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The Glycerophospho-protein Complex Envelope of *Micrococcus pyogenes*

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SUMMARY: The glycerophospho-compound which has been shown to account for more than one-quarter of the total organic phosphate of *Micrococcus pyogenes* is present almost exclusively in the particulate fractions of mechanically disintegrated cell suspensions.

The main particulate fraction corresponds to the cell envelope material of Dawson (1949). Three-quarters of the weight of this material is made up of a glycerophospho-protein complex of which the protein moiety resembles silk fibroin in amino-acid composition and in its general properties. The amount of hydroxylamino-acid in the protein of the envelope would be sufficient to bind the polyolphosphoric acids covalently, but the possibility that the polyolphosphoric acids form a polymer which is adsorbed on to a protein matrix is not excluded.

A subsidiary particulate fraction, made up of very small particles containing a high proportion of phospholipid, was found to be otherwise similar in composition to the envelope fraction. It is suggested that in the intact cell, the material of the small particle fraction may form a continuous layer (lipid membrane?) lying beneath the glycerophospho-protein complex envelope (cell wall?).

It has been inferred from observations on the release of glycerophosphate from *M. pyogenes* by hydrolysis in dilute acid or alkali, that a large amount of a readily hydrolysed glycerophospho-compound is present (Mitchell & Moyle, 1951*b*). It was desirable to determine the nature of the complex in which the glycerophosphate is bound, and hence to isolate the intact glycerophospho-compound. The present paper describes the isolation and partial characterization of two particulate fractions of *M. pyogenes* var. *aureus* (strain Duncan) which contain the major part of the glycerophosphate. Some implications of this work have been published (Mitchell & Moyle, 1950).

EXPERIMENTAL

In the course of preparing the ribonucleoprotein of *Micrococcus pyogenes* by disintegrating a cell suspension in distilled water and precipitating the nucleoprotein according to the method of Sevag & Smolens (1941) with 60-66 % saturated ammonium sulphate, it was noted that most of the phosphate in excess of that which could be accounted for as nucleotide units of ribonucleic acid (RNA) (XSP = total organic P-RNA, expressed in $\mu\text{mol/g. cell dry weight}$) was contained in the water-insoluble debris centrifuged down in 1 hr. at 3000 g, while a smaller portion appeared in the centrifugate fraction precipitated by 60 % saturated ammonium sulphate. Table 1 shows the relevant data and the methods of estimation, the meaning of the symbols being as previously described (Mitchell & Moyle, 1951*b*). It thus appeared likely that

the glycerophospho-compound of *M. pyogenes* would be found in the particulate fractions of mechanically disintegrated cell suspensions. Accordingly, we proceeded to prepare and examine the appropriate fractions of *M. pyogenes* grown under the same conditions as before.

Table 1. *The 'excess phosphate' (XSP) content of water insoluble and ammonium sulphate precipitable fractions of mechanically disintegrated Micrococcus pyogenes*

Fraction	Phosphate content as $\mu\text{mol./g. cell dry wt.}$		
	Total organic P	RNA	XSP
Insoluble	230	—	230
0-60 %*	61.5	46	15.5
60-66 %	62.5	62.5	0
66-80 %	72.0	70.5	1.5

* Figures show percentage saturation with ammonium sulphate required to precipitate the fraction.

Preparation of fractions

Disintegration of cells. The cells were disintegrated by the method of Mickel (1948). The two cylindrical containers (22 mm. internal diameter, 50 mm. internal height, mounted vertically) were each filled with 4 g. of Ballotini glass-balls no. 12, (Chance Bros. Ltd., Birmingham), 10 ml. of distilled water and 250 mg. of freeze-dried organism and vibrated at 50 cyc./sec. for 1 hr. at room temperature. After this treatment, stained smears (Gram) showed less than 1 % intact cells. The froth and underlying opalescent suspension were transferred quantitatively from the containers to a centrifuge tube, together with ten washings of the Ballotini balls with 1 ml. sample of distilled water.

Separation of fractions. The froth was broken by centrifuging for 5 min. at 1000 g and the Ballotini held by the froth were released. The centrifugate was transferred quantitatively to a celluloid centrifuge tube, leaving a small pad of partially disintegrated cells with the Ballotini. The suspension was now centrifuged at 3000 g for 1 hr., and material was deposited in two distinct layers, the lower layer being white and opaque and the upper one yellow and transparent. After decanting most of the opalescent centrifugate, it was possible by gently agitating the tube to redisperse the yellow material in the remaining supernatant liquid without disturbing the underlying layer. The supernatant was removed with a bulb pipette and the surface of the underlying layer was washed with a little distilled water, the latter being added to the residue of partially disintegrated cells. The supernatant and original centrifugate were combined and centrifuged at 15,000 g for 1 hr. to deposit the yellow material from which an almost clear centrifugate could be decanted. Both the white and yellow deposits were submitted to a further similar differential centrifugation after redispersing in small volumes of distilled water. The appropriate parts were combined to give four fractions:

(i) Envelope fraction (see below), corresponding to the opaque white material centrifuging down rapidly at 3000 g.

(ii) Small particle fraction, corresponding to the yellow material centrifuging down at 15,000 g.

(iii) Soluble fraction, corresponding to the centrifugate separated from fraction (ii).

(iv) Residue fraction, consisting of partially broken cells, washings of the surface of the pad of fraction (i) and residues corresponding to fractions (ii) and (i) returned during the second differential centrifuging of fractions (i) and (ii) respectively.

The fractions were dried over P_2O_5 *in vacuo* at room temperature. The weights of the fractions obtained from 1 g. of similarly dried *M. pyogenes* are shown in Table 2.

Table 2. *The weights of the fractions of mechanically disintegrated Micrococcus pyogenes*

Fraction	Weight (mg.)
(i) Envelope	166
(ii) Small particle	84
(iii) Soluble	626
(iv) Residue	102
Total	978

The fractions were obtained from 1000 mg. of dried organism.

Morphology of the fractions

With the assistance of Mr Horne of the Cavendish laboratory, the fractions were examined in the Siemens electron microscope after shadowcasting with gold and palladium, and photographs were taken of representative specimens. Fraction (i) was found to consist of cell envelopes, closely resembling those first observed by Dawson (1949), practically uncontaminated by any other particulate material. Fraction (ii) showed mainly spherical particles 10–50 m μ . diameter. There was a relatively small proportion of flat fragments resembling parts of cell envelopes, but no intact cells or intact cell envelopes. Fraction (iv) contained mostly partially emptied cells, but also some of the material of fractions (i) and (ii).

On examining the fractions with the light microscope, it was found that the cell envelopes could be made visible by staining with amphipathic dyes such as Victoria blue, but that the material of the envelope fraction showed generally a very low affinity either for hydrophilic acidic and basic dyes or for the hydrophobic lipid-soluble ones. While the individual particles of the small particle fraction could not be discerned, the fixed material was found to stain intensely with lipid soluble dyes such as Sudan black.

Distribution of organic phosphate in whole cells and cell fractions

In the procedure adopted for determining the distribution of organic phosphate, most of the manipulations were carried out in a single test-tube to eliminate the losses inherent in transferring the small amounts of material which were available for analysis. Quickfit test-tubes 8 cm. in overall length

with B14 sockets were found to be convenient. The material (20 mg. for the whole organism and fractions (i), (ii) and (iv), and 80 mg. for fraction (iii)) was weighed into a stoppered B14 tube. One ml. of 95 % methanol was added, and the material was heated under reflux for 1 hr. to break down the lipid-protein complexes (Reichert, 1944). The methanol was evaporated, and after drying the material *in vacuo* over P_2O_5 for 15 min., it was extracted for 5 hr. with sodium-dried ether in an apparatus designed to obviate the transfer of the material from the Quickfit tube (Mitchell, 1951). The process of heating with methanol and extraction with ether was repeated until no more lipid could be extracted, not more than three extractions usually being required. After evaporating the combined ether extracts to dryness, the lipid was taken up in a small volume of ether, transferred to a weighing tube and dried *in vacuo* over P_2O_5 . It was weighed and then redissolved in ether and samples were taken for estimation of phosphate (lipid P). The cell residue in the Quickfit tube was dried and reweighed. It was then dispersed in 2.5 ml. distilled water and 0.5 ml. 30 % (w/v) trichloroacetic acid was added. The residue was centrifuged down and washed twice with 2 ml. distilled water, and the washings were combined with the trichloroacetic acid extract and made up to 10 ml. for estimation of the 'acid-soluble P' on suitable samples. The cell residue was dried and then dissolved by incubation with 2 ml. *N*-NaOH at 35° for 15 hr. The desoxyribonucleic acid (DNA) was precipitated by adding 0.2 ml. 10 *N*-HCl and 0.44 ml. 30 % (w/v) trichloroacetic acid. After centrifuging down the DNA, the ribonucleic acid (RNA) and 'excess phosphate' (XSP) were determined by estimations of organic phosphate and of the extinction at 260 $m\mu$. of the centrifugate, as previously described (Mitchell & Moyle, 1951*b*). The DNA was measured by the organic phosphate content of the residue after washing once with distilled water and dissolving in 1 ml. *N*-NaOH. The distribution of the phosphate in the cell fractions is compared with that of the intact cell in Table 3. The envelope and small particle fractions contain a total of 68 % of the XSP of the whole cell and only traces of nucleic acids, while the soluble fraction contains most of the nucleic acids. A further 18 % of the XSP is present in the residue fraction, leaving only 14 % in the soluble fraction. The lipid phosphate of the envelope fraction is very small, but the small particle fraction contains 30 % of the total lipid phosphate. In general, the values obtained for the sum of the fractions agree well with those for the whole cell—indicating that changes due to autolysis during the mechanical disintegration of the cells cannot be serious. The discrepancy is greatest in the case of the RNA values, where the sum of the values for the fractions falls 17 % below that for the intact organism. This drop, which has been repeatedly observed, is due to a decrease in the apparent purine-pyrimidine extinction coefficient, for it is not accompanied by a loss of organic phosphate. For this reason, the XSP values show a corresponding rise in the soluble fraction. Allowing for this error, the XSP value of the soluble fraction would be 35 μ mol./g. cell dry weight.

In the evaluation of the RNA content of whole cells from the extinction of the RNA fraction at 260 $m\mu$. it has been our practice not to correct for the

relatively insignificant light absorption by substances other than purines and pyrimidines. For the very small RNA values found in the envelope and small particle fractions, however, the absorption of light by substances other than

Table 3. *Distribution of phosphate in cell fractions and whole cells of Micrococcus pyogenes*

Phosphate fraction	Fractions				Sum of (i) to (iv)	Whole cell
	(i)	(ii)	(iii)	(iv)		
Acid soluble inorganic	0	2.5	157	1.6	161	166
Acid soluble organic	0	1.9	117	1.2	120	110
Lipid	0.12	11.3	20.5	3.7	35.6	38
RNA	3.1	5.2	137 (170)	19.0	164 (197)	197
XSP	126	43	61 (35)	46	276 (250)	250
DNA	3.0	1.2	59	6.8	70	73

The values given in the body of the Table are the $\mu\text{mol.}$ of phosphate present in the appropriate fraction/g. of dry organism. Values in brackets are corrected for change of apparent extinction coefficient of the RNA fraction at 260 $m\mu.$ after disintegration of the cells.

Fig. 1. Absorption spectrum of the so-called RNA of the envelope fraction in acid solution at a concentration corresponding to 1 mg. cell dry weight/ml.

purines and pyrimidines should not be neglected. This was demonstrated by the absorption spectrum of the so-called RNA of the envelope fraction, shown in Fig. 1, which does not correspond to the spectrum of a ribonucleic acid. Hence the already low RNA values for the envelope fraction and probably also for the small particle fraction are erroneously high.

Estimation of polyolphosphates of fractions (i) and (ii)

The solution containing the XSP of fractions (i) and (ii) was adjusted to pH 9 after centrifuging down the DNA and taking the small samples required for the estimations described above. Ethanol (2 vol.) was added and the fine precipitate was allowed to adhere to the glass during 2 days. The clear supernatant was decanted and the barium salt of the polyolphosphates was precipitated on adding 10 equivalents of M-BaCl_2 . The barium salt was centrifuged down, washed once with a little 60 % ethanol and dried. The polyolphosphoric acids were liberated from the barium salt by grinding with 0.1 ml. 0.1N- H_2SO_4 /mg., and after removing the barium sulphate by centrifuging, 10 μl . samples of the solution were taken for estimation of total organic phosphate and for chromatographic determination of the ratio of glycerophosphate (GP) to the unidentified polyolphosphates (IP) as previously described (Mitchell & Moyle,

Table 4. *The recovery of GP and IP from the XSP of fractions (i) and (ii)*

Component	$\mu\text{mol./g.}$ dry wt. cells	Percentage recovery of XSP of fraction	Percentage of whole cell XSP
Envelope fraction (i)			
XSP	126	—	51
GP	92	73	36
IP	18	15	8
GP + IP	110	88	44
Small particle fraction (ii)			
XSP	43	—	17
GP	29	68	12
IP	10	23	4
GP + IP	39	91	16

The XSP values are computed by the difference total organic P-RNA. The GP and IP values are determined by estimations of phosphate after isolation of the mixed barium salt and chromatographic separation of the phosphate esters.

1951*b*). The chromatograms (run in 60 ml. *n*-propanol/30 ml. 20N ammonia + 10 ml. water) showed spots corresponding only to GP ($R_F=0.30$) and IP ($R_F=0.12$). The yields and proportions of the polyolphosphates are described in Table 4. The recovery of both GP and IP is higher than was obtained from the whole cell, as would be expected. The interpretation of the proportions of the polyolphosphates obtained in the alkali hydrolysate will be dealt with in a later communication.

Purity of the cell fractions

The fractions of the disintegrated cells have been separated by taking advantage of differences in particle size and density, but the aim has been to obtain a separation of anatomical units of the cell, and it is in this sense that the purity of the fractions must be judged. The electron micrographs showed that the differences in particle size and density were accompanied by distinct morphological characteristics; but only in the case of fraction (i) (the envelope fraction) could the material be recognized as part of an anatomical unit of the

cell. The distribution of organic phosphate compounds in the fractions shows that while there are profound differences between the compositions of the envelope and soluble fractions, the small particle fraction differs significantly from the envelope fraction only in containing more phospholipid. We therefore consider it possible that the small particle fraction may have been artificially produced during disintegration of the cells, and that in the intact cell the material of this fraction constitutes part of the cell envelope. The small amounts of nucleic acids found in the envelope and small particle fractions give an indication of the possible degree of contamination of these fractions with the material of the soluble fraction, while the corrected XSP content of the soluble fraction indicates the possible degree of loss of the particulate fractions by solubilization (autolysis?) during the process of cell disintegration.

In order to shed light on the possible modes of attachment of the glycerophosphate, we now proceeded to examine the lipid, carbohydrate and nitrogen-containing components of the particulate fractions.

Composition of the cell fractions

Estimation of nitrogen. Kjeldahl nitrogen was determined by a colorimetric iodomercurate method similar to that of Umbreit, Burris & Stauffer (1949), giving values with a standard deviation of $\pm 3\%$ for samples of 1–5 μg . Estimations of Van Slyke α -amino nitrogen were kindly made by Dr Haselbach on hydrolysates prepared by heating the material with 5N-HCl at 100° for 24 hr. in sealed tubes.

Estimation of carbohydrate. Total carbohydrate was estimated by the quantitative adaptation of Dreywood's anthrone reaction method developed by Seifter, Dayton, Novic & Muntwyler (1950). For identification of the sugars, the material was hydrolysed by heating at 100° with methanolic-HCl in sealed tubes for 18 hr., evaporation to dryness *in vacuo* over NaOH pellets and heating at 100° for 18 hr. in aqueous N-HCl to hydrolyse the methyl glycosides. The hydrolysates were run on chromatograms in pyridine (1 vol.), water (2 vol.), ethyl acetate (2 vol.) at 25° on Whatman no. 1 paper, the upper layer being used as the moving phase. The chromatograms were developed with 1% aniline phthalate in butanol, the presence of glucosamine being confirmed in duplicate chromatograms by spraying with 0.2% ninhydrin in water-saturated butanol. The amount of glucosamine present was estimated very approximately by comparing the intensity of the aniline phthalate and ninhydrin spots with standards of known concentration.

Estimation of lipid. The total loosely bound lipid was estimated gravimetrically after extraction with solvents as described above. Lipid not extracted by this procedure was obtained by carrying out a similar extraction after hydrolysing the material with 5N-HCl at 100° for 24 hr. The lipid thus obtained was freed from traces of glycerol by redissolving in a small volume of ether before being dried over P_2O_5 and weighed.

Results. The estimations of protein, lipid and carbohydrate components of the fractions are shown in Table 5. The protein weight was calculated from the

non-lipid Kjeldahl nitrogen weight using a multiplying factor of 6.2, and the weight of polyolphosphoric acid was taken as five times the weight of XSP phosphorus.

It is apparent that the main components of the envelope and small particle fractions are the polyolphosphoric acids, protein and lipid. Part of the lipid is readily separated from the other two components by extraction with organic solvents, but it has not proved to be possible to separate the polyolphosphoric acids from the protein except by methods which hydrolyse the latter. The following solutions were found to exert no solvent action either on the protein or on the polyolphosphoric acids of the envelope fraction in 1 hr.: water at 25° or at 100°; hot or cold methanol, ethanol, *n*-butanol or ether; 5 or 90 % (w/v) aqueous phenol at 25°; 50 % (w/v) aqueous guanidine hydrochloride, pure *N*-dimethylformamide or 70 % (w/v) aqueous *N*-dimethylformamide at 25°; 5 % (w/v) trichloroacetic acid, *N* acetic acid or 10 *N* aqueous ammonia at 25°; 0.1 *N*-NaOH or 0.1 *N*-HCl at 0°; water/0.1 % (w/v) oxine in *n*-amyl alcohol

Table 5. *Composition of envelope and small particle fractions of Micrococcus pyogenes*

Substance estimated	Weight of substance in mg./100 mg. of fraction	
	Envelope fraction	Small particle fraction
Total Kjeldahl N	10.6	9.5
Lipid Kjeldahl N	0.70	3.0
Non-lipid Kjeldahl N	9.9	6.5
Van Slyke α -amino N	10.1	6.9
Protein	62	41
Polyolphosphoric acid	12	8
Lipid (direct extraction)	3.2	22.5
Lipid (from hydrolysate)	4.4	—
Glucose	0.5	1.7
Glucosamine	c. 1	c. 3
RNA	0.6	2.4
DNA	0.6	0.5
Total recovery	84	79

at 25°. On the other hand, when the envelope fraction was slowly hydrolysed by suspension in *N*-HCl at 35°, organic phosphate and protein were found to go into solution at a constant rate, about 40 % of the protein and 80 % of the organic phosphate being solubilized in 5 hr. Solubilization of protein and organic phosphate took place at about the same rate on hydrolysis with *N*-NaOH at 0° or 10 *N* ammonia at 100° as with *N*-HCl at 35°.

A more specific hydrolysis of components of the glycerophospho-protein complex might be obtained by enzymic degradation, but so far the material has proved to be resistant. Treatment of the envelope fraction for 3 hr. at 35° with crystalline pepsin at pH 1 or 2, crude trypsin at pH 7.5, crystalline egg-white lysozyme in 0.5 % NaCl at pH 7, intestinal alkaline phosphomono-esterase at pH 9.7 in the presence of 0.001 *M*-MgCl₂, or crystalline pancreatic ribonuclease at pH 7.5 caused no solubilization of phosphate or protein. A

preliminary treatment of the envelope fraction in water at 100° for 30 min. made no difference to the action of the above enzymes.

The possibility was considered that the polyolphosphoric acids might be present as a polymer adsorbed on to the protein of the envelope fraction. On the other hand, the evidence available did not exclude the possibility that whether present as a polymer or not the polyolphosphoric acids might be linked covalently to the protein, either directly or through one or more unknown compounds accounting for the 16 % of the weight of the envelope fraction still to be identified.

It was clear at this stage that a knowledge of the approximate amino-acid composition of the envelope fraction would materially assist in our considerations of the possible mode of attachment of the polyolphosphoric acids.

Amino-acid composition of the protein of the envelope fraction

The lipid was extracted from the envelope fraction with organic solvents as before, and 10 mg. samples were heated for 24 hr. with 5N-HCl at 100° in sealed tubes. The hydrolysate was dried *in vacuo* over NaOH pellets and P₂O₅, and redissolved in water to give a concentration of 1 %. The amino-acid composition was determined by running 5, 10 and 20 μ l. samples of the

Table 6. *Approximate amino-acid composition of the acid hydrolysate of the envelope fraction of Micrococcus pyogenes*

Amino-acid	No. of residues	μ mol./g. cell dry wt.
Glycine	20	350
Alanine	17	290
Glutamic acid	11	190
Lysine	7	120
Serine	4	70
Threonine	4	70
Leucine + isoleucine	3	50
Aspartic acid	2	35
Valine	1	15
Histidine	<1	—
Arginine	<0.5	—

Other amino-acids present in smaller amount than arginine, not determined.

hydrolysate on one-dimensional paper chromatograms and measuring the ninhydrin-reacting material eluted from the appropriate areas of the paper by the method of Boissonas (1950). The moving phase used for the chromatograms was phenol saturated with water, and in order to obtain adequate separation of the amino-acids on Whatman no. 1 paper at 25°, the equilibrating phase was varied from equal weights of phenol and water + a trace of KCN to equal parts of phenol and water + 0.1, 0.5 and 3.0 % (v/v) of 20N ammonia. A high degree of accuracy has been claimed for Boissonas's method, but owing to unaccountable variations which occasionally occurred in our results we considered that significance should not be given to determinations of molecular amounts of amino-acid less than one-twentieth of the amount of glycine present in our hydrolysates. The relative numbers of amino-acid residues are therefore given in Table 6 with glycine written as 20. It is evident that the amino-acid

composition of the whole protein of the envelope resembles that of silk fibroin more closely than that of any other protein for which analyses are available (Tristram, 1949). It differs from silk fibroin mainly in containing relatively much less tyrosine, but more threonine. There is, of course, no evidence that the protein of the envelope fraction can legitimately be regarded as homogeneous, but at all events, a composition resembling that of silk fibroin is the least unexpected of the highly resistant and insoluble material of the protein envelope.

The second column of Table 6 shows the amounts of the amino-acids of the envelope fraction expressed as $\mu\text{mol./g.}$ cell dry weight for comparison with the figures of Table 3. Since the total amount of serine and threonine in the envelope fraction is estimated as 140 $\mu\text{mol.}$ and that of polyolphosphoric acid phosphorus as 126 $\mu\text{mol./g.}$, it would be possible for the polyolphosphoric acids to be linked to the protein through the hydroxylamino-acids. This possibility is now under investigation.

DISCUSSION

Because of the preponderance of protein in the envelope of *M. pyogenes* isolated after mechanical disintegration of the cells, we propose for the present to refer to it as the protein envelope to distinguish it from other layers of less mechanically resistant material which may lie internally or externally to it in the intact cell. A more detailed knowledge of its chemical structure will probably suggest a more specific name for the protein envelope by which it may be distinguished from other envelopes also of a protein nature which may be discovered. It is evident that the polyolphosphoric acids are firmly bound to the protein envelope, either as a polymerized complex (possibly forming a continuous layer) or dispersed in the protein matrix. The phosphate radicals of the polyolphosphoric acids may be covalently linked to the protein, but until this linkage has been proved it is advisable to refer to the compound as a phospho-protein complex rather than as a phospho-protein.

When *M. pyogenes* is treated with 5 % trichloroacetic acid or with phenol or other detergents or organic solvents the osmotic barrier (Mitchell, 1949) is broken and diffusible substances of molecular size not usually exceeding that of the nucleotides escape or can be washed from the cells, although under these conditions the protein envelope is not removed. It seems likely, therefore, that the protein envelope should not be considered as an impervious sheet of peptide chains, but as a pervious network of chains or fibres with an effective maximum pore diameter of *c.* 1 $\mu\text{m.}$ In the intact cell, this protein envelope presumably acts as the support for the osmotic barrier, part of which may be built into the protein matrix. Cytological evidence indicates the presence of a lipid layer, readily stained by hydrophobic, lipid-soluble dyes such as Sudan black (Burdon, 1946), usually thought to represent the osmotic barrier. This lipid layer lies inside the 'cell wall' which is characterized by a very low affinity for dyes (Knaysi, 1951). In view of the similarity of composition of the non-lipid moiety of the small particle fraction obtained from disintegrated *M. pyogenes*, we suggest that the material of the small particle fraction may be

associated with the protein envelope in the intact cell and that the lipid of the small particle fraction may form the layer which is stained by Sudan black. Thus, the protein envelope would correspond to the 'cell wall' and the small particle fraction would contribute the underlying lipid membrane.

Taking into account the weight and composition of the residue fraction, the protein envelope can be estimated to account for *c.* 20 % of the cell dry weight and the small particle fraction for *c.* 10 %. The cells are about 0.7 μ . in diameter at the time of harvesting (Mitchell & Moyle, 1951*a*) and consequently, assuming the superficial layers to be hydrated to the same extent as the rest of the cell, the envelope fraction would form a surface layer *c.* 25 m μ . thick, while in combination with the small particle fraction it would form a layer *c.* 40 m μ . thick—well below the limit of resolution, but not, of course, below the limit of visibility of the light microscope.

The concept of the protein envelope of *M. pyogenes* as a structural component might be taken as an indication of metabolic inertness, particularly in view of the resistance of the material to enzymic degradation; but it does not follow that because a structure performs a mechanical function that it may not also be involved in a metabolic one, particularly in a system which might be concerned with the transport of metabolites such as amino-acids across an osmotic barrier. It is with this consideration in mind that we are now examining the nature of the glycerophospho-protein complex which, from the envelope fraction alone, accounts for more than 20 % of the total organic phosphate of the cell.

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