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Characterization of the Photosynthetically Synthesized " g-Keto Acid" Phosphate as a Diphosphate Ester of 2-Keto-L-Gulonic Acid

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CHARACTERIZATION OF THE PHOTOSYNTHETICALLY SYNTHESIZED  
"γ-KETO ACID" PHOSPHATE AS A DIPHOSPHATE ESTER  
OF 2-KETO-L-GULONIC ACID

V. Moses, R. J. Ferrier and M. Calvin

June 1962

CHARACTERIZATION OF THE PHOTOSYNTHETICALLY SYNTHESIZED " $\chi$ -KETO ACID"

PHOSPHATE AS A DIPHOSPHATE ESTER OF 2-KETO-L-GULONIC ACID\*

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INTRODUCTION

Some years ago Calvin<sup>1,2</sup> suggested that the primary reaction in the photosynthetic assimilation of carbon dioxide, the carboxylation of D-ribulose-1,5-diphosphate, produces, in the first instance, a labile  $\beta$ -keto acid, 2-carboxy-3-pentulose-1,5-diphosphate (3-keto-2-C-phosphohydroxymethyl-pentonic acid-5-phosphate), which is subsequently cleaved to two molecules of 3-phosphoglyceric acid. In search for such a compound, Moses and Calvin<sup>3</sup> observed the incorporation by Chlorella of <sup>14</sup>C from NaH<sup>14</sup>CO<sub>3</sub> into two substances having the general characteristics of diphosphate esters of ketohexonic acids. One of these compounds appeared to be quite stable, and was therefore unlikely to be the  $\beta$ -keto acid phosphate proposed as the carboxylation intermediate. On the basis of a number of chemical tests, this compound was tentatively assigned to the class of 2-carboxy-4-pentulose diphosphates ( $\chi$ -keto acid esters). The other substance, about which less information was obtained, was very unstable, and it appeared possible that this was indeed the sought-after intermediate.

It was not possible to arrive at any definite decisions regarding the steric configuration of either of these components, since the various isomeric compounds of these types were not available for

comparison. However, a compound obtained by the reduction of the " $\gamma$ -keto acid" was shown to have chromatographic properties very similar to, but not identical with, those of hamamelonic acid (2-C-hydroxymethyl-D-ribonic acid).

Two aspects of the findings were not fully explained at the time: (i) reduction with borohydride of the keto acid should have yielded two isomeric hydroxyacids, yet only one was obtained; (ii) the apparent inability of the " $\gamma$ -keto acid" to form a lactone in acid media.

More recently, all four potentially chromatographically resolvable 2-C-hydroxymethylpentonic acids have been prepared<sup>4</sup>, and the problem was reopened with a view to determining more precisely the chemical configuration of the " $\gamma$ -keto acid".

#### METHODS

The methods for the preparation of the <sup>14</sup>C-labelled " $\gamma$ -keto acid" and its phosphate ester were the same as those used previously<sup>3</sup>. For an investigation of the kinetics of the formation of the substance biologically, a suspension of 0.25 ml. of wet-packed Chlorella pyrenoidosa cells in 4.75 ml. of distilled water in a 125 ml. Erlenmeyer flask was incubated at 25° while illuminated from below with a light intensity of about 6000 foot candles. At the beginning of the incubation period 0.25 ml. of a solution of NaH<sup>14</sup>CO<sub>3</sub> (100  $\mu$ C.; 3  $\mu$ moles) was added to the cells. Samples (approx. 0.5 ml.) of the reaction mixture were removed at 5, 10, 20, 30, 45, 60, 90, 120 and 180 sec. and rapidly injected into tared tubes containing 2 ml. of ethanol. These tubes were stoppered and subsequently weighed to determine the precise quantity of the reaction mixture sample. The cells were centrifuged,

the supernatants removed, and the residues extracted twice with 0.25 ml. of 20% (v/v) aqueous ethanol. The supernatants were pooled, concentrated under reduced pressure below 40°, and chromatographed in two dimensions in toto on oxalic acid-washed Whatman No. 4 paper. The chromatograms were developed for 24 hr. with the first solvent (phenol-water<sup>5</sup>) and for 20 hr. in the second solvent (n-butanol-propionic acid-water<sup>5</sup>). The positions of the radioactive substances on the chromatogram were determined by radioautography, and the amount of <sup>14</sup>C in each compound measured using an automatically operating and recording radiation counter<sup>6</sup>. The labelled compounds, after removal of the phosphate groups with human seminal acid phosphatase, were identified by cochromatography with authentic non-radioactive markers. Where appropriate, substances were reduced with borohydride before cochromatography.

#### RESULTS

##### Identification of the chemical structure of the "γ-keto acid".-

In contrast with the earlier findings<sup>3</sup>, reduction of the dephosphorylated acid with potassium borohydride, followed by removal of the potassium ions with Dowex-50 (hydrogen form), of the boric acid by repeated evaporation to dryness from methanol, and lactonization of the reduction products by repeated evaporation to dryness from glacial acetic acid solution, produced two chromatographically resolvable lactones. Both of these lactones were quantitatively converted to the salts of the corresponding acids by mild alkaline treatment (0.5 N-NH<sub>4</sub>OH overnight at room temperature). That these were not two lactones (e.g. γ- and δ-) of the same acid was shown by the fact that after conversion of each lactone to the salt by alkali,

relactonization produced in each case only one compound - that from which the salt had been derived.

The reductions of the dephosphorylated keto acid were performed by dissolving the dried chromatographic eluates in 0.05 ml. of freshly prepared 0.075 M-aqueous potassium borohydride, and allowing the mixtures to stand at 37° for 2 hr. before removal of the potassium and boron. The solutions were acidified before being spotted onto chromatograms. Under these standard conditions, greatly varying ratios of the two lactones were obtained on different occasions, ranging from about 1:10 to about 10:1. The reasons for this variation have not been investigated. Under the reducing conditions used only the ketone function was reduced, and not the carboxylic acid (either free or as lactone).

Cochromatography of these two lactones with the lactones of the non-radioactive 2-hydroxymethylpentonic acids demonstrated that the reduction products of the " $\gamma$ -keto acid" did not belong to this class of compounds. Attention then turned to the possibility that they were derived from straight-chain hexonic acids. The lactone with the lower chromatographic mobility was shown to be gulonic- $\gamma$ -lactone by two-dimensional chromatography in the following pairs of solvent systems: phenol-water plus n-butanol-propionic acid-water, and ethyl acetate-pyridine-water (10:4:3) plus n-butanol-ethanol-water (4:1:5, organic layer).

Four ketogulonic acids could have given rise to gulonic acid as one of two reduction products: those with the ketone function at C(2), C(3), C(4) or C(5) (Fig. 1). The isomeric products would have been idonic, galactonic, allonic or mannonic acid, respectively.



3-Ketogulonic acid is a  $\gamma$ -keto acid and has been shown to be very unstable<sup>7</sup>; our compound, which is quite stable, was therefore not the 3-keto compound. The second reduction product was shown chromatographically in the two pairs of solvent systems to be different from allonic and mannonic acids (run as their lactones), but identical with idonic acid. The structure of the original keto acid was thus established as being 2-ketogulonic acid (Fig. 1).

The absolute configuration of the derived radioactive gulonolactone was determined as follows<sup>8</sup>. A sample of gulonolactone-<sup>14</sup>C was dissolved in 2 ml. of glacial acetic acid. To half this solution was added 50 mg. of D-gulono- $\gamma$ -lactone,  $[\alpha]_D -54^\circ$  (Pfansteil Chemical Co., catalogue no. 4764). To the other half was added 50 mg. of L-gulono- $\gamma$ -lactone,  $[\alpha]_D +53^\circ$ , prepared from D-glucuronolactone<sup>9</sup>. The added lactones were dissolved in 4 ml. of glacial acetic acid, with warming. Crystals were recovered from each of these solutions, which were washed with glacial acetic acid and dried. Measurement of the specific radioactivity of these crystals through two crystallizations (Table I) demonstrated that the gulonolactone-<sup>14</sup>C cocrystallized with the authentic L-gulono- $\gamma$ -lactone, but not with the D-isomer. The materials obtained from the second crystallizations were dissolved in 3 ml. of methanol, and converted to the amides by the addition of 3 ml. of methanol containing 15% of anhydrous ammonia. The solutions were concentrated under nitrogen, taken up in ethanol and the amides allowed to crystallize. Measurements of the specific radioactivities of the products (Table I) confirmed that the amide derived from gulonolactone-<sup>14</sup>C was the L-isomer. The original " $\gamma$ -keto acid" was thus 2-keto-L-gulonic acid.

With the establishment of the structure of this compound, the

earlier observation<sup>3</sup> that treatment of it with hot hydrochloric acid produces xylulose and not a lactone, was reinvestigated. It was found that acid treatment did indeed result in some lactone formation, while xylulose was not produced. The original error arose because the keto acid lactone and xylulose have indistinguishable chromatographic parameters in both phenol-water and in n-butanol-propionic acid-water. Chromatography in other solvent systems (ethyl acetate-pyridine-water and n-butanol-ethanol-water) permitted the 2-ketogulonolactone to be distinguished from xylulose.

Investigation of the phosphate ester of 2-keto-L-gulonic acid.- In a

phosphate ester of 2-ketogulonic acid, phosphates on the carboxyl or enolic carbonyl groups (carbons 1 and 2) might be distinguished from those on carbons 3-6, since the former, but not the latter, would be hydrolyzed in 7 min. by N-hydrochloric acid at 100°<sup>10</sup>.

A sample of the phosphate ester of 2-keto-L-gulonic acid-<sup>14</sup>C was divided into two equal portions: one aliquot was spotted onto paper for chromatography, while the other was heated at 100° for 7 min. with N-hydrochloric acid, and then chromatographed. Both chromatograms were run in the phenol-water, n-butanol-propionic acid-water system.

With the untreated sample, all the <sup>14</sup>C was recovered from the diphosphate area of the chromatogram<sup>5</sup>. After heating with hydrochloric acid, about 45% of the <sup>14</sup>C was recovered from the diphosphate area, 15% from the monophosphate area, and 40% appeared in free ketogulonic acid. On hydrolysis with acid phosphatase, all the compounds obtained from the phosphate areas of these chromatograms produced only 2-ketogulonic acid. Since treatment with hydrochloric acid left some 45% of the starting material unchanged, this evidence suggests that

none of the phosphate groups is acid labile in the conventional sense<sup>10</sup>, and hence none is attached to either carbons 1 or 2 of 2-ketogulonic acid. The formation of a monophosphate ester as well as some free acid by hydrochloric acid treatment was probably due to non-specific hydrolysis of one or both phosphate groups from carbons 3-6. Ribulose-1,5-diphosphate and fructose-1,6-diphosphate, at <sup>14</sup>C concentrations approximately equal to that of the ketogulonic acid diphosphate, when treated in a similar manner with hydrochloric acid, also partially decomposed to produce some monophosphate esters and some free sugars, though neither of the phosphate groups in these compounds is usually considered to be acid labile under the conditions used here. It seems probable, therefore, that there are two phosphate groups attached to the 2-keto-gulonic acid, that they are similarly linked, and that both are alkyl rather than acyl or enolic phosphates.

Biochemistry of 2-keto-L-gulonic acid diphosphate formation. - The rates of <sup>14</sup>C incorporation in the light into a number of phosphorylated compounds of *Chlorella* are given in Table II; the radioactivity present in each compound is expressed as a percentage of the <sup>14</sup>C found in all those compounds which were studied. Both the mono- and diphosphate areas of the chromatogram, when treated with phosphatase, yielded <sup>14</sup>C-labelled 2-ketogulonic acid; <sup>14</sup>C appeared in 2-ketogulonic acid in the diphosphate area before that from the monophosphate area. This finding indicates that in the biosynthesis of this compound it is the diphosphate ester which is formed first, presumably either from a diphosphate ester of another six-carbon compound, or perhaps by the union of two monophosphates of two three-carbon compounds, or a two-carbon compound with a four-carbon compound. The monophosphate

ester of 2-ketogulonic acid could then arise from the diphosphate by removal of one phosphate group.

As the radioactivity present in the phosphate esters of 2-ketogulonic acid was very much less than that found in other sugar phosphates which might possibly have been their precursors, it was not possible to relate the rate of formation of 2-ketogulonic acid phosphates to changes in  $^{14}\text{C}$  concentration of other components.

#### DISCUSSION

Nature of the phosphate ester of 2-keto-L-gulonic acid.— Four observations suggest that the compound originally isolated<sup>3</sup> possessed two phosphate groupings. The substance was obtained from that area of the chromatogram known to be the position of sugar diphosphates<sup>5</sup>. No chromatographic information is available about keto acid diphosphates, but it is worthy of note that 6-phosphogluconic acid runs in the monophosphate region and carbonyl groups do not have any very marked effect on mobilities of carbohydrates.

In an experiment in which the presumed diphosphate of 2-ketogulonic acid was used as a substrate for spinach chloroplasts<sup>11</sup> one of the products observed was a compound which ran in the monophosphate region of the chromatogram and which, on hydrolysis with phosphatase, yielded 2-ketogulonic acid. This supports the contention that the monophosphate ester of 2-ketogulonic acid possesses the chromatographic properties to be expected of such an ester, and does not run with the sugar diphosphates. The experiment on the kinetics of the biosynthesis of 2-ketogulonic acid reported in this communication similarly demonstrates two phosphate esters of 2-ketogulonate, one of which ran with the sugar monophosphates and the other with the sugar diphosphates.

Finally, the results described above relating to the hydrolysis of the phosphate ester by hydrochloric acid indicate that as originally isolated the compound contained two phosphate groups. The data on the lability of the phosphate groups indicates not only that both phosphate groups were similarly attached to the sugar acid and that neither was an acyl or an enolic phosphate, but also that a pyrophosphate linkage was not involved since these are also acid labile<sup>10</sup>. We are therefore confident that the "X-keto acid diphosphate" is 2-keto-L-gulonic acid diphosphate, the phosphate groups probably residing on two of the carbon atoms 3-6.

Biological origin of 2-keto-L-gulonic acid diphosphate.- The kinetics of the photosynthetic formation of 2-ketogulonic acid diphosphate from  $\text{NaH}^{14}\text{CO}_3$  reported above suggest that this substance is not closely related to the primary intermediates of the carbon reduction cycle. The maximum percentage of  $^{14}\text{C}$  incorporation into such cycle intermediates as phosphoglyceric acid, fructose diphosphate and sedoheptulose monophosphate occurred within about 20 sec. from the start of the experiment; ribulose diphosphate showed a maximum at about 60 sec. However, 2-ketogulonic acid diphosphate did not show a peak until 120 sec., suggesting that it is formed later than the cycle intermediates. Evidence obtained by Louwrier<sup>12</sup> from experiments involving the sweeping out of radioactivity from the ribulose diphosphate pool by  $^{12}\text{CO}_2$  without concomitant loss of  $^{14}\text{C}$  from 2-ketogulonate diphosphate, supports the idea that the keto acid is not formed from a direct carboxylation reaction of ribulose diphosphate unless it is the stable end product of an irreversible carboxylation with rearrangement.

In a study of the incorporation of  $^{14}\text{C}$  from  $^{14}\text{CO}_2$  by illuminated Chlorella in the presence and absence of unlabelled glucose, and of the incorporation of  $^{14}\text{C}$  from glucose-U- $^{14}\text{C}$  in the presence and absence of unlabelled  $\text{CO}_2$  in the light and dark, it was found that  $^{14}\text{C}$  appeared in 2-ketogulonic acid diphosphate from glucose-U- $^{14}\text{C}$  in the dark, even though ribulose-1,5-diphosphate was unlabelled<sup>13</sup>. (At that time the component now characterized as 2-ketogulonic acid was believed to be 2-carboxy-4-pentulose.) The effect of illumination on the incorporation of  $^{14}\text{C}$  from glucose-U- $^{14}\text{C}$  into 2-ketogulonic acid diphosphate was much less (an increase of 23%) than the effect on the  $^{14}\text{C}$  in ribulose diphosphate; in the light the latter contained 1.7 times as much  $^{14}\text{C}$  as 2-ketogulonate.

Furthermore in the light the effect of the presence of unlabelled glucose reduced the incorporation of  $^{14}\text{C}$  from  $^{14}\text{CO}_2$  into 2-ketogulonic acid diphosphate by 22% although the total  $^{14}\text{C}$  incorporated was not affected, and the incorporation into (photosynthetically produced) glucose monophosphate increased by 32%; glucose diphosphate was not affected. Thus the evidence at this stage suggests that 2-ketogulonic acid is derived from glucose or a phosphate ester thereof in the first instance, but that this glucose derivative is not functionally identical with glucose or glucose phosphate derived directly from the carbon reduction cycle. Since the  $^{14}\text{C}$  incorporated from glucose-U- $^{14}\text{C}$  plus  $^{12}\text{CO}_2$  into glucose monophosphate, fructose monophosphate, sedoheptulose monophosphate and other closely related compounds was depressed by 30-50% during illumination while the  $^{14}\text{C}$  in 2-ketogulonic acid increased by 23%, it appears probable that externally supplied glucose is metabolized by Chlorella by two mechanisms, only one of which equilibrates with intermediates formed

photosynthetically from  $\text{CO}_2$ ; it is by the other mechanism that 2-ketogulonate is formed.

2-Keto-L-gulonic acid, on enolization and lactonization, becomes L-ascorbic acid and thus is a possible precursor of the latter. Such a biosynthetic scheme for the formation of L-ascorbic acid has already been proposed<sup>14</sup>, though it was not believed that phosphorylated derivatives were involved. Studies are continuing on the possible role of 2-keto-L-gulonic acid diphosphate in the mechanism L-ascorbic acid biosynthesis.

#### SUMMARY

A substance isolated from Chlorella pyrenoidosa metabolizing  $^{14}\text{CO}_2$  in the light, previously believed to be a diphosphate ester of a 2-carboxy-4-pentulose<sup>3</sup>, has now been shown to be a diphosphate of 2-keto-L-gulonic acid. The phosphate groups appear to be attached to two of the carbon atoms 3-6. Evidence is presented suggesting that this compound arises from glucose, or a glucose phosphate, which is not in rapid equilibrium with photosynthetically produced glucose derivatives.

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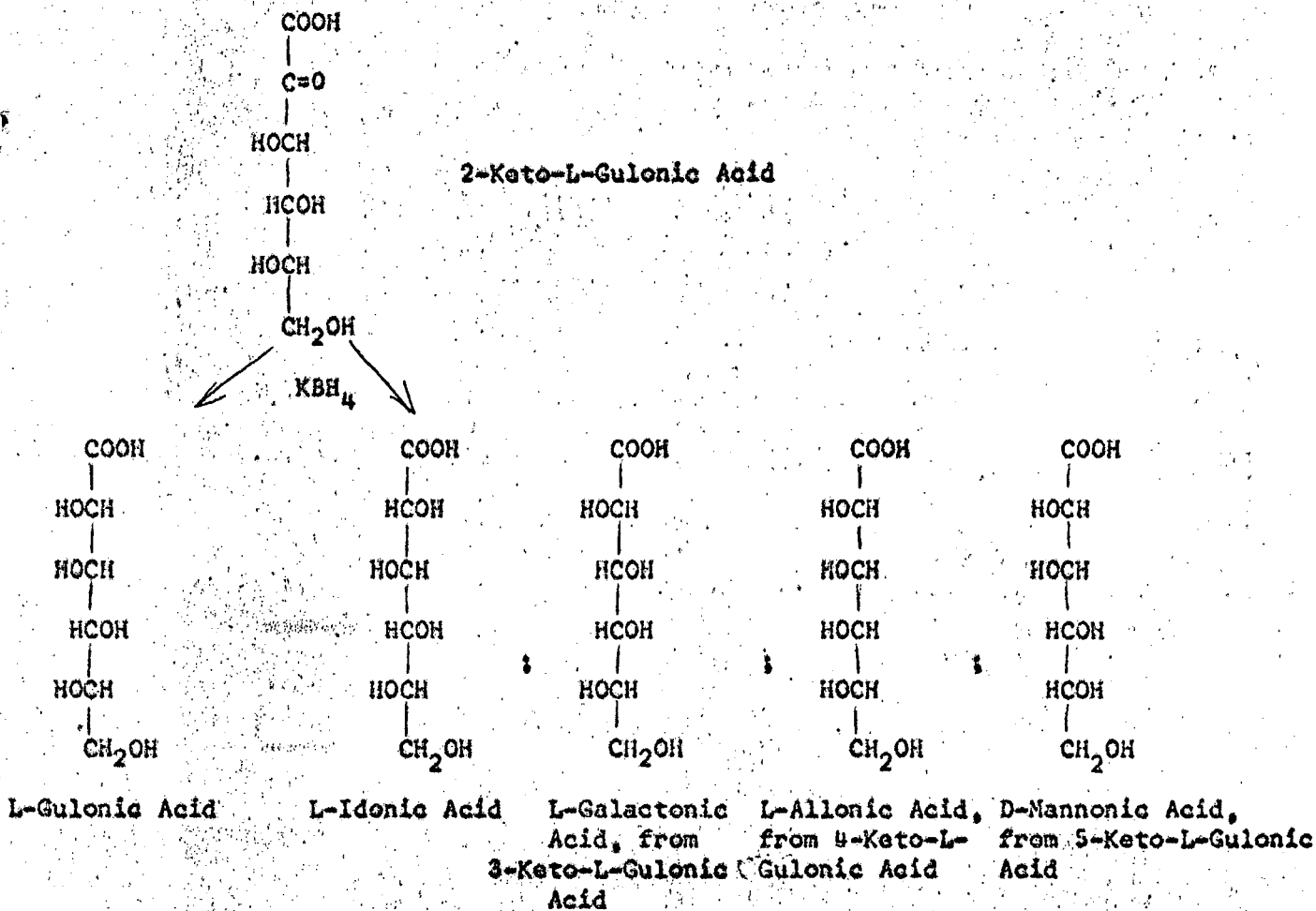


Fig. 1. The reduction of the different keto-L-gulonic acids.

TABLE I

COCRYSTALLIZATION OF GULONOLACTONE OBTAINED BY REDUCTION OF 2-KETOGULONIC ACID WITH L-GULONO- $\gamma$ -LACTONE AND D-GULONO- $\gamma$ -LACTONE

No. of crystallizations	mg. recovered	specific activity (counts/min./mg. carbon)
1. D-	47	46
L-	39	501
2. D-	39	7
L-	36	486
3. Amide		
D-	25	< 2
L-	24	484

TABLE II  
 INCORPORATION OF  $^{14}\text{C}$  FROM  $\text{NaH}^{14}\text{CO}_3$  INTO A NUMBER OF SUBSTANCES BY  
 ILLUMINATED CHLORELLA CELLS

(Expressed as percentage of activity in the components counted in each sample.)

Incubation time (sec.)	5	10	20	30	45	60	90	120	180
Phosphoglyceric acid	60.7	52.6	37.5	36.1	16.6	13.1	11.2	12.0	11.0
Glucose monophosphate	16.0	20.4	31.1	29.2	33.5	35.3	36.2	38.7	45.6
Fructose monophosphate	5.7	6.6	4.7	6.4	7.4	6.5	7.1	6.1	2.6
Sedoheptulose mono- phosphate	13.4	15.4	15.1	12.4	4.2	3.1	3.0	2.5	3.4
2-Keto-L-Gulonate monophosphate	0.0	0.0	0.0	0.0	0.0	0.02	0.04	0.05	0.1
Glucose diphosphate	0.8	1.1	1.9	1.7	1.5	1.7	2.0	1.8	1.9
Fructose diphosphate	1.2	1.5	3.1	2.9	3.0	2.7	2.2	2.1	1.9
Ribulose diphosphate	1.6	1.3	3.3	5.8	25.8	27.9	26.2	25.0	22.1
2-Keto-L-gulonate diphosphate	0.0	0.01	0.06	0.1	0.3	0.4	0.9	1.5	1.2
Uridinediphosphoglucose (glucose moiety only)	0.6	1.1	3.2	5.4	7.7	9.3	11.1	10.3	10.2
Total $^{14}\text{C}$ present in all the compounds counted (dis./min./ul wet-packed cells $\times 10^{-3}$ )	61	113	169	187	197	197	151	137	107

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