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Studies Involving Enzymic Phosphorylation

3. THE PHOSPHORYLATION OF D-RIBOSE BY EXTRACTS OF *ESCHERICHIA COLI**

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(Received 2 August 1954)

Although several notable advances have recently been made in the elucidation of the intermediary metabolism of the pentose phosphates, little information is at present available regarding their enzymic formation from free pentoses. Sable (1950, 1952) showed that ribose disappeared when incubated with ammonium sulphate fractions prepared from extracts of baker's or brewer's yeast, supplemented with adenosine triphosphate (ATP), magnesium and fluoride ions. He detected the formation of hexose phosphates by specific enzymic methods, but obtained no evidence for the intermediate formation of a phosphate ester of ribose. Cohen & Raff (1951) found that when *Escherichia coli* was grown on ribose, the washed cells specifically utilized this pentose. Hochster & Watson (1952) reported that a cell-free extract of xylose-grown *Pseudomonas hydrophila*, when incubated with xylose, ATP, magnesium and fluoride ions, liberated carbon dioxide from a bicarbonate buffer under anaerobic conditions, an observation which was consistent with the postulated phosphorylation of the xylose by ATP, but the isolation of a xylose phosphate was not attempted. More recently, Lampen (1953) has shown that when a cell-free extract of xylose-grown *Lactobacillus pentosus* was incubated with xylose and ATP, an equilibrium mixture of ribose 5-phosphate and ribulose 5-phosphate was formed.

In the work to be described, a strain of *Esch. coli* has been grown under conditions similar to those of Cohen & Raff (1951). When the washed cells were disintegrated, the cell-free extract was found to catalyse the transfer of the terminal phosphate group of ATP to ribose when magnesium ions were present. The characteristics of this enzymic phosphorylation have been investigated.

* Part 2, Long (1953).

A preliminary account of this work has already appeared (Heald & Long, 1953).

EXPERIMENTAL

Materials

D-Ribose was obtained from Roche Products Ltd.; we have found it to be chromatographically pure. Other monosaccharides were the best available commercial preparations.

Adenosine triphosphate was isolated as the barium salt from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948) and stored as a frozen 0.05M solution of the potassium salt at -20° .

A sample of synthetic barium ribose 5-phosphate was generously given by Dr Gertrude Glock, Courtauld Institute of Biochemistry, Middlesex Hospital, London. Barium was removed by treating a solution in 0.01N-HCl with a slight excess of solid K_2SO_4 , centrifuging down the $BaSO_4$ and neutralizing the supernatant.

In all experiments *Esch. coli* (National Collection of Industrial Bacteria, no. 7271) was used. The cultures were maintained on slopes of percentage (w/v) composition: agar 2.67, yeast extract ('Oxoid') 0.1, bacterial peptone ('Oxoid') 0.1, $NH_4H_2PO_4$ 0.07, NaCl 0.17, glucose 0.13.

The buffer used throughout was aminotrihydroxymethyl methane (THAM), pH 7.6 (Gomori, 1946).

Methods

Growth of Esch. coli 7271. A loop inoculum from a slope was introduced into 5 ml. of liquid medium of percentage (w/v) composition: yeast extract 0.3, bacterial peptone 0.3, $NH_4H_2PO_4$ 0.2, NaCl 0.5, glucose 0.4, brought to pH 7.5 with NaOH. After 16 hr. growth at 38° , 0.2 ml. inoculum was transferred into each of about twenty-five flat bottles containing 15 ml. of solid culture medium of percentage (w/v) composition: agar 2, yeast extract 0.5, ribose 0.25, NaCl 0.07, K_2HPO_4 0.45, KH_2PO_4 0.12, $CaCl_2$ 0.0007, $MgCl_2$ 0.0007. In the preparation of this medium, the ribose was autoclaved separately and then added to the other sterilized components. After 16 hr. growth at 38° , the

bacteria were washed off the agar with a total of about 45 ml. 0.1 M THAM buffer, pH 7.6, and the suspension was filtered through gauze. The bacteria were centrifuged at 3° in an angle centrifuge at 8000 g, washed twice with buffer and finally suspended in buffer to a final concentration of 20 mg. dry wt./ml. Eight successive experiments gave an average yield of *Esch. coli* of 15.9 mg. dry wt./bottle (range 12.6–20.2); this is equivalent to slightly more than 1 g. dry wt. of bacteria/l. solid culture medium.

For comparative purposes, *Esch. coli* 7271 was also grown on solid medium containing glucose in place of ribose. The amount of growth was similar with these two sugars.

Preparation of cell-free extracts. About 2–3 g. of washed Pyrex ballotini (grade 12) were added to 5–10 ml. of buffered *Esch. coli* suspension, and the mixture shaken at 3° in a Mickle (1948) disintegration apparatus for 20 min. The suspension of broken cells was decanted from the ballotini, centrifuged at 3° and the cloudy supernatant frozen at –20°. When the latter was allowed to thaw, much inactive material flocculated and was removed by centrifuging. The final supernatant, which was still somewhat opalescent, was stored in the frozen state at –20°.

Utilization of ribose by Esch. coli 7271. In experiments with intact cells, 0.05–0.10 ml. *Esch. coli* suspension (20 mg. dry wt./ml.) was incubated at 38° for 10 min. in 0.08 M THAM buffer, pH 7.6, containing 1 μmole ribose in a total volume of 0.5 ml. The reaction mixture was then chilled to 0°, immediately diluted to 5.0 ml. with ice-cold water, mixed thoroughly and centrifuged at 3°. The ribose content of 1 ml. of clear supernatant was determined by the method of Mejbaum (1939), as modified by LePage (1949), using orcinol recrystallized from benzene. Initial ribose and blank determinations were made on unincubated cells.

When cell-free preparations were employed, 0.1 ml. of bacterial extract was incubated for 5–20 min. at 38° in a reaction mixture of total volume 0.5 ml. having the following composition (final concentrations in brackets): ribose (0.002–0.006 M), ATP (0.005 M), MgCl₂ (0.005 M), THAM buffer, pH 7.6 (0.04 M), with or without (NH₄)₂SO₄ (0.28 M). (The reason for including (NH₄)₂SO₄ is explained in the Results section.) Enzyme action was stopped by treating the incubated reaction mixture with 0.5 ml. 5% (w/v) ZnSO₄ followed by 0.5 ml. 0.3 N-Ba(OH)₂ (Somogyi, 1945), while 0.07 ml. 2 M barium acetate was also added to pre-

cipitate the excess sulphate ion, whenever (NH₄)₂SO₄ was used. In such cases the addition of the ZnSO₄ before the Ba(OH)₂ was necessary, for otherwise destruction of ribose by the liberated NH₄OH occurred. The mixture was diluted with water to 5 ml., centrifuged and the supernatant filtered through a no. 42 Whatman paper. This method of deproteinization is well known to precipitate adenine nucleotides and has also been found to adsorb ribose phosphates completely (see Table 1). Ribose concentration was determined on 1 ml. of the filtrate and on both a zero-time reaction mixture and a control without ribose, similarly treated.

When it was required to precipitate the ATP only and to determine the sum of the concentrations of ribose and pentose phosphate, enzyme action was stopped by addition of 0.3 ml. of 20% (w/v) mercuric acetate in 0.1% (v/v) acetic acid. The volume was made up to 5 ml. with 0.1% acetic acid, and after standing for about 30 min. to ensure complete precipitation, the mixture was centrifuged and the supernatant filtered; 1 ml. of the filtrate was taken for pentose determination. The presence of (NH₄)₂SO₄ in the reaction mixture was found to be advantageous, for it caused the simultaneous precipitation of a crystalline mercuric ammonium compound which assisted in carrying down the mercuric complexes of the adenine nucleotides. Separate experiments showed that ribose and ribose 5-phosphate were not removed from solution by the mercuric acetate treatment, while ATP was completely precipitated under these conditions. The presence of excess mercuric ions had no effect on the determination of the pentose content of the filtrate by the orcinol method. The concentration of pentose phosphate was taken as the difference between the total pentose concentrations of the mercury and barium–zinc filtrates. This mercury-precipitation procedure is based on the observations of Kerr (1940, 1941). The specificity of the mercury and barium–zinc precipitations is shown in Table 1.

Other analytical methods. Glucose and other hexoses were determined as reducing substances by the copper reduction–arsenomolybdate method of Nelson (1944). Phosphate was determined by the micromodification (Long, 1943) of the method of Berenblum & Chain (1938). Acid-labile phosphorus of ATP was determined after hydrolysis in N-HClO₄ for 10 min. on a boiling-water bath.

Table 1. *Determination of ribose, ribose 5-phosphate and adenosine triphosphate separately and in mixtures*

Solutions of ribose, ribose 5-phosphate (R 5-P) and ATP were used singly or in mixtures in total vol. 0.5 ml. In (a) the solution was diluted to 5.0 ml. with water; in (b) 0.3 ml. of 20% (w/v) mercuric acetate in 0.1% (v/v) acetic acid was added, followed by 0.1% acetic acid to 5.0 ml., and the mixture centrifuged and filtered; in (c) 0.5 ml. of 0.3 N-Ba(OH)₂ and 0.5 ml. of 5% (w/v) ZnSO₄ were added, followed by water to 5.0 ml., and the mixture was centrifuged and filtered. 0.4 ml. samples of filtrate were taken for pentose determination by the orcinol method. The calculated values are based on the assumption that treatment (b) precipitates ATP only, while treatment (c) precipitates both ATP and R 5-P.

Composition of original solution (μmoles)			Pentose (μmoles) found after					
			(a) No pptn.		(b) Mercury pptn.		(c) Barium-zinc pptn.	
Ribose	R 5-P	ATP	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
1.03	0	0	1.00	1.03	1.04	1.03	0.97	1.03
1.03	0.91	0	2.05	1.94	1.94	1.94	1.05	1.03
1.03	0	2.69	3.71	3.72	1.01	1.03	0.99	1.03
1.03	0.91	2.69	4.81	4.63	1.95	1.94	0.95	1.03
0	0.91	0	0.92	0.91	0.84	0.91	0.01	0
0	0.91	2.69	3.54	3.60	0.84	0.91	0	0
0	0	2.69	2.61	2.69	0	0	0	0

RESULTS

The utilization of monosaccharides by intact cells of Esch. coli 7271

Washed intact cells of glucose-grown and ribose-grown *Esch. coli* 7271 were found to utilize glucose, mannose and glucosamine rapidly, galactose less so and fructose only slowly. Ribose was fermented only by the cells grown on this pentose, while other pentoses were not utilized by either kind of cells.

In a Warburg experiment, using ribose-grown *Esch. coli* cells (4 mg. dry wt.) in 1 ml. 0.05M THAM buffer, pH 7.6, and 2 μ moles of pentose, the O₂ uptakes (μ l.) in 1 hr. were: no substrate, 43; D-xylose, 44; D-arabinose, 45; D-ribose, 166 and 167 (duplicate).

The utilization of ribose by cell-free preparations of Esch. coli 7271

Cells of ribose-grown *Esch. coli* 7271, suspended in 0.1M THAM buffer, pH 7.6, were readily disintegrated in a Mickle (1948) apparatus. It was established by microscopic examination after Gram staining that this procedure gave complete disintegration of the cells. This preparation of broken cells was now able to utilize D-ribose only when supplemented with ATP and magnesium ions. When the glass ballotini and cell debris were removed by centrifuging at about 8000 g for 10 min. at 3°, the whole of the phosphorylating activity was found in the supernatant. Maximal rates of ribose utilization were obtained with 0.005M-ATP. Similar active supernatants were obtained when the cells were disintegrated in the following media, pH 7.6; 0.12M potassium phosphate buffer; 0.04M veronal (King & Delory, 1940); 0.05M bicarbonate containing 0.15M-KCl. Other pentoses were not utilized under any of these conditions.

Fluoride, which was found necessary for inhibiting phosphatase activity in 'homogenates' of animal tissues in enzymic phosphorylation experiments (Case & McIlwain, 1951; Long, 1951), was not required. However, the extracts had slight adeno-

sinetriphosphatase activity. When present at a concentration of 0.05M, potassium fluoride was found to inhibit ribose disappearance by about 25%. Sodium iodoacetate (0.01M) also inhibited to about the same extent. Some of these results are shown in Table 2.

Dialysis in a collodion sac for 2.5 hr. at 4° against 0.1M THAM buffer, pH 7.6, in one experiment caused a 10% loss of activity, which was not restored by addition of an equal volume of the boiled extract from an undialysed preparation. In another experiment, carried out under similar conditions, no loss of activity was observed. Thus there is no evidence for the requirement of any dialysable coenzyme.

The enzymic formation of a pentose phosphate from ribose by extracts of Esch. coli

Duplicate reaction mixtures containing ATP, ribose and magnesium ions were incubated with an extract of ribose-grown *Esch. coli*, under conditions similar to those described in Table 2. One sample was treated with the Ba(OH)₂-ZnSO₄ reagents of Somogyi (1945) and the filtrate analysed for pentose; the other was treated with mercuric acetate in acetic acid and the sum of the free and phosphorylated pentose was determined in the filtrate. The fraction of the metabolized ribose which could be accounted for in terms of phosphorylated pentose was thus determined.

When ribose-grown *Esch. coli* cells were freshly disintegrated and the suspension centrifuged, the fraction accumulating as pentose phosphate in the presence of the supernatant extract was usually only about 0.5-0.6. However, when the extract was frozen and thawed several times and insoluble material removed by centrifuging, the fraction accumulating as pentose phosphate was appreciably increased, although the rate of utilization of free pentose was practically unchanged. It seems, therefore, that the enzyme systems leading to the breakdown of pentose phosphates are either destroyed by storage or else become insoluble on freezing and thawing and are then removed by centrifuging.

It has been found that in the presence of high concentrations of ammonium sulphate the fraction of the ribose disappearing which accumulates as pentose phosphate is uniformly high and does not differ from unity within the experimental error of the determinations. This is shown in Table 3, from which it is seen that the best results are obtained with 0.28M ammonium sulphate. The ammonium sulphate appears to act by inhibiting the breakdown of the accumulated pentose phosphate, a view which is supported by the finding that the rate of disappearance of a sample of added ribose 5-phosphate was markedly decreased in the presence

Table 2. *Utilization of D-ribose by extracts of ribose-grown Esch. coli*

The complete system contained 0.1 ml. of extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells), 0.005M ribose, 0.005M-ATP, 0.005M-MgCl₂ and 0.02M THAM buffer, pH 7.6; total vol. 0.5 ml. 30 min. at 38°.

	Ribose disappearing (μ moles)
Complete system	1.38
Same without MgCl ₂	0.06
Same without ATP	0.00
Same with 0.01M iodoacetate	1.11
Same with 0.05M-KF	1.04

Table 3. Accumulation of pentose phosphate after enzymic phosphorylation of ribose by ATP in the presence of extracts of ribose-grown *Esch. coli*

The reaction mixtures contained 0.1 ml. of extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells), 0.005M-ATP, 0.005M-MgCl₂ and 0.04M THAM buffer, pH 7.6, with or without added salts at the concentrations specified. Ribose concentration 0.004M (Expt. 1), 0.002M (Expt. 2); total vol. 0.5 ml. Incubated at 38° for 20 min. (Expt. 1) or 14 min. (Expt. 2). Ribose was determined in Ba(OH)₂-ZnSO₄ filtrates; the sum of the ribose and pentose phosphate was determined in mercuric acetate filtrates.

Expt. no.	Salt added	Concn. (M)	Ribose utilized (μmoles)	Pentose phosphate accumulated (μmoles)	Fraction accumulating as pentose phosphate
1	None	—	1.60	1.35	0.84
	(NH ₄) ₂ SO ₄	0.10	1.68	1.44	0.86
	(NH ₄) ₂ SO ₄	0.20	1.42	1.33	0.94
	(NH ₄) ₂ SO ₄	0.28	1.40	1.44	1.04
	(NH ₄) ₂ SO ₄	0.40	1.20	1.25	1.04
2	None	—	0.91	0.42	0.46
	(NH ₄) ₂ SO ₄	0.28	0.79	0.73	0.92
	K ₂ SO ₄	0.28	0.98	0.83	0.83
	Na ₂ SO ₄	0.28	0.56	0.48	0.86

Table 4. Dependence of extent of ribose utilization and pentose phosphate accumulation on the amount of adenosine triphosphate present

The reaction mixtures contained 0.1 ml. extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells) in 0.04M THAM buffer, pH 7.6, 0.005M-MgCl₂, 0.28M-(NH₄)₂SO₄, 0.002M ribose and the amounts of ATP shown. The reactions were allowed to proceed to completion (30 min.). Control experiments in which incubation was continued for 60 min. showed no increased disappearance of ribose. Ribose was determined in ZnSO₄-Ba(OH)₂ filtrates; ribose plus pentose phosphate was determined in mercuric acetate filtrates. The amounts of ATP were determined on separate samples by measurement of acid-labile phosphorus and assuming two atoms of acid-labile P/molecule of ATP.

Expt. no.	Adenosine triphosphate (μmole)	Ribose utilization (μmole)	Pentose phosphate accumulation (μmole)
1	0.74	0.65 (0.64*)	0.74 (0.61*)
	0.49	0.47	0.46
	0.25	0.13	0.15
2	0.31	0.32 (0.29*)	0.37 (0.32*)
	0.15	0.11	0.13
	0.08	0.02	0.03

* Values found in presence of a mixture of 0.008M-KF and 0.00012M adenosine monophosphate, which strongly inhibits myokinase activity.

of 0.28M ammonium sulphate. Thus, high yields of accumulated pentose phosphate may be obtained in the presence of ammonium sulphate with either fresh or aged extracts of *Esch. coli*.

It is probable that the sulphate ion is responsible for this inhibition of the breakdown of pentose phosphate, for, as also shown in Table 3, both sodium and potassium sulphates produced qualitatively similar effects. Unfortunately, the effects of other anions could not be properly assessed, for when ammonium sulphate was replaced by am-

monium chloride or ammonium acetate, the method of determining free plus phosphorylated pentose in the mercuric acetate filtrates failed to work.

Enzymic phosphate transfer from adenosine triphosphate to ribose

The need for the presence of ATP and magnesium ions in reaction mixtures for ribose utilization by extracts of ribose-grown *Esch. coli* (Table 2) may be taken as evidence that the reaction is a typical enzymic phosphorylation. Further support for this is provided by the fact that the extent of ribose disappearance and pentose phosphate accumulation are dependent on the amount of ATP present, as shown in Table 4. It is clear from these experiments also that one molecule of ATP is required for each molecule of ribose utilized, in contrast with the results of Hochster & Watson (1952) with *Pseud. hydrophila* and Lampen (1953) with *Lb. pentosus*, where the presence of myokinase in the bacterial extracts was found to bring about the overall transfer of both energy-rich phosphate groups of ATP in the phosphorylation of xylose. Furthermore, the amount of ribose disappearing and pentose phosphate accumulating is unaffected by the addition of 0.008M fluoride and 0.00012M adenosine monophosphate; this combination is known to inhibit strongly the myokinase reaction in animal tissues (Slater, 1953).

The equivalence of the phosphate transferred from ATP to ribose is shown by the experiment of Table 5. In the absence of ribose, a small amount of acid-labile phosphorus was found to disappear during the incubation while an approximately equivalent amount of inorganic phosphate was formed. This would be expected if the bacterial extract contained adenosinetriphosphatase, for the conditions of the experiment were likely to favour the activity of this enzyme, i.e. presence of

magnesium ions and absence of fluoride. Some acid-stable phosphorus was also formed during the incubation, but the source of this material has not been determined. These values were used for correcting the data obtained when ribose was present in the reaction mixtures. Under the latter conditions, both the loss of acid-labile phosphorus and gain of acid-stable phosphorus were much increased, and when corrected for the control values, there was equivalence within experimental error. The amount of ribose disappearing slightly exceeded the corrected amounts of phosphate transferred, but this would be expected since ammonium sulphate was not present in these reaction mixtures so that some decomposition of pentose phosphate would inevitably take place.

Table 5. *Phosphate equivalence between adenosine triphosphate utilized and pentose phosphate accumulated*

Ribose (2.0 μ moles) and ATP (2.5 μ moles) were incubated with 0.1 ml. extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells) in 0.04M THAM buffer, pH 7.6, and 0.005M-MgCl₂ for 20 min. at 38°; total vol. 0.5 ml. One reaction mixture was treated with the ZnSO₄-Ba(OH)₂ reagents of Somogyi (1945) and free ribose determined in the filtrate; another reaction mixture was deproteinized with HClO₄ (final concn. 5%) and the filtrate analysed for inorganic, acid-labile (10 min. at 100° in N-HClO₄) and total phosphorus. A control reaction mixture without ribose was incubated under similar conditions and the HClO₄ filtrate analysed for phosphate fractions. Zero-time reaction mixtures were analysed for initial ribose and phosphate fractions.

	Change in amount after 20 min. incubation		Difference due to ribose (μ mole)
	Ribose absent (μ mole)	Ribose present (μ moles)	
Inorganic P	+0.54	+0.66	+0.12
Acid-labile P	-0.55	-1.50	-0.95
Acid-stable P	+0.42	+1.30	+0.88
Ribose	—	-0.98	-0.98

Table 6. *Optical densities at 580 and 670 m μ . of orcinol-reaction products of pentoses and of the product of enzymic phosphorylation of ribose by *Esch. coli* extracts*

1.0 μ mole of ribose was incubated for 20 min. at 38° with 0.1 ml. extract of ribose-grown *Esch. coli* (derived from 2 mg. dry wt. of cells) in 0.04M THAM buffer, pH 7.6, 0.005M-ATP, 0.005M-MgCl₂ and 0.28M-(NH₄)₂SO₄. Pentose-reacting material was absent from Ba(OH)₂-ZnSO₄ filtrates. 1.0 ml. of the mercuric acetate filtrate was treated with the orcinol reagent and found to contain 0.192 μ mole of pentose phosphate corresponding to a total yield of 0.96 μ mole. This solution was examined in a Unicam SP. 500 spectrophotometer at 580 and 670 m μ . against an unincubated control free from ribose and an unincubated reaction mixture containing ribose, both subjected to similar mercuric acetate precipitations, followed by the orcinol reaction.

	Amount (μ mole)	(a) $E_{580 \text{ m}\mu}$	(b) $E_{670 \text{ m}\mu}$	Ratio b/a
Ribose	0.200	0.123	0.463	3.76
Enzymically produced pentose phosphate	0.192	0.117	0.441	3.76
Arabinose	—	—	—	3.78*
Sedoheptulosan	—	—	—	0.29*

* Data of Horecker *et al.* (1953).

The experiment in Table 5 is interpreted as demonstrating the conversion of acid-labile phosphorus of ATP into acid-stable pentose phosphate. It does not distinguish between (a) the transfer of the terminal group of ATP to ribose to give an acid-stable pentose monophosphate and (b) the transfer of both acid-labile groups of ATP or the terminal groups of two molecules of ATP to ribose to give a pentose diphosphate containing one acid-labile and one acid-stable phosphate group. This is due to the fact that the phosphate determinations were carried out on perchloric acid filtrates which contained both the pentose phosphate and the nucleotides. In view of the finding by Klenow (1953) of ribose 1:5-diphosphate as a coenzyme-intermediate of the phosphoribomutase reaction, it became necessary to distinguish between these possibilities.

In another experiment, therefore, phosphate determinations were made on mercury filtrates, which contain the pentose phosphate uncontaminated by nucleotides. It was found that the formation of 0.74 μ mole of pentose phosphate, determined as the difference between the pentose-reacting substances in mercury and ZnSO₄-Ba(OH)₂ filtrates, was accompanied by the production of 0.73 μ mole of acid-stable organic phosphate. No acid-labile phosphate was present. Thus it can be concluded that a pentose monophosphate is the product of the enzymic phosphorylation of ribose by ATP.

*Absence of heptulose derivatives from reaction mixtures containing the products of phosphorylation of ribose by ATP in extracts of ribose-grown *Esch. coli**

The conversion of ribose 5-phosphate by enzymes of liver and spinach (Horecker, Smyrniotis & Klenow, 1953) and of erythrocytes (Dische & Pollaczek, 1952) into sedoheptulose 7-phosphate raised the possibility that phosphorylated seven-carbon sugars might be formed secondarily in the present experiments, following the initial enzymic

phosphorylation of ribose. The fact that sedoheptulose and its derivatives react in the orcinol test enabled us to investigate this possibility. Horecker *et al.* (1953) have reported that after carrying out the orcinol reaction, the ratio $E_{670 \text{ m}\mu} : E_{580 \text{ m}\mu}$ for pentoses is 3.78, while for sedoheptulosan it is 0.29. In the experiment reported in Table 6, ribose was incubated with ATP and *Esch. coli* extract under the usual conditions until all the free ribose had disappeared, as shown by the absence of pentose-reacting material in the $\text{ZnSO}_4\text{-Ba(OH)}_2$ filtrates. A sample of the mercury filtrate, containing pentose phosphate, was then subjected to the orcinol reaction and the optical densities at 580 and 670 $\text{m}\mu$. were determined. As will be seen from Table 6, the observed ratio was in good agreement with the value reported by Horecker *et al.* (1953) for arabinose and with that found by us for ribose. Thus, it can be concluded that no seven-carbon sugar is formed under the conditions of our experiments.

DISCUSSION

When *Esch. coli* 7271 was grown on a medium with D-ribose as the carbon source, the washed cells were able to utilize ribose but not other pentoses. Disintegration of a suspension of the cells in buffer yielded an extract which also had the power to utilize ribose, always provided that adenosine triphosphate and magnesium ions were present in the reaction mixture. We have shown by the use of a precipitant, consisting of mercuric acetate in dilute acetic acid, that it is possible to remove the adenine nucleotides from an incubated reaction mixture and at the same time to leave in solution both unchanged ribose and its reaction products. The pentose content of such a mercuric acetate filtrate was always greater than the amount of free ribose, as determined in a filtrate from a similar reaction mixture after barium hydroxide-zinc sulphate precipitation, the difference between the two quantitatively representing an accumulation of pentose-containing products. It follows that under the conditions employed, the rate of formation of pentose-containing products exceeds their rate of degradation. This appears not to have been the case in the experiments of Sable (1950, 1952), in which no pentose-containing products could be detected following the action on ribose of a protein fraction prepared from an extract of yeast, supplemented with adenosine triphosphate and magnesium ions. The rate of removal of the pentose-containing products is low in our experiments, perhaps partly through the deliberate ageing of the extracts and because the enzymes involved become insoluble after freezing and thawing. However, the major factor responsible for the depression of their rate of removal is undoubtedly the presence of the high

concentration of ammonium sulphate which was added to the reaction mixture, and which seems to inhibit the activity of the enzymes involved. Lampen (1953) also achieved some success in inhibiting ribose 5-phosphate degradation by cell-free extracts of *Lb. pentosus*, both by working at pH 8.0 and by a preliminary treatment of the enzyme extract with manganous chloride, followed by removal of the precipitate.

It appears that the reaction being studied is a simple enzymic transfer of the terminal phosphate group from adenosine triphosphate to ribose, giving a pentose monophosphate as the reaction product. This follows from the demonstration that there is equivalence between the amounts of ribose and acid-labile phosphate disappearing and of acid-stable phosphate accumulating. This work incidentally proves also that the pentose phosphate which is formed is not an aldopentose 1-phosphate, for such compounds are highly acid-labile. In the next paper of this series (Long, 1955), it will be shown that the product is in fact ribose 5-phosphate.

SUMMARY

1. Washed cells of ribose-grown *Esch. coli* 7271 are able to utilize ribose *in vitro*.
2. Cell-free extracts prepared from these bacteria will also bring about the disappearance of ribose when supplemented with adenosine triphosphate and magnesium ions.
3. The reaction consists of the enzymic transfer of the terminal phosphate group of adenosine triphosphate to ribose, yielding an acid-stable pentose monophosphate.

We would like to express our thanks to the Agricultural Research Council for a full-time studentship for one of us (K.H.). We are also much indebted to Miss Iris Creighton for skilled technical assistance.

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Studies Involving Enzymic Phosphorylation

4. THE CONVERSION OF D-RIBOSE INTO D-RIBOSE 5-PHOSPHATE BY EXTRACTS OF *ESCHERICHIA COLI*

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(Received 2 August 1954)

In the previous paper of this series (Heald & Long, 1955), the enzymic phosphorylation of D-ribose by extracts of ribose-grown *Escherichia coli* in the presence of adenosine triphosphate and magnesium ions was described. Large-scale experiments under optimal conditions have now been carried out and the product of phosphorylation has been isolated as the barium salt. Although the substance obtained is not completely pure, its analysis, properties and reactions identify it as the barium salt of ribose 5-phosphate. A preliminary report of some of this work has already been given (Heald & Long, 1953).

EXPERIMENTAL

Materials

In addition to the D-ribose, adenosine triphosphate (ATP), synthetic barium ribose 5-phosphate and *Esch. coli* strain 7271, already described (Heald & Long, 1955), the following ribose phosphates were also studied for comparison:

Ribose 5-phosphate. This sample was obtained by hydrolysing dibarium ATP (1.7 g.) in 0.5N-H₂SO₄ (25 ml.) for 4-5 hr. on a boiling-water bath; excess barium acetate was added and BaSO₄ removed. Barium phosphate was precipitated by raising the pH to 8.0 with 10N-NaOH and barium ribose 5-phosphate was precipitated from the filtrate by addition of 4 vol. ethanol; purification was effected by solution in 0.01N-HCl and reprecipitation with 4 vol. of ethanol.

Ribose 2- and 3-phosphates. A mixture of the 2- and 3-phosphates of ribose (previously considered to be ribose 3-phosphate only) was prepared by acid hydrolysis of adenosine 3'-phosphoric acid (L. Light and Co. Ltd.) by the procedure of LePage & Umbreit (1943). The material was isolated as the barium salt.

Methods

The procedures for growing, washing and distintegrating the bacteria have already been described (Heald & Long,

1955); pentose was determined by the method of Mejbaum (1939) and phosphorus by the method of Long (1943).

The action of alkaline phosphatase on pentose phosphates. Alkaline phosphatase was prepared from rabbit intestinal mucosa by the *n*-butanol method of Morton (1950). The enzyme solution was dialysed against 0.025M ammonium acetate buffer, pH 8.9, and stored in the frozen state at -20°. When incubated at 37° for 15 min., 0.1 ml. of a 1 in 50 dilution of this stock enzyme solution liberated 0.15 μmole of inorganic phosphate from a reaction mixture containing 0.0125M diethylbarbiturate (veronal) buffer, pH 9.2 (King & Delory, 1940), 0.01M-MgCl₂ and 0.015M disodium β-glycerophosphate.

The enzymic hydrolysis of the pentose phosphates was studied as follows: a weighed amount of the barium salt (about 8 μmoles) was dissolved in 0.4 ml. 0.01N-HCl and the barium precipitated by addition of about 5 mg. of solid K₂SO₄. The suspension was centrifuged and the residue washed with 0.1 ml. 0.01N-HCl. The combined supernatants were adjusted to pH 9.2 with N-KOH and to the solution were added 0.016 ml. 0.5M-MgCl₂, 0.1 ml. 0.025M veronal buffer, pH 9.2, and 0.2 ml. of the stock phosphatase solution. The reaction mixture was incubated at 37° for 45 min., during which time a white precipitate, which was possibly MgNH₄PO₄, separated out. The tube was placed in a boiling-water bath for 5 min. to inactivate the phosphatase and then centrifuged. The clear supernatant was used for pentose analysis and for enzymic phosphorylation experiments.

Reducing power of ribose phosphates. Samples of the barium salts of the ribose phosphates (about 0.2 μmole; amount determined accurately as total pentose) were dissolved in 0.05-0.10 ml. 0.01N-HCl and the reducing power determined by the method of Nelson (1944). The colours were read in a photoelectric colorimeter (Evans Electro-selenium Ltd.) against a ribose standard and water blank treated similarly.

Acid-lability of ribose phosphates. When ribose phosphates are heated on a boiling-water bath in N-HClO₄, both inorganic phosphate and free ribose are liberated. Ribose 5-phosphate, however, is much less acid-labile than is a mixture of ribose 2- and 3-phosphates. The procedure was as follows: to a known amount of the barium salt of the