Isolation of Hydrolytic Products of a Glycerophospho-compound from *Micrococcus pyogenes*

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SUMMARY: The ribonucleic acid fraction of *Micrococcus pyogenes* var. *aureus* (strain Duncan), separated by the method of Schmidt & Thannhauser (1945), contains organic phosphate in excess of that which can be accounted for by a polynucleotide nucleic acid structure. This 'excess phosphate' can be separated from the other phospho-compounds after fractional extraction of *M. pyogenes* in dilute acid or in dilute alkali. Four polyolphosphate compounds occur in the extracts. The main components are α - and β -glycerophosphate, the two other polyolphosphates (giving water-insoluble barium salts; separable chromatographically) occurring in smaller amount. The yields of glycerophosphate show that at least 75 % of the 'excess phosphate' is present in an easily hydrolysed glycerophospho-compound which may also be the origin of the unidentified polyolphosphates. The glycerophospho-compound of *M. pyogenes* accounts for more than one-quarter of the total organic phosphate of the organism. The presence of 'excess phosphate' in a number of Gram-positive organisms suggests that a glycerophospho-compound similar to that of *M. pyogenes* may be of general occurrence in Gram-positive bacteria and in yeasts.

In the course of checking a spectrophotometric method of estimating nucleic acid in intact cell suspensions (Mitchell, 1950*a*) against an adaptation of the method of Schmidt & Thannhauser (1945) developed by Stephenson & Moyle (1949), it was found that although the two methods were in substantial agreement when applied to *Escherichia coli* (strain H), they did not agree when applied to *Micrococcus pyogenes* var. *aureus* (strain Duncan). Comparison of the light absorption at a wave-length of 260 m μ . with the organic phosphate content of the ribonucleic acid (RNA) fraction of the Schmidt & Thannhauser method indicated that in *M. pyogenes* there was an excess of organic phosphate amounting to more than 30 % of that which could be accounted for by a polynucleotide RNA structure (Mitchell, 1950*a*). The occurrence of 'excess phosphate' in the RNA fractions of a number of micro-organisms showed a strong correlation with the capacity for retaining Gram's stain (Mitchell & Moyle, 1950), and it was observed that the amount of 'excess phosphate' in *M. pyogenes* was decreased during growth in the presence of penicillin (Mitchell, 1950*b*; Mitchell & Moyle, 1951).

It was desirable to begin a study of the origin of the 'excess phosphate' of the RNA fraction of Gram-positive bacteria not only to facilitate the elimination of errors in the estimation of RNA, but also to account for the occurrence of a large fraction of the total organic phosphate of these organisms. The present paper describes work on the separation and characterization of fragments of a phospho-compound containing the major part of the 'excess phosphate' of the RNA fraction of *M. pyogenes* var. *aureus* (strain Duncan), cultivated as described by Mitchell (1950*a*).

Definition and measurement of the 'excess phosphate'

In the Schmidt & Thannhauser (1945) method of estimating nucleic acids the cells are treated with cold 5 % trichloroacetic acid to remove the inorganic phosphate and diffusible organic phosphate compounds. The lipid is then extracted with organic solvents and the residue is treated with *N* alkali at 37° for 15 hr. to depolymerize the RNA. The deoxyribonucleic acid (DNA), which is resistant to the alkali treatment, is reprecipitated with 5 % trichloroacetic acid, leaving the organic phosphate of RNA and inorganic phosphate of phosphoprotein in solution. This solution, referred to as the RNA fraction, contains the 'excess phosphate' in which we are interested.

It was convenient to express the quantity of 'excess phosphate' (XSP) as the number of $\mu\text{mol.}$ organic phosphate in excess of the number of $\mu\text{mol.}$ purine-pyrimidine in the RNA fraction from 1 g. of organism dried *in vacuo* over P_2O_5 at room temperature. The organic phosphate was measured by the difference between determinations of total and inorganic phosphate by micro-adaptations of the methods of Fiske & Subbarow (1925) giving values with a standard deviation of ± 1 % for samples of 3–30 $\mu\text{g. P}$ and ± 5 % for samples as small as 0.5 $\mu\text{g. P}$. The measurement of purine-pyrimidine on a comparable micro scale could be carried out only by absorption spectrophotometry. The extinction of the appropriately diluted solutions was determined at 260 $\text{m}\mu$. with the Beckman spectrophotometer using quartz cuvettes giving a light path of 1 cm. Owing to lack of information about the proportions of the purine and pyrimidine bases present in the RNA of *M. pyogenes*, there was doubt about the value of the mean molecular extinction coefficient to be used to relate the extinction at 260 $\text{m}\mu$. to the mean molecular concentration of purine and pyrimidine base in the nucleotide fragments of the RNA fraction. The published values for the molecular extinction coefficient of the hypothetical nucleotide unit (identical with the mean molecular purine-pyrimidine extinction coefficient) of nucleic acids range from 6500 (Malmgren & Hedén, 1947) to 9000 (Wyatt, 1950). It was therefore desirable to determine the mean molecular extinction coefficient for the purine-pyrimidine as present in the RNA of *M. pyogenes*.

The whole ribonucleoprotein of *M. pyogenes* was isolated by the sonic disintegration and ammonium sulphate precipitation method of Sevag & Smolens (1941), and the organic phosphate content and extinction coefficient at 260 $\text{m}\mu$. were measured in neutral aqueous solution. Assuming the molecular concentrations of phosphate and base to be equal, the molecular extinction coefficient was 8200. After depolymerizing the ribonucleo-protein with *N*-NaOH at 35° for 15 hr., neutralization with HCl and precipitation of the protein with 5 % trichloroacetic acid, the molecular extinction coefficient was found to be 10,000, in agreement with the value of 10,000 found by Wyatt (1950) for a pure yeast RNA preparation after a similar treatment. The mean molecular concentration of purine-pyrimidine in the RNA fraction was therefore estimated using the coefficient 10,000. The standard deviation of the purine-pyrimidine measurements was usually $c. \pm 3$ %, the main cause of

variation being the occasional occurrence of slight turbidity. We did not consider it advisable to correct for what has been cryptically described by certain workers as 'non-specific absorption'.

As a subsidiary datum, pentose determinations were carried out as by Mejbaum (1939), the period of heating with the orcinol reagent being extended from 15 to 60 min. in order to obtain a more stable colour, the colour being determined photometrically. Pure crystalline ribose was used as a standard, and it was assumed that 65 % of the pentose of the RNA of *M. pyogenes* would be estimated (Schneider, 1945). We confirmed the finding of Valyi-Nagy (1947) that the presence of phosphate causes a depression of the estimated pentose values. In addition to this relatively small effect, however, unknown substance present in *Corynebacterium*, *Neisseria*, certain *Lactobacillus* and *Escherichia* spp., and baker's yeast, changed the colour given by the orcinol reagent and diminished the extinction coefficient by as much as 50 %. It therefore seemed probable that in samples of unknown composition, such as those from the so-called nucleic acid fractions of bacteria, the estimation of pentose would be liable to unaccountable errors. Nevertheless, estimations of pentose were considered to be of use in confirming those of purine-pyrimidine, particularly when significance could be attached to relative changes in the pentose values.

Separation of 'excess phosphate' from the nucleotide components

The 'excess phosphate' might be present in the RNA fraction either as part of the RNA fragments or as a separate compound or group of compounds. Several attempts were made to separate phospho-compounds other than nucleotides from the mono- and poly-nucleotides of the RNA fraction, using ethanol fractionation of the barium and silver salts at various pH values; the separation being followed by the ratio of the extinction at 260 m μ . to the organic phosphate content of the fractions. These attempts, however, gave no indication of the existence of a phospho-compound not containing purine-pyrimidine; nor were we able to obtain paper chromatographic separation of the components of the barium salt fractions.

An alternative procedure to the isolation of components of the whole RNA fraction was the fractional extraction of these components from the cells; for it would be expected that if the 'excess phosphate' were firmly bound to the RNA, the rates of extraction of organic phosphate, purine-pyrimidine and pentose by a given reagent would be the same, whereas if the rates were different the presence of some other phospho-compound might be indicated. The most convenient method for fractional extraction was by hydrolysis in dilute acid or alkaline solution.

Fractional extraction by alkali. In order to minimize the complicating effects of autolytic activity, it was advisable to carry out the extraction at a low temperature and at the highest pH giving a rate of extraction convenient for attempting to observe fractionation. Preliminary experiments showed that 0.1 N-NaOH at 0° gave a suitable rate of extraction of organic phosphate.

In order to stop the hydrolysis when each sample of cell suspension in

0.1N-NaOH was withdrawn, it was desirable to neutralize with acid. It was found, however, that neutralization with HCl gave erratic results which suggested reprecipitation of the extracted RNA. The effect of bringing the samples to different pH values was therefore examined. A washed suspension of *M. pyogenes* was extracted with 5 % trichloroacetic acid to remove diffusible purine-pyrimidine and phosphate compounds, and washed on the centrifuge with distilled water. The cells were then resuspended at a dry-weight concentration of 10 mg./ml. in 0.1N-NaOH at 0° for 30 min. Samples of the suspension were neutralized with an equal volume of various acids and centrifuged, and the pH (glass electrode) and organic phosphate content of the centrifugate were measured. The results given in Table 1 showed that at pH values below 6 organic phosphate was removed from the solution. It was observed, however, that this removal was not a simple precipitation of nucleic acid, because, when the cells were centrifuged down from the 0.1N-NaOH before the pH was lowered, precipitation of organic phosphate did not occur.

Table 1. *Effect of pH value on re-absorption of RNA fragments extracted in 30 min. from a suspension of Micrococcus pyogenes in 0.1N-NaOH at 0°*

Solution added to an equal volume of suspension	Final pH value	Amount of organic P left in solution (μ mol./g. dry wt. cells)
Water	12.5	86
0.1M boric acid	9.73	86
0.1M acetic acid	9.24	84
0.2M boric acid	8.86	84
0.033M citric acid	5.90	84
0.05M citric acid	4.85	59
0.2M acetic acid	4.70	41
0.1N HCl containing 10 % (w/v) trichloroacetic acid	3.0	16

Evidently the nucleic acid fragments were adsorbed by components of the cells. This interesting but troublesome effect was eliminated by using an equal volume of 0.1N acetic acid to neutralize the 0.1N-NaOH.

The time course of the extraction of purine-pyrimidine, pentose and organic phosphate of the RNA fraction of *M. pyogenes* could now be determined. A washed cell suspension (dry weight at harvesting 4 mg./ml.) was extracted with 5 % trichloroacetic acid at 0° for 30 min. at a dry-weight concentration of 10 mg./ml., centrifuged, and the cell residue was washed once with distilled water. The cell residue was resuspended at the original dry-weight concentration in 0.1N-NaOH at 0°. Samples, taken at intervals, were neutralized with an equal volume of 0.1N acetic acid and the cells were centrifuged down from the extracts. Estimations of deoxypentose by the Dische (1930) reaction showed that DNA was not extracted in 0.1N-NaOH at 0°. The components of the RNA fraction occurring in the extracts were therefore examined directly by determinations of organic phosphate, purine-pyrimidine and pentose. The components of the RNA fraction remaining in the cell residue were similarly examined in the solution obtained by treating the residue with N-NaOH at 35° for 15 hr., neutralizing with N-HCl and reprecipitating the DNA by the

addition of 0.2 vol. of 80 % trichloroacetic acid. The progress of the extraction is described in Fig. 1 in terms of $\mu\text{mol.}$ of pentose, purine-pyrimidine and 'excess phosphate' (XSP) per g. of dry organism found in cell residue and extract. Although the pentose values were consistently low for the cell residues, it is evident that the purine-pyrimidine and pentose were extracted about ten times as quickly as the 'excess phosphate', the half-extraction times being *c.* 5 and 50 hr. respectively.

Fractional extraction by acid. Preliminary experiments showed that washed suspensions of *M. pyogenes* were considerably more resistant to acid than to alkaline hydrolysis. Extraction of organic phosphate proceeded slowly in

Fig. 1. The time course of the extraction of organic phosphate, purine-pyrimidine and pentose from *M. pyogenes* suspended at a concentration of 10 mg. dry wt./ml. in 0.1 N-NaOH at 0°, observed by determinations on both cells and extract. Expressed as $\mu\text{mol.}$ of purine-pyrimidine or RNA nucleotide units (○—○); $\mu\text{mol.}$ 'excess phosphate' (XSP = total organic P-RNA) (●—●); and $\mu\text{mol.}$ pentose (+ + +) per g. of dry organism.

N-HCl at 0°, and as there was little likelihood of autolytic activity in such an acid solution, the rate of extraction was brought to a convenient level by raising the temperature to 35°. The time course of the extraction of purine-pyrimidine and organic phosphate was determined as follows. Washed *M. pyogenes* (dry weight at harvesting 1 mg./ml.) was suspended in N-HCl at 35° at a dry-weight concentration of 10 mg./ml. Samples were taken at intervals, and the cell residue was centrifuged rapidly from the extract without neutralizing the HCl. The estimations on the cell residues and extracts were carried out as before, pentose estimations being omitted. The initial values for the organic phosphate and purine-pyrimidine content of the extract represented the 'acid soluble' fraction which is usually extracted in 5 % trichloroacetic acid; these values were therefore subtracted from the values subsequently

obtained, to give the progress curves. Fig. 2 shows that the half-extraction times were about 10 hr. for the 'excess phosphate' and 2 hr. for the purine-pyrimidine. The total organic phosphate and purine-pyrimidine increased with time because the DNA is extracted by the N -HCl in *c.* 3 hr. This does not, however, affect the relative rates of extraction of phosphate and purine-pyrimidine, for the ratio of phosphate to purine-pyrimidine in the relatively small DNA fraction is near unity. The total 'excess phosphate' of the cells extracted in alkali (Fig. 1) was greater than that of the cells extracted in acid (Fig. 2), because in the latter case the culture of *M. pyogenes* used was harvested at an earlier phase of growth (Mitchell, 1950b).

Fig. 2. The time course of the extraction of organic phosphate and purine-pyrimidine from *M. pyogenes* suspended at a concentration of 10 mg. dry wt./ml. in N -HCl at 35°, observed by determinations on both cells and extract. Expressed as μ mol. of purine-pyrimidine (○—○) and μ mol. of 'excess phosphate' (●—●) per g. of dry organism.

Isolation and characterization of the carriers of the 'excess phosphate'

It was likely that the separation of the carriers of the 'excess phosphate' in *M. pyogenes* would be facilitated by a preliminary extraction of most of the nucleic acid with dilute acid or alkali.

Isolation of barium salt. A crude solution of the 'excess phosphate' carriers was obtained by extracting 10 g. dry *M. pyogenes* with 1 l. 0.1N-NaOH at 0° for 24 hr. to remove RNA, solution of the cell residue in 100 ml. N -NaOH at 35°, neutralizing with 10 ml. 10N-HCl and precipitation of DNA and other contaminants by adding 22 ml. of 30 % trichloroacetic acid followed by 530 ml. ethanol. The solution yielded a water-soluble ethanol-precipitable barium salt containing 6 % P and 5 % N. The nitrogen was present almost exclusively in surface-active, biuret-reacting, non-dialysable substances which could be removed either by foam fractionation or, more easily, by adsorption on activated charcoal. After treatment with 20 g. activated charcoal, the barium salt was repeatedly reprecipitated from water with 2 vol. ethanol. The white

barium salt thus obtained, free from sulphur, nitrogen and halogen, and containing 9 % P and 40 % Ba, weighed 230 mg., and represented 670 μ mol. of PO_4 out of a total 2500 μ mol. of XSP.

General properties of the 'excess phosphate' carriers. The barium salt was soluble in water to the extent of *c.* 5 %. The free acid, prepared by the quantitative precipitation of the barium with sulphuric acid, showed the following properties. It gave no orcinol reaction for pentose (Mejbaum, 1939) or Dische (1930) reaction for deoxy sugars. A faint Molisch reaction suggested a carbohydrate phosphoric ester, but there was no reducing activity towards either alkaline copper reagents, ferricyanide or dinitrosalicylic acid before or after acid or enzymic hydrolysis. There was no 'rapid furfural', Seliwanoff or Foulger (1932) reaction for sugars or Scherer (1850) reaction for inositol. There was no light absorption between wave-lengths of 2 μ . and 240 $m\mu$. There was a positive acrolein reaction for glycerol.

Table 2. *Titration data for polyolphosphoric acid*

[M]	pH	[Na ⁺]	pK'
3.65	2.48	0.00	1.49
3.62	2.62	0.99	1.45
3.59	2.85	1.98	1.62
3.55	3.40	2.90	—
3.52	5.05	3.85	—
3.48	5.94	4.75	6.17
3.45	6.51	5.65	6.27
3.42	7.16	6.55	6.13
3.38	9.13	7.40	—

[M] = total concentration of polyolphosphoric acid + sodium salt in mg. atoms P per litre.

[Na⁺] = concentration of sodium ion added to the polyolphosphoric acid as NaOH, in mg. ions per litre.

$$\text{pH} = -\log_{10}[\text{H}^+]$$

$$\text{pK}' = \text{pH} + \log_{10}([\text{M}] + [\text{OH}'] - [\text{Na}^+] - [\text{H}^+]) - \log_{10}([\text{Na}^+] + [\text{H}^+] - [\text{OH}']).$$

The ionization constants were determined by the method of Kumler (1938). A solution of 1.26 mg. of the acid in 1 ml. distilled water was titrated in a polystyrene electrode vessel with CO_2 -free 0.1 N-NaOH using 0.01 ml. additions. The pH values were determined with a bulb type glass electrode giving a linear response over the range from pH 1 to 9. The results in Table 2 show two pK' values, *c.* 1.5 and 6.2, each corresponding to the dissociation of one hydrogen ion per phosphorus atom. In view of the small amount of material titrated, the pK' values might be in error by as much as ± 0.2 pH unit. According to Kumler & Eiler (1943), glycerophosphoric acid gives pK' values of 1.4 and 6.4.

Hydrolysis at 100° for 2 hr. in N-HCl or N-NaOH liberated less than 10 % of the phosphate as inorganic phosphate, thus indicating polyhydric alcohol phosphates (Kaplan & Greenberg, 1944). Treatment of a solution at pH 9.5 in 0.02M bicarbonate buffer containing 0.01 M-MgCl₂ with a highly purified preparation of the alkaline phosphomonoesterase of milk (kindly supplied by R. K. Morton) at 35° liberated inorganic phosphate at a rate *c.* 10 % lower than from a control sample of pure β -glycerophosphate, the reaction going to completion.

It was concluded that the unknown phosphate-containing material was

a polyhydric alcohol monophosphoric ester (or a mixture of such esters) contaminated by a small amount of carbohydrate. Henceforward this material is referred to as the polyolphosphate fraction.

Chromatographic analysis of the polyolphosphate fraction. Samples of the polyolphosphate fraction, freed from barium by the theoretical amount of sulphuric acid, were run on paper chromatograms following in detail the methods of Hanes & Isherwood (1949), with the results shown in Table 3. The main polyolphosphate corresponded to glycerophosphate, but there was a subsidiary component, representing *c.* 10 % of the total phosphate, running with an R_F value of 0.11 in propanol/ammonia.

Table 3. *Chromatography of the polyolphosphates*

Substance	R_F value
(Solvent: 80 ml. tertiary butanol/20 ml. water + 2 g. picric acid)	
Polyolphosphate fraction	0.69–0.65
α -Glycerophosphate	0.655
β -Glycerophosphate	0.69
Glyceric acid 3-phosphate	0.655
Glyceric acid 2-phosphate	0.68
(Solvent: 60 ml. <i>n</i> -propanol/10 ml. water + 30 ml. 20 <i>N</i> ammonia)	
Polyolphosphate fraction	0.305 (strong) 0.110 (weak)
α -Glycerophosphate	0.31
β -Glycerophosphate	0.30
Glyceric acid 3-phosphate	0.21
Glyceric acid 2-phosphate	0.29
Fructofuranose 1:6-diphosphate	0.093
Orthophosphate	0.14

Chromatograms were run at 25° on Whatman no. 1 paper washed with dilute acetic acid. For comparison of the above R_F values with those for other phosphate esters see Hanes & Isherwood (1949).

Chromatographic analysis of the polyols. Dephosphorylation of 25 mg. of the polyolphosphoric acid was carried out with the milk phosphatase under the same conditions as before. The inorganic phosphate was precipitated with excess barium hydroxide, and barium and magnesium removed by passing the solution through a small acid-washed Zeocarb column. The solution was then dried *in vacuo* over P_2O_5 and sodium hydroxide pellets, the latter being required to take up HCl and CO_2 . A yield of 12 mg. of a viscous liquid, miscible with water and with ethanol, was obtained. This material was run on paper chromatograms in *n*-butanol/water, using development with ammoniacal silver nitrate according to the method of Hough (1950). Table 4 shows that the main component behaved like glycerol, but there was a subsidiary component, approximately corresponding in amount to the subsidiary polyolphosphate, running with an R_F value of 0.13, the same as xylose. When developed with ammoniacal silver nitrate, the reducing sugars were found to give rise to rapidly appearing brown spots, whereas the polyhydric alcohols developed more slowly, giving black spots. The subsidiary polyol behaved like a polyhydric alcohol during development.

A water-insoluble barium polyolphosphate fraction. During the purification of the polyolphosphate fraction by repeated precipitation of the barium salt

from aqueous solution with 2 vol. ethanol, a water-insoluble barium salt fraction was eliminated. This fraction consisted mainly of barium phosphate but some organic phosphate was also present. When examined on propanol/ammonia chromatograms, the organic component of the water-insoluble barium salt fraction was found to correspond to the subsidiary polyolphosphate of the water-soluble barium polyolphosphate fraction ($R_F=0.11$). After hydrolysis with the milk phosphatase it gave rise to a polyol chromatographically identical with the subsidiary polyol derived from the water-soluble polyolphosphate fraction ($R_F=0.13$ in *n*-butanol/water) and a smaller quantity of a substance running like glycerol. Like the water-soluble barium salt fraction, the water-insoluble barium polyolphosphate fraction showed no reducing activity even towards dinitrosalicylic acid before or after acid or enzymic hydrolysis.

Table 4. *Chromatography of the polyols*

Solvent: <i>n</i> -butanol/water	
Substance	R_F value
Polyol fraction	0.350 (strong) Bl. 0.130 (weak) Bl.
Propylene glycol	0.64 Bl.
Ethylene glycol	0.57 Bl.
Glycerol	0.35 Bl.
Diglycerol	0.25 Bl.
<i>meso</i> -Erythritol	0.22 Bl.
D-Ribose	0.17 Br.
D-Xylose	0.13 Br.
Mannitol	0.085 Bl.
D-Glucose	0.073 Br.
D-Mannose	0.021 Br.
<i>meso</i> -Inositol	0.007 Bl.

Chromatograms run at 25° on Whatman no. 1 paper washed with dilute acetic acid. Bl. = black spot developing slowly. Br. = brown spot developing rapidly.

Identification of the water-soluble barium polyolphosphate. The water-soluble barium salt fraction was freed from 'water-insoluble' barium polyolphosphate and contaminating carbohydrate by treatment of a 5 % aqueous solution with activated charcoal followed by talc, precipitating the barium salt with 2 vol. ethanol, twice reforming the 5 % aqueous solution (discarding insoluble material) and precipitating the barium salt with 2 vol. ethanol. After drying to constant weight *in vacuo* at 100° over P_2O_5 , two different preparations, obtained in a yield of 120 mg. of barium salt from the equivalent to 10 g. of *M. pyogenes* gave on analysis: 9.5 % P; 43.2 % Ba, and 10.1 % P; 44.0 % Ba, corresponding satisfactorily to barium glycerophosphate, $C_3H_7O_6P\text{Ba} + \frac{1}{2}H_2O$: 9.79 % P; 43.42 % Ba. The identity of the water-soluble barium polyolphosphate as barium glycerophosphate was considered to be proved.

Further chromatographic analysis of the water-insoluble barium polyolphosphates. Samples of the water-insoluble barium polyolphosphate fraction which were judged to be free from glycerophosphate on the basis of chromatograms in propanol/ammonia were found to give as much as 20 % of glycerol

after dephosphorylation with the milk phosphatase. This fraction was therefore suspected of being complex. A separation into two components with R_F values of 0.62 and 0.73 was obtained in the *tert*.-butanol/picric acid solvent of Hanes & Isherwood (1949). Using a more acidic solvent consisting of equal volumes of *n*-butanol and *N*-HCl, a better separation into components with R_F values of 0.22 and 0.41 was achieved. The R_F value of β -glycerophosphate in *n*-butanol/*N*-HCl was 0.39, and it therefore seemed possible that the spot with $R_F=0.41$ might represent glycerophosphate formed from the unknown polyol phosphate by the *N*-HCl. Two-dimensional chromatograms, run first in *n*-butanol/*N*-HCl and then in propanol/ammonia, showed that both the components appearing on the butanol/HCl chromatograms gave an R_F value of 0.11 when subsequently run in propanol/ammonia. Neither component could therefore have corresponded to glycerophosphate, which has an R_F value of 0.30–0.31 in propanol/ammonia. Further characterization of the water-insoluble polyolphosphate fraction was postponed while larger amounts of material were collected. The work now in progress on this material will be published in due course.

Conclusions. It was evident that *M. pyogenes* must contain a phospho-compound, not extracted by organic solvents, from which glycerophosphate is released on hydrolysis with alkali. The components of the water-insoluble barium polyolphosphate fraction must also have been similarly released either from the same or from some other phospho-compound. In view of the low yields and of the obvious selectivity of our fractionation procedures, it was not known whether the major proportion of the 'excess phosphate' might be accounted for in this way. Moreover, it was uncertain to what extent the treatment with alkali might determine the nature of the phosphate-containing fragments. It was therefore desirable to carry out the fractionation in a more quantitative manner, using as starting material both the crude extract obtained by alkali treatment of *M. pyogenes* and that obtained by an analogous acid extraction.

Quantitative fractionation of alkali- and acid-extracted 'excess phosphate'

Growth and organic phosphate distribution of M. pyogenes. A 4 l. culture of *M. pyogenes* was grown at 25° to a dry-weight concentration of 3 μ g./ml. in a 10 l. rotated flask (Mitchell, 1949) in our casein digest medium containing 1 % (w/v) glucose and 0.1 % Marmite. After centrifuging, the cells were washed twice in distilled water, cooled with solid CO₂ in acetone and dried in the frozen state over P₂O₅ in a vacuum desiccator. Estimation of the distribution of the organic phosphate gave the results shown in Table 5, expressed as μ mol. phosphate per g. dry-weight organism. For comparison with Table 5, the yields obtained in the extraction and fractionation procedures described below are expressed as μ mol. RNA nucleotide units (estimated as purine-pyrimidine) and μ mol. XSP (=total organic P–RNA) per g. dry-weight organism. In preparation for the alkali and acid extraction, lipid was exhaustively extracted from two 5 g. samples of dried organism by repeatedly heating under reflux with 95 % aqueous methanol for 1 hr., evaporating to

dryness and extracting in a Soxhlet apparatus with sodium-dried ether (Reichert, 1944).

Alkali extraction. A 5 g. sample of dried organism, freed from lipid, was dispersed in 100 ml. 0.1 N-NaOH and held at 0° for 19 hr., neutralized with 100 ml. 0.1 N acetic acid, centrifuged, and the centrifugate discarded. The cell residue was washed once with 0.1 N sodium acetate and dissolved in 50 ml. N-NaOH by incubation at 35° for 15 hr. To this solution, 5 ml. 10 N-HCl and 9 ml. 30 % (w/v) trichloroacetic acid were added, and after 30 min. at room temperature, the precipitate was centrifuged down and discarded. The centrifuged liquid was adjusted to pH 8 with 4 N-NaOH and made up to 150 ml. with distilled water. Yield: RNA = 191, XSP = 246, the units being $\mu\text{mol./g.}$ as defined above.

Table 5. *Distribution of organic phosphate in Micrococcus pyogenes*

Component	Phosphorus distribution	
	$\mu\text{mol. P/g.}$ cell dry wt.	% of total P
Free nucleotides and other components extracted in cold 5 % trichloroacetic acid	119	17
Phospholipid	38	6
'Phosphoprotein'	< 1	
DNA	77	11
RNA (as defined in text)	197	29
XSP (as defined in text)	250	37
Total	681	100

Acid extraction. 5 g. sample of dried organism, freed from lipid, was dispersed in 250 ml. N-HCl and held at 35° for 4 hr., centrifuged, and the centrifugate discarded. The cell residue was washed once with N-HCl and resuspended in 90 ml. N-HCl at 35° for 20 hr. The residue was centrifuged down and discarded, and to the centrifuged liquid 22 ml. 4 N-NaOH and 20 ml. 30 % (w/v) trichloroacetic acid were added. The precipitate was removed by centrifuging and the supernatant liquid adjusted to pH 8 with 4 N-NaOH and made up to 150 ml. with distilled water. Yield: RNA = 25, XSP = 198.

Fractionation. The same fractionation procedure was used for both alkali and acid extracts. To the extract in a 500 ml. flask, 300 ml. ethanol was added. After 2 days, the fine precipitate formed by the ethanol had become attached to the glass and the clear supernatant was quantitatively decanted. To this supernatant, 10 ml. M barium acetate was added; the precipitate of barium salt allowed to aggregate overnight, and was then collected on the centrifuge, washed with 60 % (v/v) aqueous ethanol and dried *in vacuo* over P_2O_5 . The barium salt was extracted by grinding 10 times with 5 ml. volumes of distilled water, adjusting to pH 6 with HCl during extraction. Yields thus obtained from the barium salt were: from alkali extract, RNA = 147, XSP = 214; from acid extract, RNA = 25, XSP = 151. The barium salt solution was adjusted to pH 8 in preparation for passage through an acid-washed charcoal column prepared as follows. A suspension of 10 g. of 'B.D.H. animal charcoal' (British Drug Houses Ltd.) in water was packed onto two thicknesses of Whatman

no. 1 filter-paper previously covered with a thin layer of talc in a 10 cm. Buchner funnel, and successively washed under gentle suction with 250 ml. 0.1 N-HCl, 100 ml. 5 % KCl, 10 ml. 0.1 M-Ba(OH)₂ and 100 ml. 5 % KCl. The solution of the barium salt was drawn slowly through the charcoal column, followed by five lots of 50 ml. of 5 % KCl. The solution was readjusted to pH 8 with barium hydroxide, and the barium salt precipitated by the addition of 4 vol. ethanol and dried *in vacuo* over P₂O₅. Yields: from alkali extract, 145 mg. of barium salt per g. of organism, containing RNA < 1, XSP = 214; from acid extract, 94 mg. of barium salt per g. of organism, containing RNA < 1, XSP = 116.

The barium salt was extracted with 5 ml. of 0.1 N-HCl/100 mg. and the residue was centrifuged down and discarded. The solution was partially separated into water-insoluble and water-soluble barium salt fractions by three successive precipitations of the acidified barium salt with barium hydroxide and precipitation of the soluble barium salt from the supernatant with 4 vol. ethanol. Yields: (as μ mol. organic phosphate per g. dry organism) from alkali extract, soluble fraction = 187, insoluble fraction = 17; from acid extract, soluble fraction = 88, insoluble fraction = 31.

In order to obviate the losses inherent in obtaining a complete separation of the soluble and insoluble barium salts, the relative amounts were estimated by determining chromatographically the degree of mutual contamination of the partially separated fractions. The fractions were run on duplicate paper chromatograms in propanol/ammonia, using 0.01 ml. samples of 1 % solutions of the barium salt fractions converted to the free acids by treatment with the theoretical amounts of sulphuric acid. One chromatogram was developed to show the positions of the spots so that the appropriate areas (1 $\times$ 1.5 cm.) could be cut out of the duplicate chromatogram, eluted in 5 ml. water, and the organic phosphate determined in the usual way. The weight of phosphorus required in each spot fell in a convenient range between 0.5 and 10 μ g. The chromatograms showed that only glycerophosphate and the unidentified polyolphosphates with R_F = 0.11 were present. The ratio of the weight of phosphate in the glycerophosphate (GP) spot to that in the spot given by the water-insoluble barium polyolphosphates (IP) was as follows: from alkali extract, water-soluble fraction GP/IP > 100, water-insoluble fraction GP/IP = 0.09; from acid extract, water-soluble fraction GP/IP = 9.3, water-insoluble fraction GP/IP < 0.01. Of the 250 μ mol. total XSP, the yields of GP and IP were thus: from alkali extract GP = 188, IP = 16, giving a total recovery of 81 %; and from acid extract GP = 80, IP = 39, giving a total recovery of 48 %.

Chromatograms of the water-insoluble barium polyolphosphate fraction in *n*-butanol/*N*-HCl showed that the two components (R_F values 0.22 and 0.41) were present in this fraction both after alkali and after acid extraction.

Determination of α - and β -glycerophosphate. The glycerophosphate fraction from the acid extract was submitted to further fractionation to remove the remaining water-insoluble barium polyolphosphate. This material and the glycerophosphate fraction from the alkali extract (already free from other polyolphosphates) were assayed for α - and β -glycerophosphate following in

detail the periodate oxidation method of Burmaster (1946). The glycerophosphate from the alkali extract assayed as 60 % β - and 40 % α -, while that from the acid extract assayed as 43 % β - and 57 % α -glycerophosphate.

Collection of data. The quantities and yields of the polyolphosphates representing the 'excess phosphate' are set out in Table 6.

Table 6. *The yields of the carriers of the 'excess phosphate' (XSP)*

Component	$\mu\text{mol. P/g.}$ cell dry wt.	Recovery of total XSP (%)
From alkali extract		
α -GP	113	45
β -GP	75	30
Total GP	188	75
IP	16	6
Total polyolphosphate	204	81
From acid extract		
α -GP	46	18
β -GP	34	14
Total GP	80	32
IP	39	16
Total polyolphosphate	119	48

GP = glycerophosphate; IP = water-insoluble polyolphosphates.

DISCUSSION

Our experimental observations lead to the conclusion that there is present in *M. pyogenes* an easily hydrolysed glycerophospho-compound of which the phosphate accounts for at least 75 % of the 'excess phosphate' of the RNA fraction and for at least 27 % of the total organic phosphate of the organism harvested towards the end of growth. It is difficult to believe that so large a phosphate fraction should have remained unobserved, but we have been unable to find any previous reference to its existence.

It was not possible to determine the proportions of α - and β -glycerophosphate in the glycerophospho-compound because of the migration of the phosphate between the α and β positions during extraction. The proportions of α - and β -glycerophosphate found after alkali extraction (60 % β and 40 % α) may be compared with the observation of Bailly & Gaumé (1934) that the methyl ester of either α - or β -glycerophosphate gives rise to 2/3 β - and 1/3 α -glycerophosphate on alkaline hydrolysis. In acid, on the other hand, Bailly (1938) found that β -glycerophosphate is converted almost quantitatively to the α -glycerophosphate form. The glycerophosphate isolated from the acid extract (43 % β and 57 % α) might have contained β -glycerophosphate produced during hydrolysis of esters of α -glycerophosphate, or α -glycerophosphate produced from free β -glycerophosphate or both.

The yield of glycerophosphate from the acid extraction was about half that obtained from the alkali extraction. Most of the loss of glycerophosphate from the acid extraction is accounted for by the loss of XSP which occurs during the preliminary acid extraction of RNA. In the alkali extraction, on the other hand, the preliminary extraction of alkali-soluble components

resulted in practically no loss of XSP and very little extraction of RNA, the latter being removed on the activated charcoal column at a later stage. This unintentional improvement in the extraction procedure was caused by raising the concentration of the cell suspension in the 0.1N-NaOH from 10 to 50 mg./ml.

The yield of the two unidentified polyolphosphates was about three times as great from the acid extraction as from the alkali extraction. This difference is susceptible of two possible explanations. It is possible that, as with the relative formation of α - and β -glycerophosphate, the hydrolytic conditions of the extraction procedure determine the amount of the unidentified polyolphosphates formed relative to that of glycerophosphate from a common precursor. On the other hand, the difference between the yields might be due entirely to the selectivity of the extraction procedures. If the latter be the case, the total amount of the 'excess phosphate' which has been accounted for is the sum of the GP obtained from the alkali extraction and the IP obtained from the acid extraction, namely, 227 μ mol./g. or 91 % of the total XSP.

The presence of 'excess phosphate' in a number of Gram-positive organisms (Mitchell, 1950*b*; Mitchell & Moyle, 1950) suggests that a glycerophospho-compound similar to that of *M. pyogenes* may be of general occurrence in Gram-positive bacteria and in yeasts, a possibility now under examination in our laboratory.

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