UCRL 2334

UNCLASSIFIED

UNIVERSITY OF CALIFORNIA

Radiation Laboratory

BERKELEY, CALIFORNIA

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

UCRL-2334
Unclassified-Chemistry-Distribution

UNIVERSITY OF CALIFORNIA

Radiation Laboratory

Contract No. W-7405-eng-48

THE DISTRIBUTION OF C^{14} IN THE CARBON ATOMS OF PHOTOSYNTHETICALLY-PRODUCED RIBULOSE

Anne Grace Zweifler
(Thesis)

September, 1953

Berkeley, California

THE DISTRIBUTION OF C^{14} IN THE CARBON ATOMS OF PHOTOSYNTHETICALLY-PRODUCED RIBULOSE

TABLE OF CONTENTS

Abstract. 3

Introduction. 4

Sources of Photosynthetically Produced Ribulose 10

Degradations. 12

A. Periodic Acid Oxidation of Ribulose

B. Periodic Acid Oxidation of Ribulosazone

C. Attempted Degradations of 1,2 Bisphenylhydrazone of Mesoxaldehyde

D. Lead Tetra-acetate Oxidation of Ribulose

E. Periodic Acid Oxidation of Adonitol

F. Cerate Oxidation of Ribulose

Results and Discussion. 29

Appendix I

Bibliography

THE DISTRIBUTION OF C^{14} IN THE CARBON ATOMS OF PHOTOSYNTHETICALLY-PRODUCED RIBULOSE

Anne Grace Zweifler

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

September, 1953

ABSTRACT

Methods have been developed for a complete degradation of ribulose. These methods have been applied to labeled ribulose, formed during a variety of photosynthetic experiments with $C^{14}O_2$. The experiments included both the batch and flow type experiments, experiments performed with different organisms, and experiments of different duration.

The results of the ribulose degradations, in conjunction with sedoheptulose degradations from the same and different photosynthetic experiments, indicate that modifications of the previously proposed photosynthetic cycle may be in order. The changes postulated are: (a) No free diose or tetrose is involved; (b) sedoheptulose is formed from hexose, eliminating the second carboxylation; (c) ribulose is formed both from sedoheptulose and from C_2 plus C_3 combination; (d) ribulose is the carbon dioxide acceptor.

THE DISTRIBUTION OF C^{14} IN THE CARBON ATOMS OF PHOTOSYNTHETICALLY-PRODUCED RIBULOSE

Anne Grace Zweifler

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

September, 1953

INTRODUCTION

The path of carbon in photosynthesis has been studied by allowing a plant (an algae suspension) to undergo photosynthesis in what approaches an idealized steady state as closely as possible, given $C^{14}O_2$ from a time t = 0, and analyzing the products with respect to the distribution of radioactivity among the compounds, and among the carbon atoms of each compound, after suitable periods of time.[1] Although the idealized steady state experiment in which the mass concentration of each compound remains the same has not been achieved, due to external variables such as light intensity and CO_2 pressure, as well as variables intrinsic to the algae culture itself, considerable progress has been made recently in this respect by controlling fluctuations in CO_2 pressure prior to and during a run. Assuming ideal conditions, if the rate of appearance of radioactivity in each of the various compounds is plotted against time, only those compounds which have no appreciable stable reservoirs lying between them and the $C^{14}O_2$ should show a finite slope at zero time. All others must have an initial zero slope, since the reservoirs lying between them and the $C^{14}O_2$ must first become labeled. Similarly, the precursor-product relationship can be

established by plotting the percentage of activity of each compound in a particular group of compounds, with respect to the total amount of radioactivity in the group of compounds, against time. The earliest labeled compounds in the group will have negative slopes and extrapolate to a finite value at t = 0, whereas all the others will have positive slopes that approach zero at t = 0. From degradation data of the compounds involved, it is possible to trace the path of labeled carbon not only through successive compounds, but through the atomic positions of those compounds. It can then be determined just where the label first enters each compound by degrading compounds from shorter and shorter photosynthetic experiments, and extrapolating to zero time. It is theoretically possible to test the validity of a proposed photosynthetic sequence by a comparison of the actual appearance and distribution curves with those calculated for the proposed sequence. These curves may be calculated by setting up a system of linear differential equations for the rate of change of the specific activity of each atom of every compound in a particular model. The equations may be solved explicitly by means of a differential analyzer provided that the total rate of entry of carbon into the system, and the steady-state concentration of each atom are known. Although these calculated curves are of interest, experimental data concerning steady-state concentrations, as well as experimental appearance and distribution curves, are not sufficiently refined to make strict mathematical treatment useful.

In very short photosynthetic experiments, phosphoglyceric acid was found to contain most of the radioactivity. Since its appearance curve has a finite slope at very short times, it is probable that there

is no stable reservoir between it and CO_2. Upon degradation, it was found that the carboxyl group is first to become labeled, followed by the α- and β- carbons, which are equally labeled. This evidence led to the hypothesis that CO_2 reacts with a two-carbon compound to give phosphoglyceric acid. When the hexose, obtained from short-term sucrose was degraded, it was found that the distribution of radioactivity in this molecule was similar to the distribution in the phosphoglyceric acid. That is, most of the activity was found in carbons 3 and 4, with the rest distributed equally between 1 and 6, and 2 and 5. This led to the suggestion that the hexose was made indirectly from the phosphoglyceric acid by a process similar to the reversal of the well-known glycolytic sequence. There is now considerable evidence to support this theory.

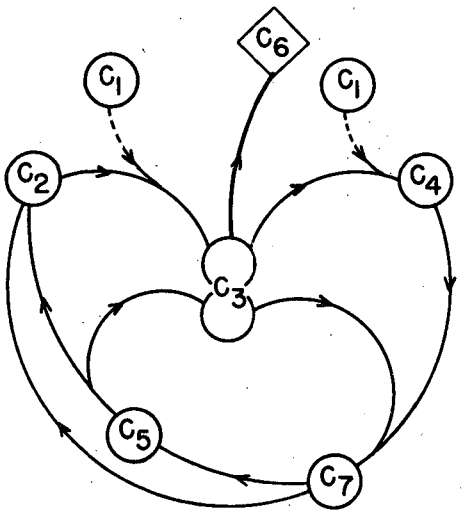
There was still to determine the nature of the two-carbon acceptor, as well as the sequence of reactions that leads to its regeneration. To date, only two compounds containing two carbon atoms have been found, namely, glycine and glycolic acid. At short times, the distribution of radioactivity in the two carbon atoms of glycolic acid corresponds to the distribution of radioactivity in the α- and β- carbon atoms of glyceric acid. Feeding experiments with α-labeled glycolic acid resulted in glyceric acid which was equally labeled in the α- and β- carbon atoms, with little activity in the carboxyl group. With carboxyl-labeled glycolic, the glyceric acid was again equally labeled in the α- and β- carbon, although there was considerable activity in the carboxyl carbon, presumably due to partial oxidation of the glycolic acid to radioactive CO_2. The data

above indicate that glycolic acid is somehow related to the C_2 CO_2 acceptor, either on a direct line or as a side product, the C_2 acceptor being a symmetrical molecule. This C_2 compound can only arise from the combination of two one-carbon compounds, or from the degradation of a molecule containing four or more carbon atoms. It is unlikely that it arises from a one plus one combination, since all attempts to find a one-carbon compound more reduced than CO_2, such as formic acid or formaldehyde, which is labeled in the early stages of photosynthesis, have failed. Also, the amount of radioactivity in glycolic acid is increased under conditions of low CO_2 concentrations. If glycolic acid is related to the two-carbon CO_2 acceptor, one plus one combination would mean a decrease in the amount of radioactive glycolic acid under these conditions. It seems likely, then, that the C_2 compound is formed from the splitting of a larger molecule. The following data, presented here in only the briefest form, is part of the evidence which led to a postulated sequence for the regeneration of the C_2 acceptor.

Malic acid is rapidly labeled, and its slope seems to indicate that it, too, is a primary carboxylation product. Malonate inhibition experiments, however, indicate that malic acid is not directly involved as an intermediate in the C_2 regenerative cycle. Nevertheless, it is still possible that malic acid is in rapid equilibrium with a compound arising from a second carboxylation, the carboxylation of a C_3 fragment. Among the earliest sugar phosphates formed during photosynthesis are sedoheptulose phosphate and ribulose phosphate.[2] They appear to be present in all plants, and have small pools which are rapidly saturated with labeled carbon. While sedoheptulose is sterochemically unrelated to glucose by a simple sequence of reactions, it is related to

ribulose, the configuration of carbons 3 and 4 of ribulose being identical with that of carbons 5 and 6 of sedoheptulose. There is evidence that ribulose phosphate and sedoheptulose phosphate are enzymically interconvertible, and that ribose phosphate and ribulose phosphate can undergo scission to give C_2 and C_3 compounds. The rise in phosphoglyceric acid and the decrease in ribulose and sedoheptulose phosphates when the light is shut off is a further indication that the C_5 and C_7 sugars are precursors of the CO_2 acceptor.[3] The sedoheptulose itself could arise by an aldolase reaction between triose phosphate and tetrose, (presumably erythrose, produce of reduction of a secondary carboxylation product) analogous to the formation of fructose from two C_3 fragments. Kinetic studies which show that sedoheptulose and fructose appear to be labeled very nearly simultaneously (suggesting that they are formed in parallel reactions from the same precursor) are in accord with this hypothesis.[4]

Up until the present time, the ideas concerning the regenerative cycle for the C_2 CO_2 acceptor could be summarized as in Figure 1. The ribulose degradations to be described here, along with the sedoheptulose degradations performed in this laboratory by Mrs. Lorel L. Kay, were undertaken in order to more definitely establish this cycle. From the results, as will be discussed later, it seems possible that modifications may be in order.



PROPOSED CARBON CYCLE FOR REGENERATION OF TWO-CARBON CO_2 ACCEPTOR

Figure 1

SOURCES OF PHOTOSYNTHETICALLY PRODUCED RIBULOSE

In general, the ribulose that was degraded was isolated from two types of experiments, the usual photosynthetic experiment, and a flow-system* or so-called "steady-state" experiment. In the usual experiment, air is bubbled through an algae suspension in a flat vessel illuminated from both sides. At the beginning of the experiment, the air is discontinued, radioactive bicarbonate is introduced,, and the vessel agitated. After a suitable period of time, the algae are killed by draining the suspension into boiling 80% alcohol. The flow experiment was designed to eliminate the sudden change in CO_2 concentration when the radioactivity is introduced, as well as variations during the run. An algae suspension, continuously aerated with a gas mixture of 4% CO_2 in air is forced through several hundred centimeters of an illuminated tube. It takes approximately 15-20 seconds for the algae to traverse the tube. A stream of $C^{14}O_2$, dissolved in water at pH 6, is injected into this tube at various points of known distance from the end, the stream of radioactivity entering at a constant rate, regardless of the point of injection. The amount of radioactive carbon is negligible compared to the total amount of carbon present. At the end of the tube, the algae flow into 80% alcohol, as in the previous experiment.

---

* This experiment was designed and performed by Dr. James A. Bassham.

80% and 20% alcohol extracts of the algae from both types of experiments were analyzed by means of paper chromatography and radioautographs.[5] The ribulose diphosphate areas on the chromatograms of the original extracts were eluted with water, concentrated to about 50 λ, and hydrolyzed with approximately 200 γ of Polidase in 10 λ of water for two days at 35°C. The products were rechromatographed, and the free radioactive ribulose located on radioautographs. The eluates from the radioactive ribulose areas were used in the following degradations.

DEGRADATIONS

A. Periodic Acid Oxidation of Ribulose

Periodic acid oxidation is applicable to compounds having two hydroxyl groups attached to adjacent carbon atoms, and results in the cleavage of the carbon-carbon bond. Carbonyl compounds in which the carbonyl group is adjacent to a second carbonyl or hydroxyl group are oxidized also. Although this oxidation is straightforward in the case of simple aldoses, in which the molecule is completely degraded, the reaction can follow two courses in the case of ketoses.

(1) $CH_2OH \cdot CO \cdot (CHOH)_n \cdot CH_2OH + (n + 1)HIO_4 \longrightarrow$

$CH_2OH \cdot COOH + nHCOOH + HCHO + (n + 1)HIO_3$ glycolic acid

(2) a) $CH_2OH \cdot CO \cdot (CHOH)_n \cdot CH_2OH + (n + 1)HIO_4 \longrightarrow$

$CHO \cdot COOH + (n - 1)HCOOH + 2HCHO + H_2O + (n + 1)HIO_3$

glyoxylic acid

b) $CHO \cdot COOH + HIO_4 \xrightarrow{\text{Slow}} CO_2 + HCOOH + HIO_3$

It is assumed[6] that the glycolic acid is formed from the splitting of the first two carbon atoms of the semiacetal form of the ketose, and glyoxylic acid from the splitting of the carbonyl carboxyl group and the neighboring primary alcohol group. An attempt[7] was made, using uniformly labeled 1-6 C^{14} fructose to find conditions in which reaction (1) took place almost exclusively. The amount of radioactivity recovered in CO_2 was a measure of the extent of reaction (2). The best conditions

found were an acidic medium (iodic acid was added to the reaction mixture) and a reaction time of three hours. Under these conditions, 2.4% of the total activity was recovered in CO_2, equivalent to 14.4% of the fructose having been degraded according to equation (2). Since it is known, however[6], that reaction (2b) is slow, it is possible that more than 14.4% of the fructose reacted according to equation (2a), and that the reaction described in equation (2b) had not reached completion in three hours. In one experiment, iodic acid was added to the reaction mixture and the reaction allowed to proceed overnight. In this case, 9.5% of the total activity was found in CO_2. Assuming the reaction (2b) is the only source of CO_2, 56.9% of the fructose was degraded according to reaction (2). With a long reaction time, however, over oxidation of other degradation products might have occurred. Although periodic acid does not further degrade glycolic acid, lead tetra-acetate oxidizes it to formaldehyde and CO_2. When this oxidation was performed on the glycolic acid resulting from reaction (1), the radioactivity in the CO_2 was appreciably lower than the theoretical amount.

In applying the periodic acid followed by lead tetra-acetate oxidations to radioactive ribulose in tracer quantities, several other difficulties presented themselves. Since a pure, standard ribulose solution was unavailable for use as carrier, carrier formic acid, formaldehyde and glycolic acid were employed. This involved the assumption that the carrier compounds were not affected in the course of the reaction, and that the reaction proceeded on a microscopic scale in exactly the same way as it would on a macroscopic scale. Furthermore, any contamination of the ribulose with ribose, two compounds which are difficultly separable chromatographically, would lead to an erroneous result.

B. Periodic Acid Oxidation of Ribulosazone

Periodic acid degradation of the osazone was an improvement over that of the free sugar in several ways. In the first place, by making this derivative, there was no longer the possibility of two competing reactions. In addition, D- ribose or D- arabinose, which react with phenylhydrazine to give the same osazone as ribulose, could be used for carrier. Contamination of the radioactive ribulose with ribose could cause no error, except in the event that the two compounds were labeled differently.

Preparation of the Osazone

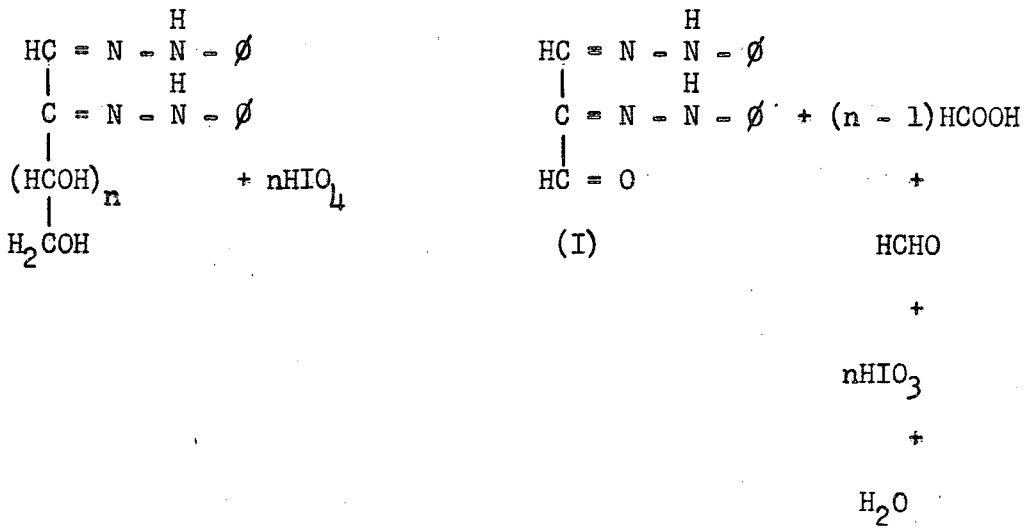
The osazone was prepared according to the procedure of Hastkins, Hann and Hudson[8], with the necessary adaptations for small scale synthesis. To the eluate from a radioactive ribulose spot were added in a small test tube 10 mgm. of arabinose, 13 λ of acetic acid, 40 λ of methyl cellosolve and 26 λ of phenylhydrazine, and the mixture heated on the steam bath for one hour. One milliliter of cold water was then added, and the osazone separated as a voluminous yellow precipitate. This precipitate was collected by centrifugation followed by decantation of the supernatant liquid. The osazone was washed with two 25 λ portions of 10% acetic acid and four 50 λ portions of water. It was then dissolved in 50 λ of hot absolute alcohol. Upon cooling, the osazone crystallized out in 53% yield. This radioactive osazone was diluted, as desired for each degradation, with pure crystalline, non-radioactive arabinosazone, the supply of which was prepared similarly, but on a large scale.

Degradation of the Osazone

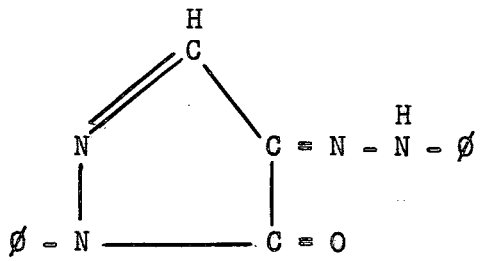
The osazone was oxidized according to the method of Chargaff and

Magasanik[9], as modified by Topper and Hastings.[10] Chargaff and Magasanik described the action of periodic acid on glucosazone at room temperature. This reaction yields the 1,2 bisphenylhydrazone of mesoxaldehyde (I) as illustrated in equation (3).

(3)



On the other hand, more vigorous oxidation in boiling periodic acid and alcohol yields the often reported 1-phenyl-4phenylhydrazone pyrazalons-5 (II) m.p. 149-50°.



(II)

The oxidation, as described by Chargaff, was carried out in acid solution. However, Topper and Hastings, noting that in alkaline solution

formaldehyde is obtained quantitatively from glucose[11], carried out the osazone degradation under similar conditions in order to insure a quantitative yield of formaldehyde.

17 mgm. of arabinosazone (0.05 millimoles) were dissolved by warming in 6 ml. of 66% alcohol and 500 λ of 1 N sodium bicarbonate, and the solution cooled to 30°. 200 λ of 1 N paraperiodic acid (0.10 millimoles) were introduced and an orange-yellow precipitate of (I) formed immediately. After 15 minutes, the mixture was centrifuged, and the centrifugate washed several times with 66% ethanol. The precipitate, after being recrystallized from 66% ethanol was counted as such. The percentage of activity in carbon atoms 1, 2 and 3 can be calculated from the specific activity and the theoretical yield. The supernate and washings were distilled to dryness *in vacuo*. To the distillate, which contained the formaldehyde, were added 35 mgm. of dimedon reagent (dimethylhydroresorcin) dissolved in a ml. of ethanol, and a drop of piperidine. After warming the mixture for 10 minutes on the steam bath, 500 λ of glacial acetic acid were added. The formaldimedon that precipitated upon standing was recrystallized from an ethanol-water mixture and its specific activity measured. From this, the activity in carbon atom 5 can be determined.

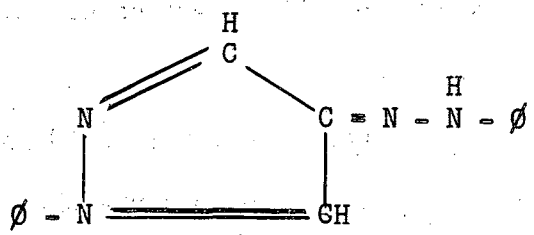
The residue from the previous distillation contained sodium formate, sodium bicarbonate and sodium iodate. This residue was dissolved in 5 ml. of water and then 100 mgm. of iodic acid were added. The solution was distilled to dryness *in vacuo*. The formic acid in the distillate was neutralized with barium hydroxide to a phenolphthalein end point, and after evaporation on the steam bath to 1 ml.,

the barium formate was precipitated by the addition of absolute alcohol. The salt was recrystallized several times from water by the addition of alcohol, and counted. From its specific activity the percentage of ribulose activity in carbon atom 4 can be calculated.

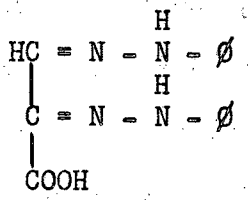
C. Attempted Degradations of the 1,2 Bisphenylhydrazone of Mesoxaldehyde

Aronoff and Vernon[12] reported that the 1,2 bisphenylhydrazone of mesoxaldehyde (I) resulting from the periodic acid oxidation of glucosazone could be further oxidized to glyoxalosazone and CO_2 by a reaction described by Diels.[13] Several attempts were made in this laboratory* to repeat the work described by Aronoff and Vernon, by following the procedure used by Diels for the oxidation of glucosazone to glyoxalosazone. Upon oxidation for five hours in potassium hydroxide (1% in absolute ethanol), the only reaction product of (I) which could be isolated was the 1-phenyl-4phenylhydrazone pyrazole, m.p. 125 (III), a ring closure, and not a degradation product. An earlier attempt by Vittorio, Krotkov and Reed[14] to oxidize (I) to glyoxalosazone yielded an unidentified compound with a melting point of 123-125°, presumably the same pyrazole.

Since (I) was easily oxidized by iodic acid in boiling alcohol, or by silver nitrate in dilute potassium hydroxide[9], to the pyrazolone (II) and not to the 1,2-bisphenylhydrazone of mesoxalic acid (IV),



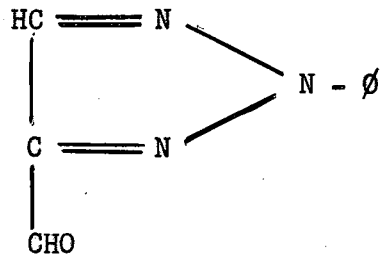
(III)



(IV)

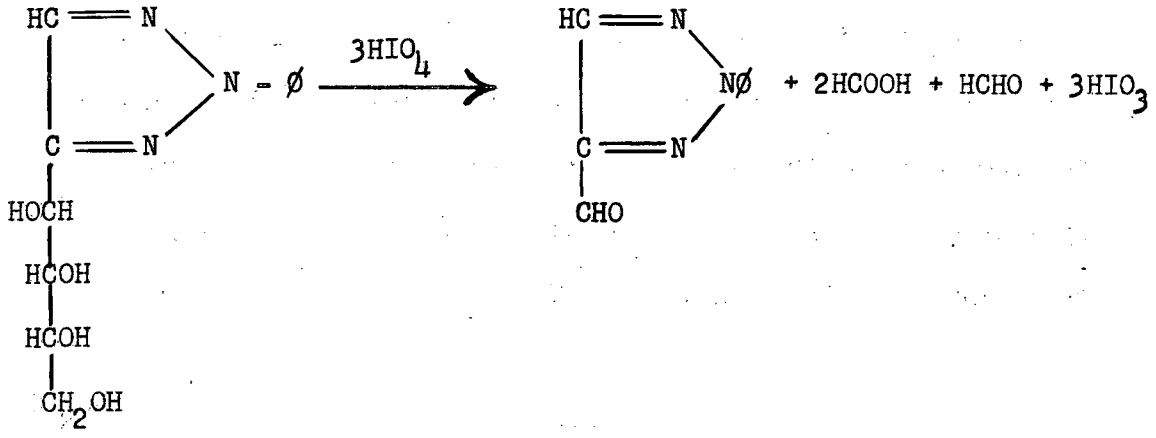
* Attempts to repeat Aronoff's work were made both by Mrs. Lorel L. Kay and the author.

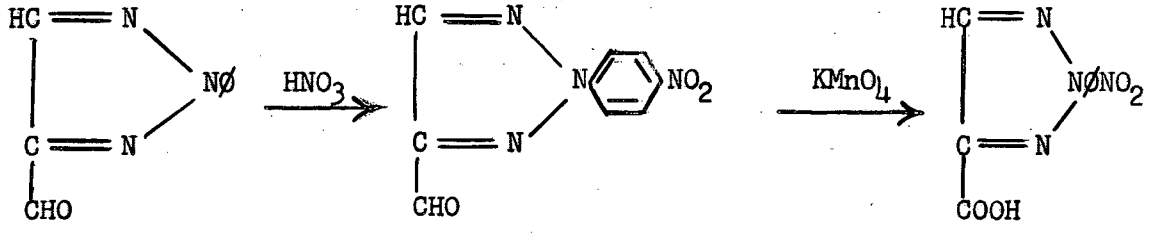
it was decided that in order to degrade the aldehyde by oxidation and decarboxylation, the opportunity for ring closure to the pyrazolone must first be removed. For this purpose, an attempt was made to convert the mesoxaldehyde osazone to an osatriazole (V) by the action of copper sulfate according to Hann and Hudson[15]. This reaction yielded



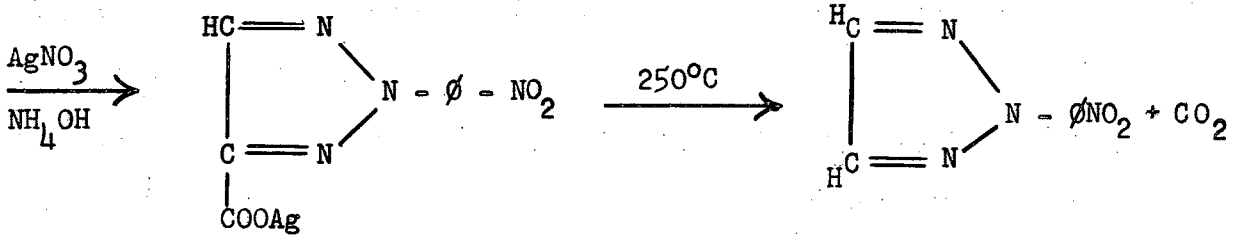
(V)

the same pyrazole (III) as that resulting from the Diels reaction. The alternative procedure of preparing the osatriazole from arabinosazone[8] before the periodic acid oxidation was rejected because of the difficulty of isolating crystalline arabinosatriazole on the small scale necessary for the degradations. A degradation for glucose based upon osatriazole formation has been accomplished by Bishop.[16] The scheme of the degradation is as follows:





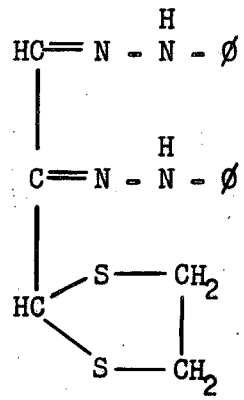
m.p. 69° - 70° m.p. 136° - 137°



If Hudson's synthesis of arabinosatriazole could be adapted to small scale synthesis, Bishop's degradation would lead to a direct determination of the activity in carbon atom 3.

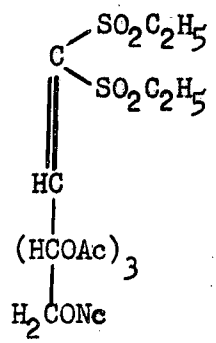
A second attempt to further degrade (I) was made by blocking the carbonyl group, and thus preventing ring closure. Accordingly, the

cyclic thioacetal (VI) of the mesoxaldehyde osazone and ethanediol was prepared and characterized, according to methods given in Appendix I. Attempts were then made to degrade the thioacetal (VI), both by conversion to a disulfone and by hydrolysis of the phenylhydrazone groups.

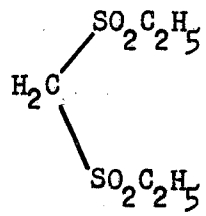


(VI)

D. E. McDonald and H.O.L. Fischer[17] have degraded glucose by oxidation of glucose diethylmercaptal penta-acetate with monoperphthalic acid to the disulfone, D-arabo-3,4,5,6-tetra-acetoxy-1,1-bis(ethansulfonyl)-hexene-1, (VII). This was hydrolyzed in hydrazine hydrate to give arabinose

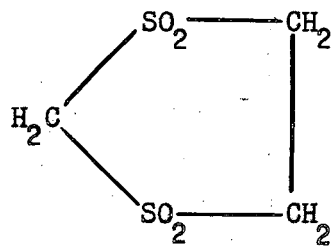


(VII)



(VIII)

tetra-acetate and the disulfone (VIII). If the cyclic thioacetal (VI) could be oxidized in a manner similar to the procedure of McDonald and Fischer, ethylene methylene disulfone (IX), m.p. 204-205°, would be the expected product, along with the oxidation products of the phenylhydrazone groups. In order to learn more about the properties of (IX) so as to



(IX)

facilitate its isolation from an oxidation mixture, (IX) was synthesized[18] by condensation of formaldehyde and ethandithiol, followed by oxidation of the resulting thioacetal in hydrogen peroxide and glacial acetic acid above 50°C. The disulfone (IX) was a white crystalline substance, difficultly soluble in cold water, alcohol, and ether, somewhat soluble in hot water. It dissolved in 1 N sodium hydroxide, but was not recovered upon acidification. It did not dissolve in sodium carbonate or sodium bicarbonate.

When the cyclic thioacetal (VI) was oxidized with monoperphthalic acid, a tarry black gum was produced. The hot water extracts of the gum before and after treatment with hydrazine hydrate precipitated nothing upon cooling except a small amount of tar. This tar was easily adsorbed upon charcoal. When the now clear water extracts were evaporated to dryness, there was no residue at all. Similar results

were obtained when the oxidation was repeated with perpropionic acid.

In the belief that more vigorous oxidation was necessary to produce the disulfone (IX) from the thioacetal (VI), (VI) was oxidized in a large excess of glacial acetic acid and hydrogen peroxide by heating on a steam bath, in an analogous manner to the successful oxidation of the formladehyde thioacetal to the disulfone (IX). In this oxidation (VI) dissolved rapidly upon heating, forming a dark reddish-brown solution. After 15 minutes on the steam bath the color of the solution began to fade, and after an hour, it was a pale yellow. It was then cooled and distilled to dryness <u>in vacuo</u>. The residue consisted of a white crystalline substance and a light yellow syrup. The crystals were separated from the syrup by washing with cold water, in which the syrup was soluble but the crystals were not. The crystalline material was then recrystallized from hot water. This compound, however, contained no sulfur, and was presumed to be an oxidation product of the phenyl groups. The syrup did contain sulfur, and a sulfur-containing barium salt could be precipitated from its water solution. It was thus evident that although the disulfone (IX) was prepared in a similar manner to the present experiment, in this case a sulfonic acid and not a disulfone was produced. Since the possibility that each mole of sulfonic acid contained a carbon atom from the original sugar molecule was small (i.e. only $OH-CH_2-CH_2SO_2CH_2SO_2H$ and not $HSO_2-CH_2-CH_2SO_2H$ and other sulfonic acids were formed), the reaction was abandoned as a degradative method.

An attempt was made to synthesize the diethylmercaptal of the 1,2-bisphenylhydrazone of the mesoxaldehyde, in hopes that the non-cyclic disulfone (VIII) would be more readily isolated than the corresponding cyclic disulfone (IX). However, when the method used for synthesizing the cyclic thioacetal (VI) was applied to the condensation of (I) and ethyl mercaptan, the only product isolated was the pyrazole (III).

It was apparent that much of the difficulty encountered in this oxidation of the cyclic acetal (VI) was caused by the presence of the phenylhydrazone groups. For this reason, it was attempted to hydrolyze the phenylhydrazone groups with hydrochloric acid[19], a reagent used in the preparation of sugar osones from osazones, and toward which thioacetals are stable. An attempt with acetaldehyde[20], a mild hydrazone splitting reagent, was also made. After treatment with either cold concentrated hydrochloric acid or acetaldehyde, all of compound (VI) was recovered unchanged.

D. Lead Tetra-acetate Oxidation of Ribulose

The bond between two hydroxy-carrying carbon atoms can be broken by lead tetra-acetate, as well as by periodic acid, the resulting carbonyl products being the same in both cases. There are, however, several differences between the two oxidizing agents. Periodic acid oxidations are usually carried out in aqueous solution. Lead tetra-acetate is hydrolyzed by water, and is best adapted to organic solvents such as glacial acetic acid. Oxidation with lead tetra-acetate can be carried out in the presence of water, provided that the oxidative

rate exceeds its hydrolytic rate. Lead tetra-acetate readily oxidizes α-hydroxy acids, whereas periodic acid reacts only slowly with α-hydroxy acids, even at elevated temperature. Formic acid is not oxidized by periodic acid at room temperature, whereas with lead tetra-acetate in aqueous media, formic acid is oxidized to carbon dioxide.

Thus, the oxidation of ketoses with lead tetra-acetate must be in accord with the single reaction

$$(4) \qquad CH_2OH{\bullet}CO{\bullet}(CHOH)_n{\bullet}CH_2OH + (n+1)H_2O - (4n+4)e^- \longrightarrow$$

$$2HCHO + (n+1)CO_2 + (4n+4)H^+$$

as opposed to the two competing reactions resulting from the oxidation of a ketose with periodic acid. Lead tetra-acetate oxidation of ketoses results in 2 moles of formaldehyde from the primary alcohol groups, and carbon dioxide from the other carbon atoms.

When a tracer sample of radioactive ribulose (from a 10 minute photosynthetic experiment) was oxidized with lead tetra-acetate in an acetic acid-water mixture at 70°C., using glucose carrier, 16% of the total radioactivity was recovered in the formaldehyde. Since it was known from a previous osazone degradation on this 10 minute ribulose sample that 16% of the total activity was in carbon atom 5, it appeared that only one of the primary alcohol groups was being oxidized to formaldehyde. It is possible that at the elevated temperature used, epimerization to ribose had occurred. Also, Rapoport[21] reports that lead tetra-acetate in acetic acid and water liberates aldehydic material

at elevated temperatures. Since calculations are based upon specific activity and theoretical yield, extraneous aldehyde would make the recovery of activity in the formaldehyde appear to be lower. The reaction was not tried at the more usual 40°C.[22]

E. Periodic Acid Oxidation of Adonitol (Ribitol)

When it appeared that ribulose was unstable in acetic acid at elevated temperatures, a more dependable determination of carbon atoms 1 and 5 was undertaken. The radioactive ribulose was hydrogenated to an alcohol and then oxidized with periodic acid at room temperature to formaldehyde and formic acid.

The eluate for a radioactive ribulose spot from a chromatogram of a photosynthetic experiment was hydrogenated in a small bomb with a few mgms. of platinum oxide and 25 γ of ribose at 2000 lbs. of hydrogen and 100°C. for 15 hours. The ribose was added to cut down the adsorption of radioactivity on the catalyst. The resulting solution was co-chromatographed with 100 γ of adonitol, and the chromatogram sprayed with Tollens reagent. The single resulting spot coincided perfectly with the radioactivity as shown by the single spot on the radioautograph.

A radioactive adonitol sample, prepared according to the procedure outlined above, was diluted with 20 mgm. of readily available adonitol carrier, and 700 λ of 1 N paraperiodic acid were added. After nine hours at room temperature, the solution was distilled to dryness *in vacuo*, and the distillate titrated to a phenolphthalein end point with barium hydroxide. This solution was now distilled to dryness *in vacuo*, and the residual barium formate recrystallized from water

by the addition of alcohol. To the distillate were added 10 ml. of dimedon reagent (10 mgm. of dimethylhydroresorcin per ml. of water adjusted to pH 6). Upon acidification flocculent formaldimedon precipitated. From the specific activity of the formaldimedon and the theoretical yield of formaldehyde, the radioactivity in carbon atoms 1 and 5 can be calculated. A similar calculation with barium formate results in the radioactivity in carbon atoms 2, 3 and 4.

F. Cerate Oxidation of Ribulose

A relatively new procedure for the oxidation of organix compounds using perchloric acid solutions of the perchlorato-cerate ion has been developed, as described in G. Frederick Smith's "Cerate Oxidimetry".[23]

In general, only 1,2 oxygen containing compounds are readily oxidized. Formaldehyde, which is rapidly hydrated and oxidized to formic acid, is an exception. Formic acid is not appreciably oxidized under conditions for the usual oxidation. Accordingly, the oxidation of a ketose molecule is as follows:

(5) $$CH_2OH{\cdot}CO{\cdot}(CHOH)_n{\cdot}CH_2OH + (n+3)H_2O - (2n+8)e^- \longrightarrow$$

$$(n+2)HCOOH + (2n+8)H^+ + CO_2$$

Smith reports that samples of pure glucose were oxidized using a 25% excess of perchlorato-cerate ion in 4 molar perchloric acid at reaction temperatures of 45°C. for 15 minutes and 26°C. for 45 minutes. The amount of glucose was determined by the amount of oxidizing agent

used. At 45° the results were somewhat high (approximately 2%). Smith postulates that since formic acid is not appreciably oxidized under these conditions, a side reaction, such as the oxidation of the aldehyde to a carboxyl* with subsequent oxidation to carbon dioxide might have taken place due to the elevated temperature. At 26° the reaction was quantitative. Very accurate results were also obtained for sucorse at the lower temperature.

For purposes of the present degradation, the carbon dioxide resulting from the oxidation of a fructose sample was collected by means of a nitrogen sweep into carbonate-free sodium hydroxide. No attempt was made to isolate the other degradation product, formic acid, from the reaction mixture.

Uniformly labeled 1-6 C^{14} fructose, and 30.9 mgms. of fructose carrier were dissolved in 3 ml. of water. 5.8 ml. of 0.5 M perchloratoceric acid in 6 M perchloric acid were added. The reaction vessel was kept at 24° and swept with nitrogen into carbonate-free base. After one hour, the base was buffered with ammonium chloride, and barium chloride was added. The resulting barium carbonate was filtered, washed with water and dried. 38.6 mgms. of barium carbonate were recovered as opposed to a theoretical yield of 33.6 mgms. Since it is mechanically difficult to exclude all atmospheric carbon dioxide, the results were taken as evidence that no appreciable side reaction had occurred. The percentage of total activity in the carbon dioxide, based upon the specific activity and total recovery of barium carbonate, was 17.5%.

---

* On p. 108 of "Cerate Oxidimetry" the following statement appears: "Such a side reaction as the oxidation of the aldehyde to a carbonyl...". It is presumed that the author meant carboxyl.

This is within experimental error of the theoretical value, 16.7%. When the reaction was repeated with 1,6 C^{14} fructose, less than 1% of the total activity was recovered in the barium carbonate. This is direct evidence that contamination of the carbon dioxide, at least from carbons 1 and 6, is negligible.

In applying this reaction to the oxidation of tracer quantities of a radioactive ribulose sample, difficulties similar to those involved in the periodic acid oxidation of ribulose were encountered. Since ribulose carrier was not available, carrier fructose was employed. It was assumed that the oxidation of ribulose proceeded in exactly the same way as the oxidation of fructose, although this was never shown by the oxidation of a macroscopic ribulose sample. Instead, a radioactive ribulose sample from a 10 minute photosynthetic experiment, was oxidized with fructose carrier as described above. Previous degradations of this sample (periodic acid oxidations of the osazone and alcohol) showed that the distribution of C^{14} in the molecule approached uniform labeling. The carbon dioxide in the present experiment contained 17.1% of the total activity. If the molecule were uniformly labeled, carbon number 2 would contain 20% of the total activity. It thus appeared that the reaction was proceeding as predicted.

RESULTS AND DISCUSSION

Comparison of ribulose and sedoheptulose degradations from the same experiment (chart 3) show that there is not a direct correspondence in the distribution of radioactivity between the ribulose and any five consecutive carbon atoms of the sedoheptulose. Therefore, it is apparent that ribulose is not formed uniquely from sedoheptulose by a transketolase reaction. For a similar reason, ribulose cannot be formed from the hexose through oxidation to gluconic acid. However, if ribulose is formed both from sedoheptulose and from the combination of C_2 and C_3 fragments, a radioactive distribution similar to the observed distribution can result for two of the experiments in chart 3. The exception is the five second non-steady state experiment, in which carbons 3 and 5 of the sedoheptulose contain approximately twice as much activity as carbon 4. In this experiment, even if a C_2 plus and C_3 combination does contribute to the ribulose formation, the inequality in labeling of carbons 3 and 4 of sedoheptulose should show up in an inequality between carbons 1 and 2 of ribulose. The observed fact is that carbons 1 and 2 of the ribulose are labeled equally. A possible explanation may lie in the nature of this five second experiment. It was performed as a stockpile experiment, in which many consecutive batches of algae were allowed to photosynthesize with $C^{14}O_2$ for five seconds and the extracts of all the batches combined. Since the algae cultures were of different ages, having different histories (some were used right after harvesting while others were in aqueous suspension without nutrient for relatively long periods of time) it cannot be

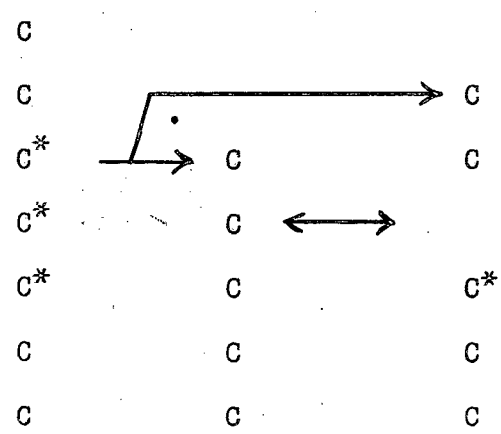
assumed that all the experiments that were combined were equivalent. Degradations of sedoheptulose from photosynthetic experiments with soy beans indicate that at very short times (less than one second) the amount of radioactivity in carbon 4 is considerably less than in carbons 3 and 5. However, in any experiment in which there was less activity in carbon 4 of the sedoheptulose than in carbons 3 and 5, no appreciable amount of radioactive ribulose was found. If conditions were such in the five second experiment that some batches of algae were photosynthesizing at a lower rate, these algae might have produced sedoheptulose with the low carbon 4 label and no appreciable amounts of labeled ribulose. Other batches of algae, photosynthesizing at a faster rate, might have produced sedoheptulose with an equal amount of activity in carbons 3,4 and 5, and appreciable amounts of ribulose with an equal amount of activity in carbons 1 and 2. It is possible, then, that in this case the distribution of activity in the ribulose and sedoheptulose of the combined extracts is not a valid indication of the relationship between the two sugars.

In the short-term "steady state" photosynthetic experiments for which ribulose and sedoheptulose degradations are available, carbons 3, 4 and 5 of the sedoheptulose are approximately equal in activity, and carbons 1 and 2 of the ribulose are equal in activity. If we now assume that the ribulose can result from five consecutive carbon atoms of the sedoheptulose (I), as well as from some combination of C_2 and C_3 fragments (II), the center carbon of the resulting ribulose will be the most radioactive, with the top two carbon atoms being more radioactive than the bottom two (III).

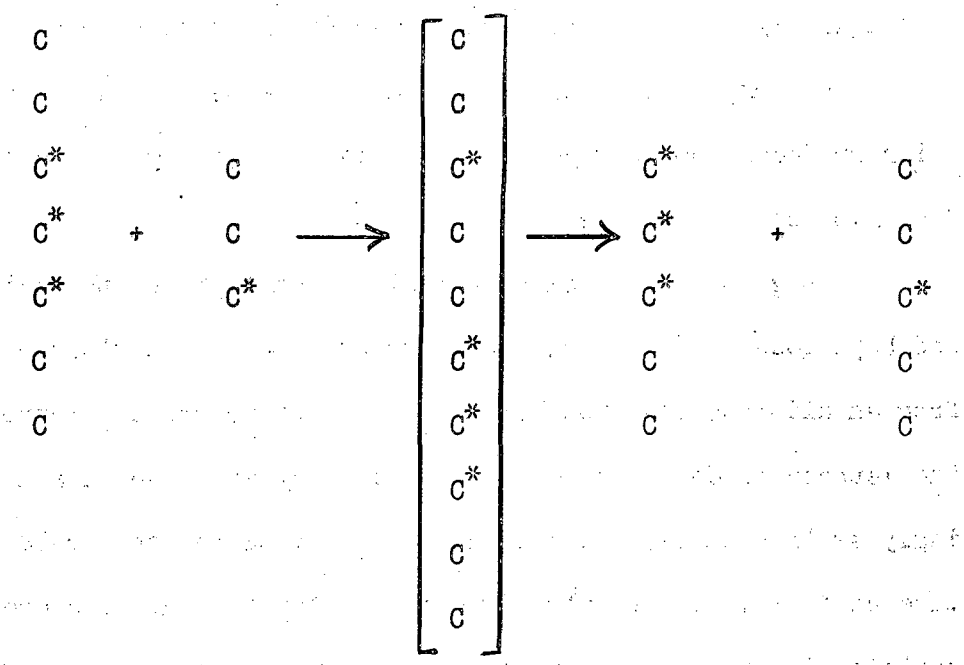
C*	C	C*
C*	C	C*
C*	C*	C**
C	C	C
C	C	C
(I)	(II)	(III)

Since at all times previous to and up to the time of the observation the specific activity per carbon atom of phosphoglyceric acid is higher than the specific activity of sedoheptulose, it is to be expected that carbon 3 of the resulting ribulose will contain more radioactivity than if there were an equal contribution of (I) and (II). The experimental data is qualitatively in agreement with this proposal.

Two essentially similar mechanisms can be suggested for the formation of ribulose. The first of these provides for the ribulose to result both from the splitting of the sedoheptulose to pentose and diose by a transketolase reaction, and the recombination of free diose with triose. The latter reaction is a reversal of the transketolase splitting of ribulose.



The second mechanism provides for the production of ribulose by allowing the heptose to undergo an aldolase reaction with dihydroxyacetone, forming a transient C_{10} piece which immediately breaks down to two pentoses.



Only small quantitative differences could arise between the two mechanisms, and those only if there were an appreciable C_2 pool, or if there were some source of C_2 other than the splitting of the heptose and pentose. The second mechanism would eliminate the necessity for any free C_2 compound in this step. This elimination of C_2 is of interest in connection with an experiment performed recently in this laboratory[24]. In this experiment algae were allowed to photosynthesize in a steady state at a certain carbon dioxide pressure for long enough to saturate all pools of finite size. At a given time the carbon dioxide pressure was drastically lowered, and the changes in pool sizes followed at short intervals thereafter. As was expected, the amount of phosphoglyceric acid decreased rapidly, and eventually

leveled off. The amount of ribulose increased rapidly before decreasing and leveling off at a lower value than it had originally at the high carbon dioxide pressure. The amount of glycolic acid increased, although at a slower rate than ribulose, and leveled off at a higher value than it originally had. A reasonable inference is that ribulose, and not an unknown C_2 compound, is the carbon dioxide acceptor in the carboxylation which produces phosphoglyceric acid, and that the glycolic acid is formed from ribulose by an irreversible reaction.

Degradation studies have also shed light on the origin of the sedoheptulose. If, as has been proposed, the sedoheptulose results from an aldolase reaction between dihydroxyacetone and tetrose, and the tetrose is derivedfrom a second carboxylation and has a vanishingly small pool size, carbon 4 of the sedoheptulose should become labeled first. It has already been pointed out that the amount of activity in carbon 4 is approximately equal to the amount of activity in carbons 3 and 5, and in some cases, it is even less than the amount of activity in carbons 3 and 5. The fact that the amount of activity in carbon 4 is not greater than the activity in carbons 3 and 5 appears to be evidence against a second carboxylation.

One possible explanation involves postulating a mechanism for the second carboxylation in which the bicarbonate ion and not carbon dioxide is the reacting species. One can then assume that there is a relatively large bicarbonate pool within the cell which prevents the immediate entry of labeled bicarbonate. This could qualitatively explain the low initial activity in carbon 4, followed by an increase to

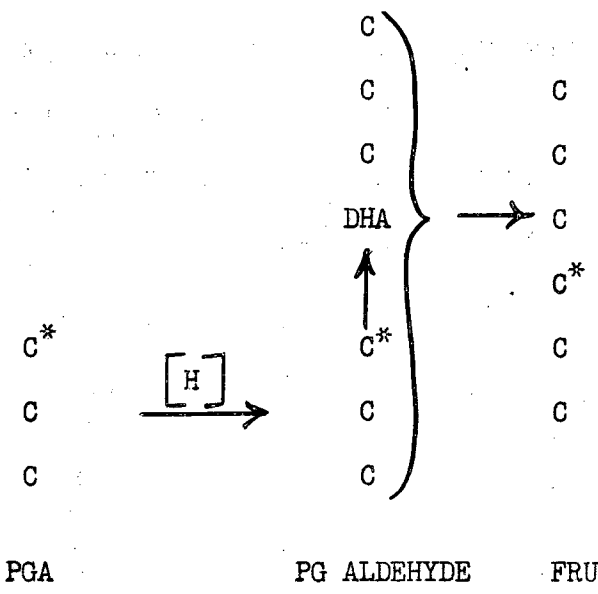
activity comparable with that of carbons 3 and 5. There is, however, no experimental evidence to substantiate the bicarbonate hypothesis.

Another explanation involves postulating a means of production of the C_4 fragment other than a carboxylation. In the short photosynthetic experiments in which the activity in carbons 3,4, and 5 of the sedoheptulose are approximately equal, the correspondence between the activity in four consecutive carbon atoms of the hexose (IV) and the activity in four consecutive carbon atoms of sedoheptulose (V) is evident. It is possible, therefore, that the hexose provides the source for the C_4 fragment, which unites with a C_3 fragment. Mechanisms for these reactions will be discussed later.

	C	
C	C	C
C	C*	C
C*		C*
C*	C*	C*
C	C*	C*
C	C	C
	C	C
(IV)		(V)

However, explanation of the results which show carbon 4 of the sedoheptulose to be less active than carbons 3 and 5 require the additional assumption of an unsymmetrical hexose at extremely short times. This hexose could be produced in the following manner. Fructose 1,6 diphosphate is formed by an aldolase reaction between phosphodihydroxyacetone

and phosphoglyceraldehyde. Phosphoglyceric acid, which is the product of the primary carboxylation, is reduced to phosphoglyceraldehyde, which in turn is isomerized to phosphodihydroxyacetone. Consider now that the first moelcules of radioactive phosphoglyceraldehyde react with phosphodehydroxyacetone to form fructose 1,6 diphosphate before radioactive phosphoglyceraldehyde completely equilibrates with phosphodihydroxy-. acetone. This lag would produce an unsymmetrical hexose.



If this unsymmetrical hexose provides the C_4 fragment which unites with a C_3 fragment (either a later dihydroxyacetone or a C_3 fragment derived from a later dihydroxyacetone) the resulting sedoheptulose has less activity in carbon 4 than in carbons 3 and 5 (VI).

	C	
	C	C
C	C*	C
C		C*
C	C	C
C*	C*	C*
C	C	C
C	C	C

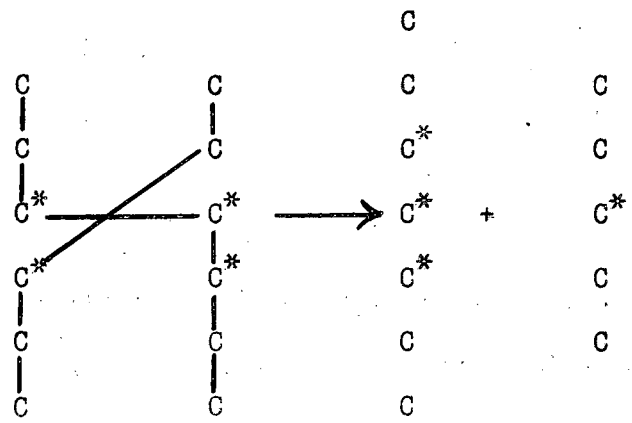
(VI)

As yet, there has been no experimental evidence to substantiate the unsymmetrical hexose hypothesis. The shortest hexose that has been degraded, the fructose from the 0.4 second soy bean experiment, appeared to be symmetrically labeled. However, 0.4 seconds may be too long a time for the phenomenon to persist. Horecker[25] has recently performed an experiment in which he enzymatically converts a specifically labeled pentose to hexose, presumably through heptose and determines the labeling in the resulting hexose. His results can best be explained by assuming the formation of hexose by a combination of C_2 and C_4 fragments, the reverse of what is proposed here.

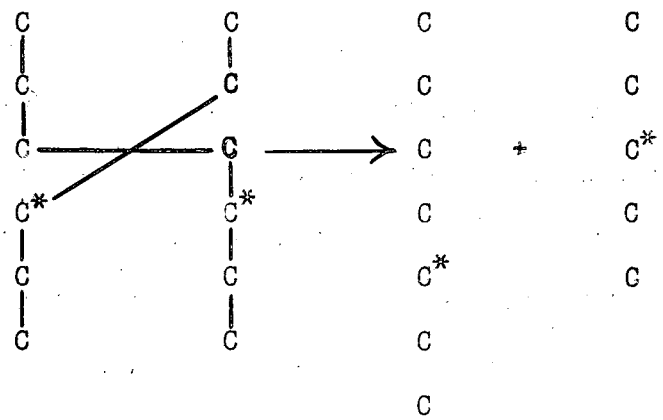
Several mechanisms can be suggested for the formation of sedoheptulose from hexose. First of all, one can assume a transketolase splitting of hexose into free diose and tetrose, similar to the transketolase splitting of pentose and heptose. The tetrose can then react with dihydroxyacetone to form sedoheptulose. This mechanism, of course, involves the existence of free diose and tetrose. The question as to whether or not the C_2 compound is the carbon dioxide acceptor

has already been discussed.

A second mechanism involves the reaction of two hexoses, one splitting by an aldolase reaction, the other by a transketolase reaction, to form pentose and heptose. If sedoheptulose is formed from symmetrical hexose, this mechanism is essentially the same as the first.

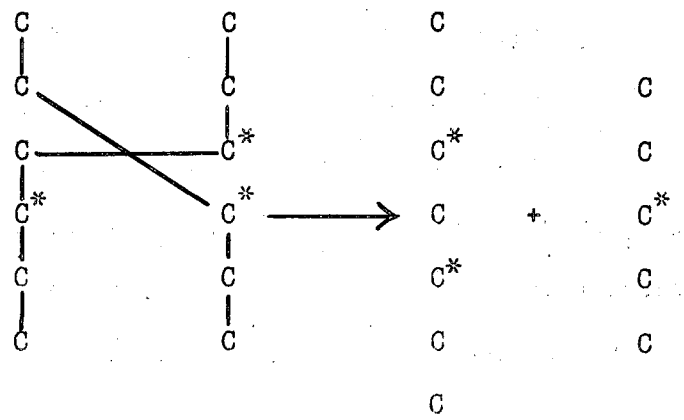


However, in the case of sedoheptulose formation from unsymmetrical hexose, added assumptions must be made, or sedoheptulose with more activity in carbons 3 and 5 than in carbon 4 cannot result.



It must also be assumed that the two hexose molecules which react are not the same, fructose 1,6 phosphate supplying the C_3 fragments, and

either fructose 1 phosphate or glucose 1 phosphate supplying the C_4 and C_2 fragments. Since fructose 1 phosphate or glucose 1 phosphate are formed from fructose 1,6 phosphate, they reflect an earlier labeling. A further assumption must be made that there is a lag in these reactions at very short times, similar to the one which originally produced the unsymmetrical hexose. Under this assumption, more symmetrical fructose 1,6 phosphate would react with less symmetrical fructose or glucose 1 phosphate.



GLUCOSE or FRUCTOSE

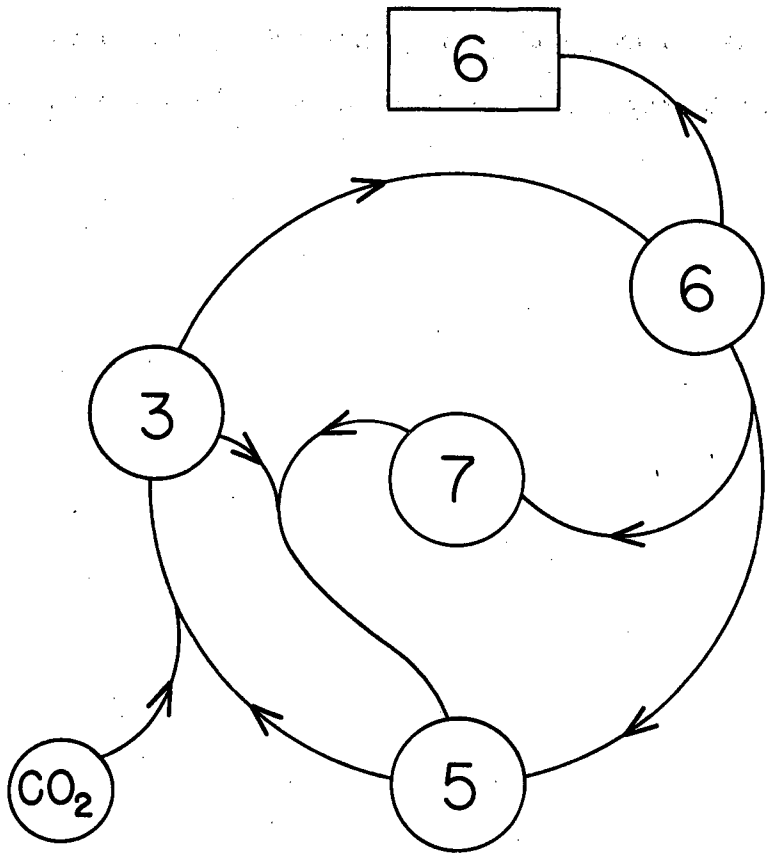
FRUCTOSE 1 PHOSPHATE 1,6 PHOSPHATE

A third mechanism, essentially similar to the first except that it does not involve the formation of free diose and tetrose, provides for a fructose molecule capable of transketolase splitting, to be attacked by two C_3 molecules which can pick up the C_2 and C_4 fragments from the fructose, forming ribulose and sedoheptulose. This mechanism would insure that the label in carbon 3 of the sedoheptulose would be comparable to that of carbon 5, at times short enough to produce an unsymmetrical hexose. The possibility of a

trimolecular reaction is, however, smaller than the possibility of a dimolecular reaction.

It should be noted that regardless of mechanism, if sedoheptulose is formed from hexose, additional ribulose with the C_2 plus C_3 distribution is formed. As was previously observed, it appears that this distribution (II) contributes more to the observed ribulose distribution than does the sedoheptulose distribution (I). In this respect, the hexose hypothesis is in accord with experimental data.

A modified cycle can be proposed, as shown in Figure 2, with the following characteristics: (1) No free diose or tetrose is involved; (2) sedoheptulose is formed from hexose, eliminating the second carboxylation; (3) ribulose is formed both from sedoheptulose and from C_2 plus C_3 combination; (4) ribulose is the carbon dioxide acceptor. The present results seem to indicate these changes, and lead the way for further experiments in these directions.



MU-6083

Figure 2

ACKNOWLEDGMENTS

I should like to express my sincere appreciation to Professor Melvin Calvin for the advice and encouragement he has given me throughout the course of this work. I am grateful to the staff of the Bio-Organic Group of the Radiation Laboratory for their many helpful suggestions. I am particularly indebted to Dr. James A. Bassham and Mrs. Lorel L. Kay for their invaluable cooperation and assistance.

Degradations Performed	Carbon Atoms	Degradation Product
Periodic acid oxidation of osazone	1 + 2 + 3	mesoxaldehyde osazone
	4	formic acid
	5	formaldehyde
Periodic acid oxidation of ribitol	1 + 5	formaldehyde
	2 + 3 + 4	formic acid
Ce^{+4} oxidation of ribulose	2	CO_2

Determination of the Carbon Atoms of the Ribulose Molecule	
Carbon No. 1	(1 + 5) = 5
2	direct measurement
3	(1 + 2 + 3) - 1 = 2 and (2 + 3 + 4) - 2 = 4
4	direct measurement
5	direct measurement

Chart 1

RIBULOSE DEGRADATIONS

	SCENEDESMUS 8.8 sec.	SCENEDESMUS 5.4 sec.	SCENEDESMUS 5 sec.	CHLORELLA 1 min.	SCENEDESMUS 2 min.	SCENEDESMUS 5 min.	CHLORELLA 10 min.
	← steady state →						
carbon 1	11	11	16	14			18
2	11	10	16	15			17
	87	90	95	81	72	74	65
3	63-65	64-69	61-63	47-52			30
4	7.7	5.3	2.5	9.2	13	15	17
5	5.4	3.1	1.8	10	12	16	16
Recovery	98--100%	93--98%	98--100%	95--100%	97%	105%	98%

Chart 2

COMPARISON OF SUGAR DEGRADATIONS

	5 SEC. SCENEDESMUS		5.4 SEC. SCENEDESMUS			8.8 SEC. SCENEDESMUS		0.4 SEC. SOY	
	Non-Steady State		Steady State			Steady State		Non-Steady state	
	Sed.	Rib.	Sed.	Rib.	Fructose	Sed.	Rib.	Sed.	Fructose
	2		2					---	
	2		2		3	3		---	---
	34	16	28	11	3		11	33	---
	18	16	25	10	43	22	11	8	45;50*
	34	62	26	69	42		65	49	56;48
	2 assume	2	2	5	3		8	--- assume	---
	2	2	2	3	3		5	---	---
Recovery	94%	98%	87%	98%	97%		100%	90%	

* Two degradations on the same sample

Chart 3

SEDOHEPTULOSE DEGRADATIONS - SOY BEAN

	0.4 SEC.	0.8 SEC.	1.5 SEC.	5 SEC.	2 DAY
	---	2		3	14
	---	2		4	13
	33	39		29	13
	8	18	24	32	12
	49	40		32	12
	---	2		3	12
	---	2		3	14
Recovery	90%	105%		103%	90%

APPENDIX I

Preparation of the Thioacetal of Mesoxaldehyde Osazone and Ethandithiol

To 500 mgms. of mesoxaldehyde osazone dissolved in the minimum amount of chloroform at room temperature, were added 500 λ of ethandithiol. Dry HCl was bubbled through the solution for 30 minutes at room temperature. A dark color formed almost immediately with the addition of HCl. The reaction vessel was allowed to stand for four hours, at the end of which time the solution was washed several times with 1 N sodium hydroxide and then with water. The now yellow chloroform solution was distilled to dryness *in vacuo*. The residue was recrystallized from ethanol-water after treatment with charcoal. Approximately 350 mgms. (50% yield) of recrystallized product were recovered. The compound, as recrystallized from ethanol-water, was in the form of long, light-yellow needles, melting at 195°-196°C.

	Theoretical	Found
C	59.6	59.37
H	5.3	5.21
N	16.4	16.49
S	18.7	18.83

BIBLIOGRAPHY

1. M. Calvin, The Harvey Lectures, Series XLVI, 218 (1950-1951), Charles C. Thomas, Springfield, Illinois.

2. A. A. Benson, J. A. Bassham, M. Calvin, A. G. Hall, H. Hirsch, S. Kawaguchi, V. Lynch and N. E. Tolbert, J. Biol. Chem. 196, 703 (1952).

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

4. A. A. Benson, S. Kawaguchi, P. M. Hayes and M. Calvin, J. Am. Chem. Soc., 74, 4477 (1952).

5. A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, J. Am. Chem. Soc., 72, 1710 (1950).

6. Yvonne Khouvine and George Arragon, Compt. rend., 212, 167 (1941)

7. Lorel L. Kay, unpublished work.

8. W. T. Hastkins, Raymond M. Hann and C. S. Hudson, J. Am. Chem. Soc., 68, 1766 (1946).

9. Erwin Chargaff and Boris Magasanik, J. Am. Chem. Soc., 69, 1459 (1947).

10. Yale J. Topper and A. Baird Hastings, J. Biol. Chem. , 179, 1255 (1949)

11. Richard E. Reeves, J. Am. Chem. Soc., 63, 1476 (1941).

12. S. Aronoff and L. Vernon, Arch. Biochem., 28, 424 (1950).

13. O. Diels, R. Meyer and O. Onnen, Ann., 525, 94 (1936).

14. P. V. Vittorio, G. Krotkov and G. B. Reed, Science, 115, 567 (1952).

15. R. M. Hann and C. S. Hudson, J. Am. Chem. Soc., 66, 735 (1944).

16. C. T. Bishop, Science, 117, 715 (1953).

17. D. L. McDonald and H. O. L. Fischer, J. Am. Chem. Soc., 74, 2087 (1952).

18. David T. Gibson, J. Chem. Soc., 12 (1930).

19. E. Fischer, Ber., 22, 87 (1889).

20. H. Collatz and I. S. Neuberg, Biochem. Z., 255, 27 (1932).

21. Henry Rapoport, personal communication.

22. Samuel Abraham, J. Am. Chem. Soc., 72, 4050 (1950).

23. G. Frederick Smith, "Cerate Oxidimetry", G. Frederick Smith Chemical Company, Columbus, Ohio, (1942).

24. Alexander T. Wilson, unpublished work.

25. B. L. Horecker, unpublished work.