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Utilization of Free Sedoheptulose by Green Leaf Preparations*

By

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Since the first identification of a phosphate ester of sedoheptulose as a photosynthetic product in green plants[1], evidence has rapidly been accumulated indicating that phosphorylated forms of this seven-carbon sugar play inportant metabolic roles in a variety of organisms. Especially, the isolation and characterization of the enzymes, transketolase[2-7] and transaldolase[8,9], have produced a great deal of informations as to the metabolic origin and fate of the heptose phosphates.

In spite of these achievements with sedoheptulose phosphates, knowledge concerning the metabolic behavior of the heptose in its free form is so far rather scanty. Free sedoheptulose was originally discovered in the succlent plant *Sedum spectabile*[10] and later detected also in other plants belonging to several families such as *Crassulaceae* and *Saxifragaceae*[11-14].

The existence in a sedoheptulose-adapted bacterium of a metabolic pathway utilizing the free heptose was first reported from our laboratory[15]. It was further revealed that the adapted bacterial cells contain a phosphokinase which catalyzes the phosphorylation of sedoheptulose with adenosine triphosphate (ATP)[16]. These observations with bacteria have now invited us to extend similar studies to higher plants. In this paper, we wish to report that spinach leaf preparations possess an enzyme system capable of utilizing free sedoheptulose and this utilization process, too, appears to involve the initial phosphorylation of the heptose molecule with ATP. Recently, Tolbert and Zill[17] have also reported the utilization of C^{14}-labeled sedoheptulose by green leaves under various conditions.

EXPERIMENTAL

Materials and Methods

Three different spinach leaf preparations were employed in the present investigation. (a) **Leaf slices.** Fresh spinach leaves** were washed, chilled to 0°C, and cut into small

*) Contribution from the Department of Chemistry, Faculty of Science, Kanazawa University. The material reported in this paper will also be published elsewhere in Japanese [Seikagaku, **28**, 24 (1956)].

**) Leaves should be used within 5-6 hours after being collected from fields. Otherwise, no or little activity could be detected in any of the preparations.

slices (about 3 × 3 mm). (b) **Preparation I.** Washed leaves were chilled to 0°C, ground in a mortar with 3 parts of powdered glass, extracted with 2 parts of 0.05 *M* bicarbonate buffer (pH 9.4), and centrifuged for 15 minutes at 2,000 × *g* to remove the insoluble residue. An equal volume of cold ethanol (96%) was then added to the bicarbonate extract and the resultant greenish precipitate was collected by centrifugation. The precipitate was rapidly washed two times with cold ethanol and finally suspended in distilled water. (c) **Preparation II.** The bicarbonate extract obtained in the above preparation was made 50% saturated with respect to ammonium sulfate. The precipitate formed was collected, and dissolved in bicarbonate buffer. The ammonium sulfate precipitation was effected once more and the precipitate was dissolved in distilled water. This preparation contained only small amounts of inorganic and acid-labile phosphates (inorganic P, 0.6% and acid-labile P, 0.1%). All these preparations were rather unstable and had to be used immediately after preparation.

Sedoheptulose was obtained as a concentrated syrup from the succlent plant *Sedum japonicum* according to the procedure described by Nordal[18]. This syrup was further purified with the aid of paper chromatography. Five to ten milligrams of the syrup were dissolved in 0.5 ml. of distilled water and applied on a paper strip (Toyo Roshi No. 50, 25 × 25 cm) along a line drawn at an end of the strip (5 cm from the edge.). After developing the chromatogram using a mixture of *n*-butanol, water and acetic acid (4:1:2) as solvent, the area corresponding to Rf values between 0.3 and 0.4 was cut off and eluted with water. The eluate was concentrated to a syrup under reduced pressure (about 50°C). The purity of this syrup was 92-96%; it was free from any inpurities of carbohydrate nature when examined by paper chromatography.

Barium salt of ATP was prepared from rabbit muscles by the method of Szent-Györgyi[19] and converted to sodium salt before use. Purity, 80-85%.

The determination of sedoheptulose was carried out by the method previously described[16]. The procedure for separating sedoheptulose phosphate from the free sugar was, however, omitted in the present investigation.

Both inorganic and acid-labile phosphates were assayed as previously described[16].

A mixture of 20% mercuric acetate and 10% sodium acetate was used as a deproteinizing reagent when the leaf slices were employed. In experiments in which preparations I and II were used, deproteinization was performed by 20% trichloroacetic acid.

Results

Sedoheptulose Consumption by Spinach Leaf Preparations: As can be seen from Fig.1, spinach leaf slices were found to consume an appreciable amount of free sedoheptulose when they were shaken at 35°C with the heptose and inorganic phosphate in citrate buffer (pH 6.4). Their activity was, however, considerably lower than that of the heptose-adapted bacterial cells reported in a previous paper[15]. The heptose-consuming reaction seemed to be aerobic in nature and was greatly diminished in the absence of molecular oxygen. As will be shown below, Preparations I and II were also active in metabolizing

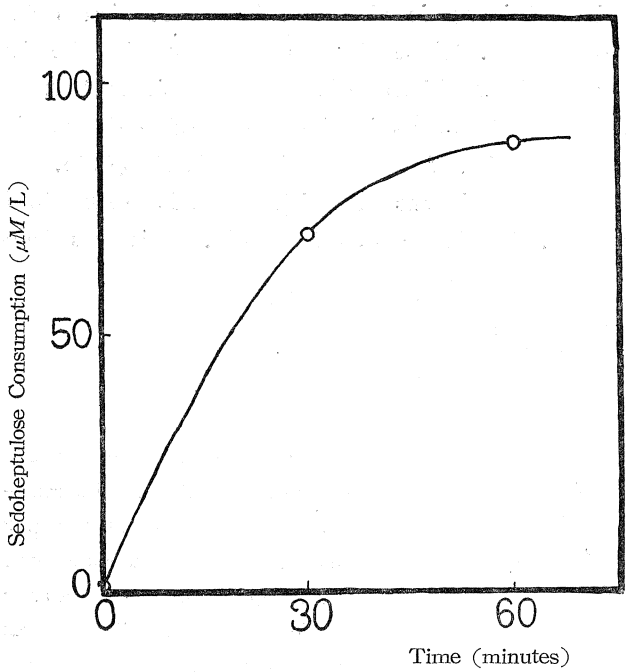

Fig. 1 Sedoheptulose consumption by spinach leaf slices. The reaction mixture consisted of 2 ml. of 0.008 *M* sedoheptulose, 2 ml. of 0.05 *M* citrate buffer containing 10^{-3} *M* inorganic phosphate (pH 6.4), and 3 g. of slices. Shaken at 35°C.

the free sugar if appropriate conditions (*e. g.*, the presence of Mg^{++}) were assured.

Effect of Mg^{++}, Inorganic Phosphate and ATP : Contrary to the leaf slices which were active even in the absence of added cofactors, the presence of Mg^{++} was indispensable for Preparation I (and II) to utilize the free heptose, as is shown in Fig. 2. It will also be

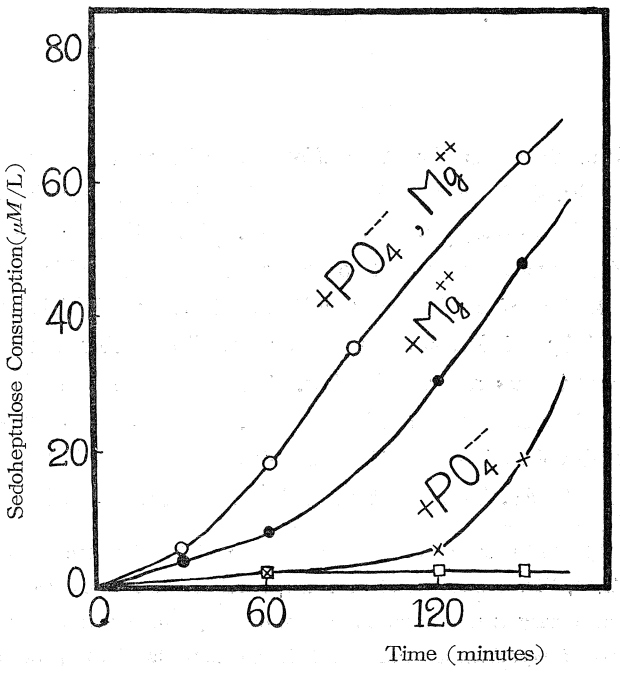

Fig. 2 Effects of Mg^{++} and inorganic phosphate upon sedoheptulose consumption by Preparation I. The complete system contained in a total volume of 4 ml. the followings : Preparation I, 5.6 mg.(dry weight) ; sedoheptulose, 2×10^{-4} *M*; and PO_4^{---}, 5×10^{-4} *M*. In the other systems $MgCl_2$ or PO_4^{---} or the both are omitted as indicated. Shaken at 35°C.

evident from Fig. 2 that the addition of inorganic phosphate in the reaction system greatly stimulated the activity of preparation I. These findings, together with the fact that the consumption is favored under aerobic conditions, point to the possiblity that the consumption of sedoheptulose in the plant systems, as in the heptose-adapted bacteria, is dependent upon the oxidation-coupled formation of ATP from inorganic phosphate.

This assumption received a support from experiments in which inorganic phosphate in the reaction mixture was replaced by ATP. As will be seen from Fig. 3, it was found

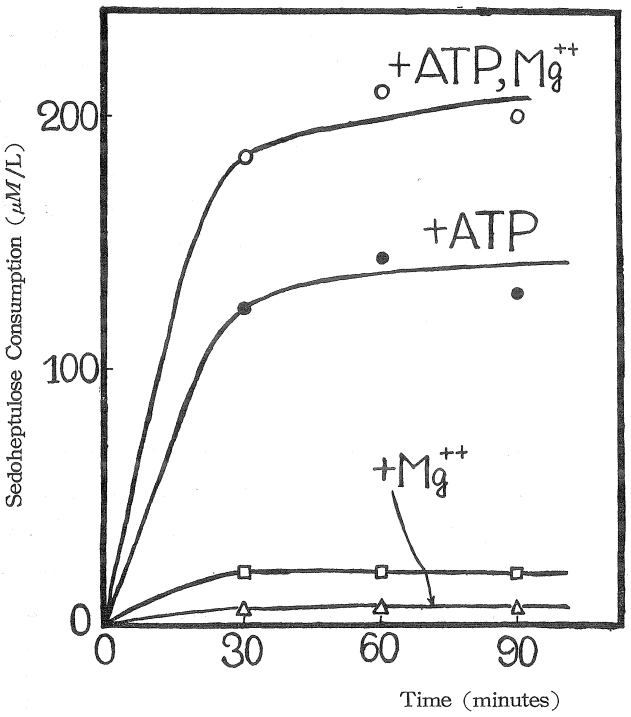

Fig. 3 Replacement of inorganic phosphate by ATP. The conditions were the same as in Fig. 2 except that each reaction mixture contained 8.4 mg. of Preparation I (dry weight), PO_4^{---} was replaced by 5×10^{-4} *M* ATP, and the concentration of $MgCl_2$ was raised to 5×10^{-4} *M*.

that ATP was almost as effective as inorganic phosphate in stimulating the reaction*.

Fig. 4 illustrates the effect of varied concentrations of inorganic phosphate and Mg^{++} on the sedoheptulose consumption. The optimal concentrations for inorganic phoshate and Mg^{++} were both found to be around 10^{-3} *M* under the experimental conditions employed.

Effect of Inhibitors : Effects of several inhibitors upon the sedoheptulose-consuming activity of Preparation I were studied under the same conditions as employed in the experiments recorded in Fig. 2 (both $MgCl_2$ and inorganic phosphate were added to a final concentration of 10^{-3} *M*). It was thus revealed that NaF, Na_2HAsO_4, 2,4-dinitrophenol and $HgCl_2$ were all completely inhibitory to the reaction at a concentration of 10^{-2} *M*. The effect of varied concentrations of each inhibitor has, however, not yet

*) While an appreciable consumption of the heptose is seen in Fig. 2 with the sole addition of Mg^{++}, Fig. 3 shows that no reaction takes place at all under the same condition. The reason for this discrepancy is not clear. It is, however, possible that the enzyme preparations employed in these two sets of experiments contained different amounts of endogenous phosphates.

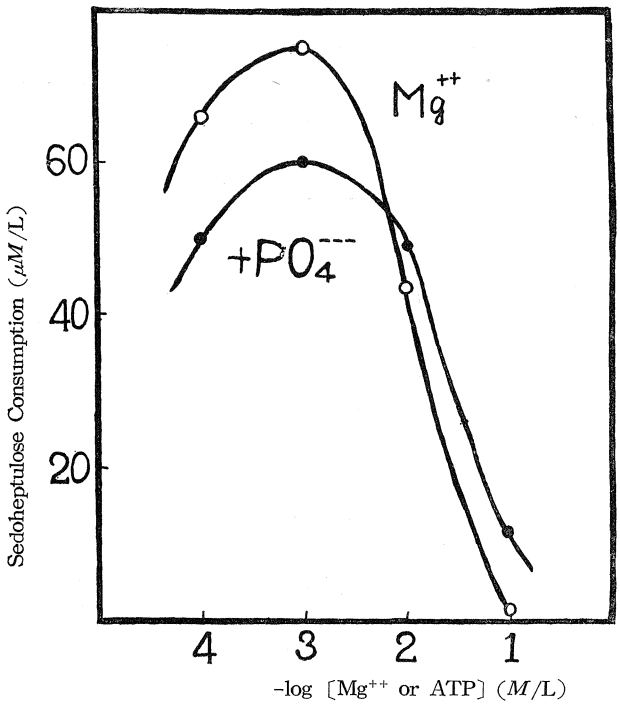

Fig. 4 Effects of varied concentrations of Mg^{++} and inorganic phosphate. The conditions were the same as in Fig. 2 except that varied concentrations of $MgCl_2$ or PO_4^{---} were used. The concentration of PO_4^{---} in the Mg^{++} experiments was fixed at 5×10^{-4} *M*, and that of Mg^{++} in the PO_4^{---} experiments at 2.5×10^{-4} *M*. Shaken for 60 minutes.

been investigated. The inhibitions caused by fluoride, arsenate and dinitrophenol are consistent with the aforementioned hypothesis that the formation of ATP is a preliminary requisite for the reaction.

Demonstration of Phosphokinase Activity : From the evidence presented above and by analogy to the bacterial system reported in the previous paper[16], we were provisionally led to an assumption that the sedoheptulose utilization in the leaf preparations is also initiated by the phosphorylation of the free sugar with ATP. The ATP may be either provided by the *de novo* synthesis from inorganic phosphate coupled to the oxidation or readily oxidizable substrates contaminated in the preparations, or it is probable that a minute amount of preformed ATP is contained in the system.

If the above assumption is correct, we have to postulate the existence in the preparation of a phosphokinase capable of catalyzing the following reaction,

$$\text{Sedoheptulose} + \text{ATP} \longrightarrow \text{Sedoheptulose phosphate} + \text{ADP}.$$

It is, in principle, possible to follow this type of phosphorylation by determining the decrease of acid-labile phosphate, since one of the two acid-labile phosphate groups of ATP should be converted in this reaction into the acid-stable phosphate of the heptose phosphate. The situation may, however, not be so simple because of the further oxidation of the sugar ester which is, in all probability, coupled to the resynthesis of ATP. The possible presence of ATP-ase activity in the preparations will also interfere the measurements.

Being aware of all these drawbacks, we tried to see if such a decrease of acid-labile

phosphate could be observed when the enzyme preparations were incubated with ATP, sedoheptulose and Mg^{++}. It was thus found that no definite results could be obtained with Preparation I, presumably due to its relatively high content of phosphate compounds. We were, however, able to demonstrate a satisfactory decrease of acid-labile phosphate using a rather large amount of Preparation II which contained much less phosphate compounds than Preparation I. A typical result is illustraled in Fig. 5. This finding appears to

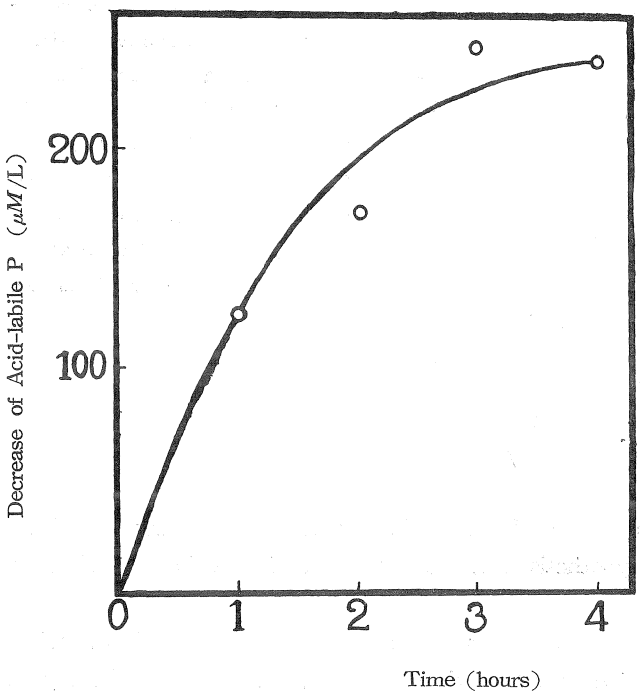

Fig. 5 Decrease of acid-labile phosphate in the ATP-sedoheptulose-Preparation II system. The reaction system contained the followings in a total volume of 6 ml.: sedoheptulose, 2×10^{-3} *M*; ATP, 2.15×10^{-3} *M*; $MgCl_2$, 1.25×10^{-3} M; citrate buffer (pH6.4), 0.05 *M*; Preparation II, 79.6 mg. (dry weight). Allowed to stand at 35°C.

provide some indirect support for the action of the phosphokinase, although this could not necessarily be justified owing to the difficult situations mentioned above.

The decrease of acid-labile phosphate under these conditions was further found to be inhibited by iodoacetate and alloxan ; the latter inhibition being recovered by cysteine.

Effect of pH on Sedoheptulose Consumption : ——Fig. 6 shows the effect of varied pH values on the heptose utilization by Preparation I. As can be seen from the figure, the pH curve obtained had two maxima at around pH 6 and 8. This somewhat unusual curve could be confirmed in repeated experiments, although there were some minor differences from one experiment to the other. It should be recalled here that such two-peaked pH curves were also obtained with the heptose utilization by the adapted bacterial cells[15] and with the phosphokinase enzyme extracted from them[16]. The mechanism underlying these phenomena were to be elucidated in future. A possible explanation may, however, be that in the overall utilization process are involved two enzymes which have different pH optima and limit the overall reaction rate at different pH regions.

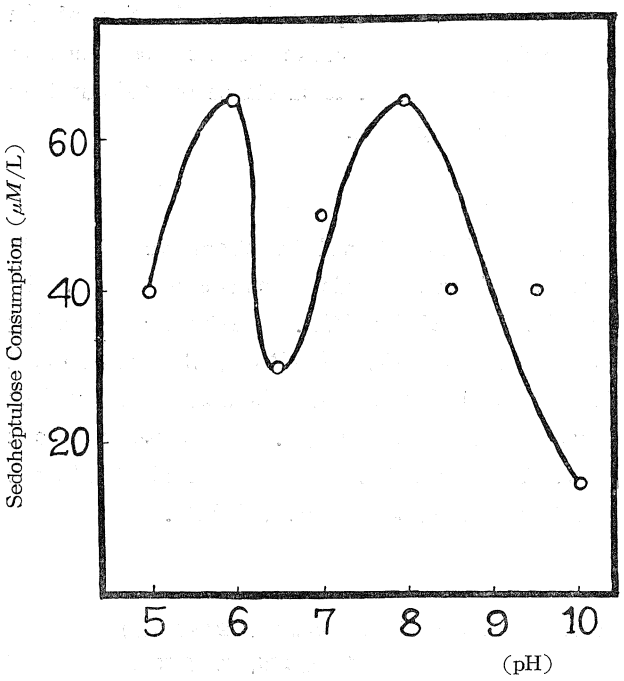

Fig. 6 Effect of pH on sedoheptulose consumption by Preparation I. The conditions were the same as in Fig. 2 except that pH was varied with the use of the following buffers (final, 0.05*M*); citrate, pH 5-7 ; veronal, pH 7-9 ; borax, pH 9-10.

DISCUSSION

Although the experimental results described in this paper are still insufficient to permit any decisive conclusion concerning the mechanism of the sedoheptulose utilization, it is likely that an enzyme system similar to what has been demonstated in bacterial cells is also functioning in spinach leaves. In other words, the utilization in the leaf preparations appears to require a preliminary phosphorylation of the heptose molecule with ATP catalyzed by a phosphokinase. The heptose is thereby converted to a metabolically active phosphate ester, preseumably sedoheptulose-7-phosphate. As has been reported in the experimental part, we have, in fact, some evidence for the occurrence of such a phosphokinase in Preparation II. It is, however, not yet clear whether this phosphokinase is specific to sedoheptulose or not. The requirement of Mg^{++} for the heptose-utilizing system could be accounted for by the fact that all the phosphokinases hitherto reported are inactive in the absence of this metal ion. It is, of course, probable that Mg^{++} is needed not only for the phosphokinase reaction but also for the further degradation of the heptose phosphate.

At any rate, if the initial formation of sedoheptulose-7-phosphate is actually the case, it seems very possible that the sugar phosphate is then either decomposed into ribose-5-phosphate and "active glycolaldehyde" by the action of transketolase or reacts with glyceradehyde-3-phosphate to yield fructose-6-phosphate and a tetrosephosphate under the influence of transaldolase. (Both transketolase and transaldolase have been shown to be present in spinach leaves). These transformations will eventually lead to more extensive degradations of the sugar by a number of oxidizing systems known to occur in the leaves.

Finally, we are at present not able to say much about the physiological function of the utilization process in green leaves until more data will be available on the distribution of the free heptose in higher plants and on many other points. It should be pointed out here that the activity in spinach leaves is by no means very high.

SUMMARY

Spinach leaf slices as well as enzyme preparations obtained therefrom were found to consume free sedoheptulose in the presence of both inorganic phosphate and Mg^{++}. The requirement of inorganic phosphate could be replaced by ATP. The heptose-consuming activity of the enzyme preparations was inhibited by NaF, Na_2HAsO_4, 2,4-dinitrophenol and $HgCl_2$. A decrease of acid-labile phosphate was observed when the enzyme preparations were incubated with the heptose in the presence of ATP and Mg^{++}. From these findings it was tentatively concluded that the heptulose utilization in the leaves may be initiated by the phosphorylation of the free sugar by the action of a phosphokinase and ATP.

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