4) hydroxamate of C^{14}-V with the hydroxamate of carbamic acid.

Analysis of compounds II, III, IV and V for C, H, N, O and molecular weight determination showed the following results.

Analysis for	%C	%H	%N	%O (Calcd)	Mol.Wt.
Found for comp. II	39.81	6.72	---	53.47	88.0
Calcd for $C_3H_6O_3$ (DMC)	40.00	6.66	---	53.34	90.0
Found for comp. III	46.48	8.86	13.25	31.41	109.0
Calcd for $C_4H_9NO_2$(NDMMCR)	46.60	8.74	13.59	31.07	103.0
Found for comp. IV	40.63	7.82	15.49	36.06	84.0
Calcd for $C_3H_7NO_2$(NMMCR)	40.45	7.86	15.73	35.95	89.0
Found for comp. V	31.61	6.71	17.75	43.93	74.0
Calcd for $C_2H_5NO_2$(MCR)	32.00	6.66	18.66	42.66	75.0

The mass spectra of compounds II, III, IV and V had a highest mass value of 90, 103, 89 and 75, respectively. (Calcd mol. wt. of DMC, 90; of NDMMCR, 103; of NMMCR, 89; and of MCR, 75) The comparison of the mass spectra of compounds II, III, IV and V with the mass spectra of DMC, NDMMCR, NMMCR and MCR, respectively, showed an exact coincidence in the place and number of peaks, and in the relative abundance of each peak.

Figs. 32, 33, 34 and 35 show the infrared spectra of compounds II, III, IV and V and DMC, NDMMCR, NMMCR and MCR, respectively. The study of these spectra clearly confirms that compound II is dimethyl carbonate, compound III is N-dimethyl-methyl carbamate, compound IV is N-methyl-methyl carbamate, and compound V is methyl carbamate.

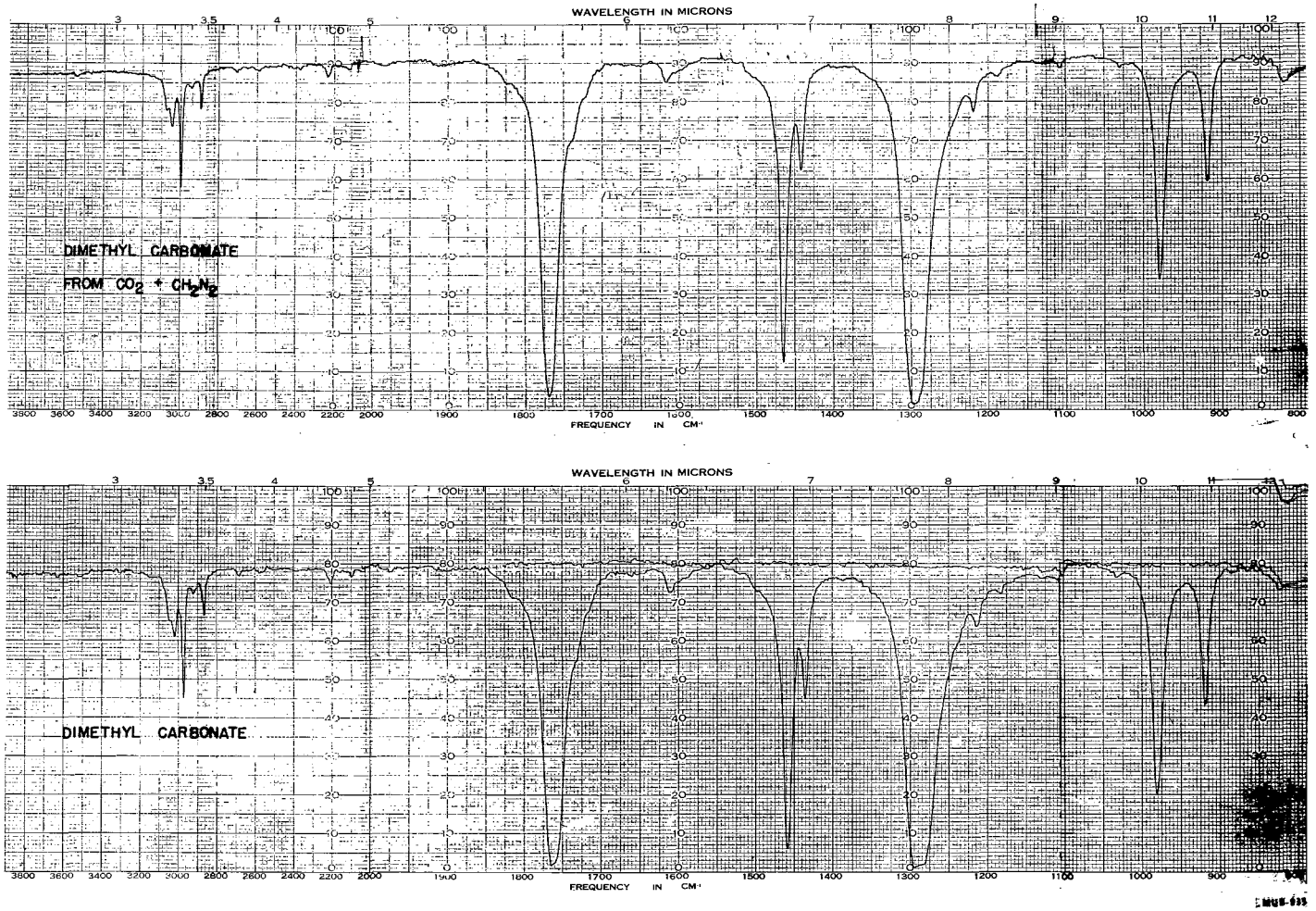


Fig. 32. Infrared spectra of dimethyl carbonate.

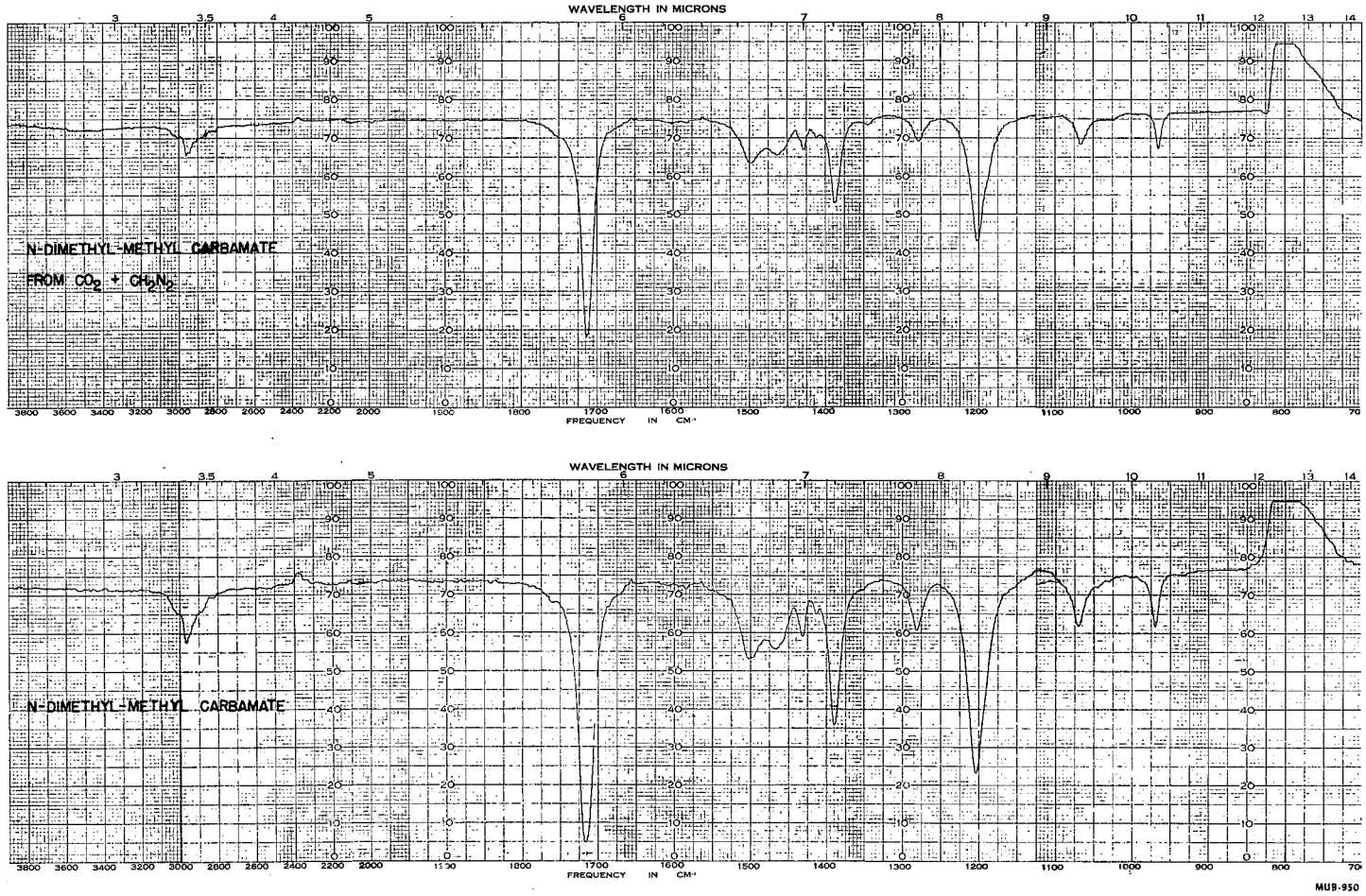


Fig. 33. Infrared spectra of N-dimethyl-methyl carbamate.

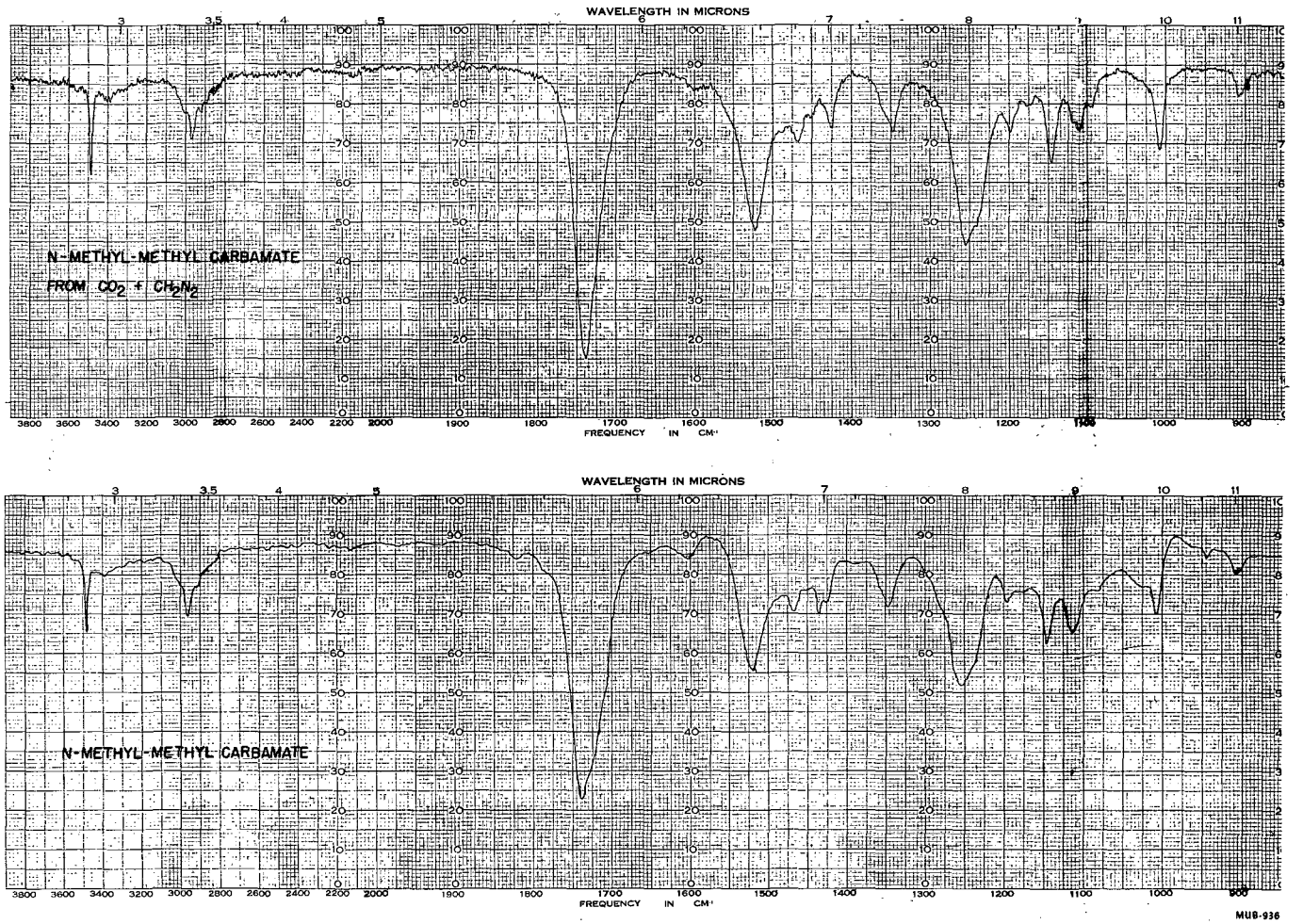


Fig. 34. Infrared spectra of N-methyl-methyl carbamate.

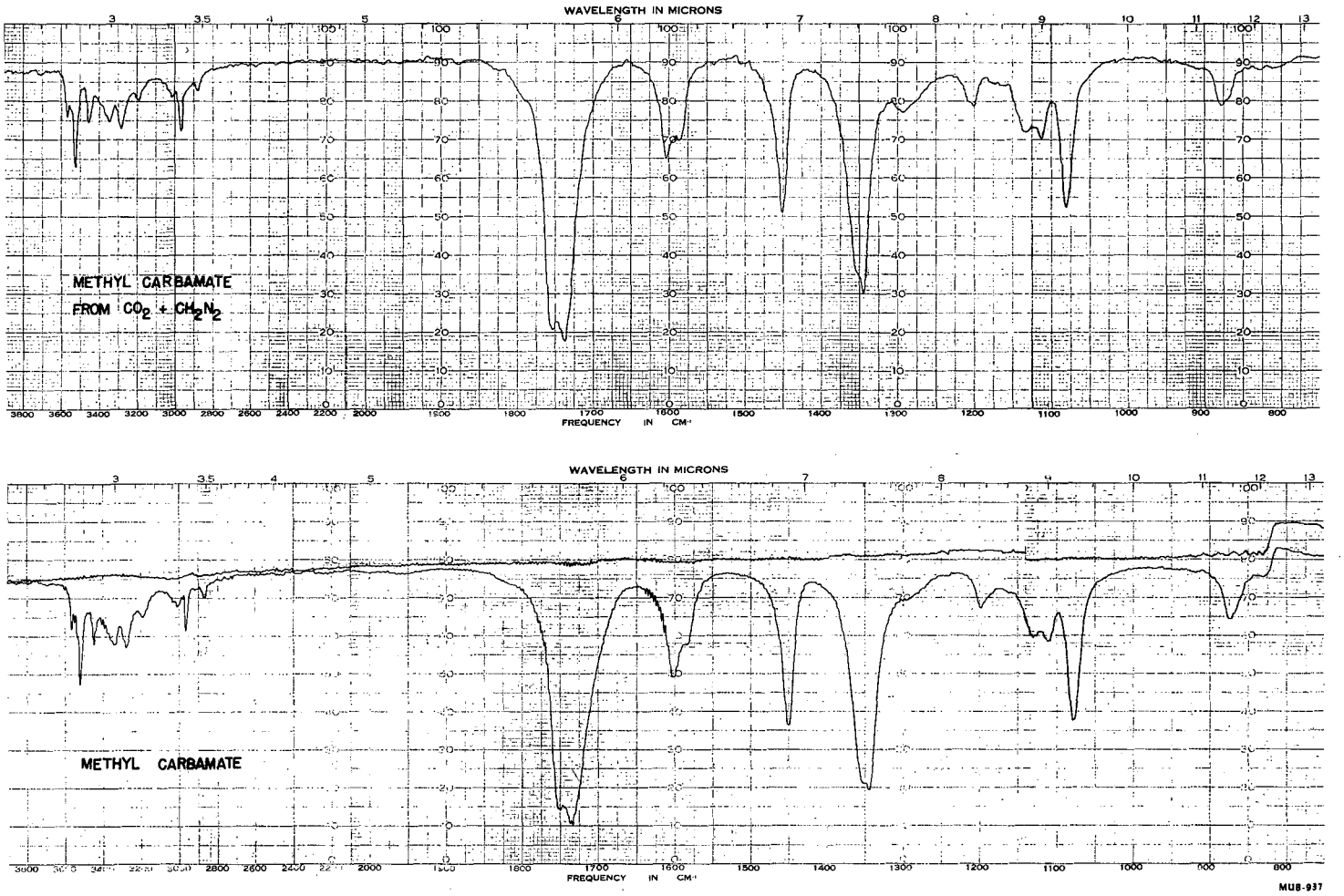


Fig. 35. Infrared spectra of methyl carbamate.

As was mentioned earlier, compound V is solid at room temperature. The melting point was found to be 51.5°C. The melting point of methyl carbamate is 53°C. A mixed melting point determination of compound V and methyl carbamate showed no change in its value.

All the above results show conclusively that the products of the reaction of carbon dioxide and diazomethane (prepared from N-methyl-N-nitroso-N'-nitroguanidine), in the presence of small amounts of water, are dimethyl carbonate, methyl carbamate, N-methyl-methyl carbamate, and N-dimethyl-methyl carbamate. The yield of each product is approximately 40% DMC, 35% MCR, 20% NMMCR and 5% NDMMCR.

V. DISCUSSION

Formation of Products

As far as it is known, in all reactions of diazomethane, either the products contain both nitrogens of the diazomethane molecule or none, in which case nitrogen gas is evolved. In the case of the reaction under study, three of the products (MCR, NMMCR and NDMMCR) contain one nitrogen atom in their molecules and one (DMC), none. If the nitrogen atom originated from the diazomethane molecule, then a splitting of the two nitrogens had to occur and a new kind of diazomethane reaction had to take place. On the contrary, if the nitrogen atom did not originate from diazomethane, then a by-product of the diazomethane formation reaction had to be present in the ethereal solution of diazomethane, which could react with carbon dioxide and diazomethane to form the nitrogenous products.

In order to elucidate the above problem, diazomethane was prepared by alkaline hydrolysis of an alcoholic solution of N-methyl-N-nitroso-p-toluenesulfonamide, as described in the experimental section, and its ethereal solution reacted with gaseous carbon dioxide. After evaporation of the solvent, the products were separated by gas chromatography. Figure 36 is a gas chromatogram of the products of the above reaction. As it can be seen, the only common product of the two reactions is dimethyl carbonate. Compounds, labeled unknown I and II, are different

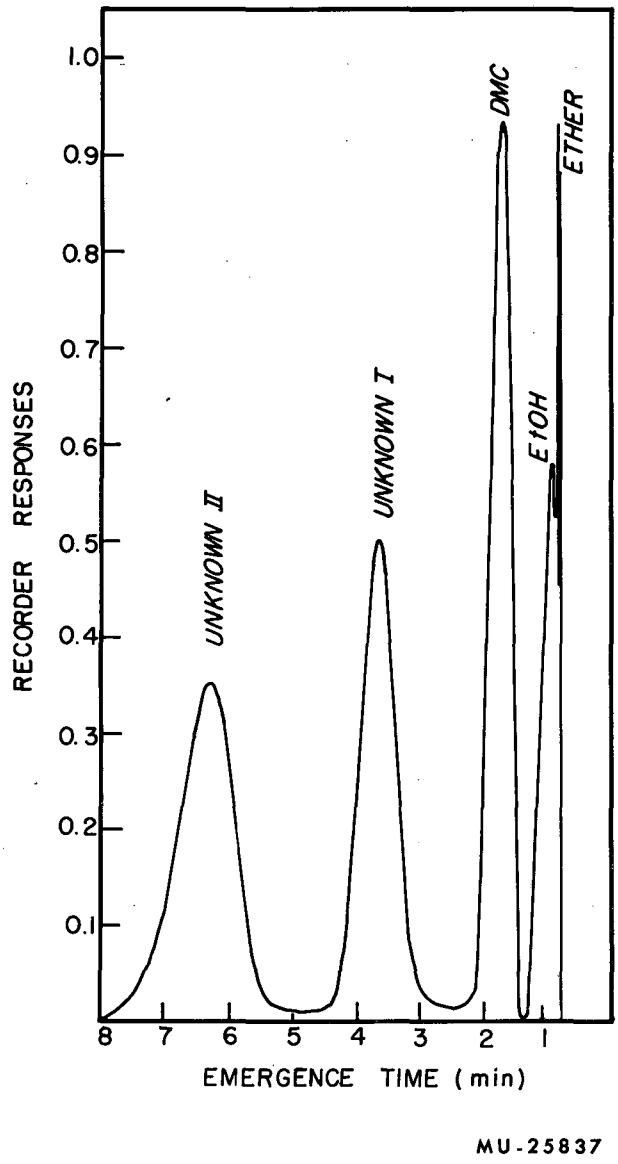


Fig. 36. Gas chromatogram of the nonradioactive mixture of the products of the reaction of carbon dioxide with diazomethane. Diazomethane was prepared by alkaline hydrolysis of N-methyl-N-nitroso-p-toluene-sulfonamide.

from NDMMCR, NMMCR or MCR, as their emergence time in the VPC is different from the emergence time of any of the other compounds. This was confirmed by comparing the IR spectra of the unknown compounds I and II (Fig. 37) with the IR spectra of the other compounds (Figs. 33, 34, 35). Using $NaHC^{14}O_3$ instead of $C^{12}O_2$ in the above reaction, it was established that carbon dioxide was not the precursor of the unknown compounds I and II. The gas chromatogram of the radioactive mixture showed carbon dioxide and dimethyl carbonate as the only radioactive components present. Unknown compounds I and II were not radioactive and they were only by-products of the diazomethane formation reaction.

It can be concluded from the above experiments that the three nitrogenous compounds (MCR, NMMCR and NDMMCR) are not formed by the action of diazomethane on carbon dioxide alone, but also another compound is necessary. This compound is formed by the alkaline hydrolysis of N-methyl-N-nitroso-N'-nitroguanidine, as a by-product of the diazomethane formation reaction. The only product that is always formed by the action of pure diazomethane on pure carbon dioxide is dimethyl carbonate.

It is known that salts of carbamic acid or N-derivatives of carbamic acid are formed by the action of NH_3 or amines on carbon dioxide[35,36], according to the reactions:

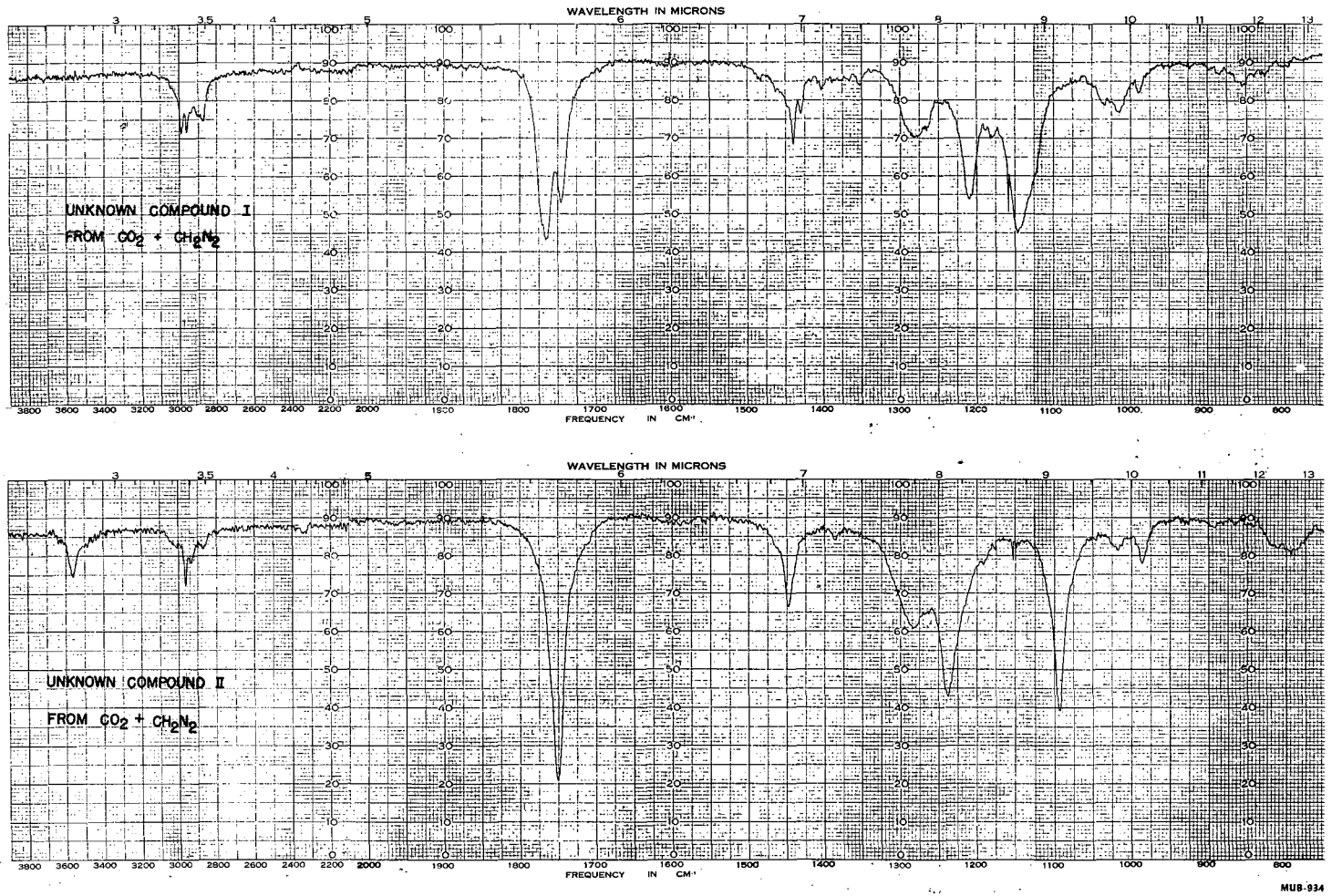


Fig. 37. Infared spectra of unknown compounds I and II.

Fig. 37. Infrared spectra of unknown compounds I and II

$$2\ NH_3 + CO_2 \rightleftharpoons H_2NCOONH_4$$

$$2\ RNH_2 + CO_2 \rightleftharpoons RNHCOONH_3R$$

$$2\ R_2NH + CO_2 \rightleftharpoons R_2NCOONH_2R_2$$

where R is any organic group. It is also known[37,38] that diazomethane reacts with ammonium, amine or other salts of organic or inorganic acids to form the corresponding methyl esters. Ammonia or the corresponding amine or base is evolved. Thus, ammonium carbamate and amine salts of N-derivatives of carbamic acid react with diazomethane to give methyl carbamate and N-derivatives of methyl carbamate, respectively.

Addition of a small amount of dimethylamine in the ethereal solution of diazomethane, before the addition of carbon dioxide, resulted in an increase in the amount of NDMMCR formed, compared with the orther products. An increase in the amount of NMMCR was noticed when methylamine was added in the ethereal solution. In the case of ammonia, a large increase in the amount of MCR formed was obvious, but there was also a large increase in the amount of NMMCR, and a smaller increase in the amount of NDMMCR, compared with the amount of DMC formed.

It was mentioned earlier that the only radioactive product formed by the action of diazomethane (prepared from N-methyl-N-nitroso-p-toluenesulfonamide) on $NaHC^{14}O_3$ was dimethyl carbonate. When a small amount of concentrated ammonia solution was added

in the ethereal solution of diazomethane, before the addition of bicarbonate, then MCR, NMMCR and NDMMCR were formed in addition to DMC. The gas chromatogram showed five radioactive peaks. In other words, the products of a reaction mixture, containing ammonia, carbon dioxide and diazomethane, all of them containing carbon atom originated from CO_2, are: (1) Dimethyl carbonate, (2) methyl carbamate, (3) N-methyl-methyl carbamate, and (4) N-dimethyl-methyl carbamate. The above experiments suggested the possibility of ammonia being the by-product present in the ethereal solution of diazomethane, and formed by the alkaline hydrolysis of MNNG. Search for ammonia in the ethereal solution of diazomethane, prepared from MNNG, gave positive results. Ammonia can be explained as being formed by the alkaline hydrolysis of the imide group of N-methyl-N-nitroso-N'-nitroguanidine. The hydrolysis can take place before or after the formation of diazomethane. In the first case, N-methyl-N-nitroso-N'-nitrourea is formed according to the reaction

$$CH_3N(NO)C(NH)NHNO_2 + H_2O \longrightarrow CH_3N(NO)CONHNO_2 + NH_3$$

which is further hydrolyzed to yield diazomethane. In the second case, diazomethane is formed first, and the residue is further hydrolyzed to give ammonia.

Mechanism of the Reaction

The reaction of carbon dioxide with diazomethane in the presence of ammonia yielded MCR, NMMCR and NDMMCR in addition to DMC. DMC was formed by the action of diazomethane on carbonic acid (reaction of diazomethane with acidic hydrogen) according to Arndt or Eister mechanisms as described in the introductory section. Methyl carbamate was formed by the action of diazomethane on carbamic acid or ammonium carbamate, which are present in a mixture of carbon dioxide and ammonia. Three hypotheses were considered for the explanation of NMMCR and NDMMCR formation from ammonia, carbon dioxide and diazomethane:

(a) Methyl carbamate was formed first, which then reacted with diazomethane to give its N-methyl derivatives.

(b) Diazomethane reacted with ammonia to give methylamine and dimethylamine, which formed N-methyl carbamic acid and N-dimethyl carbamic acid inthe presence of carbon dioxide. The acids were further methylated by the action of diazomethane, to yield NMMCR and NDMMCR.

(c) The carbamic acid, present in a mixture of ammonia and carbon dioxide, reacted with diazomethane to form its N-methyl derivatives (N-methyl carbamic acid and N-dimethyl carbamic acid), and the products were further methylated to yield the corresponding methyl esters.

The first (a) hypothesis was not correct, because: (1) extensive action of diazomethane on the reaction mixture did not show

an increase in the amount of NMMCR and NDMMCR, as would be the case if they were formed by methylation of methyl carbamate; (2) action of diazomethane on C^{14}-MCR, or unlabeled MCR, showed no formation of any N-methyl derivative of methyl carbamate.

The second (b) hypothesis also was not correct because: (1) The same relative amount of each product was formed when either a new or an old preparation of diazomethane was used; (2) all attempts to prepare methyl derivatives of ammonia by the action of diazomethane on gaseous ammonia, or ammoniacal solution, or solutions of ammonium salts, were unsuccessful.

The third hypothesis was the only one which remained to be used for the explanation of the NMMCR and NDMMCR formation from ammonia, carbon dioxide and diazomethane.

Biltz and Paetzole[38] have observed that betaine was formed by the action of diazomethane on an aqueous solution of glycine. In the absence of water no reaction took place. Kohn and Reulius[18] studied more systematically the reaction of diazomethane with amino acids in the presence of water or alcohol. They found that an extensive action of diazomethane on the aqueous solution of amino acid produced as final product the corresponding betaine. Some intermediates were present in the case when the reaction was allowed to proceed for a short time only. They followed only the rate of formation of the methyl ester of the amino acid, and they observed that there was an increase in the concentration of the methyl ester up to a maximum and then, as the reaction con-

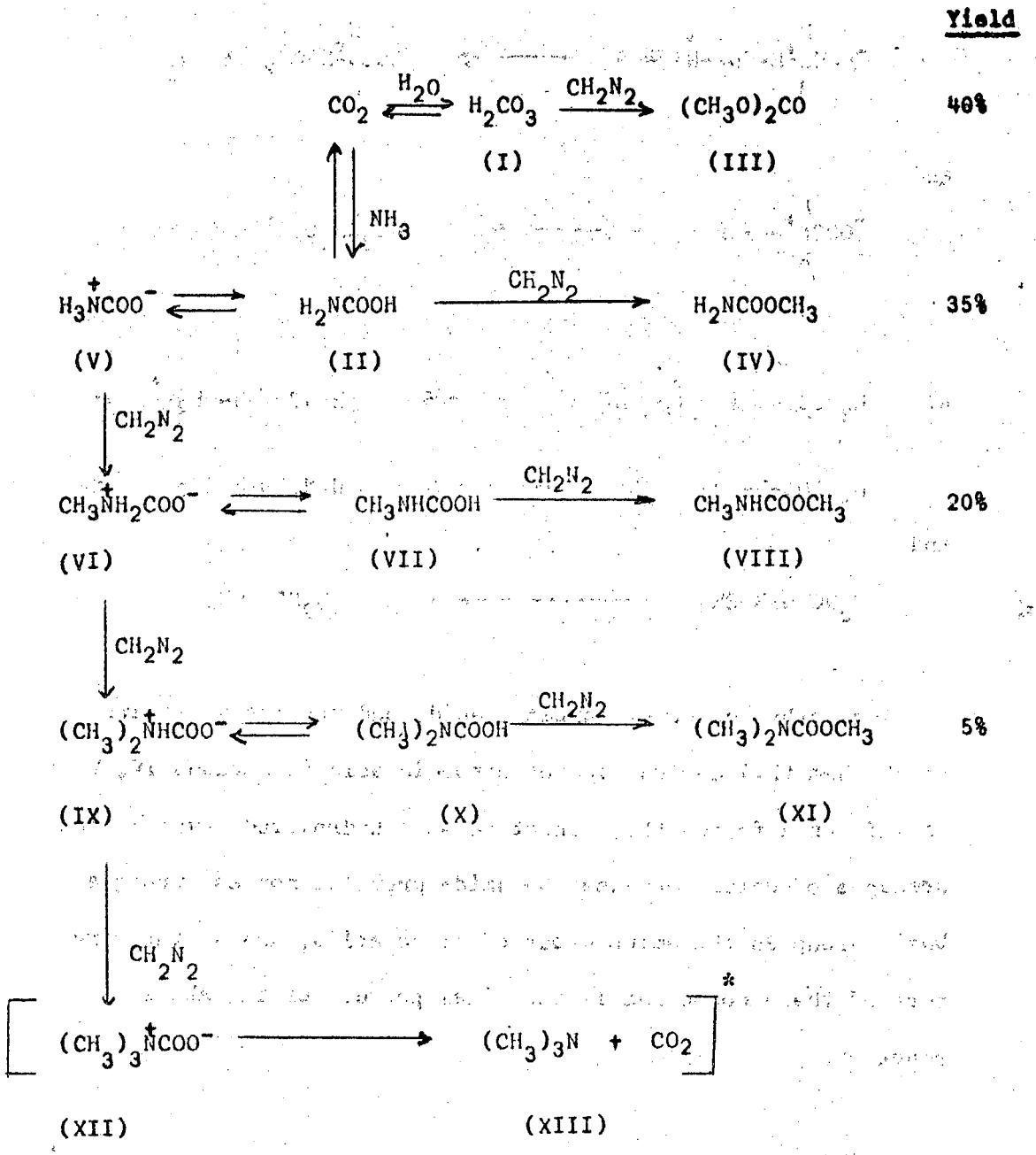
tinued, it decreased slowly to zero. The time for reaching the maximum was 30 min for glycine and about 45 min for alanine. After approximately 90 min for glycine and 120 min for alanine from the beginning of the reaction, no ester was present.

When the methyl ester of an amino acid and diazomethane were used, no reaction occurred in the absence of water, but the addition of a small amount of water caused the slow conversion of all the methyl ester to the corresponding betaine. The reaction did not start immediately after the addition of water, but a short time later.

For the explanation of the above reactions, they proposed that diazomethane reacted with the zwitterion ion of the amino acid to form first the N-methyl derivatives and finally the betaine. The action of diazomethane on the carboxylic group caused the formation of the ester, and at the beginning there was a competitionbetween these two reactions. In the presence of water, because of the basicity of the amino group, a self-hydrolysis of the methyl ester occurred, forming again the free acid, and causing the quantitative transformation of the amino acid to betaine. When the methyl ester was used in the presence of water, a self-hydrolysis first occurred to form the free amino acid, which consequently reacted with the diazomethane to yield the betaine.

Table VI shows the probable reactions that take place between diazomethane and glycine in the presence of water, which lead to the formation of betaine as the final product.

Table VII Mechanism of the Reaction of Diazomethane with an Aqueous Solution of a Mixture of Carbon Dioxide and Ammonia



* It is not known if trimethylamine is really formed because no attempts were made for detecting it.

A. $^{-}OOC\overset{+}{N}H_3 + :\overset{-}{C}H_2-\overset{+}{N}\equiv N: \longrightarrow {}^{-}OOC\overset{H}{\underset{H}{N}}\overset{+}{-}H-CH_2-\overset{+}{N}\equiv N:$

$$^{-}OOC\overset{H}{\underset{H}{N}}\overset{+}{-}H-CH_2-\overset{+}{N}\equiv N \longrightarrow {}^{-}OOC\overset{H}{\underset{H}{N}}\overset{+}{-}H-CH_2 + N_2$$

and

$$^{-}OOC\overset{H}{\underset{H}{N}}{}^{+}-H-CH_2 \longrightarrow CH_3\overset{+}{N}H_2COO^{-}$$

B. $H_2NCOOH + :\overset{-}{C}H_2-\overset{+}{N}\equiv N \longrightarrow H_2NCOO-H-CH_2-\overset{+}{N}\ N:$

$$H_2NCOO-H-CH_2-\overset{+}{N}\equiv N: \longrightarrow H_2NCOO-H-CH_2 + N_2$$

and

$$H_2NCOO-H-CH_2 \longrightarrow H_2NCOOCH_3$$

The methyl ester of carbamic acid, and the methyl esters of the N-methyl derivatives of carbamic acid (compounds IV, VIII and XI) once formed they cannot be self-hydrolyzed, even in the presence of water, because the amide group is not as strong a basic group as the amino group of amino acids, and so the mixture of these compounds is the final product of the above reaction.

VI. SUMMARY

Carbon dioxide was allowed to react with diazomethane, prepared by alkaline hydrolysis of N-methyl-N-nitroso-N'-nitroguanidine. The different products were separated by gas chromatography, collected separately, and identified as dimethyl carbonate, methyl carbamate, N-methyl-methyl carbamate, and N-dimethyl-methyl carbamate. The presence of ammonia was necessary for the formation of MCR, NMMCR and NDMMCR when carbon dioxide reacted with diazomethane which was prepared by alkaline hydrolysis of N-methyl-N-nitroso-p-toluenesulfonamide. Ammonia was found in the ethereal solution of diazomethane, formed as a by-product of the alkaline hydrolysis of N-methyl-N-nitroso-N'-nitroguanidine. Dimethyl carbonate, methyl carbamate, N-methyl-methyl carbamate and N-dimethyl-methyl carbamate were found to be the products of the reaction between ammonia, carbon dioxide and diazomethane, in aqueous solution. A mechanism for the formation of N-methyl-methyl carbamate, and N-dimethyl-methyl carbamate from ammonia, carbon dioxide and diazomethane in the presence of water is proposed.

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