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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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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-H_2SO_4 (25 ml.) for 4·5 hr. on a boiling-water bath; excess barium acetate was added and $BaSO_4$ 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-$MgCl_2$ 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 K_2SO_4. 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-$MgCl_2$, 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 $MgNH_4PO_4$, 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 Electroselenium 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-$HClO_4$, 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

appropriate pentose phosphate (about 1 μmole), dissolved in 0·2 ml. 0·01 N-HCl contained in a graduated centrifuge tube, were added 1·45 ml. water and 0·15 ml. 72% $HClO_4$ (giving a final concn. of N-$HClO_4$). The solution was heated on a boiling-water bath for a predetermined period not exceeding 7 hr. After the requisite time, the tube was cooled, and the contents neutralized to pH 7 (internal indicator, bromothymol blue) with 40% (w/v) NaOH and diluted to 2·5 ml.; a 0·5 ml. sample was taken for inorganic phosphate determination. The remaining 2 ml. were treated with 0·5 ml. 5% (w/v) $ZnSO_4$ and 0·5 ml. 0·3 N-$Ba(OH)_2$ (Somogyi, 1945), diluted with water to 5 ml., mixed, centrifuged and filtered; 3·0 ml. of clear filtrate were taken for free-ribose determination. Total ribose and total phosphorus were determined on samples of the unhydrolysed ribose phosphates. Zero-time samples, standards and blanks were analysed simultaneously.

Bromine oxidation. This method has been used to detect and determine ketose derivatives in the pentose phosphate samples, and is based on the procedure of Lampen (1953). However, the $BaCO_3$ used by the latter author was replaced here by $MgCO_3$. The final procedure is described in Table 3.

Periodate oxidation. The experimental procedure is described in Table 6.

Spectrophotometric determinations. Contamination by purine derivatives was tested for by examining the ultraviolet absorption of the pentose phosphates over the range 230–290 mμ. (Kalckar, 1947).

The presence of ketopentoses and of heptoses was tested for by carrying out the orcinol reaction of Mejbaum (1939) and examining the green solution over the range 500–700 mμ. In addition to the usual peak given by aldopentoses at 670 mμ., ketopentoses show a subsidiary peak at 540 mμ. (Horecker, Smyrniotis & Seegmiller, 1951); ketoheptoses, when treated in this way, do not possess an absorption band at 670 mμ. but instead show maximum absorption at 580 mμ. (Horecker, Smyrniotis & Klenow, 1953).

A Unicam spectrophotometer SP. 500 was used for these studies, using 1 cm. cells.

RESULTS

Large-scale enzymic phosphorylation of D-*ribose*

About ten large-scale enzymic phosphorylations have been carried out, with varying yields and purities of the product. In the earliest experiments, ammonium sulphate was not present in the enzyme reaction mixtures, with the result that the yields of pentose phosphate were low (Heald & Long, 1955), and the material isolated was grossly contaminated with phosphorus-containing products of further metabolism. When ammonium sulphate was later included in the reaction mixtures, however, the presence of the sulphate ion introduced an additional problem, namely, the separation of barium pentose phosphate from barium sulphate in the barium-fractionation procedure. It was eventually found that the best method of purification consisted in removing the adenine nucleotides by mercuric acetate precipitation and then adding barium acetate at a slightly alkaline pH, whereby the barium pentose phosphate became strongly adsorbed upon the barium sulphate precipitate. The pentose phosphate was then eluted by several treatments with dilute acid and finally purified by barium and ethanol fractionation. The most successful procedure is described below.

A cell-free extract of ribose-grown *Esch. coli* was prepared by the method given in the previous paper (Heald & Long, 1955); the extract was 0·1 M with respect to aminotrishydroxymethylmethane (THAM) buffer, pH 7·6, and 1 ml. contained the soluble material derived from 20 mg. dry wt. of bacteria. Before use, it was dialysed for 70 min. at 3° in a collodion sac against 8 vol. of 0·1 M THAM buffer, changing the buffer after 35 min. and stirring occasionally.

The dialysed enzyme (30 ml.) was incubated for 45 min. at 38° in a total volume of 150 ml. with a reaction mixture of the following composition (final concentrations in brackets): THAM buffer, pH 7·6 (0·04 M); $MgCl_2$ (0·005 M); potassium ATP (0·005 M); $(NH_4)_2SO_4$ (0·28 M) and D-ribose (0·005 M). Mercuric acetate (20% (w/v) in 0·1% (v/v) acetic acid; 90 ml.) was then added and the mixture kept overnight at 2°. After centrifuging, the residue was washed twice with 100 ml. portions of ice-cold 0·5% (w/v) mercuric acetate in 0·1% (v/v) acetic acid. The original supernatant and washings were combined and filtered; H_2S was passed through the clear filtrate for 30 min. The HgS precipitate was spun down and discarded, and the supernatant was filtered. The clear filtrate was evaporated *in vacuo* to about 50 ml. A sample of this solution on analysis, indicated that the total volume contained 232 μ-moles ribose and 450 μmoles pentose phosphate.

Isolation and purification of the product of enzymic phosphorylation

The solution, together with 5 ml. washings, was transferred to a 250 ml. centrifuge bottle. Barium acetate (2 M; 15 ml.) in slight excess was added, followed by 2·5 N-NaOH (about 12 ml.) until just alkaline to phenolphthalein. The mixture was centrifuged and the residue washed 3 times with 20 ml. portions of 0·1 M barium acetate made pink to phenolphthalein. The supernatant and washings on analysis were found to contain only free pentose and were therefore discarded.

The barium sulphate precipitate was extracted 3 times with 20 ml. portions of 0·1 N-HCl, the suspensions being centrifuged in an angle centrifuge at 8000 *g* for 15 min. each time. The supernatants were then filtered. Most of the pentose phosphate was present in the second fraction; a total of 287 μmoles pentose phosphate were recovered in this way.

The combined HCl supernatants (55 ml.) were treated with 1 ml. of 2 M barium acetate and 40%

(w/v) NaOH (about 1 ml.) until pink to phenolphthalein. The mixture was chilled overnight and the insoluble residue, which contained little pentose-reacting material, was discarded. The 'barium soluble' fraction was acidified to pH 6·0 and treated with 4 vol. ethanol. After chilling for 2 hr., the 'barium soluble–alcohol insoluble' material was spun down; it contained 197 μmoles pentose phosphate. The 'barium soluble–alcohol soluble' supernatant contained practically no pentose-reacting material and was discarded.

The 'barium soluble–alcohol insoluble' precipitate was dried *in vacuo* at room temperature, to remove ethanol, and dissolved in 10 ml. 0·1 N-HCl. Barium acetate (2 M; 0·1 ml.) was added and N-NaOH until the solution reacted just pink to phenolphthalein. The mixture was chilled for 1·5 hr., the small amount of precipitate spun down and discarded, the supernatant adjusted to pH 6·0 with conc. HCl and 40 ml. ethanol were added. After 2 hr. at 3°, the precipitate was spun down and washed with 10 ml. ethanol followed by 10 ml. ether. It was dried *in vacuo* at room temperature. Yield, 115·1 mg. of barium salt. On analysis, 1·485 mg. contained 2·29 μmoles pentose-reacting material, 2·75 μmoles organic phosphorus and 0·13 μmole inorganic phosphorus. When a sample was subjected to acid hydrolysis (N-$HClO_4$ on a boiling-water bath for 10 min.), it was found to contain about 10 % of its organic phosphorus in an acid-labile form; a sample of synthetic ribose 5-phosphate, by contrast, contained no detectable acid-labile phosphorus under these conditions. The contaminating organic phosphate was believed to have originated from the enzyme extract used.

The crude biosynthetic barium pentose phosphate (110 mg.) was dissolved in a mixture of 0·9 ml. 72 % $HClO_4$ and 9·9 ml. water, and the solution heated on a boiling-water bath for 1 hr. After cooling and neutralizing with 10 N-NaOH, 0·2 ml. 2 M barium acetate was added, the pH adjusted to 8·3 (pink to phenolphthalein) and the mixture placed in a refrigerator overnight. After centrifuging down the precipitate, which consisted largely of barium phosphate and was discarded, the supernatant was treated with 3 vol. ethanol and chilled. The 'barium soluble–alcohol insoluble' material was washed with 30 ml. ethanol, followed by 30 ml. ether. The product was dried *in vacuo* at room temperature. The yield was very low (about 35 mg.), but this was partly due to a mechanical loss.

On analysis, 1·60 mg. contained 2·94 μmoles of bound ribose and 3·41 μmoles total phosphorus. There was no inorganic phosphate and no acid-labile (10 min. in N acid at 100°) phosphorus. However, the pentose:phosphate ratio of 0·86 indicates that the expected purification had not been achieved and that there was present some acid-stable phosphate contaminant. The material gave no absorption in the 260 mμ. region, and was taken to be free from any contamination with purine derivatives. No further efforts were made to purify the material owing to the small amount available.

Properties and reactions of the biosynthetic barium pentose phosphate

The biosynthetic pentose phosphate, authentic ribose 5-phosphate and the mixture of ribose 2- and 3-phosphates when examined by paper chromatography, using different solvent systems invariably gave identical R_F values. As a result, this method could not shed any light on the position of the phosphate group in the biosynthetic material.

Examination for possible contamination by sedoheptulose 7-phosphate. The product of enzymic phosphorylation has been examined spectrophotometrically for contamination by seven-carbon sugar derivatives, although it has been shown in the previous paper (Heald & Long, 1955) that in small-scale experiments such contamination was not detectable.

The results of an experiment to test for sedoheptulose contamination (Table 1) do not provide any spectrophotometric evidence to suggest that this impurity is present in the biosynthetic pentose phosphate. For comparison, the results obtained with the sample of synthetic barium ribose 5-phosphate are also given.

The nature of the pentose in the biosynthetic pentose phosphate. So far, no evidence has been presented regarding the nature of the pentose in the biosynthetic product. The pentose itself was found to be readily liberated from its phosphate ester after incubation with alkaline phosphatase at 37° and pH 9·2, as shown by the presence of orcinol-reacting material in filtrates obtained after treatment with the $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945). In view of the small amount of material available, polarimetric examination was not feasible, but paper chromatography strongly

Table 1. *Examination of the biosynthetic pentose phosphate for contamination with sedoheptulose phosphate*

Samples (about 0·2 μmole) of the barium salts of the biosynthetic pentose phosphate and of synthetic ribose 5-phosphate were subjected to the orcinol reaction and optical densities measured at 580 and 670 mμ. in a Unicam spectrophotometer.

	Amount taken (μmole)	Optical density at (*a*) 580 mμ.	(*b*) 670 mμ.	Ratio *b*/*a*
Synthetic ribose 5-phosphate	0·146	0·101	0·379	3·76
Biosynthetic pentose phosphate	0·218	0·158	0·590	3·74

suggested that the pentose was in fact ribose. When a chromatogram was run with phenol as solvent (Partridge, 1948), followed by spraying with aniline hydrogen phthalate, the pentose was readily separated from admixture with D-arabinose or D-xylose, but travelled as a single spot when mixed with authentic D-ribose.

This conclusion was confirmed by making use of the known specificity of the ribokinase present in extracts of ribose-grown *Esch. coli*. As shown in the previous paper (Heald & Long, 1955), these bacterial extracts will utilize D-ribose when supplemented with ATP and magnesium ions, but have no effect on D-xylose or D- or L-arabinose. When the biosynthetic pentose phosphate was incubated first with alkaline phosphatase and then, after inactivating this enzyme, with the ribokinase and ATP, the free pentose formed by the action of the first enzyme was found to be well utilized by the second. This experiment (Table 2) also strongly suggests that the pentose phosphate is in fact a ribose phosphate. The similar results, obtained in a parallel experiment with a sample of synthetic ribose 5-phosphate, are also shown for comparison in Table 2. It should, of course, be pointed out that this experiment does not exclude the possibility that the pentose may be ribulose, which perhaps is also a substrate for ribokinase; however, as shown in the next section, there is strong evidence against the presence of ketopentose.

Examination of the biosynthetic product for ketose. Since any sugar containing a potential aldehyde group may be oxidized by bromine water to the corresponding carboxylic acid, whereas ketoses and their derivatives are not oxidizable under these conditions, one may readily determine the proportions of aldose and ketose derivatives in a mixture. In the pentose series, both aldoses and ketoses give a positive orcinol reaction, but the carboxylic acid formed by oxidation of an aldopentose will not react with the reagent. It follows, then, that bromine oxidation of ribose phosphates (except ribose 1-phosphate) will result in material giving a negative orcinol reaction, whereas bromine treatment will not affect the ribulose phosphates. The action of bromine water, in the presence of magnesium carbonate, has been applied to the biosynthetic pentose phosphate and to the other ribose phosphates, with the results shown in Table 3.

Table 2. *The nature of the pentose in the biosynthetic pentose phosphate*

Authentic D-ribose or the pentose solutions (about 0·6 μmole), obtained by the action of rabbit intestinal mucosa alkaline phosphatase on synthetic ribose 5-phosphate or the biosynthetic pentose phosphate (see Methods section), were incubated for 30 min. at 37° with 0·2 ml. of an extract of ribose-grown *Esch. coli* (derived from 4 mg. dry wt. of cells) in a reaction medium of the following composition: 0·005 M-ATP, 0·005 M-$MgCl_2$, 0·28 M-$(NH_4)_2SO_4$ and 0·04 M THAM buffer, pH 7·6; total vol., 0·5 ml. Reaction mixtures were deproteinized with either the $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945) or with 20% (w/v) mercuric acetate in 0·1% (v/v) acetic acid (Heald & Long, 1955) and the filtrates analysed for free pentose and for free + esterified pentose, respectively. The results are given as μmoles of pentose or pentose phosphate.

Substrate	Amount present initially: Free pentose	Amount present initially: Pentose phosphate	Amount present at end of incubation: Free pentose	Amount present at end of incubation: Pentose phosphate	Pentose utilized	Pentose phosphate formed
Authentic D-ribose	0·70	0	0·29	0·53	0·41	0·53
Ribose derived from synthetic ribose 5-phosphate	0·60	0·17	0·27	0·50	0·33	0·33
Pentose derived from biosynthetic pentose phosphate	0·60	0·06	0·33	0·43	0·27	0·37

Table 3. *Bromine oxidation of pentose phosphates*

The barium salt of the pentose phosphate (about 1 μmole), dissolved in 0·5 ml. 0·01 N-HCl, was treated with about 20 mg. $MgCO_3$ and 0·25 ml. 1·2% bromine water. The mixture was incubated for 1·3 hr. at 25° and the orcinol reaction carried out directly on the suspension.

	Amount taken* for bromine oxidation (μmoles)	Amount remaining after bromine oxidation* (μmole)	Apparent ketose (%)
Biosynthetic pentose phosphate	1·09	0	0
Synthetic ribose 5-phosphate	0·73	0·10	13
Ribose 5-phosphate prepared by acid hydrolysis of ATP	0·99	0·02	2
Mixture of ribose 2- and 3-phosphates	0·93	0·02	2
Ribose	0·96	0	0

* These values are based on pentose estimation by the orcinol reaction.

These findings demonstrate that ketose derivatives were absent from the biosynthetic product, and that negligible amounts were present in the ribose 5-phosphate prepared by acid hydrolysis, or in the mixture of ribose 2- and 3-phosphates. However, about 13 % ketose appeared to be present in the specimen of synthetic ribose 5-phosphate; this value has been repeatedly confirmed, but no explanation for the result can be offered. That the latter material does not in fact contain ketopentose, however, is shown by the observation that the green solution obtained in the orcinol reaction showed no enhanced absorption in the region of 540 mμ. as compared with a ribose standard; the absorption at 540 mμ. is characteristic of ketopentoses (Horecker *et al.* 1951). Furthermore, the bromine-resistant material from the sample of synthetic ribose 5-phosphate did not show any spectrophotometric evidence for the presence of ketopentose. In fact, when examined by this spectrophotometric method, neither the biosynthetic pentose phosphate nor any of the other pentose phosphates used in this work showed any indication of contamination with ketose derivatives. These experiments, therefore, indicate that the biosynthetic pentose phosphate is a ribose phosphate.

Reducing properties of the biosynthetic ribose phosphate. Reducing power was determined with the Nelson (1944) reagent, using ribose as standard. The results for the biosynthetic ribose phosphate and for the various reference ribose phosphates are shown in Table 4. From these it will be seen that the two samples of ribose 5-phosphate have reducing powers close to that of ribose itself, and that the biosynthetic ribose phosphate is similarly strongly reducing. By contrast, the mixture of ribose 2- and 3-phosphates is only weakly reducing. These observations suggest that the biosynthetic material is ribose 5-phosphate.

Acid hydrolysis. Samples of the barium salts of the two ribose 5-phosphate samples, the mixture of ribose 2- and 3-phosphates and the biosynthetic ribose phosphate were heated with N perchloric acid on a boiling-water bath for periods up to 7 hr. The hydrolysates were then analysed for free ribose and inorganic phosphate after the different time intervals. The results are given in Table 5.

Table 4. *Reducing power of ribose phosphates*

Samples of the barium ribose phosphates (about 0·2 μmole) were quantitatively examined for reducing power by the method of Nelson (1944).

	Amount taken* (μmole)	Reducing equivalent† (μmole)	Ratio‡: reducing equivalent / ribose content
Biosynthetic ribose phosphate	0·219	0·181	0·87
Synthetic ribose 5-phosphate	0·184	0·186	1·01
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	0·198	0·184	0·93
Mixture of ribose 2- and 3-phosphates	0·161	0·060	0·37

* Based on total pentose content by orcinol reaction.
† Free ribose as standard for reducing power.
‡ Ratio for free ribose is unity.

Table 5. *Acid hydrolysis of ribose phosphates*

Samples of ribose phosphates (about 1 μmole) were heated on a boiling-water bath with N-$HClO_4$ and the amounts of free ribose and inorganic phosphate were determined after different time intervals, as described in the Experimental section.

	Percentage of total liberated after (hr.)			
	1	2	4	7
Free ribose analyses*				
Biosynthetic ribose phosphate	9	—	22	32
Synthetic ribose 5-phosphate	12	20	24	31
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	14	17	23	28
Mixture of ribose 2- and 3-phosphates	94	96	94	—
Inorganic phosphate analyses				
Biosynthetic ribose phosphate	27	32	54	73
Synthetic ribose 5-phosphate	20	31	56	74
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	17	27	60	70
Mixture of ribose 2- and 3-phosphates	91	97	100	—

* Corrected for decomposition of ribose, when heated in N acid. For explanation, see text.

It was found in a parallel control experiment with ribose that, under the conditions of acid hydrolysis used here, the extent of its decomposition, as measured by the orcinol reaction, amounted to 4, 6, 7 and 15 % after 1, 2, 4 and 7 hr., respectively. The values for free ribose in Table 5 have therefore been corrected for this slight decomposition, although it is clear that the correction should be rather smaller, since there is no free ribose at zero time.

It will be observed that the liberation of free ribose and inorganic phosphate from the mixture of ribose 2- and 3-phosphates is more than 90 % complete after 1 hr., whereas in the case of the two ribose 5-phosphate samples only 12–14 % of the ribose and 17–20 % of the phosphate appear to be liberated during this period. It is not clear why the rates of liberation of ribose and inorganic phosphate do not run parallel during the hydrolysis of ribose 5-phosphate, but the very considerable discrepancy is evident after all time intervals, the ratio pentose: inorganic phosphate averaging about 0·4. A possible explanation of this behaviour is that prolonged treatment with hot mineral acid first converts some of the ribose 5-phosphate into an unknown phosphorylated intermediate, and that when this subsequently undergoes hydrolysis, inorganic phosphate is formed together with a degradation product of ribose which does not give a positive orcinol reaction.

The rate of hydrolysis of the biosynthetic ribose phosphate is clearly very similar to that of the two ribose 5-phosphate samples, and quite different from that of the ribose 2- and 3-phosphate mixture. The fact that the authentic ribose 5-phosphate and the biosynthetic ribose phosphate both show the discrepancy between the rates of ribose and phosphate liberation gives added support to the likelihood that they are identical.

Periodate oxidation. Ribose 5-phosphate should react with 3 molecular proportions of periodic acid, whereas both ribose 2- and 3-phosphates should react with only two. When the barium salts of the biosynthetic ribose phosphate and of the other ribose phosphates were treated with about a twofold excess of periodic acid for 30–120 min., the results in Table 6 were obtained. It will be seen that the ribose 5-phosphate samples gave, within experimental error, the theoretical value for the molar ratio, periodic acid:ribose. The biosynthetic ribose phosphate also required about 3 moles periodic acid/mole ribose. The mixture of ribose 2- and 3-phosphates gave rather lower than the theoretical value. This experiment supports the view that the biosynthetic material is ribose 5-phosphate.

Further evidence for this conclusion was obtained by determining the formaldehyde production after treatment with periodate, using the procedure of O'Dea & Gibbons (1953). It was found that yields of 0·10 and 0·08 mole of formaldehyde were obtained from 1 mole of biosynthetic ribose phosphate and synthetic ribose 5-phosphate, respectively. It can be inferred from these results that the—CH_2OH group is not free in position 5 in the biosynthetic material, for otherwise the yield of formaldehyde would have been 1·0 mole/mole ribose phosphate.

Rate of colour development in the orcinol reaction. The rate at which the green colour develops in the orcinol reaction for pentoses has long been known to depend on the structure of the pentose derivative. Thus, pentoses with a phosphate substituent in C_5, as in ATP, ADP, AMP and ribose 5-phosphate, react more readily than do the pentoses themselves or their derivatives with phosphate substituents in C_2 or C_3. In the present work, samples of the ribose phosphates (about 0·2 μmole) were heated for different periods with the orcinol reagent and the extent of colour development, expressed as a percentage of that found after 40 min., was determined.

Table 6. *Periodate oxidation of ribose phosphates*

The barium salt of each ribose phosphate (about 1 μmole), dissolved in 0·5 ml. 0·01 N-HCl, was treated with 1 ml. approx. 0·0067 M-HIO_4 (in 0·1 M acetate buffer, pH 5·0). After 30, 60 or 120 min., 1 ml. 2% (w/v) KI in 4·5% (w/v) $NaHCO_3$ and one drop sat. aqueous Na_2SO_4 were added, and the mixture titrated with standard 0·1 N arsenite from a Conway burette, with one drop of starch indicator added near the end point. Controls with water instead of ribose phosphate, at the same time intervals, and with ribose phosphates added after the HIO_4, KI, $NaHCO_3$ and Na_2SO_4 were simultaneously carried out. The values given are corrected for these controls. Since there were no differences in the titration readings obtained after 30, 60 and 120 min. in the experimental tubes, the figures have been averaged.

	Amount* taken (μmole)	0·1 N arsenite required (ml.)	Molar ratio, HIO_4:ribose	
			Obs.	Calc.
Synthetic ribose 5-phosphate	0·824	0·0504	3·05	3·0
Ribose 5-phosphate, prepared by acid hydrolysis of ATP	0·794	0·0473	2·97	3·0
Mixture of ribose 2- and 3-phosphates	0·931	0·0300	1·61	2·0
Biosynthetic ribose phosphate	0·750	0·0471	3·14	—

* Based on pentose content by orcinol determination.

The results are represented graphically in Fig. 1. It will be seen that ribose and the mixture of ribose 2- and 3-phosphates reacted relatively slowly with the reagent, whereas the two ribose 5-phosphate samples and the biosynthetic ribose phosphate all reacted much more rapidly. These results also confirm the view that the biosynthetic product is ribose 5-phosphate.

DISCUSSION

Although the biosynthetic pentose phosphate isolated in this work has not been obtained completely pure, the evidence strongly favours the structure ribose 5-phosphate being assigned to it. The barium salt after purification contained no material absorbing at 260 mμ., indicating its freedom from contamination by the adenosine triphosphate used in its preparation. It was also quite free from inorganic phosphate and had a pentose:phosphorus ratio of 0·86. Furthermore, it was uncontaminated by heptulose phosphate, as shown spectrophotometrically on the green solution formed in the orcinol reaction (Table 1), nor did it contain any ketopentose derivative, as indicated by oxidation studies with bromine water (Table 3). The pentose was almost certainly ribose, for alkaline phosphatase liberated a free pentose, which could be rephosphorylated by a ribokinase preparation in the presence of adenosine triphosphate. It has already been shown in the previous paper (Heald & Long, 1955) that D-xylose and D- and L-arabinose were not enzymically phosphorylated under these conditions, whereas D-ribose reacted readily.

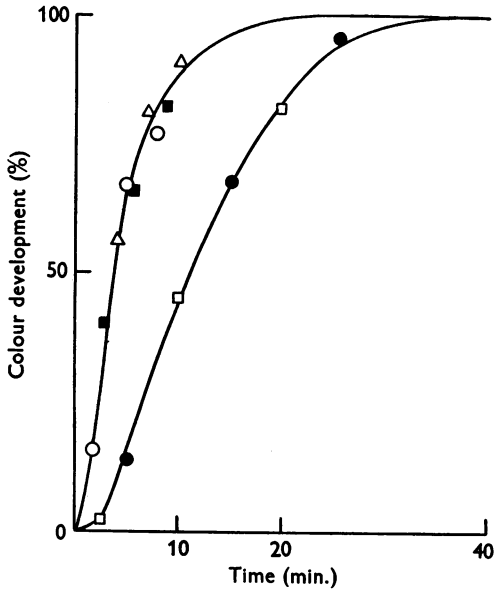

Fig. 1. Rates of colour development with ribose and with different ribose phosphates in the orcinol reaction. Ribose phosphate (about 0·2 μmole) heated with the orcinol reagent on a boiling-water bath for various periods. Colour intensity measured in a photoelectric colorimeter and expressed as a percentage of the maximum obtainable at 40 min. □, ribose; ●, mixture of ribose 2- and 3-phosphates; ○, biosynthetic ribose phosphate; ■, synthetic ribose 5-phosphate; △, ribose 5-phosphate, prepared by acid hydrolysis of ATP.

Evidence for the position of the phosphate group at C_5 is equally convincing. Thus, the stability in N acid at 100° of ribose 5-phosphate is much greater than that of the mixture of ribose 2- and 3-phosphates (Table 5), and the biosynthetic ribose phosphate had similar stability to that of the ribose 5-phosphate samples. Ribose 1-phosphate, of course, is excluded because of its very great acid lability (Lowry & Lopez, 1946). The action of periodic acid strongly supports the ribose 5-phosphate structure (Table 6), one molecule of the biosynthetic product reacting with but slightly more than the three molecules of periodic acid required by theory. The rate of colour development with the orcinol reagent (Fig. 1) is also consistent with this conclusion. Finally, the reducing power accords well with the ribose 5-phosphate structure (Table 4), the latter being a much stronger reducing agent under the conditions employed than the mixture of ribose 2- and 3-phosphates, although the reason for this difference is obscure. The reducing properties also effectively rule out the possibility of the material being ribose 1-phosphate, which is non-reducing under alkaline conditions.

SUMMARY

1. Large-scale enzymic phosphorylations of D-ribose, by means of adenosine triphosphate in the presence of a cell-free extract of ribose-grown *Escherichia coli*, have been carried out. A biosynthetic pentose monophosphate has been isolated from the reaction mixture and partially purified.

2. This biosynthetic material is almost certainly the barium salt of ribose 5-phosphate, as shown by the liberation of ribose after the action of alkaline phosphatase, and by studies of reducing power, acid stability, periodate oxidation and rate of colour development in the orcinol reaction.

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The Occurrence of a Cyanocobalamin-binding Protein in Milk and the Isolation of a Cyanocobalamin–Protein Complex from Sow's Milk

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A preliminary communication from this laboratory has described the occurrence of a vitamin B_{12}-binding substance in sow's milk whey (Gregory, Ford & Kon, 1952). More recently, we have shown that this binding substance, and an intrinsic-factor concentrate prepared from pig stomach, combined with other vitamin B_{12}-like substances in the same way as with cyanocobalamin (vitamin B_{12}) (Gregory & Holdsworth, 1953). The similarity between the effect on cyanocobalamin of the sow's milk whey and of the intrinsic-factor concentrates, led us to attempt the isolation of the binding substance from sow's milk, since a study of its properties may help to explain some aspects of the utilization and metabolism of cyanocobalamin.

The first part of this paper presents evidence that the milk of the cow, goat, pig, rat and woman possesses the property of combining with added cyanocobalamin. The second part describes the isolation from sow's milk of a cyanocobalamin–protein complex.

THE OCCURRENCE OF A CYANOCOBALAMIN-BINDING PROTEIN IN MILK

Experimental

Materials

The samples of milk from the cow, goat, pig, rat and woman and of colostrum from the cow and goat were the same as those described by Gregory (1954).

The sow's whey concentrate (SF 8) and the 'intrinsic factor' concentrate from pig stomach were prepared as described by Gregory & Holdsworth (1953).

Microbiological methods

Method of assay. The method of assay with *Lactobacillus leichmannii* ATCC 4797 was a modification of that of Skeggs, Nepple, Valentik, Huff & Wright (1950) and is fully described by Gregory (1954).

Measurement of cyanocobalamin in the cyanocobalamin–protein complex. Cyanocobalamin was quantitatively released from the protein complex by digestion with papain as described by Gregory (1954) and measured by *Lb. leichmannii*.

Measurement of cyanocobalamin-binding activity. Two methods were used. (*a*) The growth-inhibition method, described by Gregory & Holdsworth (1953). (*b*) The ultrafiltration method. A bag of 'Visking' cellulose tubing (The Visking Corporation, Chicago), diameter $\frac{1}{4}$ in., was suspended from the stem of a glass funnel held in the neck of a filtration tube by means of a rubber bung. The bag was made by knotting one end of the tubing tightly and tying the other, with cotton, over a piece of Polythene tubing fitted over the stem of the funnel. (A diagram of this apparatus will be found in the paper by Gregory, 1954.) A known amount of cyanocobalamin in 1 ml. of solution was added to a measured amount of the binding substance, and the mixture (total volume 10 ml.) poured into the cellophan bag. The filtration tube was evacuated, sealed off and left in the refrigerator for 1–2 hr. In this time, 1–2 ml. of ultrafiltrate could be collected.

The free cyanocobalamin, which was able to pass into the ultrafiltrate, was measured by *Lb. leichmannii*. The amount of cyanocobalamin bound by the binding substance was then calculated by subtracting the free cyanocobalamin from the total amount added.

This method was applied to the measurement of the binding of cyanocobalamin by the milks of different animal species. The approximate amount of cyanocobalamin bound by each of the milks was first estimated and then a more exact determination made. Three different levels of cyanocobalamin within a narrow range (e.g. 0·05, 0·10 and 0·20 μg.) were added to 10 ml. milk, so that at least two of the levels gave an estimation of the binding activity.

Comparison of the bound form of cyanocobalamin in the milk of different species and in extracts of desiccated pig stomach

In order to establish whether the cyanocobalamin in the different milks was associated with one particular protein, the whey proteins were separated by electrophoresis on paper and the paper strips examined for proteins and