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A Thesis

I. THERMAL DECARBONYLATION OF PYRUVIC ACID AND ETHYL PYRUVATE

II. PYRUVATE METABOLISM IN GREEN ALGAE

by

Richard Millington Lemmon

Berkeley, California

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PART I.

THERMAL DECARBONYLATION OF PYRUVIC ACID AND ETHYL PYRUVATE

I. Thermal Decarbonylation of Pyruvic Acid and Ethyl Pyruvate

II. Pyruvate Metabolism in Green Algae*

by

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ABSTRACT

Pyruvic acid and ethyl pyruvate were synthesized with the α-carbon atom labeled with Carbon-14. It was found that the carbon oxides which are evolved when these compounds are heated at moderate temperatures contained no radioactivity. Therefore, it was conclusively established that the oxides of carbon come from the carboxyl, and not the carbonyl, group.

Pyruvic acid labeled in the methyl position with Carbon-14 was administered to green algae. One experiment was carried out while the plant was subjected to strong illumination for five minutes, the other experiment was conducted in the dark for the same period of time. It was found that the ability of the plant to form labeled compounds from the radioactive pyruvic acid was much greater in the light than in the dark. In both cases most of the radioactivity appeared as a single compound in the lipid fraction obtained from the algae. Very little

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* Work described in this paper was sponsored by the Atomic Energy Commission.

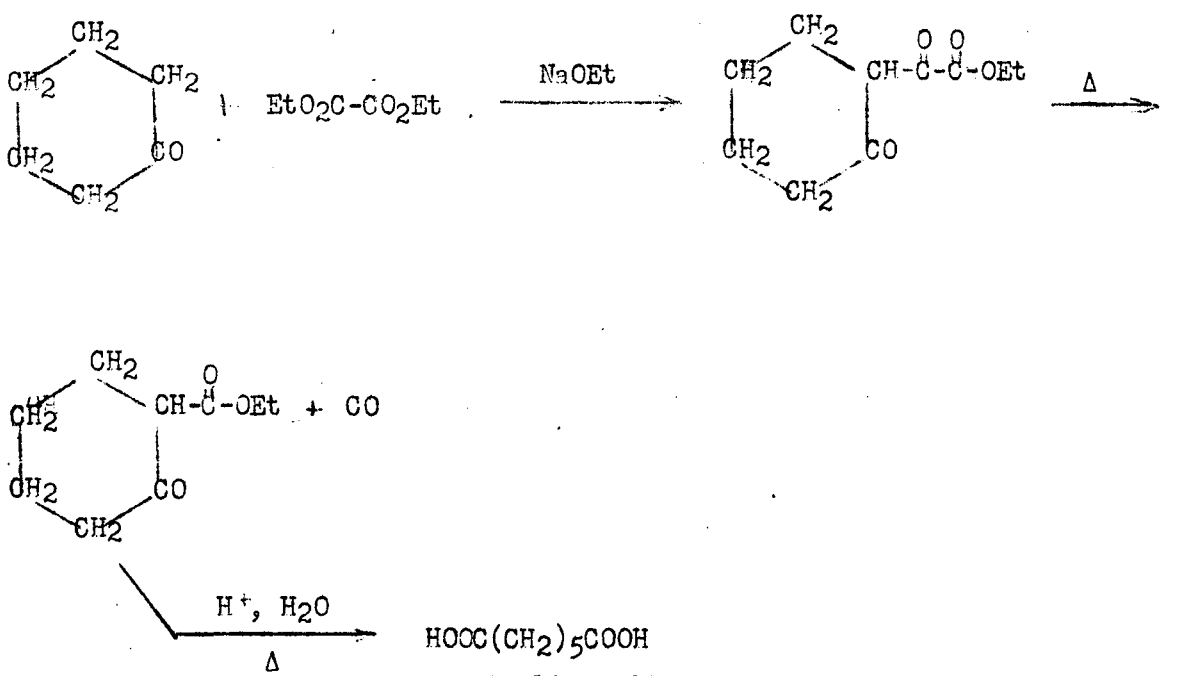
labeled sugar was formed in the light and none appeared in the dark. It was established that radioactive alanine and aspartic acid were formed under both conditions.

A. INTRODUCTION

The thermal decomposition of esters of α, γ-diketo acids, $R\text{-}CO\text{-}CH_2\text{-}CO\text{-}CO_2R$, with the evolution of carbon monoxide is a well-known reaction. The resulting product is the ester of a β-keto acid:

$$R\text{-}CO\text{-}CH_2\text{-}CO\text{-}CO_2R \xrightarrow{\Delta} CO + R\text{-}CO\text{-}CH_2\text{-}CO_2R$$

This reaction has found considerable application as a synthetic tool. For example, a convenient synthesis of pimelic acid which is described in "Organic Syntheses" (1) employs a Claisen condensation to prepare an α, γ-diketo-ester which is thermally decarbonylated to give a β-keto-ester. The latter ester may, in turn, be hydrolyzed and hydrated to give the desired pimelic acid. The reaction proceeds as follows:



It is of interest to speculate as to which of the adjacent carbonyl groups in the α, γ-diketo-ester is the group which leaves the mole-

cule as carbon monoxide. Heretofore there has been available little evidence either way on which to base any conjecture. However, the ready availability since 1945 of the long-lived Carbon 14 isotope has provided a most simple and powerful tool for the organic chemist to solve such mechanistic problems as this. Consequently, it was decided to synthesize one of these compounds with one of its adjacent carbonyl groups labeled with Carbon 14 and then to determine whether the evolved carbon monoxide was radioactive.

In searching for an appropriate ester to use for this study it was immediately evident that the above synthesis using ethyl oxalate could not be employed. If the labeled oxalate is used in a synthesis the radioactivity will be distributed between the two adjacent carbonyl groups. Also, it appeared that a simpler compound such as pyruvic ester, CH_3-CO-CO_2Et, would be not only much easier to synthesize but also would be quite as interesting for a study of the evolution of carbon monoxide from a compound containing adjacent carbonyl groups. The decarbonylation of pyruvic ester is of further interest as it is a widely postulated reaction in biochemical mechanisms. It was, therefore, decided to attempt the synthesis of pyruvic acid labeled in the α-carbon atom and to determine whether there was activity in the evolved carbon monoxide and carbon dioxide.

B. SYNTHESIS OF PYRUVIC ACID-2-C^{14} AND ETHYL PYRUVATE-2-C^{14}

The determination of the relative amount of Carbon 14 in all of the organic compounds involved in the syntheses described below was carried out by converting the compounds to barium carbonate and measuring the radioactivity of the carbonate. All of the apparatus and techniques used for this purpose were exactly those described by Prof. Calvin and his group at the Radiation Laboratory (2,3).

The radioactive carbon which was used in this work was supplied by the Atomic Energy Commission in the form of $BaC^{14}O_3$. Although Cramer and Kistiakowsky (4) have developed a method for converting labeled carbon dioxide to cyanide with good yields, an even better way to synthesize labeled pyruvic acid appeared to be the utilization of a Grignard reaction to obtain labeled acetic acid from $C^{14}O_2$ followed by a cyanide chain-lengthening step to pyruvic acid. Consequently, carbonyl-labeled pyruvic acid and ethyl pyruvate were synthesized through the following series of reactions.

(1) $BaC^{14}O_3 \xrightarrow{H_2SO_4} C^{14}O_2$

(2) $C^{14}O_2 + CH_3MgI \xrightarrow{-20^\circ} CH_3C^{14}O_2MgI \xrightarrow{H^+,\ H_2O} CH_3C^{14}O_2H$

(3) $CH_3C^{14}O_2H \xrightarrow{PBr_3} CH_3C^{14}OBr$

(4) $CH_3C^{14}OBr \xrightarrow{CH_2(CN)_2} CH_3C^{14}OCN$

(5) $CH_3C^{14}OCN \xrightarrow{H^+,\ H_2O} CH_3C^{14}OCO_2H$

(6) $CH_3C^{14}OCO_2H + C_2H_5OH \xrightarrow{H_2SO_4} CH_3C^{14}OCO_2C_2H_5 + H_2O$

<u>Conversion of $BaC^{14}O_3$ to $C^{14}O_2$.</u> Step 1 was carried out in an evacuated closed system (see Figure 1). Concentrated sulfuric acid was placed in a pressure-compensated funnel connected to a flask containing the radioactive $BaCO_3$. The flask was connected through the funnel to a trap which served to collect the radioactive carbon dioxide. The system was evacuated to a pressure of about 10^{-4} mm. Hg and the trap immersed in a bath of liquid nitrogen. The concentrated sulfuric acid was then allowed to run down into the flask containing the carbonate and the contents of the flask was heated to complete the evolution of $C^{14}O_2$ which was transferred quickly to the trap. The yield of $C^{14}O_2$ is 100% for this step.

Conversion of $C^{14}O_2$ to $CH_3C^{14}O_2H$. Step 2 was carried out in the same vacuum line shown in Figure 1. The complete apparatus for the Grignard reaction is shown in Figure 2. The reaction was run in a 150 ml. conical reaction flask which was fitted with an induction stirrer and a dry ice-acetone reflux condenser as is shown in Figure 2. Into the flask was placed 50 ml. of dry ether and 1 g. of magnesium turnings. The flask was chilled with liquid nitrogen and 2 ml. (4.6 g.) of methyl iodide distilled in from the storage vessel. The reaction vessel was closed off and warmed in a water bath and the ether allowed to reflux for one hour. The reaction flask was then cooled to -20° in an ice-acetone bath and into it was slowly run 0.0153 mole of radioactive carbon dioxide (containing 10.0 millicuries of Carbon 14) which had been previously generated, dried by passing through a spiral immersed in dry ice-acetone and freed of oxygen and nitrogen by condensing with liquid nitrogen and evacuating at low pressures. The complete reaction of all the carbon dioxide was indicated when the pressure of the system reached that of ether at the -20° temperature (70 mm. Hg). Stirring was then continued for 20 minutes. The reaction flask was removed from the vacuum line and opened in the hood and the cold Grignard complex was decomposed by adding, with stirring, 30 ml. of 6 N sulfuric acid. After decomposition an additional 60 ml. of water was added, together with 7 g. of silver sulfate to precipitate the iodide present. The acetic acid was recovered as sodium acetate as follows.

The ether was distilled off and the acetic acid was steam-distilled from the reaction mixture with about 600 ml. of water. This distillate was exactly neutralized with N sodium hydroxide solution; a Beckman pH meter was used to follow the titration. The solution was evaporated to a small

volume on a steam bath, filtered and evaporated to dryness. The residue was dried *in vacuo* at 10^{-3} mm. pressure. The yield of anhydrous sodium acetate was 90-93% based on CO_2, the titrations and weighings agreeing within a few tenths of a percent.

Conversion of $CH_3C^{14}O_2H$ to $CH_3C^{14}OCN$. Steps 3 and 4 were performed on a 0.25 mole scale by dissolving a small amount of the labeled sodium acetate in inactive acetic acid and placing the solution in a very small three-necked, moisture-protected flask fitted with a stirrer, condenser and dropping funnel. Phosphorus tribromide (about 20% in excess of the theoretical quantity) was added slowly through the dropping funnel. An oil bath was then placed beneath the reaction flask and its temperature maintained at 50° for one hour; the contents of the flask were stirred continuously during these operations. The reaction flask was then cooled and provided with boiling chips and a small fractionating column. The temperature of the bath was raised slowly and the acetyl bromide distilled over in the temperature range of 70-76°. The acetyl bromide was distilled directly into a second small three-necked flask which contained 0.25 mole of cuprous bromide and which was fitted with a stirrer and reflux condenser equipped with a calcium chloride tube. After all the acetyl bromide had distilled over, an oil bath was placed under the flask and the temperature maintained at 70-80° for one hour. The reaction flask was then attached to the vacuum line and chilled in a liquid nitrogen bath. The system was evacuated to 10^{-4}mm. pressure, the nitrogen bath was removed and the pyruvonitrile allowed to distill over into a second vessel which was immersed in liquid nitrogen. The yield of impure pyruvonitrile was 70-75% based on acetic acid.

Conversion of $CH_3C^{14}OCN$ to $CH_3C^{14}OCO_2H$. This reaction was carried out with approximately 0.2 mole of the labeled pyruvonitrile by the addition of 13 cc. of 12 N hydrochloric acid with constant stirring on an ice bath. After 15-30 minutes a vigorous hydrolysis took place accompanied by considerable evolution of heat and the contents of the flask solidified to a thick, crystalline mass. Enough water to dissolve the ammonium chloride was then added and the mixture was warmed under a reflux condenser for one hour on a water bath maintained at 70°.

The pyruvic acid was recovered from the reaction mixture by means of an overnight continuous extraction with ether. After the solution was dried and the ether evaporated, the residual liquid was placed in a small vessel which was connected to the vacuum line. The liquid was frozen in a liquid nitrogen bath and the system evacuated to a pressure of 10^{-4} mm. The bath was then removed and placed under a second vessel which served as a receiver and the volatile components of the liquid allowed to distill from the first vessel to the second. The liquid which distilled over was found to contain considerable acetic acid as well as the expected pyruvic acid. The acetic acid was removed by steam distillation of the mixture; a volume of water approximately equal to ten times the volume of the mixture was distilled over. This operation removes all of the acetic acid and only 10% of the pyruvic acid. The water solution remaining in the still was again extracted with ether, dried and the ether evaporated. The pyruvic acid was distilled from the residual liquid by means of the vacuum-line operation described above. The distillate was found to be pyruvic acid of better than 95% purity as shown by its equivalent weight, elementary analyses of the free acid and its potassium salt and the specific activity of both the

acid and its salt. In one preparation, however, the specific activity of the acid was only about one-fifth of the calculated value. No explanation for this anomaly could be found.

Considerable difficulty was experienced in this step of the synthesis, not only because of the appearance of large amounts of acetic acid when the pyruvonitrile is hydrolyzed but also because of the tendency of pyruvic acid to polymerize, a tendency probably increased by the presence of cyanide ion. Yields for the conversion of pyruvonitrile varied from 25-52%. The overall yields of pyruvic acid, based on the radioactive carbon dioxide, varied from 17-36%.

Conversion of $CH_3C^{14}OCO_2H$ to $CH_3C^{14}OCO_2C_2H_5$. The esterification was accomplished by adding an equal volume of absolute ethyl alcohol and 1 cc. of concentrated sulfuric acid to 1 cc. of the labeled pyruvic acid; the mixture was allowed to stand overnight at room temperature. The solution was then extracted continuously with ether for several hours, the extract was dried with calcium chloride and a very small amount of solid sodium bicarbonate was slowly added until the evolution of carbon dioxide abated. After the ether was evaporated, the residual liquid was fractionally distilled at low pressure. Ethyl pyruvate was collegted in the temperature range of 50-55° at 19 mm. pressure. The identity of the ethyl pyruvate was shown by its boiling point, specific activity, elementary analysis and equivalent weight as determined by titration with standard sodium hydroxide solution. The yields of ethyl pyruvate from pyruvic acid varied from 65-80%.

C. DECARBONYLATION EXPERIMENTS

1. Experimental Details

The decarbonylation of ethyl pyruvate and pyruvic acid took place in the apparatus shown in Figure 3. All connections were of glass. The sample of ethyl pyruvate or pyruvic acid was placed in the reaction flask C together with carbonate-free powdered glass which was used for the purpose of increasing the surface area. Before the gas washing bottles E and F were placed in position, the system was swept free of air by nitrogen. Organic impurities in the nitrogen were removed by passing the nitrogen through the first copper oxide combustion furnace A (maintained at 525°) and the gas washing bottle B which was filled with sodium hydroxide solution. After air was removed from the system the gas washing bottles E and F, which were filled with carbonate-free sodium hydroxide solution, were placed in position. The oil bath temperature was then raised to 110-130° and maintained at that value for five hours. During this time a very slow stream of nitrogen was kept moving through the system. Undecomposed pyruvate was kept in the reaction flask C by the reflux condenser D. Any evolved carbon dioxide was trapped in the gas washing bottle E while evolved carbon monoxide passed through this bottle, was burned to carbon dioxide in the second furnace A (maintained at 525°) and finally was absorbed in the gas washing bottle F. This latter bottle was protected from atmospheric carbon dioxide by the soda-lime tube G.

2. Decarbonylation of Ethyl Pyruvate

Two samples of labeled ethyl pyruvate were decomposed thermally in the apparatus described above. In both of these samples the radioactivity of the α-carbon atom was 3180 counts/min./mg. C (12.9 disintegrations per count). It had been found earlier that four hours at 120° was sufficient to

complete the decarbonylation. The samples of labeled pyruvate were kept in the reaction flask at the elevated temperature for five hours. The data for these samples are summarized in Table 1.

Table 1

Yields of Carbon Monoxide and Carbon Dioxide Obtained on Thermal Decarbonylation of Ethyl Pyruvate for Five Hours

Wt. pyruvate sample	Temp. of Decomposition	Wt. CO_2 as $BaCO_3$	% of theor. CO_2	Wt. CO as $BaCO_3$	% of theor. CO
204 mg.	110-120°	4.3 mg	1.2	304 mg.	88.0
180	120-130°	10.9	3.6	309	101.0

It is probable that the recovery of the carbon oxides in excess of the theoretical yield in the case of the second sample is due to the presence of small amounts of those gases in the system at the start of the experiment.

The two barium carbonate samples for each experiment were precipitated from the hydroxide solution in the two gas washing bottles E and F (Figure 3) on the addition of barium chloride solution. The carbonate samples were recovered quantitatively, dried and weighed. No detectable radioactivity could be found in any of the carbonate samples.

3. Decarbonylation of Pyruvic Acid

Thermal decomposition was carried out on two samples of pyruvic acid. In both of these samples the radioactivity of the α-carbon atom was 37,400 counts/min./mg. C (12.9 disintegrations per count). The results of these decarbonylations are summarized in Table 2.

Table 2

Yields of Carbon Monoxide and Carbon Dioxide Obtained on Thermal Decarbonylation of Pyruvic Acid for Five Hours

Wt. pyruvic acid sample	Temp. of Decomposition	Wt. CO_2 as $BaCO_3$	% of theor. CO_2	Wt. CO as $BaCO_3$	% of theor. CO
81 mg.	110-120°	22 mg.	12.2	153 mg.	84.0
198	110-120°	74	16.7	359	81.0

The two barium carbonate samples from each experiment were counted for radioactivity in the usual fashion. Both samples from each experiment were completely inactive.

4. Investigation of Products

Many authors (5,6,7) have reported that the residue left behind after thermal decomposition of pyruvate in the presence of sulfuric acid was, for the most part, either acetaldehyde or acetic acid. The following brief experiments were carried out to investigate the nature of the products remaining after decarbonylation.

The residue from one of the ester decarbonylations was hydrolyzed with dilute (1 N) sodium hydroxide solution at room temperature for one day after which the solution was heated to boiling for 15 minutes. The solution was then cooled, acidified with sulfuric acid and steam distilled until a volume equal to about five times the original volume had been distilled over. The distillate was titrated with standard sodium hydroxide solution; a Beckman pH meter was used to follow the titration. For each millimole of ethyl pyruvate used in the experiment 0.87 milliequivalent of acid was found in the steam distillate. This result indicates that the principal product of the decomposition was ethyl acetate. That the salt which was obtained

was sodium acetate is indicated by the fact that its specific activity was within 2% of that calculated from the specific activity of the starting pyruvate. Of the total activity used in the experiment, 96% was recovered as sodium acetate. The remaining activity was present in a dark, tarry material which was left behind in the reaction flask. No investigation was carried out to determine the nature of this material. The literature (8,9) reports that α-keto-γ-valerolactone-γ-carboxylic acid, α, α'-dimethyl-β furan-β-carboxylic acid, citric acid and pyruvic acid polymer are obtained on heating pyruvic acid in addition to the previously mentioned acetaldehyde and acetic acid.

D. SUMMARY AND DISCUSSION

The results of the decarbonylation experiments on α-labeled acid and ethyl pyruvate show clearly that the carbon oxides which are evolved from these compounds on mild heating do not come from the α-carbon atom. The carbon monoxide and carbon dioxide, therefore, must come exclusively from the carboxyl group.

No kinetic studies have ever been carried out on the thermal decarbonylation of pyruvic acid or ethyl pyruvate. In the absence of such studies it is impossible to suggest a detailed mechanism for the reaction. However, if it is assumed that this reaction is a first-order or intramolecular one then the loss of carbon oxide simply occurs when the molecule reaches an energy level high enough to cause the splitting off of a CO or CO_2 fragment. In this case the loss of carbon oxide from the carboxyl group perhaps should not be unexpected since the loss from this group involves the breaking of only one carbon-carbon bond whereas loss from the

α-carbon would involve the breaking of two carbon-carbon bonds. On the other hand, the reaction may be an intermolecular or second-order one which requires an interaction between two molecules of the acid or ester in order for the carbon oxide to be lost. In these experiments wherein the pure substances were used rather than a solution, conditions were ideal for such an interaction.

The data from these experiments show the evolution of a considerably higher fraction of carbon dioxide from the acid than from the ester. In fact, the amount of carbon dioxide obtained from the ester is quite negligible; this carbon dioxide may have arisen entirely from impurities in the system. Pyruvic acid, however, gives off about 15 mole percent of carbon dioxide. The fact that considerable carbon dioxide was evolved by the acid while essentially none came from the ester is quite in accord with the prevailing ideas about decarboxylation. Apparently, the principal tendency of the pyruvate structure is to give up a carbonyl group which is evolved as a molecule of carbon monoxide. Superimposed on this tendency, however, is the relative ease with which free pyruvic acid may be decarboxylated. This tendency towards decarboxylation, is, of course, not shown by esters due to the almost complete absence of carboxylate ion. The free acid, on the other hand, always has an appreciable concentration of carboxylate ion present due to the ionization of the hydrogen atom from the carboxyl group.

The authors previously cited (5,6,7) report that pyruvic acid evolved either carbon monoxide or carbon dioxide on heating with sulfuric acid. Nothing could be found in the literature reporting the effect of heating pyruvic acid or pyruvic ester in the absence of any other compounds, as was done in the present work. In earlier background work for the experiments which have been described here,unlabeled pyruvic acid and ethyl pyruvate were heated in the presence of sulfuric acid. However, the presence of sulfuric acid was

found to have little, if any, effect on the temperature at which thermal decomposition took place. Neither did sulfuric acid appear to have any effect on the evolution of carbon monoxide from ethyl pyruvate, although it did increase the percentage of carbon dioxide which was evolved from pyruvic acid. It was found that pyruvic acid in the presence of sulfuric acid evolved about 30 mole percent of carbon dioxide as compared to about 15 mole percent in the absence of sulfuric acid.

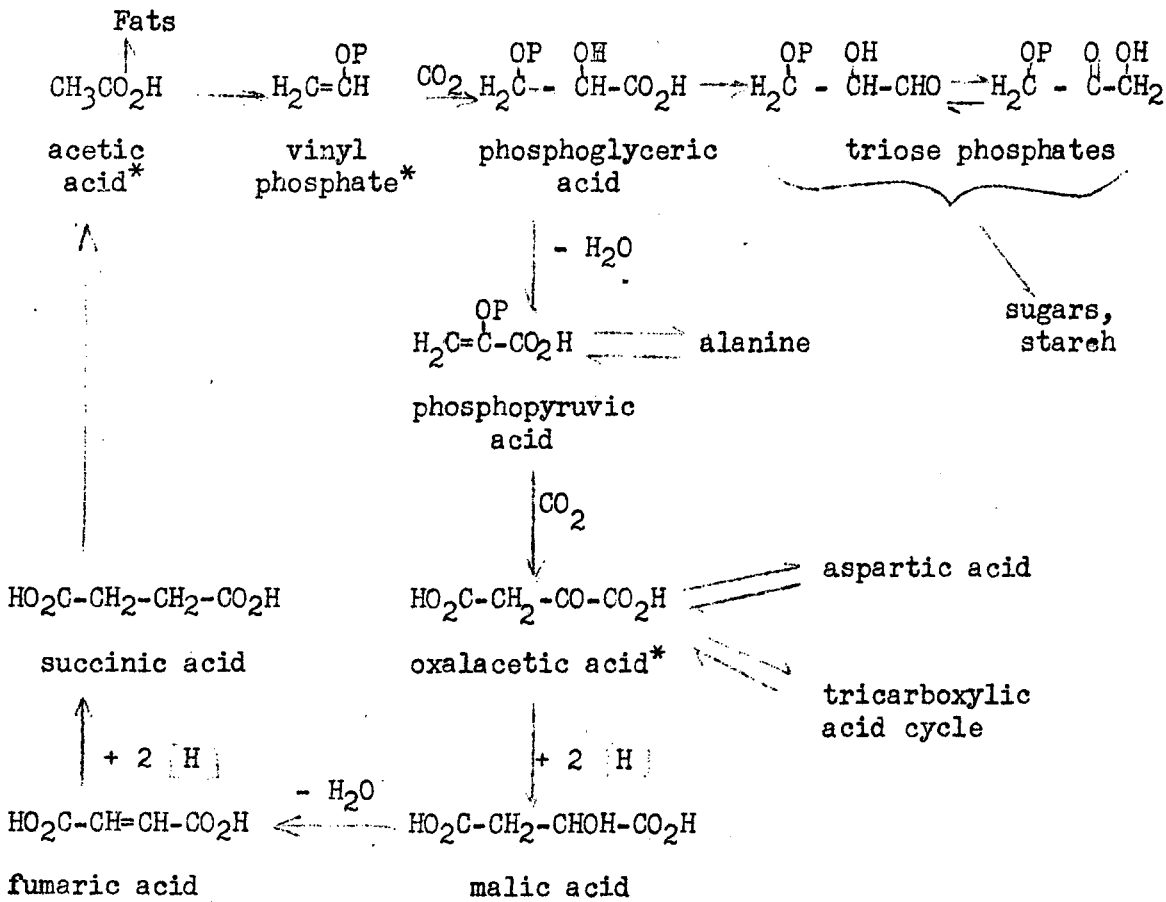
PART II

PYRUVATE METABOLISM IN GREEN ALGAE

A. INTRODUCTION

A large number of laboratories in this country are engaged in an attack on the problem of photosynthesis. The study of this vastly important biochemical process has been greatly aided, as has the investigation of so many organic chemical reactions, by the availability of the long-lived Carbon 14 isotope. One principal avenue of attack on the problem of photosynthesis is to study the way in which the light energy is transformed into chemical energy in the plant. The second principal way to attack the problem is to study the chemical "path" which is traveled by carbon in the plant during photosynthesis. This carbon is taken from the atmosphere as carbon dioxide and is finally built by the plant into fats, proteins and carbohydrates. Obviously, Carbon 14 is a most excellent means to use in studying this "path." Isotopic carbon was first used as a tracer for this purpose by Ruben and co-workers (10,11, 12,13) and their early work has been continued at the U.C. Radiation Laboratory by the group under the leadership of Prof. Calvin and Dr. Benson. The general design of their experiments has been to expose green plants to radioactive carbon dioxide and light under varying conditions and to identify the compounds into which the radioactive carbon is incorporated. They have paid particular attention to the appearance of radioactive carbon in certain compounds in relation to the time of exposure of the plant to light. This general method of attack on the problem of photosynthesis has led to considerable success in tracing the route which carbon follows in the plant during photosynthesis (14,15,16). Their results, based on the radioactive compounds identified and on the

distribution of radioactive carbon atoms in several of those compounds, led to a proposed scheme (17) for the path of carbon in photosynthesis. This scheme, which uses some of the reactions already established in animal tissue and bacteria (18), made it possible to account for the observed distribution of radiocarbon in sugar produced by a short photosynthesis as well as its distribution in the succinic acid recovered from the plant. The scheme is as follows:



The compounds which are marked with an asterisk are those which have not yet been unequivocally identified; all the other compounds have been identified and have been found to be radioactive after the algae were given labeled carbon dioxide.

The above scheme is but a tentative one and doubtless will undergo change as new data are collected which may cast additional light on the problem of the path of carbon in photosynthesis. However, the scheme does account not only for the appearance of radioactivity in the various plant metabolites but also for the rate at which the activity appears in those compounds and the rate at which activity appears in the various positions in the carbon chains (17).

As part of the continuing program to test the validity of the above scheme, it was decided to follow the distribution of the radiocarbon in the compounds appearing in green algae after labeled pyruvate was given to the actively photosynthesizing plants. The experiments herein described are but the beginning of an effort to follow this distribution and are, therefore, in the nature of preliminary or exploratory work. Their results do not in any way constitute a complete picture of pyruvate metabolism in green algae.

The carbonyl-labeled pyruvic acid which was available from the work described in Part I of this thesis did not have a sufficiently high specific activity for convenient use here. In addition, it was believed that information of a more secure nature could be obtained by giving the plants methyl-labeled pyruvate rather than the carbonyl-labeled compound since the use of the former compound would decrease possible losses of radioactivity due to decarboxylation. Consequently, it was decided to carry out a synthesis of methyl-labeled pyruvate of fairly high specific activity and to use this material in the biochemical work. The synthesis of this compound is described in the following section.

B. SYNTHESIS OF PYRUVIC ACID-3-C^{14}

The preparation of radioactive pyruvic acid labeled in the methyl position was carried out following the procedure described by Adickes and Andresen (19). Although these authors had not specifically prepared pyruvic acid they did give a good general method for the preparation of α-keto acids. Their synthesis involves a Claisen condensation between diethyl oxalate and a fatty acid ester. The resultant α-oxalo fatty acid ester is then hydrolyzed and decarboxylated to the desired α-keto acid. It was hoped that this procedure would provide a way to obtain methyl-labeled pyruvate from methyl-labeled acetate. This hope has been realized.

The starting material for this synthesis, methyl-labeled sodium acetate, was provided by the Bio-Organic Group of the Radiation Laboratory. The acetate had been prepared from radioactive barium carbonate following the method of Tolbert (20). The acetate was converted to pyruvic acid through the following reactions:

(1) $2\ C^{14}H_3CO_2Na + (C_2H_5)_2SO_4 \longrightarrow 2\ C^{14}H_3CO_2C_2H_5 + Na_2SO_4$

(2) $C^{14}H_3CO_2C_2H_5 + C_2H_5O_2C\text{-}CO_2C_2H_5 \longrightarrow C_2H_5O_2C\text{-}CO\text{-}C^{14}H_2CO_2C_2H_5$

(3) $C_2H_5O_2C\text{-}CO\text{-}C^{14}H_2CO_2C_2H_5 \xrightarrow{H^+,\ H_2O} C^{14}H_3COCO_2H$

<u>Conversion of $C^{14}H_2CO_2Na$ to $C^{14}H_3CO_2C_2H_5$</u>

Reaction (1) was carried out by refluxing the methyl-labeled sodium acetate with a 50% molal excess of redistilled diethyl sulfate. The refluxing was continued with stirring for two hours at 150-170°. The radioactive ethyl acetate was then distilled from the reaction mixture and

collected over a five degree range around its boiling point (77°). For preparations of about 0.015 moles, the yield on this reaction was 90%.

Conversion of $C^{14}H_2CO_2C_2H_5$ to $C_2H_5O_2C\text{-}CO\text{-}C^{14}H_2CO_2C_2H_5$

Reaction (2) was performed in a small three-necked flask fitted with a stirrer and a reflux condenser and surrounded by a water bath. After 0.04 moles of sodium methoxide had been placed in the flask, stirring was begun and a solution of 0.03 moles of diethyl oxalate in 15 cc. of peroxide-free ether was slowly added. To the resultant solution of ortho-ester was added 0.02 moles of radioactive ethyl acetate in a few cc. of ether. The solution was refluxed with stirring for sixteen hours, after which it was poured onto about 20 g. of an ice-sulfuric acid mixture. The resultant clear yellow solution was extracted overnight in a continuous extractor with peroxide-free ether. The ether solution was concentrated to 25 cc. and shaken with successive small portions of a 10% solution of potassium carbonate until the ether phase no longer gave a positive test for enol with ferric chloride. (The test was carried out by diluting about one cc. of the ether solution with two cc. of alcohol and then adding four or five drops of a 1% ferric chloride solution. The characteristic purple color quickly appeared if enol was present.) The combined potassium carbonate extracts were then acidified with sulfuric acid and extracted continuously for ten hours with peroxide-free ether; the ether was evaporated from the resultant solution.

Conversion of $C_2H_5O_2C\text{-}CO\text{-}C^{14}H_2CO_2C_2H_5$ to $C^{14}H_3COCO_2H$

Reaction (3) was carried out by adding about six volumes of 20% sulfuric acid to the impure material remaining after the evaporation of the ether. The solution was then heated at 65-75° for three hours. The hydrolysis

could be easily followed since the oxalacetic ester is insoluble in water while the free acid is soluble. The decarobxylation was followed by passing a slow stream of nitrogen over the reaction mixture and then through a barium hydroxide solution. When the evolution of carbon dioxide had ceased (after about three hours), the solution was extracted with peroxide-free ether and the ether was evaporated from the solution. The residual liquid was transferred to a small flask using ether to effect the transfer, the liquid was frozen in a liquid nitrogen bath and the flask pumped down on a vacuum line to a pressure of about 0.1 micron. The bath was then removed and the pyruvic acid was distilled over into a trap surrounded by a liquid nitrogen bath. After most of the volatile liquid had distilled over at room temperature, a water bath was placed under the flask and slowly warmed to a temperature of about 90°.

It was convenient for the purposes of weighing, counting and storing to convert the pyruvic acid to its potassium salt. This was done by dissolving the pyruvic acid in about five volumes of methanol; a solution of potassium hydroxide in ethanol (about a 20% excess of potassium hydroxide) was added and the precipitation of potassium pyruvate was completed by the addition of three or four volumes of ether. The salt was filtered off, washed free of potassium hydroxide with ether, and dried. The identity of the potassium pyruvate was shown by its carbon and hydrogen analysis, by its specific activity and by the specific activity of its 2,4-dinitrophenylhydrazone derivative. The preparation, however, did not give yields better than 11% for the small amounts of material used in the radioactive preparations. The bulk of the loss seemed to be in the polymerization of the pyruvic acid.

There was always a considerable quantity of non-volatile radioactive organic material left behind when the pyruvic acid was distilled over at low pressures.

C. DISTRIBUTION OF C^{14} IN ALGAE AFTER ADMINISTRATION OF METHYL-LABELED PYRUVATE

1. Fixation by Algae of Activity from Labeled Pyruvate

The algae, D_3 strain of Scenedesmus obliquus, were grown in continuous culture flasks under the conditions which were described by Benson et. al. (17) The algae were harvested immediately before using by centrifuging the cells in a refrigerated centrifuge. The cells were then dispersed in 0.05 molar KH_2PO_4 solution for the photosynthesis experiments. Two principal experiments were performed. in the first the algae were exposed to the labeled pyruvate under strong illumination for five minutes. In the second experiment the algae were exposed to the labeled pyruvate in the dark for five minutes.

The experiments were carried out as follows. One cc. of the packed cells was dispersed in 100 cc. of the phosphate solution. The solution was placed in a water-jacketed circular vessel in which the illumination or dark fixation was carried out. To the solution was added 4.4 mg. (985,000 counts/min.) of labeled potassium pyruvate. For the first experiment the illumination was provided from both sides of the vessel by 300 watt reflector spot lights; the contents of the illumination flask were protected from any significant rise in temperature by placing adequate water-cooled glass infra-red absorbing filters between the flask and the lights. During the five minutes time in which the algae were exposed to

the pyruvate a stream of nitrogen containing 4% of carbon dioxide was kept bubbling into the flask. In this way, the algae were able to continue their normal process of photosynthesis.

After five minutes in contact with the radioactive pyruvate, the algae were killed rapidly by adding 30 ml. of acetic acid - concentrated hydrochloric acid solution (4:1) to the algae suspension. The fixed non-volatile radioactivity was determined on samples prepared by evaporating 100 μl. aliquot portions of the suspension on glass or aluminum disks. The cells were then centrifuged and an aliquot portion of the clear solution was evaporated and counted in order to determine the water soluble fixed activity. Finally, the bulk of the non-water soluble activity remaining in the cells was extracted by shaking with 95% alcohol at 70° for ten minutes.

As has been indicated above, the radioactivity in the compounds obtained from the algae was determined, not by a prior conversion to barium carbonate, but by counting the material directly. Samples of 11.5 $cm.^2$ area were mounted on either aluminum or glass disks, using the small rotating table and hot-air drier which have been described elsewhere (3). It has been found that self-absorption corrections for such organic samples are negligible for sample thicknesses of less than 0.2 $mg./cm.^2$ and that the samples give reproducible (± 3%) activities.

2. Separation of Fractions and Identification of Compounds.

a. Preliminary Fractionation

The preliminary separation of fixed activity is summarized in Table 3.

Table 3

Preliminary Fractionation of Activity from Green Algae

After Administration of Methyl-Labeled Pyruvate

Fraction	% of Total Activity	
	5 min. light	5 min. dark
1. Non-volatile, water soluble	21	23
2. Non-volatile, alcohol-soluble	41	22
3. Non-extractable activity	8	1
4. Volatile compounds	30	54

The total activity given to the algae in the form of pyruvate is accounted for in four fractions: (1) non-volatile water-soluble compounds, (2) non-volatile alcohol-soluble compounds, (3) activity remaining in the cells after successive extractions with water and alcohol, (4) volatile compounds; unchanged pyruvic acid would be included in this fraction.

b. Water-soluble Components

Carboxylic Acids

The first separation of the water-soluble components from both the 5-minute illumination and dark experiments was accomplished by acidifying the aqueous solutions to pH 1 and carrying out a continuous, overnight extraction with ether. This extraction will remove all carboxylic acids from the solution. The ether extract was evaporated to dryness at reduced pressure and taken up in glacial acetic acid for determination of radioactivity. The ether-extractable activity is tabulated under I in Table 4. It was found that 42% of the water-soluble activity was present in the

form of carboxylic acid in the case of the 5 minutes illumination; 34% of this activity was carboxylic acid in the case of the dark fixation. No further separation of these compounds was carried out.

Sugars

The water-soluble components were further separated by passing them successively through two 25 cc. columns of Duolite C-3 cation exchange resin and Duolite A-3 anion resin. After the solutions had been added, the columns were washed with 250 cc. of distilled water and the total effluent was evaporated at reduced pressure to a convenient small volume for determination of radioactivity. This fraction (IV, Table 4) contained all neutral substances; therefore, all sugars from the algae would appear in this effluate. However, the activity found in this fraction was only 0.4% of the total non-volatile water-soluble activity from the experiment conducted under 5 minute illumination. No radioactive sugars could be obtained from the "dark" experiment. The very small quantity of sugar obtained in the former experiment was not enough to permit further identification.

Amino Acids

All cationic substances from the plant extract were adsorbed on the cation exchange resin. Therefore, all of the amino acids from the algae are held up on that column. The cation column was eluted with 100 ml. of 7% hydrochloric acid followed by 100 ml. of water. The total effluent was evaporated under reduced pressure and its total radioactivity determined. This fraction (II, Table 4) was found to contain 15% of the total non-volatile water-soluble activity from the "light" experiment and 51% of that activity from the "dark" experiment.

An attempt was made to identify individual radioactive amino acids using the techniques of paper chromatography. This method, which was described by Consden, et. al. (21), is proving to be a very valuable aid in elucidating the path of carbon in photosynthesis (16). The method may be described briefly as two-dimensional partition chromatography; it consists of applying the mixture to be separated at one corner of a filter paper sheet (about 18" x 22"). The mixture is then chromatographed in one direction by a water-saturated organic solvent. After the paper is dried it is chromatographed in the other direction (at right angles) with a second water-saturated organic solvent. The chromatography is accomplished by letting the paper hang down from a trough containing the solvent so that capillary action will slowly draw the solvent down the paper. The operation is carried out in a closed cabinet in which the atmosphere is saturated with respect to solvent and water vapor. The result of the paper chromatography technique is that one obtains a "map" of spots on the paper depending upon the relative partition coefficients of the different constituents of the mixture between water (the stationary phase) and the organic solvent (the moving phase). The water-saturated organic solvents which were used in the present work were phenol and 2,4-lutidine.

The identification of individual amino acids on a paper chromatogram is easily accomplished by using the well-known ninhydrin test. The completed chromatogram is sprayed evenly with a solution of ninhydrin (triketo-hydrindene) in alcohol and is then gently heated. In about half-minute's time the characteristic red-purpose spots appear at those areas of the paper which contain amino acids. In this way the relative positions on paper chromatograms of all of the simpler and well-known amino acids have been well established.

In order to determine the positions of radioactive amino acids on the paper chromatograms, radioautographs of the chromatograms may be prepared by placing the paper in contact with photographic film for a suitable period of time. After the film is developed it may then be placed over the paper chromatogram and the areas showing radioactivity can be compared with the areas which have already been shown (by the ninhydrin test) to contain amino acids. In this way all of the amino acids occurring in a plant extract may be found and, in addition, those amino acids which contain C^{14} may be determined.

Using the techniques described above, paper chromatograms and radioautographs of the cation resin eluate from both experiments have been prepared. Photographs of the two radioautographs are shown in Figures 4 and 5. These radioautographs have enabled us to determine that radioactive aspartic acid and alanine are both obtained from algae after the plants are given labeled pyruvate. This is true for the 5-minute dark fixation as well as for the experiment carried out during 5-minute illumination. An amino acid which is probably glutamic acid was also obtained in both experiments; however, the position of this spot was not such as to make its identification unequivocal. In addition, a fourth amino acid was obtained in the case of the 5-minute illumination; this amino acid was not identified.

Compounds Elutable from Anion Excange Resin

In the previous work on the path of carbon in photosynthesis (17) it has been found possible to separate and identify the triose phosphates, hexose phosphates and phosphoglyceric acids in the eluates from the anion exchange resin. The sugar phosphates were found in the effluate of the anion

column after passing through the column 100 ml. of 1.5 N ammonium hydroxide followed by 250 ml. of water. Stronger elution, employing 30 ml. of 1.5 N sodium hydroxide over a one-hour period, followed by 250 ml. of water, was found to remove 2- and 3-phosphoglyceric acid from the column. This same elution procedure was employed in the present work and the fraction of activity appearing in the ammonium hydroxide effluate (III-A) as well as that appearing in the sodium hydroxide effluate (III-B) is recorded in Table 4.

No further work was carried out on Fraction III-A from either experiment. Paper chromatograms of the III-B fractions revealed no spots which could be identified.

Summary

The following table summarizes the distribution of activity in the water-soluble fraction from both experiments.

Table 4

Distribution of C^{14} in Water-Soluble Components Obtained from *Scenedesmus* D_3 after Administration of Methyl-Labeled Pyruvate

Fraction	5-minute photosynthesis		5-minute dark fixation	
	c.p.m. x 10^{-5}	%	c.p.m. x 10^{-5}	%
Total C^{14} fixed, water-soluble	2.10	100	2.23	100
I Ether extractable (acids)	0.81	42	0.74	34
II Cationic (amino acids)	0.31	15	1.13	51
III-A Anionic. Not NH_4OH elutable	0.46	23	0.26	12
III-B Anionic. NH_4OH elutable	0.26	13	0.05	2
IV Neutral substances (sugars)	0.01	0.4	0	0

c. Alcohol-soluble Components

The alcohol-soluble non-volatile activity comprised 41 and 22% respectively of the total activity given to the algae as labeled pyruvate under 5-minute illumination and in the dark. That the bulk of this material is lipid in nature is indicated by its alcohol solubility and is further indicated by its behavior when subjected to paper chromatography. In this latter instance the radioactive material was moved to the far corner of the paper, that is, it was almost completely carried along in both the organic phases. The work which will be described in the remainder of this section is that which was carried out on the material obtained from the experiment performed under 5-minute illumination.

Most of the data characterizing the lipid material was obtained by determining distribution coefficients under various conditions. This was done by placing the two liquid phases in a 100 ml. stoppered centrifuge tube and shaking mechanically for about ten minutes. The tube was then centrifuged at about 2000 r.p.m. for ten minutes in order to remove water from the organic phase. Finally, the two liquid phases were carefully separated, evaporated and counted to determine the amount of radioactivity in each phase.

The lipid material was distributed between ether and water, first at pH 9 and then at pH 1. These distributions showed that most of the material was acidic in nature as its $\frac{\text{ether}}{\text{water}}$ distribution coefficient (determined by counting the radioactivity in both phases) changed from 0.01 to 7.0 when the pH was changed from 9 to 1. The ether phase was then re-extracted with fresh 0.1 N hydrochloric acid and this process was continued until the distribution coefficient $\left(\frac{\text{ether}}{\text{water}}\right)$ finally became constant at a value of 650.

At this point 80% of the original material still remained in the ether, the rest having been with drawn from the aqueous phases. Consequently, it appeared that 80% of the original lipid material consisted of a single acidic compound which had an $\frac{\text{ether}}{\text{water}}$ distribution coefficient of 650. Since this coefficient was inconveniently high, it was decided to distribute the compound between benzene and water at pH 1. In this latter case the distribution coefficient $\left(\frac{\text{benzene}}{\text{water}}\right)$ was found to be 95.

The acidic component was non-volatile. Aliquot portions of the ether or benzene extracts could be evaporated under a hot-air drier without any loss of radioactivity. However, it was found that labeled heptanoic acid cannot be similarly counted as, under the same conditions, most of the acid is lost because of its volatility. At the same time, the $\frac{\text{benzene}}{\text{water}}$ distribution coefficient for heptanoic acid was determined to be about 400. These data clearly show that the radioactive acidic compound obtained from the algae is not a simple fatty acid.

An attempt was made to determine if the unknown acid was unsaturated. A small amount of bromine was added to the benzene solution and the distribution between benzene and water repeated at pH 1. The distribution coefficient was found to be unchanged from its original value of 95 and it therefore appeared that the acid was not unsaturated. However, the compound is present only in very high dilution and, consequently, bromine uptake might be very slow. As a result, the fact that the distribution coefficient did not change is not unequivocal proof of a lack of unsaturation.

The $\frac{\text{benzene}}{\text{water}}$ distribution coefficient was determined for pH values ranging from 1 to 9.5. In this way a plot was prepared (see Figure 6)

showing the change in distribution coefficient as a function of pH. Such a plot is analogous to that which is obtained by neutralizing an acid with standard base and plotting volume of alkali added as a function of pH. The plot shown in Figure 6 indicates the presence of two acidic groups in the molecule, one with a pK value of 5-6 and the other with a pK value of 9-10. The first value indicates the presence of a carboxyl group; the second value is about that which is obtained if an enol grouping is present in the molecule. Therefore, in spite of the conflicting data on bromine uptake, it was reasonable to suppose that the unknown acid was a fairly long-chain β-keto acid and that it was an intermediate product between acetic acid and a simple fatty acid. This would conform with the prevalent concept of the synthesis of fatty acids in living organisms, the so-called "β-oxidation hypothesis" (22).

The benzene solution of the unknown acid (containing a total activity of 6000 counts/min.) was evaporated to a volume of about one cc. This material was transferred to a small three-necked flask equipped with a stirrer and reflux condenser. Twenty cc. of 3 N sulfuric acid was added and the contents of the flask were stirred and heated at 110° for five hours. During this time a slow stream of nitrogen was kept moving through the flask and was passed out of the system by bubbling through a gas wash bottle filled with sodium hydroxide solution. After five hours, the wash bottle was disconnected from the system and to its contents was added dilute barium chloride solution until no further precipitation of barium carbonate took place. The carbonate (about 140 mg.) had no detectable activity. Since decarboxylation of the unknown acid resulted in no loss of radioactivity, it was evident that the carboxyl group contained no C^{14}. The bulk of the

140 mg. of barium carbonate which was obtained resulted from the presence of carbonate in the hydroxide solution and from carbon dioxide in the system. In order to provide "carrier" carbon dioxide for the carbon dioxide liberated from the acid, no attempt was made to free the system of the carbon dioxide and carbonate initially present.

Extraction coefficients $\left(\frac{\text{benzene}}{\text{water}}\right)$ of the decarboxylated material were determined at pH 3.4 and 9.2. The coefficients were found to be about 4000 and 1000, respectively, showing that the material had lost its previous acid character.

If the original hypothesis of a β-keto acid was correct, the unknown compound would now be a methyl ketone which should give iodoform on reacting with hypoiodite. In order to test this supposition, an iodoform test was carried out on a portion of the unknown material following the well-known directions of Shriner and Fuson (23) except that the addition of dioxane was omitted. Eighty mg. of 2-octanone was added as carrier. After the test was carried out, 92% of the theoretical quantity of iodoform (from 80 mg. of 2-octanone) was recovered. The iodoform, however, showed no radioactivity. All of the activity devoted to the test (3000 counts/min.) was accounted for in the liquid remaining after the iodoform was precipitated and washed.

The radioactive material left behind after the iodoform test was distributed between benzene and water at various pH values from 1 to 8. A plot of the data thus obtained is shown in Figure 7. The data show that the iodoform test had, as expected, converted the ketone into a carboxylic acid, since an acidic substance with a pK value of about 5 is clearly indicated.

In a further attempt to gain insight into the nature of the unknown material, $\frac{\text{benzene}}{\text{water}}$ distribution coefficients at pH 1 were determined for 12-keto-octadecanoic acid, 12-hydroxy-octadecanoic acid and cholic acid. These acids were not labeled. The coefficients were determined by carrying out the distribution with a weighed quantity of the acid and titrating the organic phase with standard base. The coefficients were determined to be approximately 25 and 55 for the keto- and hydroxy-octadecanoic acids, respectively. These values are comparable to that (95) obtained for the original, unknown acidic compound. The distribution coefficient for cholic acid was found to be less than 1.

The data gathered on the unknown constituent of the alcohol-soluble fraction are summarized below in the order in which the data were obtained.

1. Eighty percent of the radioactivity in the alcohol-soluble fraction was incorporated in a single acidic compound. Its $\frac{\text{ether}}{\text{water}}$ distribution coefficient at pH 1 was 650; its $\frac{\text{benzene}}{\text{water}}$ distribution coefficient at the same pH was 95.
2. The value of the distribution coefficient together with the non-volatile nature of the compound showed that it could not be a simple fatty acid.
3. The compound apparently contained no unsaturation (Br_2 test).
4. A plot of the change of distribution coefficient with pH showed the presence of two acidic groups - one with a pK value of 5-6 and the other with a pK value of 9-10.
5. Decarboxylation destroyed the acidic character of the compound but the evolved carbon dioxide was not radioactive.

6. An iodoform test carried out on the decarboxylated compound failed to give radioactive iodoform. The radioactive material left after the iodoform test contained one acidic group with a pK value of about 5.

7. $\frac{\text{Benzene}}{\text{water}}$ distribution coefficients at pH 1 for 12-keto-octadecanoic acid and 12-hydroxy-octadecanoic acid were found to be about 25 and 55 respectively. The coefficient for cholic acid was less than 1.

The significance of these experimental results will be discussed in the section following.

D. SUMMARY AND DISCUSSION

The present work on pyruvate metabolism comprises no more than a first attempt to gain insight into the biochemical path of labeled pyruvate in green algae. It does not in any way constitute a complete picture of pyruvate metabolism but is rather to be considered as preliminary experiments. Further effort along these lines will be directed not only toward the identification of more individual radioactive compounds but also toward establishing the rate of appearance of those compounds.

One of the principal hopes entertained at the outset of this work was that sufficient sucrose and hexoses would be obtained from the plants to permit the chemical degradation of the sugars and thus allow us to find the position or positions in the carbon chain at which radioactivity appeared. The scheme at present advanced to explain the path of carbon in photosynthesis is dependent, in part, upon the fact that the radioactivity

in the hexoses appears first in the 3 and 4 positions, then in the 2 and 5 positions and finally in the 1 and 6 positions. The mechanism postulated to account for this fact (17) would require that radioactivity in hexose derived from methyl-labeled pyruvate would appear first in the 1 and 6 positions. Unfortunately, however, no sugar was obtained in the case of the 5-minute dark fixation and not nearly enough was obtained from the 5-minute illumination experiment to permit any degradation. It is quite probable that an increase in the length of time of these experiments would result in the appearance of sufficient quantities of sugars so as to permit us to carry out degradation studies and thus to establish the positions of the C^{14} in the sugar molecule.

No great difference was observed in the amounts of water-soluble carboxylic acid obtained in the light and in the dark, although the amount obtained under illumination was definitely larger than that obtained in the 5-minute dark fixation. These acids are probably mostly oxalacetic, malic, fumaric and succinic acids. The tentative identification of glutamic acid among the radioactive amino acids indicates that the corresponding keto acid, α-ketoglutaric acid, may also be present. No radioactive carboxylic acid containing more than four carbon atoms has been found in the algae after the plants were given labeled carbon dioxide. However, the situation in the case of labeled pyruvate may be different and some of the C^{14} may have been built into larger carboxylic acids, such as α-ketoglutaric acid, through the mechanism of the well-known "tricarboxylic acid cycle" (24).

Alanine and aspartic acid have been established definitely as containing C^{14}. These amino acids certainly are either enzymatic reduction products of their corresponding α-keto acids, pyruvic acid and oxalacetic

acid respectively, or else the plant's reservoirs of these amino acids exchange with the carboxylic acids. As was mentioned above, the glutamic acid probably comes from α-ketoglutaric acid which would arise from pyruvic or oxalacetic acid through the operation of the "tricarboxylic acid cycle." The fact that a much larger percentage of amino acids was obtained in the 5-minute dark fixation (51% of the water-soluble fraction) as compared with that obtained in the 5-minute illumination (15% of the water-soluble fraction) may be ascribed to the plant's having less energy available in the former case for the formation of a variety of compounds. For example, a smaller quantity of radioactive lipid material was formed in the dark (22%) than was formed under illumination (41%). Since the amino acids probably result from an exchange between the plant's amino acid reservoirs and the corresponding α-keto acid, this process played a relatively more important part in the "dark" experiment than it did in the "light" experiment, in which latter case the labeled acids could be more quickly converted into lipids and other materials.

No fractionation was carried out on the phosphorylated compounds obtained in either experiment. However, a greater fraction of these materials was obtained in the "light" experiment than in the "dark" experiment (see Table 3). Again we observe that the plant's ability to effect chemical transformations is greater in the light than in the dark.

The radioactivity which was recovered from the algae as the water-insoluble, alcohol-soluble fraction (from the "light" experiment) consisted, for the most part, of a single acidic compound which is not a simple fatty acid, contains no unsaturation and has two acidic groups with pK values of 5-6 and 9-10. Originally, we were led to the hypothesis that

the compound was a β-keto fatty acid; the enol form of which would account for the second acidic group. This supposition was in accord with all the data except for the failure of the addition of bromine to change the distribution coefficient. This latter fact was discounted on the basis of the extreme dilution of the compound in the solutions with which we were working. However, if we did have a β-keto acid we should expect to find radioactivity, if not in the carboxyl group, at least in the methylene group adjacent to the carboxyl. That this was not the case was shown by the fact that a decarboxylation followed by an iodoform test failed to show activity in either of these groups (if, in fact, the groups were present). Apparently, the iodoform test was carried out on a methyl ketone as no acidic group was present before the test but one was present in the product, other than iodoform, remaining after the test. Distribution coefficients for 12-hydroxy- and 12-keto-octadecanoic acid indicated that the unknown acid could be such a fatty acid with substituted oxygen-containing groups. The distribution coefficient for cholic acid indicated that the unknown acid could not be cholic acid or a similar acid; the hydrogenated cyclopentane-phenanthrene structure appears to be too insoluble in benzene. We can only state that the unknown material is apparently a β-keto acid and that the activity from the labeled pyruvate was probably attached to some of the lipid material already present in the plant. We are without data to indicate the position or positions of C^{14} in the molecule.

The greater percentage of activity appearing in the lipid material under illumination as compared to that appearing in the dark is doubtless due to the availability to the plant of greater reducing power

in the light. Before the plant can use the radioactive pyruvate, it probably must first phosphorylate the labeled compound and then carry it along as an enzyme substrate. In addition, it may be necessary to carry the pyruvate through the dicarboxylic acid cycle to a two-carbon compounds before the activity can be built into fats. The immediate availability of light energy would make it easier for the plant to accomplish these transformations under illumination than would be the case in the dark.

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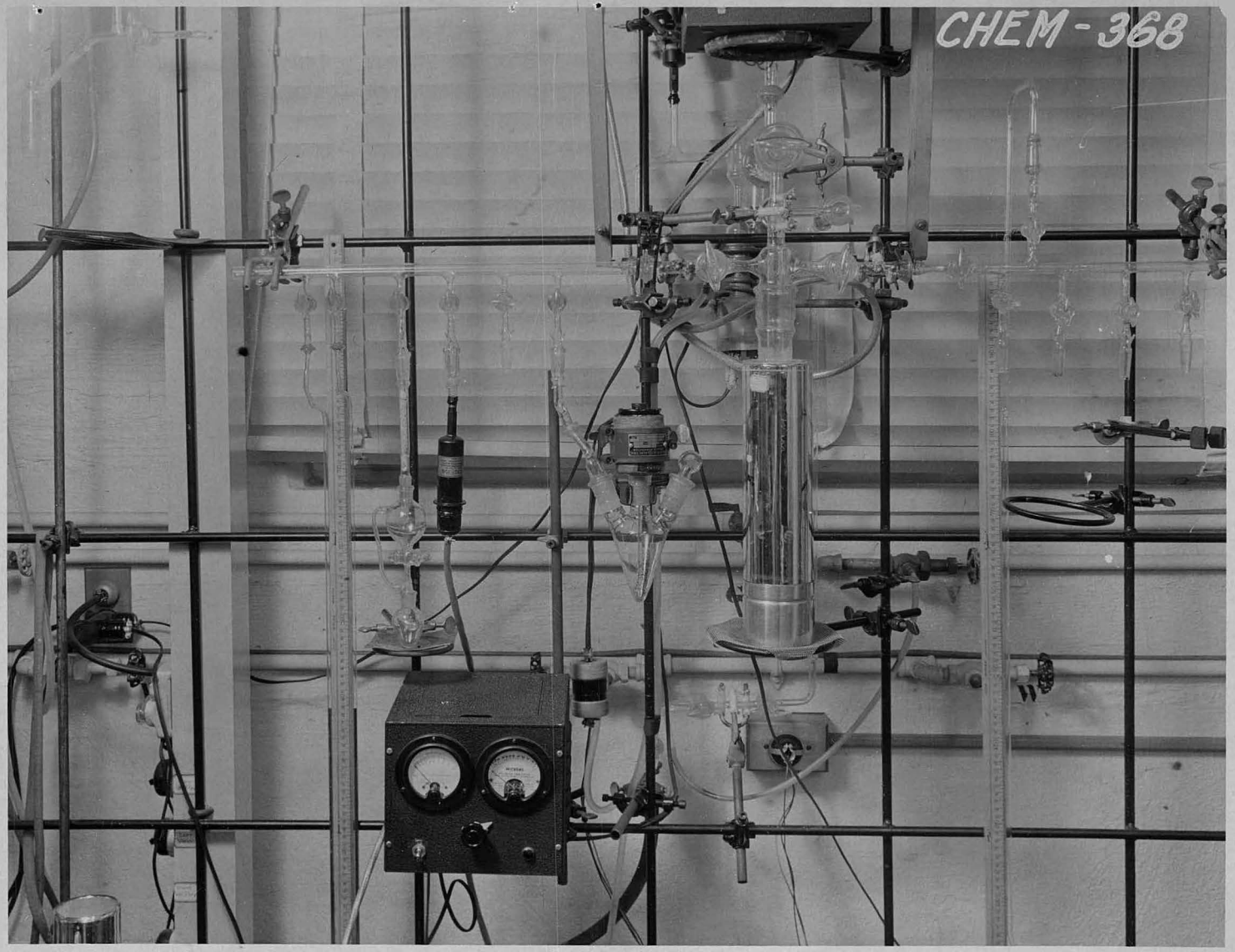

Vacuum-line apparatus for carrying out the Grignard reaction used in the preparation of acetic acid from radioactive carbon dioxide. In the left-center is a small pear-shaped flask and pressure-equalized dropping funnel used for generating radioactive carbon dioxide from the barium carbonate. The rest of the apparatus is that which is shown schematically in Fig. 2.

FIG. 1

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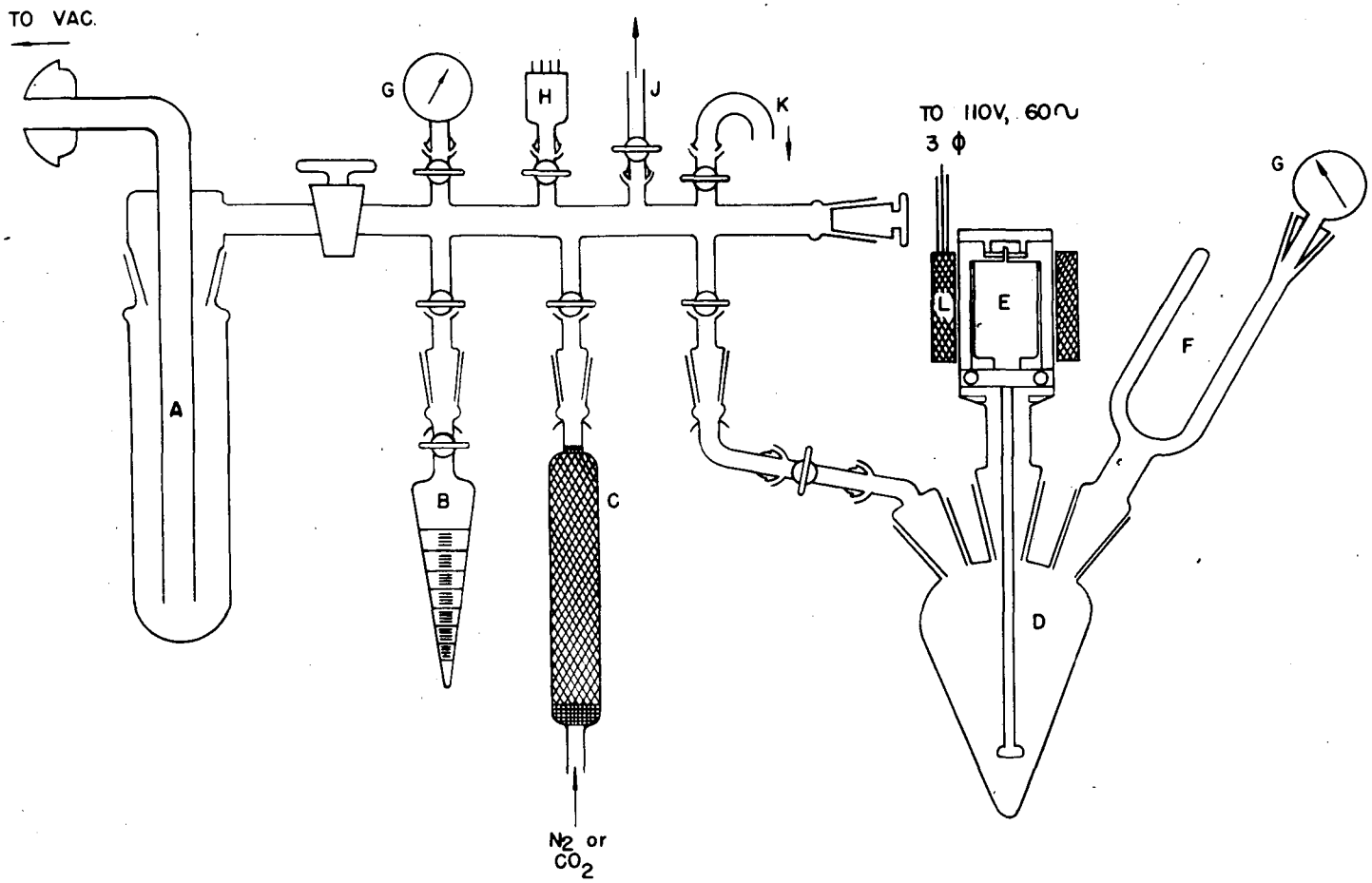

Figure 2

Grignard preparation and carbonation unit. A, large liquid nitrogen trap; B, graduated methyl iodide storage vessel; C, drying tube; D, Grignard flask; E, induction stirrer; F, dry ice-acetone reflux condenser; G, Bourdon type vacuum gages; H, thermocouple type vacuum gages; J, to CO_2 storage bulb; K, mercury manometer; L, Selsyn generator stator.

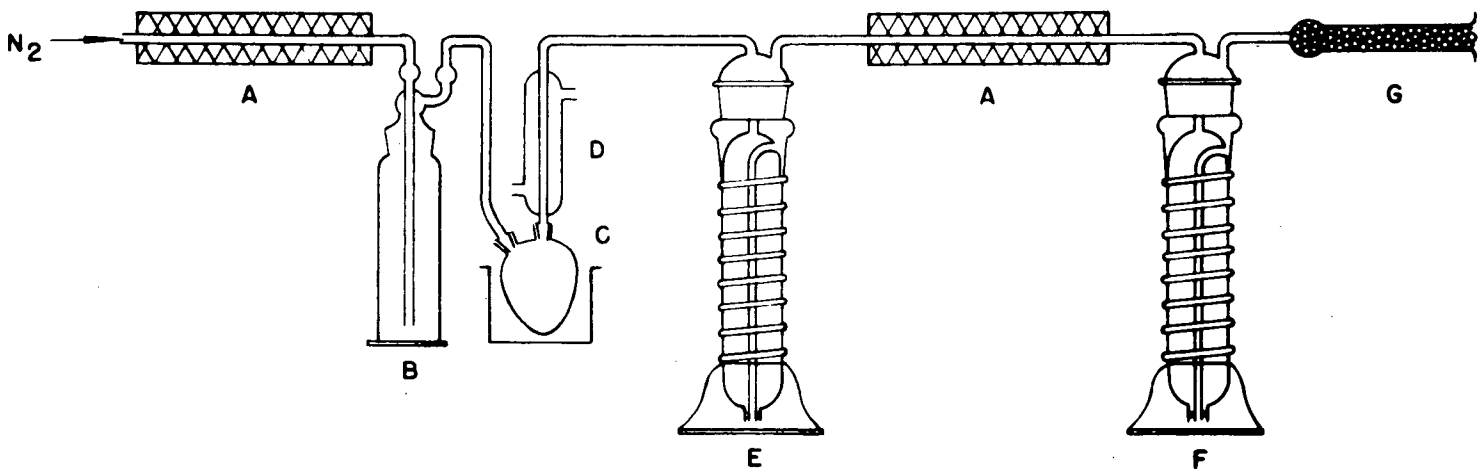

Figure 3

Apparatus used for the thermal decarbonylation of ethyl pyruvate and pyruvic acid. A, copper oxide-combustion furnace; B, gas washing bottle containing sodium hydroxide solution; C, reaction flask surrounded by oil bath; D, reflux condenser; E and F, Friedrichs gas washing bottles; G, soda lime tube.

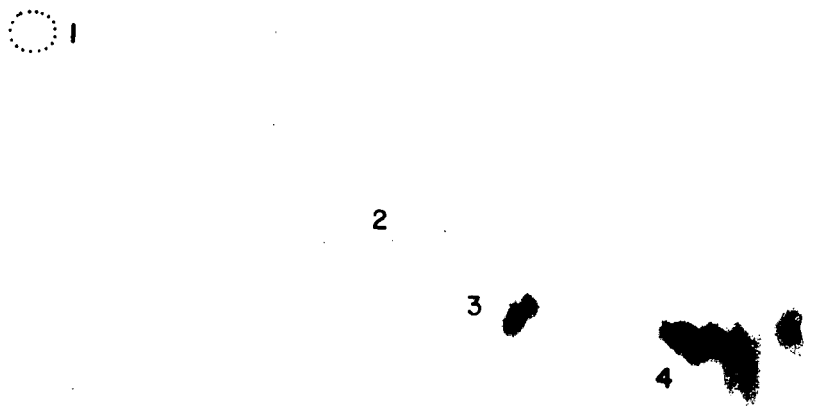

Figure 4
Radioautograph of paper chromatogram of the amino-acid fraction obtained from Scenedesmus D3 after methyl-labeled pyruvate was fed to the algae during five-minute illumination. 1) very small amount of alanine, 2) unknown amino acid, 3) probably glutamic acid, 4) aspartic acid.

Figure 5
Radioautograph of paper chromatogram of the amino-acid fraction obtained from Scenedesmus D_3 after methyl-labeled pyruvate was fed to the algae for five minutes in the dark. 1) very small amount of alanine, 2) probably glutamic acid, 3) aspartic acid.

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