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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

5. ISOLATION AND IDENTIFICATION OF RIBULOSE PHOSPHATE PRODUCED FROM 6-PHOSPHOGLUCONATE BY THE DEHYDROGENASE OF *E. COLI**

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In early enzymic studies on the oxidative decarboxylation of 6-phosphogluconate by a crude yeast system, a complex mixture of end products had been obtained (Warburg, Christian & Griese, 1935; Dickens, 1938). Work in this Laboratory demonstrated the presence of ribose phosphate and the presence of unknown Bial-reactive sugar phosphate among these products (Cohen & Scott, 1950; Scott & Cohen, 1951). The use of a considerably purified enzyme system from yeast permitted the further dissection of this reaction sequence by Horecker, Smyrniotis & Seegmiller (1951). These workers thus discovered the formation of ribulose 5-phosphate as a primary product of the oxidative decarboxylation of 6-phosphogluconate, and the subsequent isomerization of ribulose 5-phosphate to ribose 5-phosphate.

Subsequent work on this system in our Laboratory was concerned primarily with the role of this pathway in ribose formation in *Escherichia coli* under conditions of normal growth and virus infection. Nevertheless, it appeared desirable to demonstrate unequivocally that the oxidation of 6-phosphogluconate by bacterial enzymes was comparable

with that observed with yeast enzymes. Having purified the 6-phosphogluconate dehydrogenase from *Esch. coli* (Scott & Cohen, 1953), the nature of the primary reaction product using this bacterial enzyme was demonstrated to be ribulose phosphate as described below.

MATERIALS AND METHODS

The barium salt of 6-phosphogluconic acid was prepared from synthetic glucose 6-phosphate by the method of Seegmiller & Horecker (1951). Phosphogluconate (PG) was determined by the reduction of triphosphopyridine nucleotide (TPN) in the presence of phosphogluconic dehydrogenase. The barium salt of ribose 5-phosphate was prepared by the method of Groth, Mueller & LePage (1952) and always contained about 11% of ribulose 5-phosphate. Sodium pyruvate was prepared immediately before use by neutralization of freshly distilled pyruvic acid. Glycylglycine buffer and tris buffer (aminotrihydroxymethylmethane) were obtained from Sigma Chemical Co. TPN was prepared by the method of LePage & Mueller (1949) and was measured by reduction in the presence of PG and PG dehydrogenase. It was 74% pure. Pure ribulose was regenerated from the *o*-nitrophenylhydrazones of ribulose by treatment with freshly distilled benzaldehyde in the presence of benzoic acid. This derivative had been prepared from ribulose obtained

* Part 4: Scott (1956).

by chemical or enzymic isomerization of D-arabinose (Glatthaar & Reichstein, 1935; Cohen, 1953). After filtration, the solution containing ribulose was extracted with chloroform and ether and taken to a syrup by vacuum distillation. D-Arabinose was obtained from Pfanstiehl Chemical Co.

Lactic dehydrogenase was prepared from rabbit muscle according to the method of Cori, Slein & Cori (1948) and Kornberg & Pricer (1951). After several precipitations with $(\text{NH}_4)_2\text{SO}_4$ the activity in oxidizing reduced diphosphopyridine nucleotide was 41 $\mu\text{moles/min./mg.}$ of protein and in oxidizing reduced triphosphopyridine nucleotide (TPNH) was about 0.6 $\mu\text{mole/min./mg.}$ This enzyme preparation did not contain phosphatase, pentose phosphate isomerase, ribose phosphate-splitting enzyme or PG dehydrogenase.

Acid phosphatase was prepared from frozen semen by two precipitations with saturated $(\text{NH}_4)_2\text{SO}_4$ so that it gave neither a fructose reaction with carbazole nor a pentose reaction with Bial reagent. This enzyme preparation contained no phosphorus and had no pentose isomerase activity. Incubation of this enzyme with glucose 6-phosphate, PG, or ribose 5-phosphate in acetate buffer at pH 5.0 removed 100% of the phosphate in 60 min. at 37°; Mg^{2+} ions were inhibitory.

Pentose isomerase was prepared from cultures of *Esch. coli* Ba 15 (Cohen, 1953) grown in medium containing D-arabinose. The wet pellet of bacteria was ground with alumina and extracted with 0.05M tris buffer at pH 8.0. Nucleic acids were precipitated with MnCl_2 . The precipitate formed in 63% saturated $(\text{NH}_4)_2\text{SO}_4$ was discarded. The protein precipitated at pH 4.7 contained an active pentose isomerase and also phosphopentose isomerase. An activating effect of Mn^{2+} ions could be detected on this precipitated pentose isomerase; tris and ethylenediaminetetraacetic acid were inhibitory. The enzyme was used in glycylglycine buffer at pH 7.2. When either pure ribulose or pure D-arabinose was incubated with the enzyme in this buffer an equilibrium mixture resulted, containing approximately 20% of ribulose and 80% of arabinose.

Phosphogluconate dehydrogenase was prepared from 500 ml. of *Esch. coli* B, grown to a turbidity of 1400 as measured on a Klett photoelectric colorimeter fitted with a 420 $\text{m}\mu$. filter. The viable count was 7×10^8 bacteria/ml. The aerated liquid synthetic medium contained increased amounts of $(\text{NH}_4)_2\text{SO}_4$ and glucose as described earlier (Scott & Cohen, 1953). The centrifuged cells were washed with cold saline. The moist pellet weighed 4.3 g. and was ground for several minutes in a chilled mortar with 2.5 times the weight of Al_2O_3 . The resulting paste was extracted successively with 50 ml. and 25 ml. portions of cold water. The addition of 5 ml. of 0.5M- MnCl_2 to the combined extracts precipitated most of the nucleic acids after 17 hr. at 4°. The precipitate was discarded. Saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 8.0) was added to the supernatant fluid to a saturation of 62% (by vol.). This precipitate contained most of the glucose 6-phosphate dehydrogenase activity. The supernatant fluid was brought to pH 5.0 by addition of 0.5M acetate buffer at pH 3.8. This precipitate was either discarded or used for the preparation of pentose phosphate isomerase. Addition of more acetate buffer to the supernatant fluid to bring the pH to 4.3 produced a precipitate which contained most of the PG dehydrogenase and no glucose 6-phosphate dehydrogenase. This precipitate was dissolved in 6 ml. of M/8 glycylglycine buffer, pH 7.4.

Dialysis of this solution overnight against cold-running distilled water caused the formation of a further precipitate. The precipitate, which was dissolved in glycylglycine buffer, pH 7.4, had all the PG dehydrogenase activity and slight isomerase, transketolase, and phosphatase action.

The supernatant solution after dialysis of the PG dehydrogenase contained pentose phosphate isomerase with activity of 5 $\mu\text{moles/30 min./mg.}$ of protein. This preparation had no PG dehydrogenase activity, low pentose phosphate degrading activity and slow phosphatase action. This enzyme was used with ribose 5-phosphate to prepare mixtures of ribose 5-phosphate and ribulose 5-phosphate in order to determine the separation of these two components by elutions from columns.

Separation of the phosphate compounds was carried out by adsorption on a column (20 mm. $\times$ 135 mm.) of Dowex 1, washed with HCl and then with water until no further Cl^- ions were eluted. Elution was effected by increasing concentrations of monochloroacetic acid from 0.001M to M (Groth *et al.* 1952).

Pentose and pentose phosphate were determined by a modification (Miller, Golder & Miller, 1951) of the Bial reaction. Ribulose and ribulose phosphate were determined by the carbazole-cysteine- H_2SO_4 method of Dische & Borenfreund (1951) and also by the decrease in Bial-reacting substance after treatment with KOH. Ribose and ribose phosphate were determined from the Bial reaction after treatment of the solution with KOH. Spectra of the Bial reactions between 400 and 700 $\text{m}\mu$, especially the 540 $\text{m}\mu$. peak indicative of free ribulose, were very useful in characterizing the products (Cohen, 1953).

Phosphates were determined by the method of King (1932). Digestion of samples for determination of total phosphorus was performed with perchloric acid.

RESULTS

The reaction mixture for the degradation of 6-phosphogluconate was a solution (52 ml.) containing purified PG dehydrogenase, 0.0065M PG (340 μmoles), 0.0102M sodium pyruvate, 0.015M- MgCl_2 and 0.05M glycylglycine buffer, pH 7.6. A portion (2 ml.) was removed for analysis and the reaction was begun in the remaining 50 ml. of solution by adding 0.5 ml. of lactic dehydrogenase and 4 μmoles of TPN in 0.4 ml. This mixture was allowed to react at 37° for 60 min. Seven samples of 0.2 ml. each were removed at intervals for analysis. After 1 hr. approx. 320 $\mu\text{g.}$ of ribulose/ml. had been produced, i.e. 15 mg., or 100 μmoles , indicating that 30% of the phosphogluconate had reacted.

The mixture was chilled and put directly on a column of Dowex 1 (acid form) and washed through with 100 ml. of water. Elution was effected with monochloroacetic acid, starting at 0.001M and increasing in concentration. The first washing contained protein, 140 $\mu\text{g.}$ of free ribulose and no P. At 0.04M acid, Bial-reacting material and organic P started to be eluted and came off slowly in fractions 118–152. Bial-reactive and carbazole-unreactive material appeared in fractions 118–121,

amounting to approximately 0.5 mg. of pentose. Fractions 121–151 contained 15 mg. of ribulose, and 3.36 mg. of P of which 0.26 mg. was inorganic P. Thus the ratio ribulose/organic P was 1. Fractions 121–131 contained approx. 1 mg. of pentose phosphate; thus there was a slight pentose phosphate isomerase activity during the reaction, such that approx. 7% of the reaction product was pentose phosphate other than ribulose phosphate.

In fractions 152–155 pyruvate was eluted, and from 164 to 178, 0.13 mg. of organic P of unknown composition. Fractions 179–182 gave 7.55 mg. of organic P and contained the unchanged PG, which was measured by the action of PG dehydrogenase. Fractions 183–185 contained 0.37 mg. of Bial-reactive material and 0.22 mg. of organic P, and was probably TPN.

The combined fractions 121–151, consisting of 320 ml. minus 8 ml. taken for various analyses, was extracted three times with ether to remove the monochloroacetic acid and was then concentrated *in vacuo* to 17 ml. The acid solution was neutralized to pH 5.0 with KOH. To this solution was added 0.4 ml. of acid phosphatase and the mixture was incubated at 37° for 45 min., when the total P (3.36 mg. or 108 μ moles) was inorganic. Analysis indicated 10.5 mg. of ribulose and 1.6 mg. of ribose or 80 μ moles of pentose.

The mixture was brought to pH 7.0 with NaOH, and 2.3 ml. of pentose isomerase enzyme was added. The mixture was incubated at 37° for 2 hr., at which time the carbazole reaction was one-half of that of the mixture before addition of the isomerase. The Bial reaction at first increased, and after 1 hr. was equivalent to 12.8 mg. of pentose. After 2 hr. it had decreased, giving a value of 11 mg., so the reaction was stopped by chilling to avoid further destruction of the pentose.

The mixture was then de-ionized by alternate treatment with Dowex 50 in H⁺ form and Dowex 1 in CO₃²⁻ form until the phosphate had been removed and the solution had a pH of 7.0. There was a loss of pentose during this desalting procedure, and the 35 ml. of solution now contained 8.8 mg. of pentose. By vacuum distillation the volume was decreased to 3 ml. A slight precipitate was removed by centrifuging and the solution was further concentrated by a stream of dry air to 0.2 ml.

To this was added 0.4 ml. of ethanol containing 6 mg. of sodium acetate and 12.5 mg. of diphenylhydrazine hydrochloride. After standing overnight at 4° a good crop of crystals was obtained. After one recrystallization from ethanol the m.p. was 202–203° (decomp.), and after a second recrystallization, 205–206.5°. The diphenylhydrazone derivative of authentic D-arabinose melted at 204–205°, mixed m.p. 206–206.5° (decomp.). These values were obtained on a Fischer–Johns apparatus and are uncorrected (Cohen, 1953).

DISCUSSION

The reactions used in the degradation of phosphogluconate and the identification of the final product are given below:

(a) $\text{TPN} + 6\text{-phosphogluconate} \rightarrow \text{TPNH} + 90\% \text{ ribulose phosphate} + 9\% \text{ ribose phosphate} + 1.6\% \text{ unknown pentose phosphate.}$

(b) $\text{TPNH} + \text{pyruvate} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{TPN} + \text{lactic acid.}$

(c) $\text{Pentose phosphates} \xrightarrow[\text{phosphatase}]{\text{acid}} \text{pentoses} + \text{inorganic phosphate.}$

(d) $\text{Ribulose} \xrightarrow[\text{isomerase}]{\text{pentose}} \text{D-arabinose.}$

(e) $\text{D-Arabinose} + \text{diphenylhydrazine HCl} \rightarrow \text{D-arabinose diphenylhydrazone.}$

It has been demonstrated recently that ribulose 5-phosphate may be converted into xylulose 5-phosphate by a pentulose phosphate epimerase (Hochster, 1955; Stumpf & Horecker, 1956). It may be asked therefore whether xylulose 5-phosphate might have been the primary product of enzymic degradation of phosphogluconate and had been converted into ribulose 5-phosphate in this system. The following considerations argue against this possibility.

(1) The configuration of the hydroxyl groups of ribulose 5-phosphate at C-3 and C-4 are *cis*, as they are in the parent C-4 and C-5 of 6-phosphogluconate, whereas these hydroxyl groups are *trans* in xylulose 5-phosphate.

(2) The equilibrium mixture has been shown, with an enzyme from *Lactobacillus pentosus* (Stumpf & Horecker, 1956), to be a 45:55 mixture of xylulose 5-phosphate and ribulose 5-phosphate. If the epimerase had been present in the dehydrogenase preparation, an approximately equimolecular mixture of these compounds should have been obtained. Two peaks of pentulose phosphate were not observed in elution from the column, though the pentulose phosphate generated in the reaction mixture was quantitatively recovered from the column.

(3) On dephosphorylation a 70% recovery of ribulose was observed. That this pentulose was indeed ribulose may be concluded from the following evidence: (a) The spectrum of ribulose in the Bial reaction is different from that of xylulose (Stumpf & Horecker, 1956). Our material gave the spectrum of ribulose in this reaction. (b) The development of colour in the cysteine–carbazole reaction is much faster for ribulose than for xylulose (Cohen, 1953; Stumpf & Horecker, 1956). The pentulose reacted in this test as ribulose. (c) The pentose isomerase used

is specific for compounds of the ribulose configuration and does not act on xylulose (Cohen, 1953). All the pentulose detectable in the cysteine-carbazole reaction was converted into aldopentose. It may be noted that the specific enzyme is an elegant tool in distinguishing the pentuloses and in permitting the estimation of one in the presence of the other. The same enzyme may also be applied to the specific estimation of L-fucose, which is converted into L-fuculose (Green & Cohen, 1956).

Since no evidence was obtained for pentulose other than ribulose, and this compound was isolated in the form of pentulose phosphate as the only product of the degradation of 6-phosphogluconate, it has been concluded that the primary product which could be isolated after degradation of PG by the dehydrogenase of *Esch. coli* was ribulose phosphate, as has been observed for the PG dehydrogenases of yeast and liver.

SUMMARY

1. A purified 6-phosphogluconate dehydrogenase was prepared from extracts of *Escherichia coli* B. This preparation was very low in phosphatase, pentose phosphate isomerase and transketolase or other ribose phosphate-splitting enzymes.

2. After action of the enzyme on phosphogluconate, the reaction mixture was fractionated on an ion-exchange column. Ribulose 5-phosphate was isolated in high yield. It was dephosphorylated, and the free ribulose was enzymically converted into D-arabinose, which was isolated as the characteristic D-arabinose diphenylhydrazone.

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Studies on the Composition of Lipids of the Rabbit

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Of the pasture-fed animals the wild rabbit has a special interest, because linolenic acid, the main dietary fatty constituent, forms over 40% of the fatty tissues as compared with less than 1% found in the depot fats of ruminants (Shorland, 1953). Earlier studies on pasture-fed horses, which also contain a high proportion (about 16%) of linolenic acid in their fatty tissues (Brooker & Shorland, 1950; Gupta & Hilditch, 1951) have shown that this acid is differentially distributed between the tissue lipids (Shorland, Bruce & Jessop, 1952). Whereas the glyceride fractions contain trienoic (linolenic) acid as the main C_{18} unsaturated fatty acid, in the

phospholipid C_{18} unsaturated fatty acids dienoic constituents predominate. In this paper we report a similar study on the lipids of the rabbit.

EXPERIMENTAL

The rabbits used in this work were trapped on 6 and 7 June 1951 (mid-winter), in the Guavas Forest area, near Waipawa, Hawkes Bay, New Zealand, by officers of the Animal Ecology Section of the Department of Scientific and Industrial Research, New Zealand. There were twenty-five males, average wt. 1575 s.d. $\pm$ 135 g., and eighteen females, average wt. 1499 s.d. $\pm$ 160 g. A few of the females were in the early stages of pregnancy and almost all of the rabbits

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