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THE PATH OF CARBON IN PHOTOSYNTHESIS. XXII. THE IDENTIFICATION OF CARBOXY-KETOPENTITOL DIPHOSPHATES AS PRODUCTS OF PHOTOSYNTHESIS

V. Moses and M. Calvin

January 9, 1958

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V. Moses and M. Calvin

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

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ABSTRACT

Two new compounds, tentatively identified, have been isolated from chromatograms of ethanol-water extracts of cells of Chlorella pyrenoidosa which had been allowed to carry on photosynthesis for 3 minutes in the presence of $NaHC^{14}O_3$. Chemical investigation suggested that the two compounds were 1, 5 diphosphate esters of 2-carboxy-4-ketopentitol and of the lactone of 2-carboxy-3-ketopentitol. The latter has been postulated as an intermediate in the photosynthetic carboxylation of ribulose-1, 5-diphosphate to yield two molecules of 3-phosphoglyceric acid. Although the former may be an artifact resulting from the techniques used to kill, extract, and analyze the cells, possible biological roles for it have been suggested.

THE PATH OF CARBON IN PHOTOSYNTHESIS. XXII. THE IDENTIFICATION OF CARBOXY-KETOPENTITOL DIPHOSPHATES AS PRODUCTS OF PHOTOSYNTHESIS*

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INTRODUCTION

It has been well established that the initial carboxylation reaction of photosynthesis in green plants is the combination of carbon dioxide with ribulose 1,5-diphosphate followed by hydrolysis of the product to form two molecules of 3-phosphoglyceric acid.[1,2,3,4,5] Calvin has suggested that the reaction proceeds via a six-carbon sugar keto acid diphosphate intermediate, 2-carboxy-3-ketopentitol-1,5-diphosphate (2-phosphohydroxymethyl-3-ketopentonic acid-5-phosphate),[6,7] which has, however, not been identified in experiments hitherto reported that involve photosynthesis in the presence of radioactive carbon dioxide.

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*The work described in this paper was sponsored in part by the U.S. Atomic Energy Commission and in part by the Department of Chemistry, University of California, Berkeley, California.

†Postgraduate Travelling Fellow of the University of London, 1956-57.

[1]Bassham, Benson, Kay, Harris, Wilson, and Calvin, J. Am. Chem. Soc. 76, 1760 (1954).

[2]Quayle, Fuller, Benson, and Calvin, J. Am. Chem. Soc. 76, 3610 (1954).

[3]Weissbach, Horecker, and Hurwitz, J. Biol. Chem. 218, 795 (1956).

[4]Mayaudon, Benson, and Calvin; Biochim. et Biophys. Acta 23, 342 (1957).

[5]E. Racker, Arch. Biochem. and Biophys. 69, 300 (1957).

[6]M. Calvin, Fed. Proc. 13, 697 (1954).

[7]M. Calvin, J. Chem. Soc. 1895 (1956).

The standard methods used in this laboratory for the analysis of the products of short-term photosynthesis experiments performed in the presence of $C^{14}O_2$ involve killing the cells at the desired time by rapidly pouring the cell suspension into four volumes of boiling ethanol. The cell residue is then centrifuged and re-extracted with boiling 20% ethanol, followed by a final extraction with water. The pooled extracts are evaporated to a small volume in vacuo at temperatures not exceeding 40°, and aliquots are chromatographed on sheets of oxalic-acid-washed Whatman No. 4 filter paper. The solvents (phenol-water in the first dimension and butanol-propionic acid-water in the second dimension) are normally allowed to run only to the bottom edge of the paper (8 to 10 hours at 25°), or at most twice that far, before the papers are dried. The locations of radioactive substances on the chromatograms are determined by radioautography.[8]

These techniques, although very valuable for general purposes, suffer from at least two disadvantages. Firstly, the extraction of the cells by boiling ethanol-water mixtures is likely to cause breakdown of heat-labile substances, of which 2-carboxy-3-ketopentitol-1,5-diphosphate (being a β-keto acid) may be expected to be one. Secondly, on chromatograms run for the standard lengths of time, the area near the origin containing principally the sugar phosphates (and particularly the sugar diphosphates) is not well separated; it is known that the apparently single spots seen in this area are often mixtures containing several diphosphate esters of the relevant sugars.[9]

This communication reports attempts that have been made to overcome both these difficulties in an effort to isolate and identify the intermediates proposed for the reaction between ribulose diphosphate and carbon dioxide to form phosphoglyceric acid. Two substances of the carboxy-ketopentitol diphosphate type have now been tentatively identified.

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[8] Benson, Bassham, Calvin, Goodale, Haas, and Stepka; J. Am. Chem. Soc. 72, 1710 (1950).

[9] A. A. Benson, in Modern Methods of Plant Analysis, II (Springer, Berlin-Gottingen-Heidelberg, 1955), p. 113.

METHODS

Cells of Chlorella pyrenoidosa for experimental purposes were grown in the continuous-culture apparatus described by Holm-Hansen et al.[10] The cells were harvested and washed twice with distilled water, and 400 µl of wet-packed cells were resuspended in 10 ml of distilled water in a "lollipop" of suitable size. To obtain the cells in a steady metabolic state the cell suspension was flushed for 30 minutes with air containing 1% (v/v) CO_2; the lollipop was immersed in a water bath at room temperature and illuminated from both sides with 150 watt RS-P-2 photospot lamps. After 30 minutes of flushing, the gas-flushing tube was removed from the lollipop, and 1 minute later 0.25 ml of $NaHC^{14}O_3$ (100 µC; 6.6 µmoles) was added. The lollipop was stoppered and shaken, and the cells were allowed to photosynthesize between the light sources for 3 minutes. The cell suspension was then poured rapidly into 40 ml of ethanol (precooled to -15°) and was extracted overnight at -15°. After centrifugation at this temperature, the residue was extracted overnight with 20 ml of 20% ethanol at 0°, and again with 10 ml of 20% ethanol at 0° overnight. The cell residue was finally extracted with 10 ml of 20% ethanol at 60° for 30 minutes. The extracts were pooled and evaporated to a small volume in vacuo at room temperature.

Aliquots of the concentrated extract were spotted onto oxalic-acid-washed Whatman No. 4 filter paper and a current of air at room temperature was used to dry the spots; the chromatograms were developed in the first dimension with phenol-water and in the second dimension with n-butanol-propionic acid-water, the solvents being allowed to run for 48 hours in each dimension. Radioactive materials were located by radioautography on Dupont blue-sensitive single-coated x-ray film 507E.

RESULTS

Chromatography for 48 hours in each dimension of the ethanol- and water-soluble components of algal cells after 3 minutes of photosynthesis in the presence of $C^{14}O_2$ showed three distinct radioactive spots in the diphosphate area of the chromatogram (Fig. 1). Each of these spots was cut out, eluted, phosphatased, and rechromatographed. The spot labeled "RuDP" contained,

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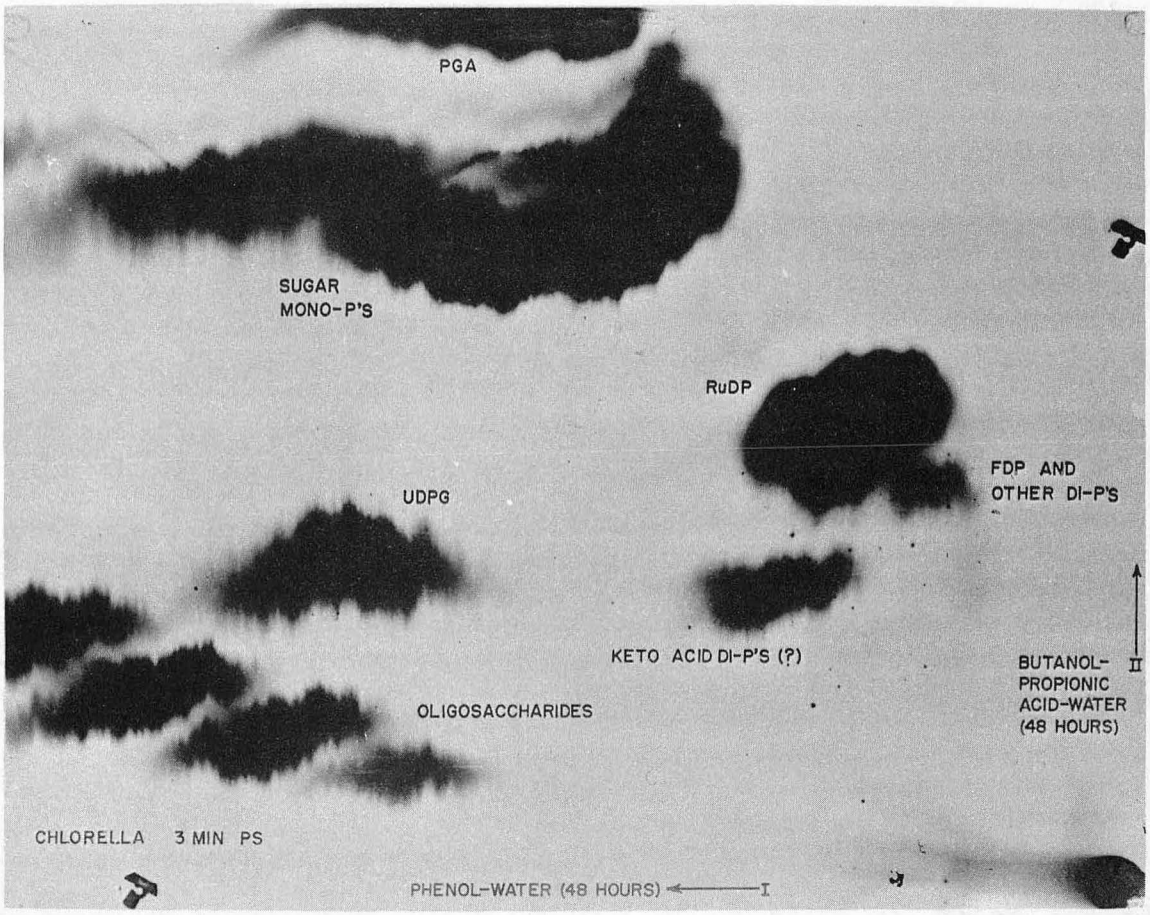
[10] Holm-Hansen, Hayes, and Smith, in Chemistry Division Quarterly Report, UCRL-3595 Oct. 1956 p. 56.



ZN-1865

Fig. 1. Radioautogram of ethanol-water extract of Chlorella after 3 minutes of photosynthesis in the presence of $C^{14}O_2$. The solvents were allowed to run for 48 hours in each dimension.

after phosphatasing, predominantly ribulose, and smaller quantities of glyceric and glycolic acids, together with twelve or more weakly radioactive substances which were not identified. The spot labeled "FDP and other Di-P's" contained, after phosphatasing, mainly fructose, glucose, and sedoheptulose, together with small quantities of glyceric and glycolic acids and one unidentified compound. The third spot ("Keto acid Di-P's"), never previously observed, ran slower in the butanol-propionic acid solvent than the other two. It seemed possible that this spot might contain a component more acidic than the other sugar diphosphates, and steps were taken to determine the nature of this substance.

After treatment with purified Polidase S,[11] or with human seminal acid phosphatase,[12] overnight in approximately 0.02 M acetate buffer, pH 5, a number of spots were seen on subsequent chromatography and radioautography (Fig. 2). Two of these appeared of particular interest, and they have now been tentatively identified (see Discussion) as 2-carboxy-4-ketopentitol ($CH_2OH.COH(COOH).CHOH.CO.CH_2OH$), and the lactone of 2-carboxy-3-keto-pentitol ($CH_2OH.COH(COOH).CO.CHOH.CH_2OH$). These substances will henceforth be referred to as the γ-keto acid and the β-keto acid, respectively.

Investigation of the γ-Keto Acid

A number of tests have been applied to determine the structure of this compound, the results of which are consistent with its being 2-carboxy-4-ketopentitol. At no time have more than tracer amounts of either the β- or the γ-keto acid been available, and all investigations have depended on following the radioactivity after various treatments.

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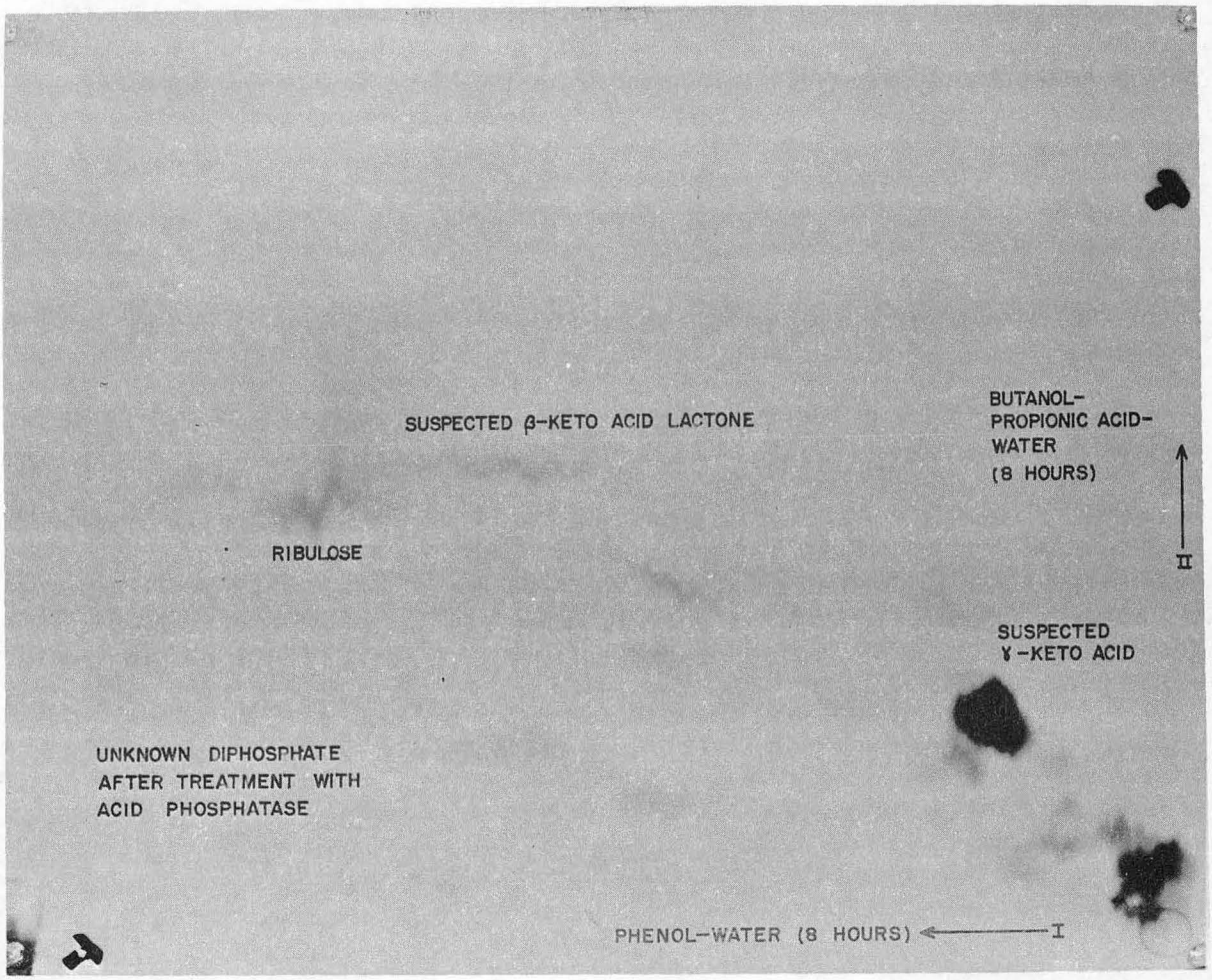
[11] S. S. Cohen, J. Biol. Chem. 201, 71 (1953).

[12] A gift from Prof. H. A. Barker.



ZN-1866

Fig. 2. Radioautogram of spot labeled "Keto acid Di-P's" from Fig. 1, after phosphatasing.

A. Electrophoresis and Chromatography With Known Substances

Electrophoresis on oxalic-acid-washed Whatman No. 4 filter paper in 0.1 M ammonium acetate buffer, pH 9, for 3 hours at 600 to 700 volts, together with authentic samples of gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid, and hamamelonic acid showed that all the available known six-carbon acids and the unknown substance ran the same distance along the paper and could be separated from five-carbon and seven-carbon sugar acids. The known six-carbon sugar acids could not be separated from one another or from the unknown substance when chromatographed in the solvent systems mentioned above, suggesting that the unknown had a generally similar structure, i.e., a polyhydroxymonocarboxylic six-carbon sugar acid.

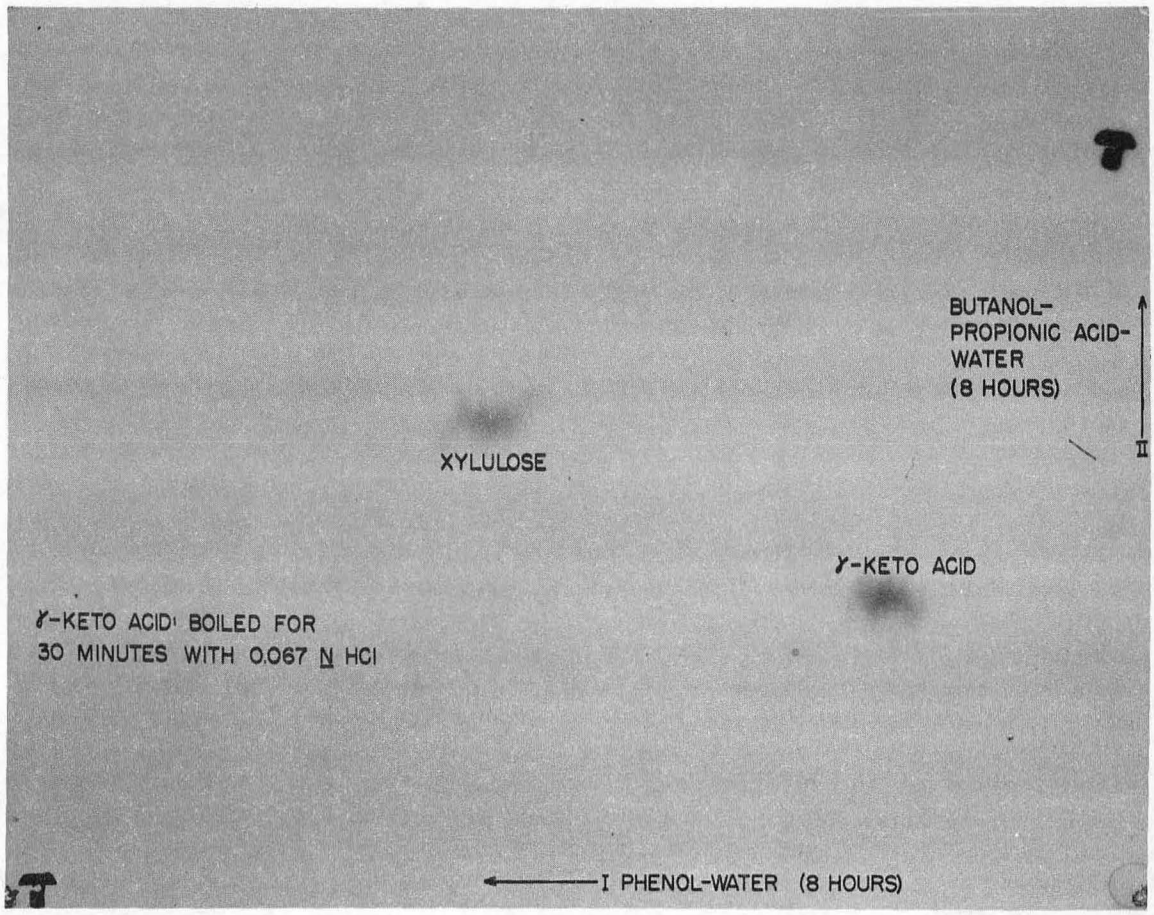
B. Lactonization

Treatment of the γ-keto acid with 0.07 M HCl at 100° for 30 minutes produced no lactone (Fig. 3), suggesting that it is not a simple aldonic acid. Polyhydroxymonocarboxylic sugar acids all readily lactonize in acid solution. However, the keto acids, 2-ketogluconic acid and 5-ketogluconic acid, undergo direct acid lactonization with much greater difficulty than the parent hydroxy acid, gluconic acid.[13]

C. Enolization and Epimerization by Alkali

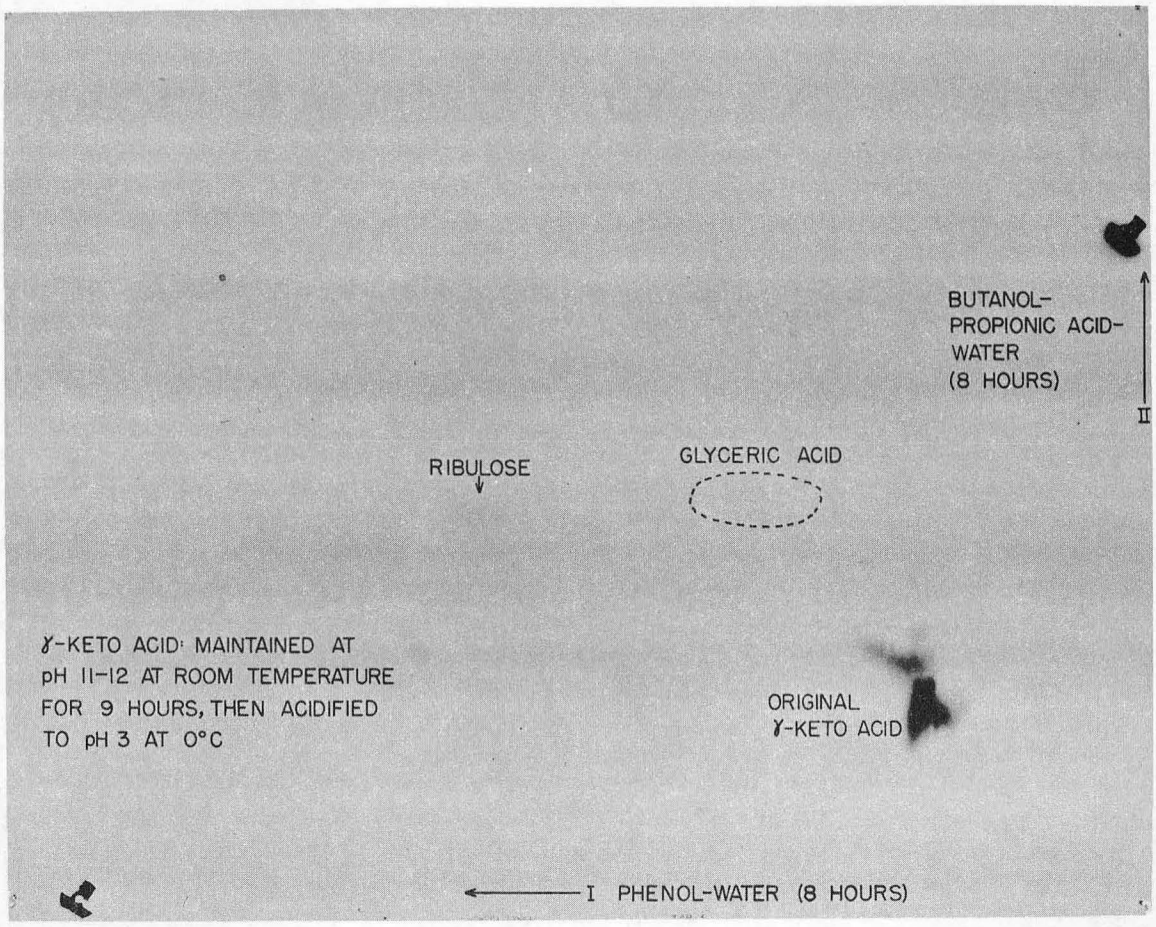
The γ-keto acid was allowed to stand for 9 hours at room temperature at pH 11-12 (NaOH), and was then acidified at 0° with HCl to pH 3. Chromatography showed the formation, by such treatment, of some ribulose and a trace of glyceric acid, while most of the original material remained apparently intact (Fig. 4); there thus appeared to be no stable epimer formed by alkali. The double spot of the γ-keto acid resulting from this treatment was probably due to the presence of sodium chloride. The question of enolization is discussed below in the section on the β-keto acid, paragraph A.

[13] P. P. Regna and B. P. Caldwell, Jr. Am. Chem. Soc. 66, 246 (1944).



ZN-1891

Fig. 3. Radioautogram of γ-keto acid after treatment with 0.07M HCl for 30 minutes at 100°.



ZN-1887

Fig. 4. Radioautogram of the γ-keto acid maintained at pH 11-12 at room temperature for 9 hours, then acidified to pH 3 at 0°.

D. Treatment with 2,4-dinitrophenylhydrazine

The γ-keto acid was treated with an excess of 2,4-dinitrophenylhydrazine in 2 M HCl for 30 minutes at 37°. Carrier 5-ketogluconic acid was also present. The hydrazones were extracted from the reaction mixture and chromatographed in one dimension on Whatman No. 1 filter paper with n-butanol saturated with 6% (w/v) aqueous ammonia.[14] Two 2,4-dinitrophenylhydrazones were produced in low yield which ran with the hydrazones of the added carrier. This suggested the presence of a carbonyl grouping.

E. Reduction with Borohydride

Reduction of the γ-keto acid by approximately 0.0075 M potassium borohydride[15,16] for 2 hours at room temperature produced a substance capable of undergoing acid-lactone interconversion at appropriate pH levels (Figs. 5 and 6). This implies the reduction of a keto group to a hydroxyl group, which is then capable of lactonization.

The product found after reduction with borohydride chromatographed very nearly, but not identically, with hamamelonic acid and hamamelonic lactone. Two samples of "hamamelonic acid" were available; one, made by Schmidt and Heintz,[17] was obtained by the action of hydrogen cyanide on d-ribulose, followed by hydrolysis of the nitrile and separation of the epimeric acids produced, by fractional crystallization of their phenylhydrazide derivatives.[18] The other

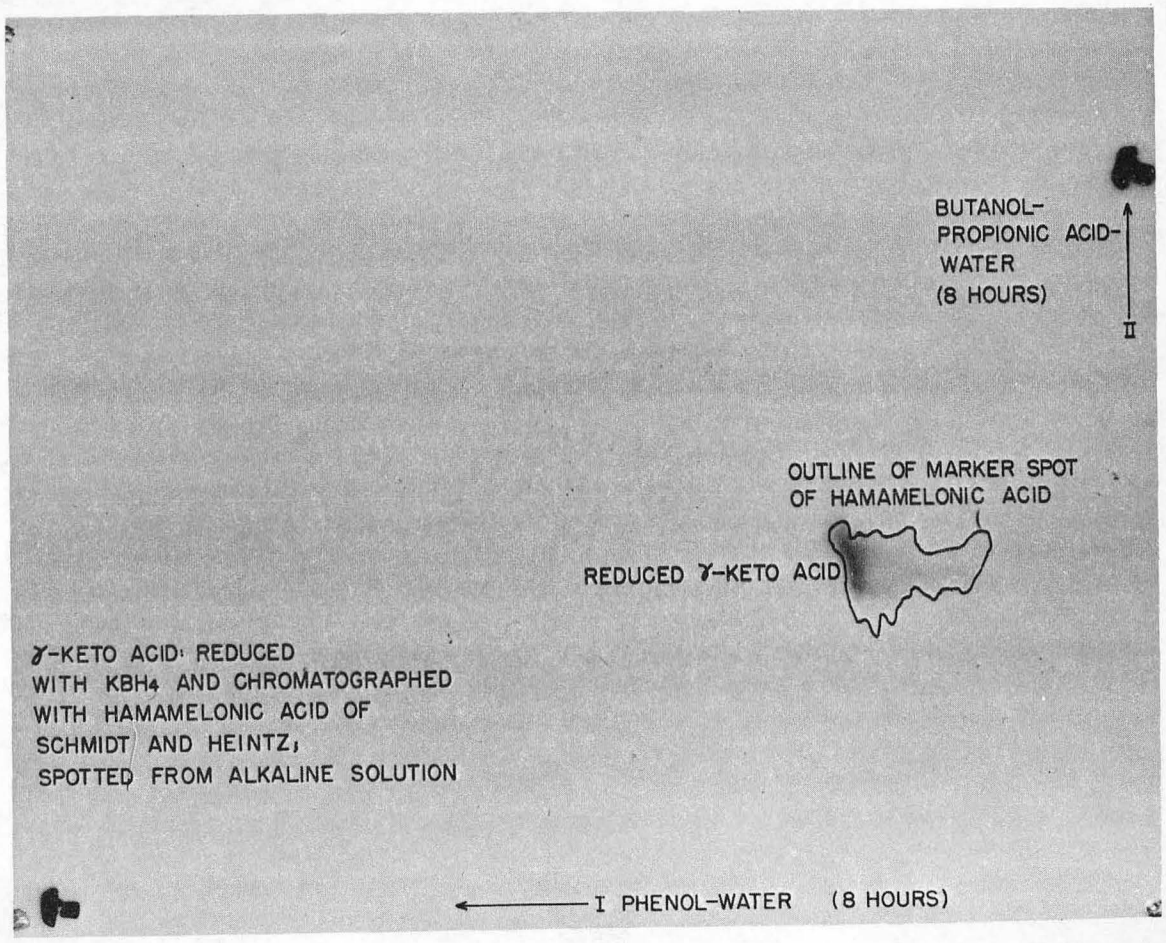
[14] V. Moses, J. Gen. Microbiol. 13, 235 (1955).

[15] P.D. Bragg and L. Hough, J. Chem. Soc. 4347 (1957).

[16] Abdel-Akher, Hamilton, and Smith, J. Am. Chem. Soc. 73, 4691 (1951).

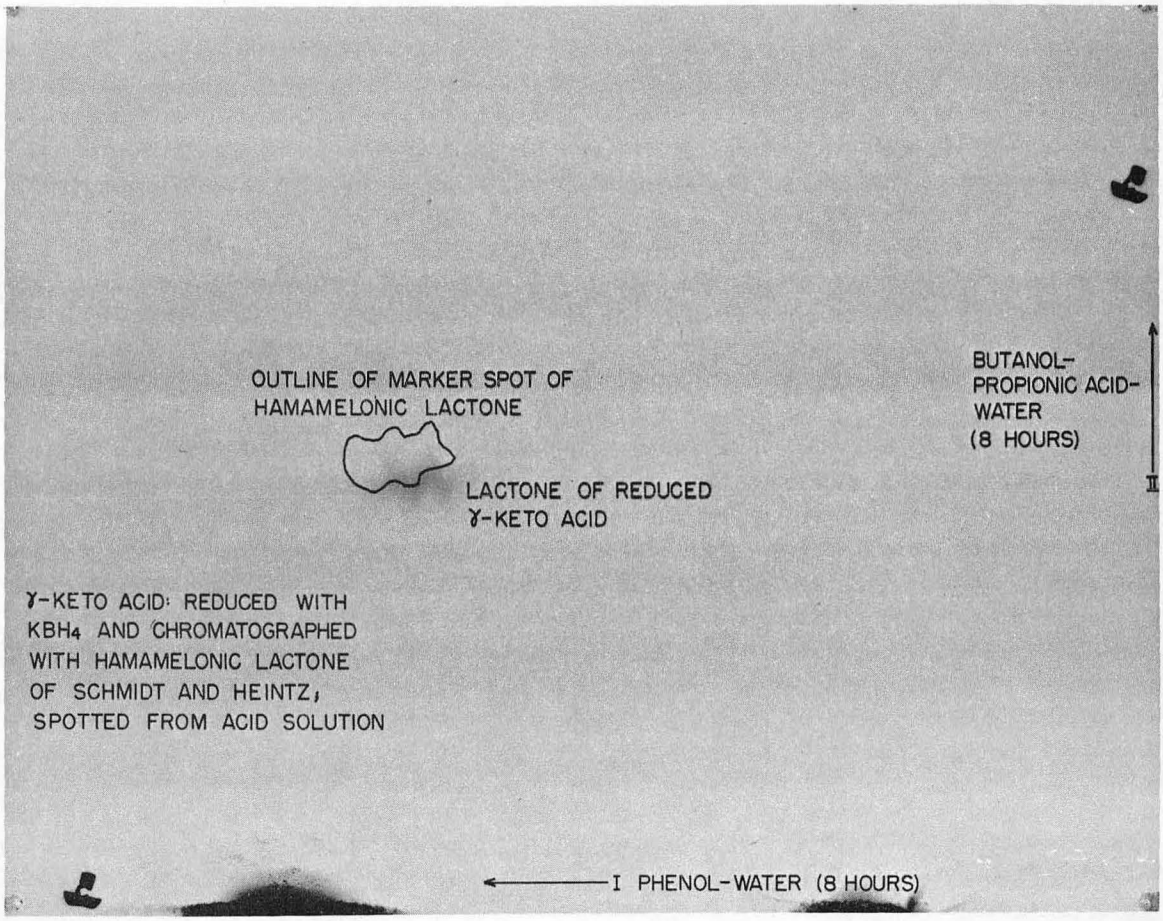
[17] O.T. Schmidt and K. Heintz, Ann. 515, 77 (1935).

[18] We are indebted to Prof. O.T. Schmidt for samples of the ammonium salt and of the phenylhydrazide of hamamelonic acid: the latter was converted by Ning G. Pon to free hamamelonic acid by refluxing with cupric sulphate and removal of excess copper and sulphate with hydrogen sulphide and barium hydroxide, respectively.



ZN-1889

Fig. 5. Radioautogram of the γ-keto acid, reduced with potassium borohydride, and co-chromatographed with the hamamelonic acid sample of Schmidt and Heintz;[17] spotted from alkaline solution (pH 13). The outline of the marker spot of authentic hamamelonic acid has been drawn in.



ZN-1886

Fig. 6. Radioautogram of the γ-keto acid, reduced with potassium borohydride, and co-chromatographed with the hamamelonic acid sample of Schmidt and Heintz;[17] spotted from acid solution (pH 1). The outline of the marker spot of authentic hamamelonic lactone has been drawn in.

sample, prepared by Rabin et al.,[19] was synthesized by treating ribulose diphosphate with potassium cyanide, with simultaneous hydrolysis of the nitrile to hamamelonic acid diphosphate, followed by enzymatic removal of the phosphate groups. This sample presumably contained hamamelonic acid and its epimer, in unknown proportions.

The γ-keto acid, after reduction with potassium borohydride, chromatographed more nearly with the sample from Rabin et al. of mixed hamamelonic acid and epimer (or their lactones) (Figs. 7 and 8), than with the pure hamamelonic acid or lactone from Schmidt and Heintz (Figs. 5 and 6). Before reduction with borohydride, the γ-keto acid ran quite distinctly from the hamamelonic acid of Schmidt and Heintz (Fig. 9), and no lactonization could be observed (Fig. 3). The explanation for these findings may be that the γ-keto acid is reduced by borohydride to a mixture of two epimers of the hamamelonic acid type, neither of which is hamamelonic acid itself, but one of which is the same as the epimer of hamamelonic acid present in the sample from Rabin et al.

F. Treatment With Acid

On heating with 0.07 M HCl for 30 minutes at 100°, some 35–45% of the γ-keto acid was converted into xylulose, presumably by decarboxylation, leaving the rest of the original compound apparently unchanged (Fig. 3).

G. Treatment With Alkali

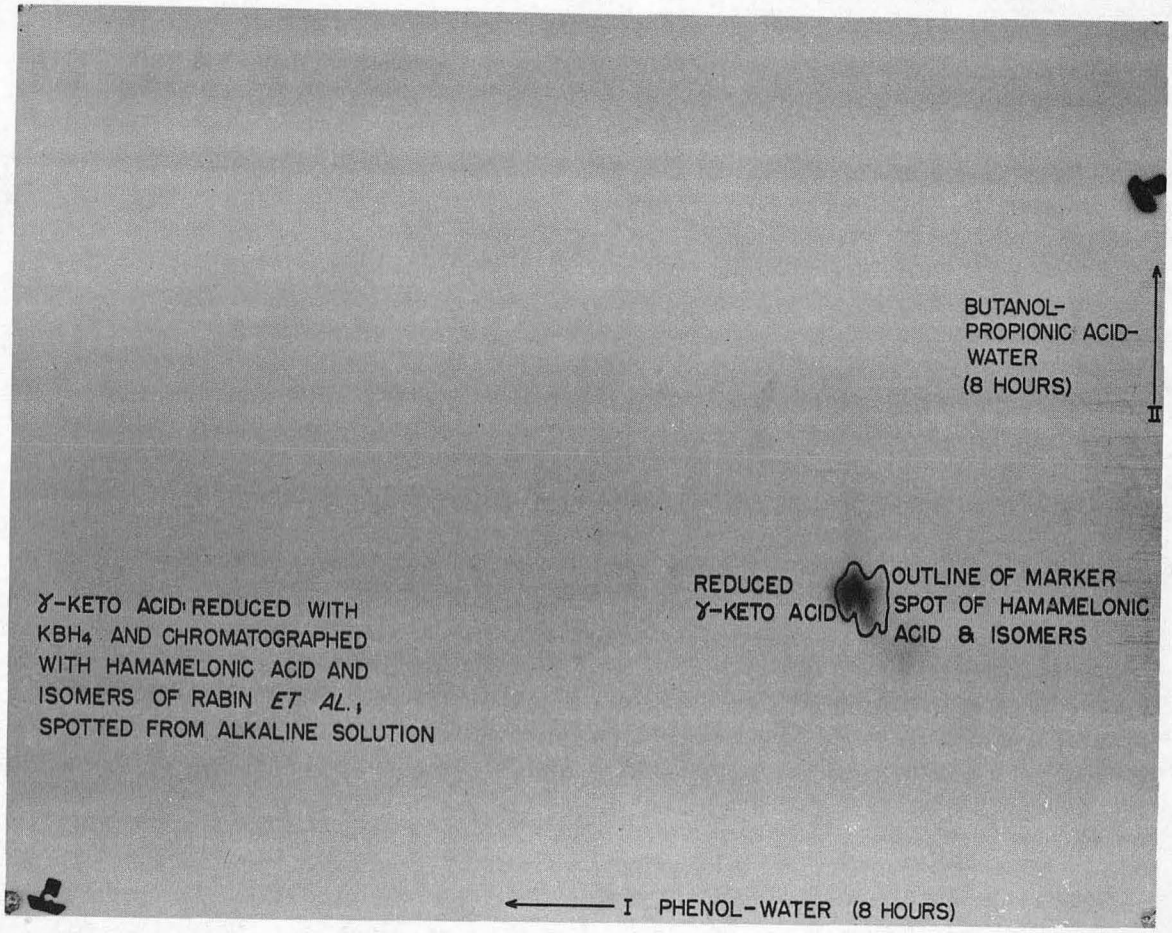
On heating of the γ-keto acid with 0.015 M NaOH for 30 minutes at 100° several substances were produced, one of which was glyceric acid (the others were not identified), and again some (about two-thirds) of the original compound was left apparently unchanged.

Investigation of the β-Keto Acid

A. Possible Formation from the γ-Keto Acid

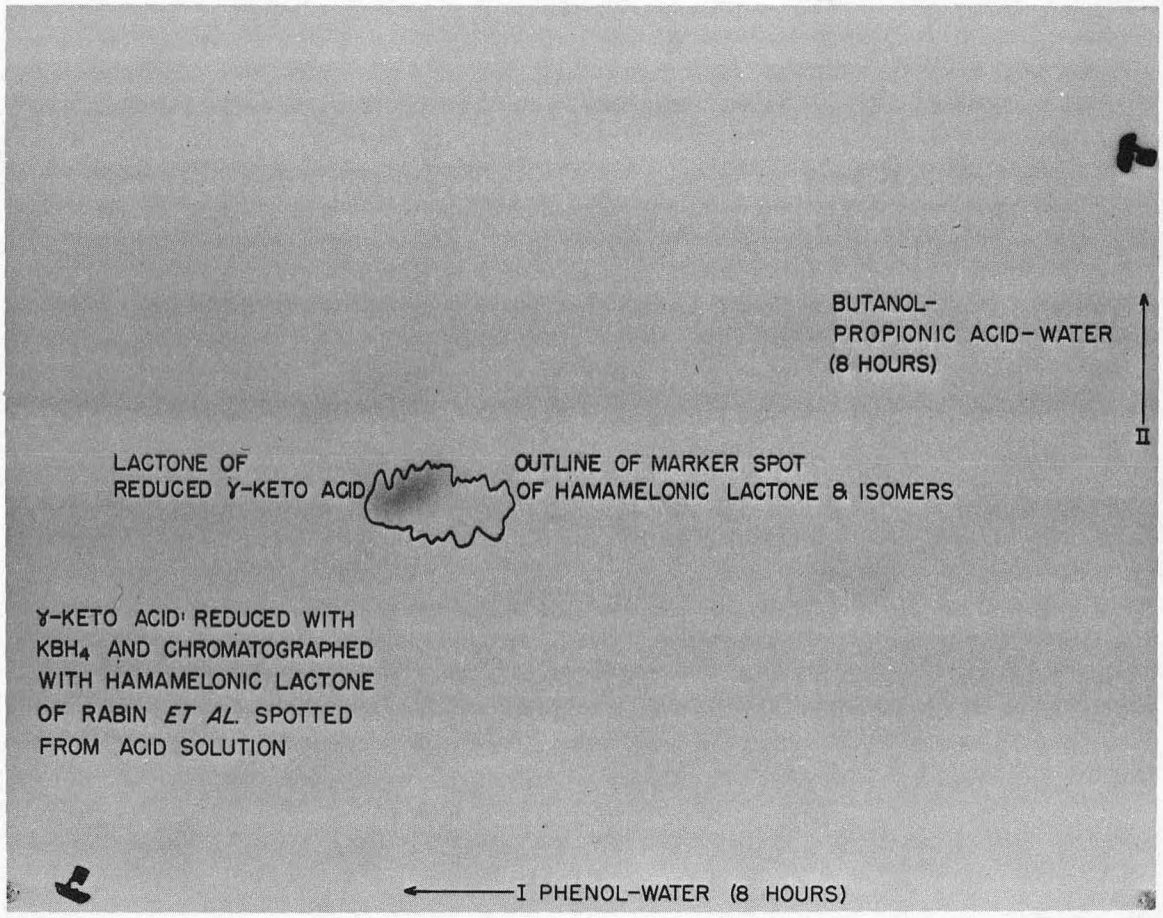
Treatment of the γ-keto acid with weak alkali, followed by acidification (see paragraph (G) above) resulted in the formation of some glyceric acid and ribulose, while most (more than 90%) of the γ-keto acid remained unchanged

[19] Rabin, Shaw, Pon, Anderson, and Calvin, J. Am. Chem. Soc., in press (1950).



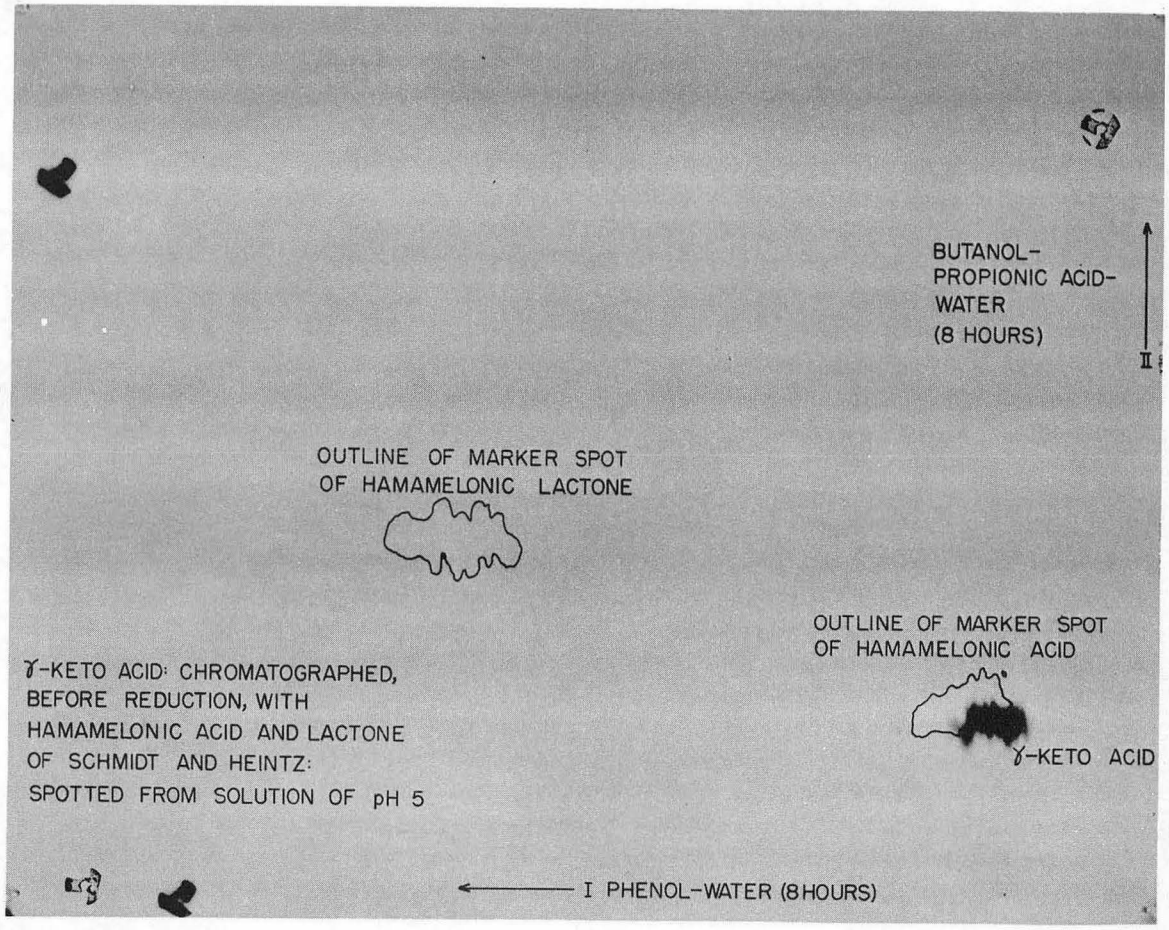
ZN-1888

Fig. 7. Radioautogram of the γ-keto acid, reduced with potassium borohydride, and co-chromatographed with the hamamelonic acid sample from Rabin et al;[19] spotted from alkaline solution (pH 13). The outline of the marker spot of authentic hamamelonic acid (and epimer) has been drawn in.



ZN-1885

Fig. 8. Radioautogram of the γ-keto acid, reduced with potassium borohydride, and co-chromatographed with the hamamelonic acid sample from Rabin et al.[19] spotted from acid solution (pH 1). The outline of the marker spot of authentic hamamelonic lactone (and epimer) has been drawn in.



ZN-1890

Fig. 9. Radioautogram of the γ-keto acid prior to reduction, co-chromatographed with the authentic sample of hamamelonic acid and lactone from Schmidt and Heintz;[17] spotted from solution of pH 5. The outlines of the marker spots of hamamelonic acid and lactone have been drawn in.

(Fig. 4). Acid treatment of the γ-keto acid results in the partial conversion to xylulose, not ribulose (Fig. 3). It seems that at pH 11-12, some β-keto acid is produced from the γ-keto acid by enolization, the former decomposing to glyceric acid in alkali and to ribulose in acid.

B. Lactonization

Treatment of the β-keto lactone with 10^{-4} M NaOH produced a substance running chromatographically similar to the γ-keto acid (i.e. just behind hamamelonic acid: Fig. 9), together with a little of what appeared to be glyceric acid (Fig. 10). Alkali treatment of the β-keto lactone apparently converted it partly into the β-keto acid, and also caused some breakdown to glyceric acid (cf. paragraph (G) above).

C. Reduction With Borohydride

When the β-keto lactone was treated with 0.0075 M potassium borohydride for 2 hours at room temperature (pH about 10), apparently some glyceric acid and a substance that ran chromatographically together with hamamelonic acid were formed (Fig. 11). However, the radioactivity was too weak to determine the exactitude of coincidence with hamamelonic acid.

A careful investigation of each of the three diphosphate spots shown in Fig. 1 has not revealed the presence of any other substances, either of this type (keto acid) or of the reduced form (hamamelonic acid and epimers).

DISCUSSION

Although the final identification of the two materials under investigation must await co-chromatography with authentic samples, the evidence accumulated here permits a tentative assignment of structure, with some degree of confidence. The position of the original spot on the chromatogram (Fig. 1) is fairly definite evidence of its diphosphate character. Its susceptibility to the enzyme phosphatase, resulting in a material apparently free from phosphate, may be taken as additional evidence for its phosphate ester character. Most of the evidence available has been obtained with the more plentiful and the more stable of the two new compounds; the course of the argument for the structure of this compound is outlined, then followed by inferences from this substance and additional evidence for the structure of the second and more unstable compound.

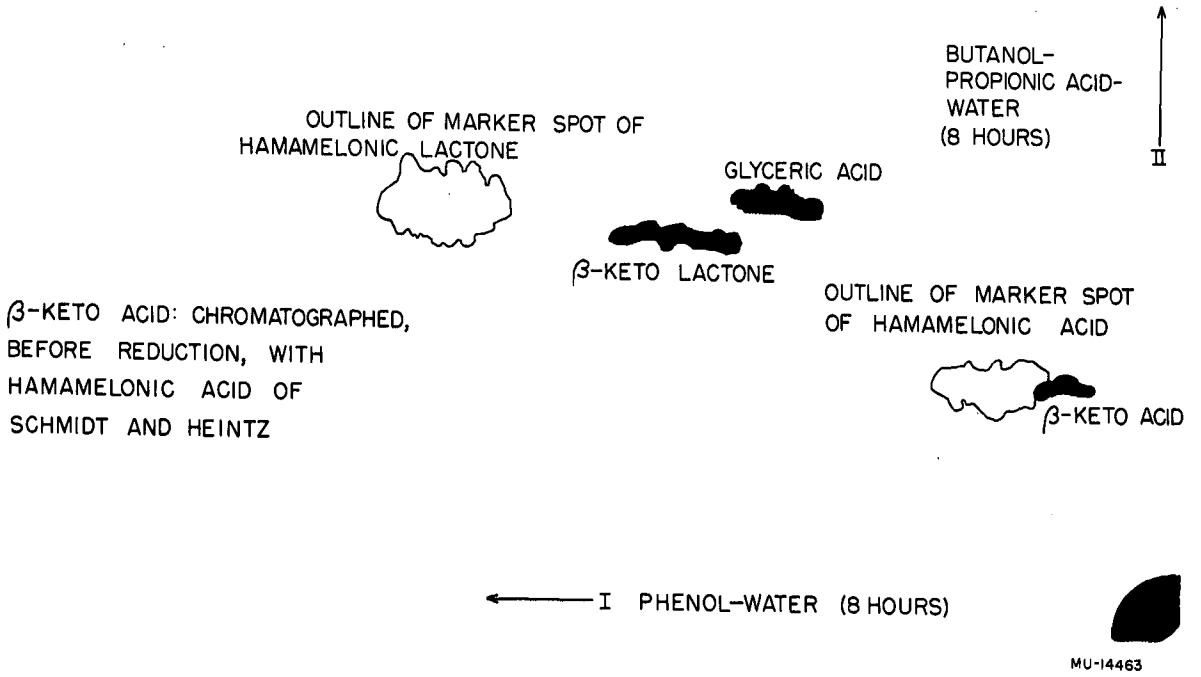


Fig. 10. Line drawing of composite radioautogram and sprayed chromatogram of β-keto lactone co-chromatographed with hamamelonic acid and lactone of Schmidt and Heintz;[17] spotted from a solution of pH 10. Solid areas represent radioactive substances; outlines are of authentic compounds.

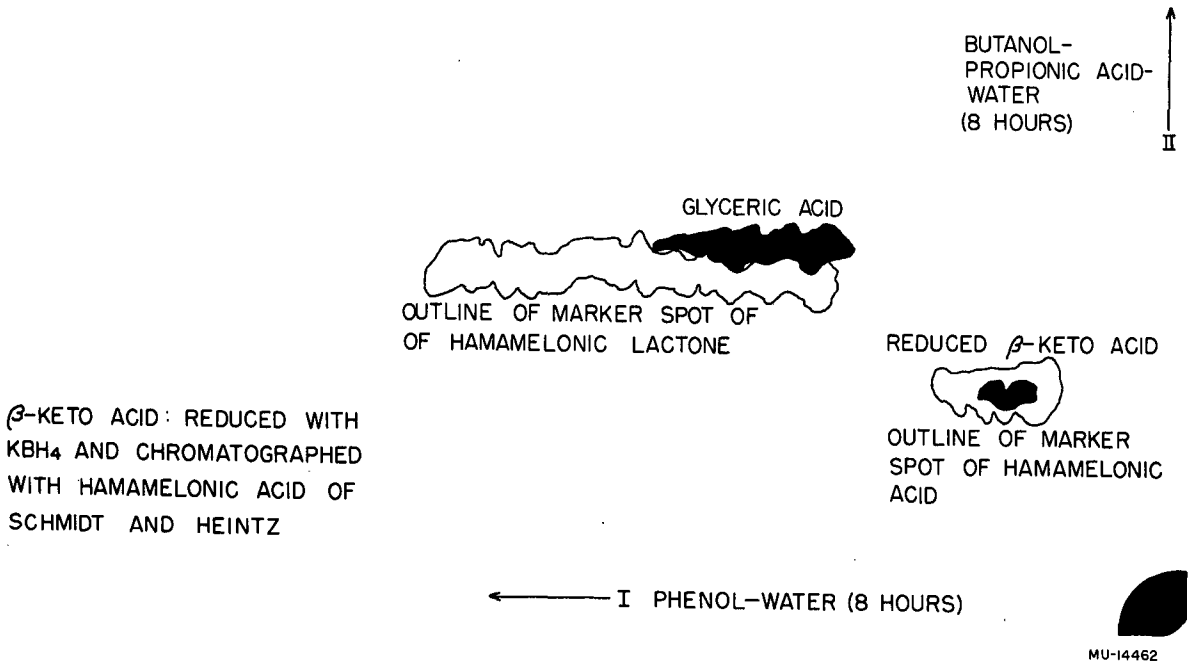


Fig. 11. Line drawing of composite radioautogram and sprayed chromatogram of β-keto lactone after reduction with potassium borohydride and chromatographed with hamamelonic acid and lactone of Schmidt and Heintz;[17] spotted from solution of pH 10. Solid areas represent radioactive substances; outlines are of authentic compounds.

The chromatographic and electrophoretic behavior of the dephosphorylated material seems to establish its character as a hydroxy sugar acid; beyond this, its location in the region of gluconic acid is fairly definite evidence that its weight corresponds to that of a six-carbon poly-hydroxy acid. Although the possibility of a heptonic acid is not eliminated on chromatographic grounds alone, the other evidence to be mentioned later appears to preclude this possibility. Electrophoretically the material moves exactly parallel with gluconic acid as well as with hamamelonic, 5-ketogluconic, and 2-ketogluconic acids. The demonstrated ability of paper electrophoresis to separate the pentonic acids from a variety of penta-oxyhexonic acids makes the value of the mobility of the unknown substance a significant quantity in establishing its penta-oxyhexonic character.

The failure of the compound to lactonize under acid conditions that lead to lactone formation in all other simple aldonic acids--such as gluconic, hamamelonic, ribonic and similar acids--indicates that the material is not a simple aldonic acid. Reduction by potassium borohydride produces a material that does exhibit the facile acid lactone interconversion so characteristic of the aldonic acids. This information, taken together with the fact that the two keto-aldonic acids available to us --2-ketogluconic and 5-ketogluconic--are also sluggish in their lactonization under the conditions used in this work, suggests that the compound originally is a keto-aldonic acid. The co-chromatograph of the reduced material, both in the free acid and in the lactone form, with hamamelonic acid and its lactone is so close as to confirm the idea that the material after reduction is indeed a six-carbon aldonic acid and therefore, before reduction, a six-carbon keto-aldonic acid. Co-chromatography of the unreduced substance with hamamelonic acid clearly demonstrated its difference from the latter. The treatment of the original keto acid with 0.07 M HCl for 30 minutes at 100° resulted in about 40% conversion to xylulose; the remaining 60% was recovered as the original acid. This stability eliminates any serious possibility that the unknown substance is a β-keto acid.

The nearest analogy available to us is β-ketogluconic acid, which has been postulated as an intermediate in the conversion of 6-phosphogluconic acid to ribulose 5-phosphate.[20,21] That is has not been possible to isolate a sample of

[20] B. L. Horecker and P. Z. Smyrniotis, Arch. Biochem. 29, 232 (1950).
[21] Gunsalus, Horecker, and Wood, Bacteriol. Revs. 19, 79 (1955).

this material (6-phospho-3-ketogluconate) has been taken as evidence of its gross instability. The chemical analogies about which we do have direct information include acetoacetic acid, α,α-dimethylacetoacetic acid, and dihydroxymaleic acid (α, hydroxyoxaloacetic acid).[22] The substitution of either methyl or hydroxyl on the α-carbon atom of a β-keto acid increases its lability considerably. Neither of the latter two acids could survive the 100° treatment for more than a few seconds to minutes. We can therefore be confident that the substitution of both methyl and hydroxyl on an α-carbon atom of a β-keto acid such as is here proposed would lead to an extremely labile compound. The possibility that we may be dealing with a 2-keto-3-carboxy pentitol stabilized against decarboxylation by a 2, 5-ketol (furanoside) formation is eliminated on the grounds that such a furanoside would not exhibit the great susceptibility to borohydride reduction that this compound does. Beyond this, the appearance of glyceric acid in the degradation of the compound speaks against the carboxyl group's being on the 3-carbon atom of a five-carbon chain.

The clean and quantitative formation of xylulose under the very mild conditions of acid treatment described above eliminates both the α- and the δ-keto acids as possibilities, since these acids would be unchanged under the mild conditions used here.[13] 2-Ketogluconic and 5-ketogluconic acids were treated with 0.07 M HCl for 30 minutes at 100° and chromatographed in the usual manner: they were found to be unchanged, and no neutral material (lactones or sugars) appeared. In stronger acid (5 M HCl) both the 2- and 5-ketogluconic acids can be converted via the corresponding ascorbic acids to decarboxylation products such as furfural.[13] The likelihood thus remains that the compound is a 4-(or γ-)keto acid.

Steric considerations apart, three six-carbon γ-keto sugar acids are possible (I, II, and III):

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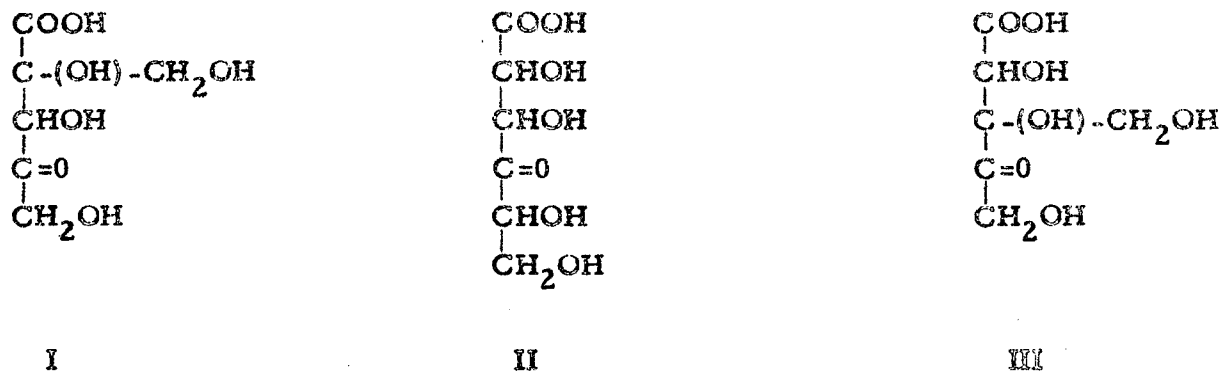
[22] B. R. Brown, Quart. Revs. (London) 5, 131 (1951).



I II III

Structure III can probably be excluded on the grounds that decarboxylation of such a branched-chain sugar acid, if it occurred at all, would most probably result in a branched-chain pentose, or a tetrose, whereas xylulose was actually the decarboxylation product. Structures I and II cannot be unequivocally distinquished, though the available evidence favors I. Decarboxylation of II would be expected to be more difficult than that of I, since it would require the elimination of a secondary hydroxyl group in II rather than a tertiary one as in I. Furthermore, the alkaline decomposition of the unknown substance (presumably via epimerization to the β-keto acid) to produce some glyceric acid is more in keeping with I than with II.

We must now provide a route for the formation of xylulose (IX) from such a γ-keto acid (IV) under the conditions where under we know this transformation occurs. Unfortunately, we have no single model compound incorporating all the structural features of this one--viz. an α-tertiary hydroxyl group, a β-hydroxyl group, and a γ-ketone function, which seem pertinent to an understanding of this transformation. One can, however, formulate it in terms of a sequence of three reactions all of which are known to take place rapidly under the conditions here required. These are: (a) the trans elimination of water from V to form the α,β-unsaturated, β-hydroxy, γ-keto acid (VI), which ketonizes to form the β,γ-diketo acid (VII); (b) the decarboxylation of this β-keto acid to form an enolic ketone (VIII); and (c) the trans addition of water to this enolic ketone, yielding xylulose (racemic) (IX) (Fig. 12). The rates of all these reactions in separate model cases are known to be sufficiently rapid to account for this sequence. The absence of any other intermediates on the chromatogram (Fig. 3) would require that the over-all rate-limiting step be the trans elimination of water (V to VI). The uncertainty lies in the possible simultaneous occurrence of the decarboxylation (VII to VIII)

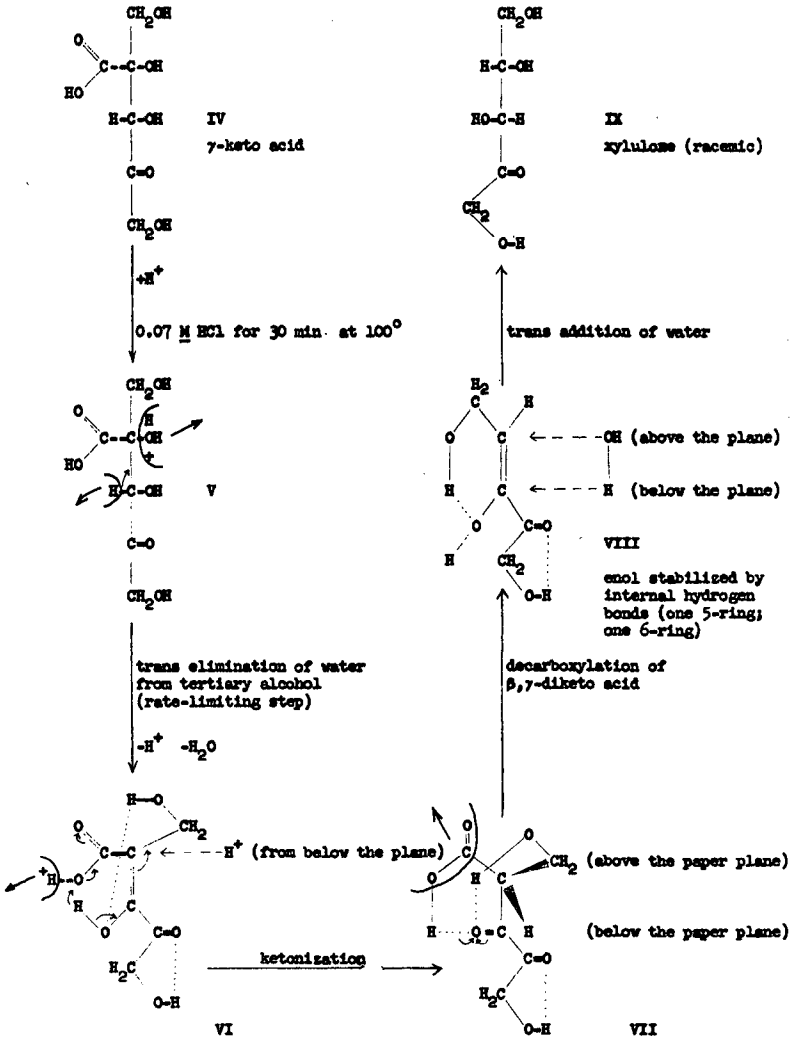


Fig. 12. Proposed mechanism for the decarboxylation of the γ-keto acid to xylulose.

and the trans addition of water (VIII to IX). One can rationalize a stabilized enolic ketone (VIII) in terms of internal hydrogen bonds that would give it a lifetime long enough to provide the configurational requirement needed for the trans addition to produce xylulose. The possibility that the series of transformations may begin by the formation of the β, γ-enol, γ-lactone of IV is recognized but considered unlikely in view of the ease of the tertiary hydroxyl elimination to form VI.

The question of the steric configuration of the γ-keto acid poses a further consideration. Figures 5 through 8 show that after reduction with borohydride the γ-keto acid co-chromatographs very closely, though not identically, with the two samples of hamamelonic acid available. Figure 13 shows the steric relationship between the two possible diastereomers of the γ-keto acid (XV and XVI) and its reduction products, epimers of hamamelonic acid (XI to XIV). The sample of hamamelonic acid obtained by Schmidt and Heintz[17] contained only one isomer, hamamelonic acid itself, structure XIV, whereas that of Rabin et al.,[19] prepared by the action of potassium cyanide on ribulose diphosphate, presumably contained structures XIII and XIV. Two possible epimeric forms of the γ-keto acid can be produced by carboxylation of ribulose, structures XV and XVI, which on reduction would result in substances XI and XIII from XV, and XII and XIV from XVI. Experimentally it was found that the reduced γ-keto acid co-chromatographed more closely with the mixture of XIII and XIV than with pure XIV itself. It could be argued from this that reduction of the γ-keto acid produced XI and XIII, neither of which was identical with the hamamelonic acid of Schmidt and Heintz (XIV),[17] and which therefore did not co-chromatograph precisely with this sample. However, one form of the reduced γ-keto acid (XIII) would correspond with one epimer in the sample from Rabin et al.[19] The lack of exact co-chromatography in this case would then result from the presence of radioactive XI derived from the γ-keto acid by reduction, and of carrier XIV present in the mixture from Rabin et al.

The γ-keto acid thus tentatively appears to be best represented by structure XV. It is interesting to note that trans elimination from this stereoisomer would produce directly the proper geometric form of the enolic ketone VI (Fig. 12). The structure XV is consistent with the inability to lactonize, as six-membered lactone rings containing a ketone group represent strained configurations and tend not to be formed.

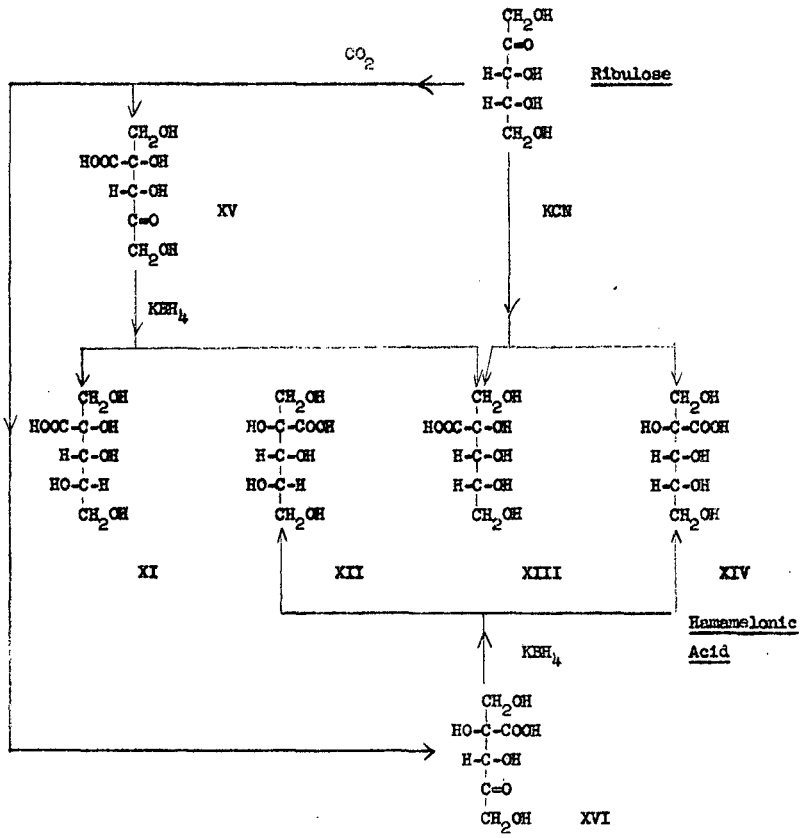
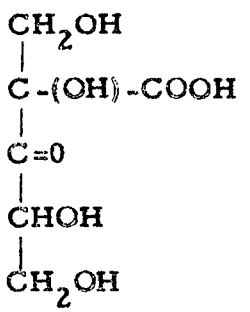


Fig. 13. The steric relationships between the two possible diastereomers of the γ-keto acid and its reduction products, epimers of hamamelonic acid.

The evidence for the structure of the β-keto acid (XVII) is less extensive. β-keto acids are known to be very unstable,[22] as this substance has been found to be,



XVII

whereas the ability of the substance to lactonize under acid conditions (indeed, it was first observed as a lactone) would, in this case, involve a five-membered lactone ring containing a ketone group, which is sterically a less strained configuration. Under mild alkaline conditions the lactone ring is easily opened to produce the free acid, and the substance readily decomposes to form glyceric acid, by hydrolysis between carbon atoms 2 and 3. The free acid, like the γ-keto acid, does not co-chromatograph with hamamelonic acid, though after reduction with borohydride, co-chromatography is very close to (and perhaps identical with) hamamelonic acid (Figs. 10 and 11).

Decarboxylation of the β-keto acid was not directly demonstrated, since the entire supply was used in the reduction experiment. However, treatment of the γ-keto acid with mild alkali, followed by acidification at 0° to pH 3, conditions which might be expected to produce some of the β-keto acid from the γ-keto acid by enolization, produced instead ribulose and glyceric acid (Fig. 4). The glyceric acid could result from hydrolytic cleavage of the β-keto acid at alkaline pH, and the ribulose by decarboxylation at acid pH. In this case decarboxylation would involve the migration of the carbonyl group (Fig. 14). No evidence is available as to the steric configuration of the β-keto acid, save that the δ carbon atom should have the same configuration as the original ribulose from which it was formed.

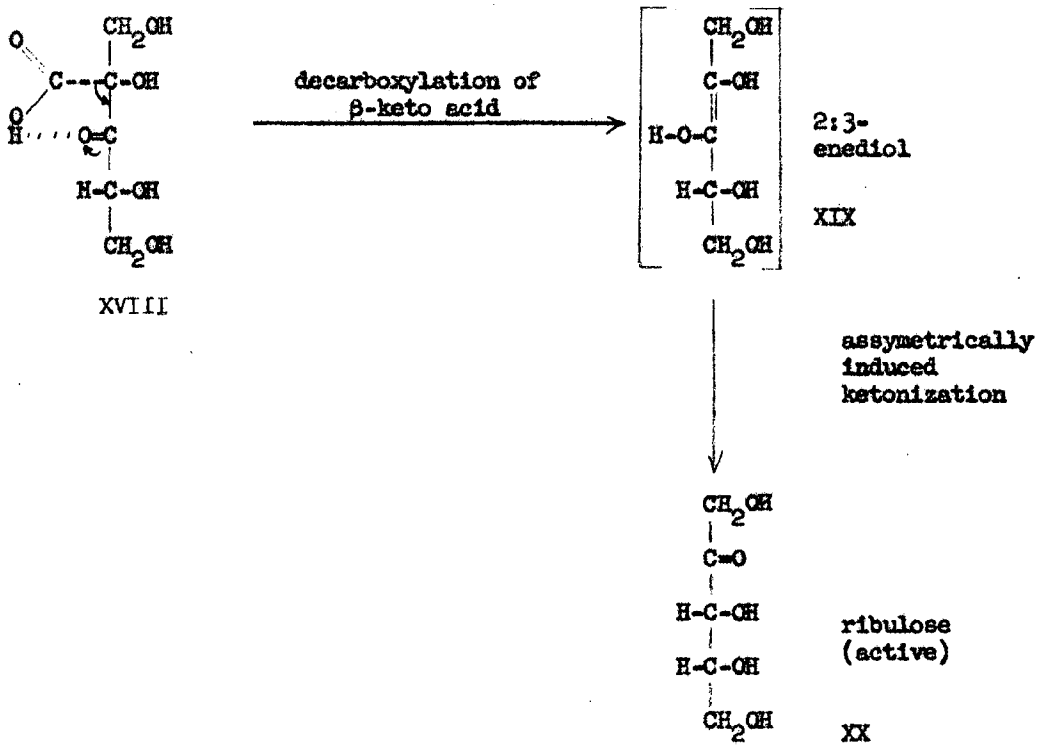
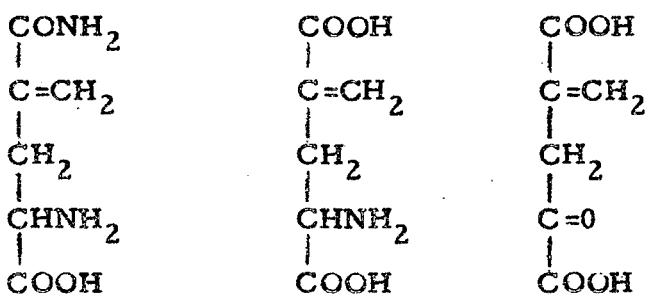


Fig. 14. Suggested mechanism for the decarboxylation of the β-keto acid to ribulose.

Biological Significance

Although the β-keto acid diphosphate has been predicted theoretically in the photosynthetic carboxylation of ribulose diphosphate,[6,7] there has been to our knowledge no previous demand for the existence of the γ-keto acid and hence no role is immediately apparent for it. It may, indeed, be an artifact resulting from the procedures of killing and extracting the cells, and from the subsequent chromatography, and thus be produced nonenzymatically from the β-keto acid, or from another compound. However, it seems that, with identical killing and chemical techniques, more radioactive γ-keto acid appears to be formed from labeled carbon dioxide in cells that have been allowed to carry on photosynthesis in the presence of 0.001 M NH_4NO_3 than in cells suspended in distilled water. The γ-keto acid may accordingly be involved in nitrogen metabolism.

It is of interest in this connection to recall the findings of Done and Fowden,[23] who observed that in peanuts the most important free amide present in these plants was γ-methylene-glutamine (XXI), while the relative amount of glutamine in relation to asparagine was very much reduced. γ-Methylene-glutamic acid (XXII) and γ-methylene-α-ketoglutaric acid (XXIII) were also subsequently found in peanuts,[24,25] and it was suggested[24] that these compounds may be the ones most intimately concerned with nitrogen transport in these plants.



XXI XXII XXIII

[23] J. Done and L. Fowden, Biochem. J. 49, xx (1951).
[24] J. Done and L. Fowden, Biochem. J. 51, 451 (1952).
[25] L. Fowden and J. A. Webb, Biochem. J. 59, 228 (1955).

The similarity between the skeletal structures of the γ-keto acid (IV) and γ-methylene-glutamic acid and amide (XXI and XXII) are immediately apparent, and it would not be difficult to conceive a metabolic pathway for the production of γ-methylene-glutamic acid from the γ-keto acid. Such a hypothetical pathway involving alternate dehydration and reduction following an initial oxidation, is illustrated in Fig. 15, though many other schemes would be possible; it is of interest to note that the γ-keto acid and γ-methylene α-ketoglutaric acid are isoximeric.

Another possible route for the further metabolism of the γ-keto acid might be an enzymatic inverse aldolase split to hydroxypyruvate and dihydroxyacetone. The hydroxypyruvate could give rise by transamination to serine, a substance known to incorporate radiocarbon very rapidly from carbon dioxide during photosynthesis. The dihydroxyacetone, during the initial stages of photosynthesis, would not incorporate isotopic carbon from labeled carbon dioxide, and such a dilution of labeled dihydroxyacetone, formed from 3-phosphoglyceric acid, by this unlabeled dihydroxyacetone might in part account for some of the anomalous labeling during photosynthesis that has been reported in the hexoses.[26]

Treatment of all the three diphosphate spots shown in Fig. 1 with phosphatase, followed by further chromatography, has confirmed the findings of Rabin et al that hamamelonic acid diphosphate is not formed during photosynthesis in the absence of inhibitors.[19] Apart from the β- and γ-keto acids that form the subject of this communication, no other acids moving chromatographically in the area of hamamelonic acid or its lactone have been found in any of the three diphosphate spots of the original chromatogram.

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[26] O. Kandler and M. Gibbs, Plant Physiol. 31, 411 (1956).

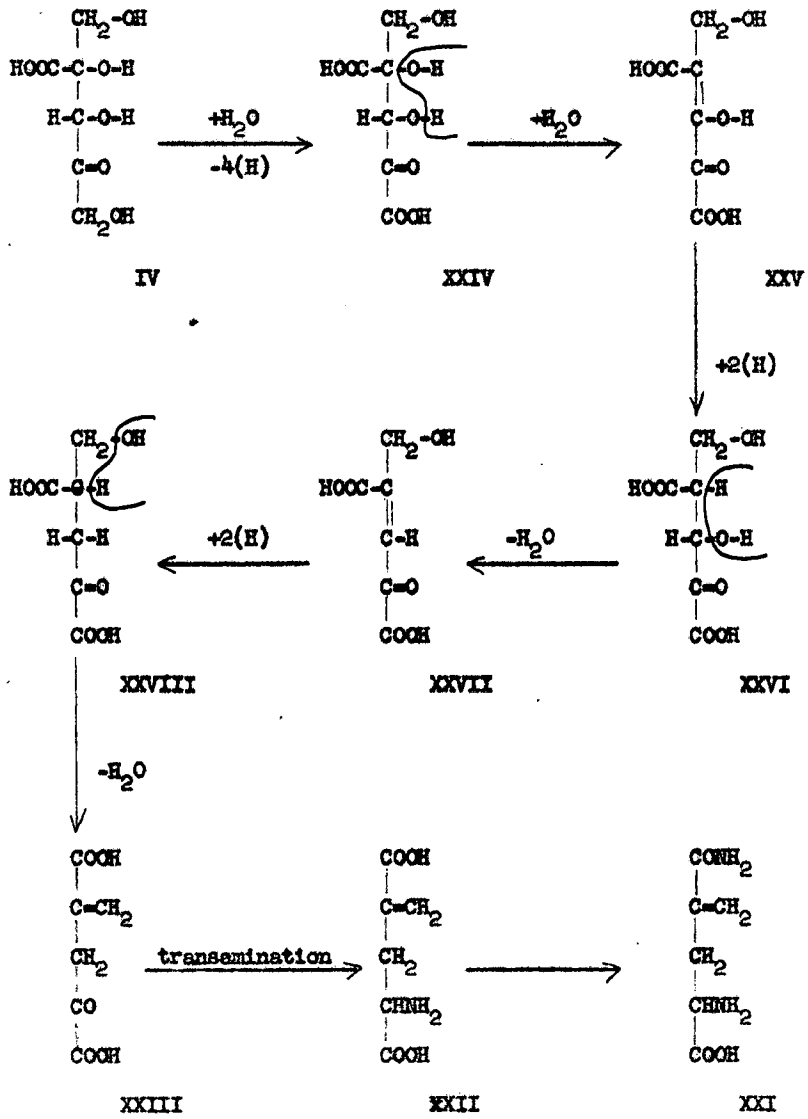

Fig. 15. Hypothetical scheme for the conversion of the γ-keto acid into γ-methyleneglutamic acid.