PARK, J. T. & HANCOCK, R. (1960). *J. gen. Microbiol.* 22, 249-258

A Fractionation Procedure for Studies of the Synthesis of Cell-Wall Mucopeptide and of Other Polymers in Cells of *Staphylococcus aureus*

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SUMMARY: A method is described for simultaneous estimation of pool constituents, protein, nucleic acids, cell-wall mucopeptide, and possibly teichoic acid in a single sample of *S. aureus*. The method makes possible isolation of the cell-wall mucopeptide contaminated by less than 5 % of the cytoplasmic protein. It is suitable for studies of biosynthesis, with radioactive precursors, and requires approximately 3 mg. cell/sample.

The role of the cell wall in maintaining the integrity of bacterial cells is now becoming well understood (Mitchell & Moyle, 1956), and studies of the synthesis of cell walls are assuming importance particularly because the reactions involved appear to be favourable targets for attack by chemotherapeutic agents (Park, 1958*a*; Park & Strominger, 1957). The present knowledge of the composition of bacterial cell walls, though limited, suggests that nearly all such walls contain a mucopolysaccharide-peptide component (hereafter called mucopeptide) which characteristically contains: *N*-acetylglucosamine, *N*-acetylmuramic acid, *D*-glutamic acid, *D*- and *L*-alanine and *L*-lysine or an isomer of diaminopimelic acid (Cummins, 1956; Work, 1957). The cell walls of many Gram-positive species contain, in addition to the mucopeptide, a polyol phosphate polymer which may represent as much as 60 % of the dry weight of the wall (Mitchell & Moyle, 1951, 1954; Armstrong, Baddiley, Buchanan & Carss, 1958*a*; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958*b*). Though in certain organisms the polyol present may be glycerol, the recent work of Armstrong *et al.* (1958*a, b*) suggests that the usual polymer of this type consists of ribitol phosphate with a sugar linked to the ribitol and *D*-alanine (Dr J. Baddiley, personal communication) bound to the sugar by an ester linkage. This type of polymer found in Gram-positive bacteria has been termed teichoic acid (Armstrong *et al.* 1958*a*). In contrast, the walls of many Gram-negative bacteria contain lipoprotein rather than teichoic acid as an additional layer, while possessing a basic structure composed of mucopeptide (Weidel & Primosigh, 1958). Since it appears that the shape and, in fact, the survival of most bacteria is dependent on the integrity of the mucopeptide component, we sought a method suitable for studying the metabolism of the

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cell in relation to cell-wall mucopeptide synthesis, and which would enable separation and estimation of mucopeptide simultaneously with other cell components.

The usual method for isolating bacterial cell walls (Dawson, 1949; Salton & Horne, 1951), although excellent for preparative purposes, is not well suited to studies of their biosynthesis, since cell walls of some organisms may undergo autolysis during the isolation procedure (Mitchell & Moyle, 1957), and the method does not lend itself readily to quantitative work involving a considerable number of small samples. This paper describes a method evolved for studies of the synthesis of the mucopeptide component of the cell wall of *Staphylococcus aureus* using ^{14}C -labelled amino acids; this method is suitable for fractionation of many small samples of cells, and has been briefly described previously (Hancock & Park, 1958). By analysis of the appropriate fractions, estimates of acid-soluble pool constituents, alcohol-soluble 'protein', nucleic acids, teichoic acid, protein and mucopeptide can be made on a single sample.

METHODS

Organisms, growth and harvesting. *Staphylococcus aureus* strain H and *S. aureus* strain Duncan were used, the strain used in any particular experiment being stated in the text. *S. aureus* Duncan was grown in medium containing per litre: 20 g. peptone (Difco Bacto); 20 g. glucose; 380 ml. inorganic salts solution (Gale, 1947). *S. aureus* H was grown in a medium containing, per litre: 5 g. peptone; 5 g. yeast extract; 3 g. K_2HPO_4 . The pH value of the media was adjusted to 7.2–7.4 before sterilization, and 2 g. glucose, sterilized separately, were added before use. Cultures were grown in flasks shaken at 37° in a constant-temperature water bath. The extent of growth was followed by determining the optical density of the culture at $700\text{ m}\mu$ in the Beckman DU spectrophotometer, and the organisms were harvested when the culture was in the exponential phase of growth at a bacterial density equiv. to c. 0.3 mg. dry weight/ml. They were centrifuged at 2000–3000 g for 15 min. at room temperature, and washed once by a similar centrifugation from distilled water. For the preparation of ^{32}P labelled cell walls, organisms were grown in medium in which tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer replaced the phosphate normally present; ^{32}P orthophosphate was added to the medium to give a final concentration of $1\text{ }\mu\text{mole/ml.}$ and specific activity of $0.1\text{ }\mu\text{C}/\mu\text{mole.}$

Incorporation of ^{14}C labelled amino acids. *Staphylococcus aureus* Duncan was harvested and washed, and resuspended in a chemically defined medium (Hancock, 1958) to give the same culture density as that at which the organisms had been harvested. The appropriate ^{14}C amino acid was added to give a final specific activity of c. $0.1\text{ }\mu\text{C}/\mu\text{mole,}$ and the culture was shaken at 37° for the specified time.

Analytical methods. Amino acid analysis were performed on the fractions after hydrolysis with 6N-HCl at 105° in a sealed tube for 16 hr. Before analysing such hydrolysates by paper chromatography, acid was removed

under an air stream at 45° and the residue was suspended 2 or 3 times in small volumes of water and redried. Amino acids were estimated by the method of Cocking & Yemm (1955), except that *m*-acetate buffer (pH 4.7) replaced citrate buffer; results are expressed relative to a glutamic acid standard.

Hexosamines were separated by the procedure of Boas (1953) after hydrolysis in 6*N*-HCl for 5 hr. at 105°; the resulting fractions were analysed by the method of Schloss (1951).

Paper chromatography. Amino acids were separated by two dimensional chromatography on squares of Whatman no. 4 paper (25 cm. × 25 cm.), with the solvents recommended by Roberts, Abelson, Cowie, Bolton & Britten (1955). The papers were sprayed with ninhydrin (0.2 %, (w/v) in acetone) and developed in an air oven at 105° for 2–3 min.; the resulting colour was preserved by spraying with 1 % (w/v) aqueous CuSO₄ solution.

For quantitative amino acid analyses, similar papers were sprayed with the ninhydrin reagent of Connell, Dixon & Hanes (1955) to reveal the positions of the amino acids; they were then sprayed with methanolic borate solution (Connell *et al.* 1955) and air-dried to remove ammonia. Each area of the paper which contained a single amino acid was cut out and divided into small pieces which were immersed in the ninhydrin reagent for quantitative estimation.

Isotope techniques. Randomly ¹⁴C-labelled amino acids, and ³²P orthophosphate, were obtained from the Radiochemical Centre, Amersham, England.

Radioactivity was determined on samples placed on aluminium planchettes (2 cm. diameter) which contained disks of lens tissue; 1 drop of 1 % (w/v) cetyltrimethylammonium bromide was added to each sample, and the samples were dried under a lamp. Samples were plated with less than 1 mg. material/sq.cm. on the planchette, so that self-absorption was less than 5 % (Coleman, 1958) and has been neglected. An end-window Geiger-Müller tube and scaler were used in the conventional manner; at least 1000 counts were recorded (standard error of counting 3.3 %). The abbreviation cpm has been used for counts/minute.

Preparation of cell walls by mechanical disintegration. The procedure of Cummins & Harris (1956) was used, except that digestion with ribonuclease was omitted. Before depositing the first crude cell-wall fraction, all manipulations were made at 5°; immediately after this point the preparation was heated in a boiling water bath for 10 min. to inactivate any autolytic systems present (Mitchell & Moyle, 1957). Cell walls containing ³²P were washed twice in 0.2 *M*-phosphate buffer (pH 6.5) to remove contaminating inorganic ³²P before treatment with trypsin.

Enzyme preparations. Crystalline trypsin and pepsin were obtained from the Nutritional Biochemicals Corp.

The lysopeptidase preparation was a gift from Dr M. R. J. Salton and consisted of pure F1 and crude F2 fractions, as used by Salton & Ghuysen (1957). Potassium phosphate buffer (0.01 *M*, pH 7.0), and a temperature of 37° were used for digestions with this preparation.

Ribonuclease (RNAase) was obtained from the Sigma Chemical Corp. and

from L. P. Light and Co.; 'Streptodernase' (Lederle Laboratories) was used as a crude deoxyribonuclease (DNAase) preparation. Both enzymes were used at 500 μ g./ml. in 0.2M-phosphate buffer (pH 6.5) at 37°.

RESULTS

The method devised is similar to the procedure of Roberts *et al.* (1955) with the important addition that protein and cell-wall mucopeptide in the residual fraction are separated. The method as outlined in Table 1 yields 5 fractions which have been characterized as follows.

Table 1. *Scheme for fractionation of Staphylococcus aureus*

Fraction	Treatment*	Contents of fraction
1. Cold TCA	Suspend 2.5–3.0 mg. (dry weight) of washed organism in 2 ml. cold water, add 0.5 ml. cold 25 % (w/v) trichloroacetic acid (TCA); after 10 min. at 0° centrifuge at 4000 <i>g</i> for 5 min., and decant extract	All low-molecular weight compounds soluble in 5 % (w/v) TCA
2. Aqueous ethanol-soluble	Suspend residue in 2.5 ml. 75 % (v/v) ethanol in water; after 10 min. at room temperature centrifuge (4000 <i>g</i> , 10 min.) and decant extract	Ethanol-soluble 'protein' and lipid
3. Hot TCA	Suspend residue in 2.5 ml. 5 % (w/v) TCA, heat 6 min. at 90°, cool, centrifuge (4000 <i>g</i> , 10 min.) and decant extract	Breakdown products of nucleic acid and teichoic acid
4. Trypsin-solubilized	Suspend residue in 0.95 ml. 0.05M-NH ₄ HCO ₃ containing 0.005N-NH ₄ OH; add 0.05 ml. of solution containing 1 mg. crystalline trypsin/ml. Incubate 2 hr. at 37° or until digestion is complete. Centrifuge (4000 <i>g</i> , 10 min.) and decant extract	Trypsin-degraded proteins
5. Residue	Suspend residue in 1.0 ml. of water	Mucopeptide of wall

* The wall of the centrifuge tube is wiped dry after each decantation so as to decrease contamination of the subsequent extract to negligible amounts.

(1) Fraction 1 is the cold trichloroacetic acid (TCA) extract of the cells, which contains the pool amino acids (Hancock, 1958) and other acid-soluble components. A minimum of 2.5 mg. (dry weight) cells is used in order to assure well-packed pellets upon centrifugation. After decantation of each extract, residual liquid adhering to the walls of the centrifuge tube is removed by absorption on cotton or paper swabs. In this way, when 2.5 ml. of extractant is used, contamination of each extract by material from the previous one should be less than 1 %, so that washing of the residue between each extraction is usually unnecessary.

(2) Extraction of the TCA-insoluble residue with 75% (v/v) ethanol in water yields a small amount of lipid and 'protein'. In *Staphylococcus aureus* this 'protein' corresponds to about 0.5% of the dry weight of the cell, and contains glycine, lysine, alanine and glutamic acid in the approximate proportions 1:1:0.6:0.6; these 4 amino acids account for 60% of the total ninhydrin-reacting material in the fraction after hydrolysis. No other amino acid or amino sugar is present in comparable amount.

(3) Extraction with hot TCA removes nucleic acids from the cells (Park & Johnson, 1949). This extraction also removes virtually all the teichoic acid from the cell wall, as measured by release of radioactivity from ^{32}P -labelled walls. As shown in Fig. 1a, over 90% of the phosphate of isolated cell walls of *Staphylococcus aureus* Duncan was released by 5% TCA in 6 min. at 90°. No radioactivity was released from these walls on treatment with RNAase or DNAase. The results in Fig. 1b suggests that some ninhydrin-reacting material

Fig. 1. Release of constituents of cell walls (prepared by Mickle disintegration) of *Staphylococcus aureus* Duncan during treatment with 5% (w/v) TCA at 90° or 100°. At intervals samples of the suspension were cooled rapidly in ice, and centrifuged (8000 g, 15 min.); samples of the supernatant fluid were taken for estimations. A. Release of radioactivity from walls containing ^{32}P . B. Release of ninhydrin-reacting materials, estimated on samples of the supernatant fluid directly (+, O) and on further samples after acid hydrolysis (x, ●).

was released rapidly by hot TCA, and that a slower continued release occurred at a rate of about 5% of the total ninydrin-reacting material per 6 min. period at 90°. Thus it is possible that this fraction contains as much as 5% of the amino acids from the cell wall mucopeptide. Since Armstrong *et al.* (1958) showed that the teichoic acid from *S. aureus* H probably contains *O*-alanyl groups and *N*-acyl-glucosamine linked together to form a side chain to the ribitol phosphate polymer, measurement of the total alanine or glucosamine released with hot TCA may serve to measure the amount of teichoic acid present. Indeed as the data in Table 2 indicate, considerable amounts of glucosamine and alanine were liberated from the cells by hot TCA, while muramic acid and glutamic acid which are representative of the mucopeptide component,

remained with the cell residue. However, though the release of a portion of the glucosamine and alanine paralleled the release of phosphate, quantitative measurements of the release of these three components from isolated cell walls with hot TCA indicated that for every mole of phosphate present in teichoic acid there was only about one-third of a mole each of glucosamine and the associated ester-linked alanine. The amount of ester-linked alanine, measured as the hydroxamate, was approximately equivalent to the glucosamine released by hot TCA. The additional alanine released by hot TCA (Table 2) may have come from the mucopeptide since only about 2 moles of alanine remained in the wall residue per mole of glutamic acid, instead of the 3 moles that might have been expected (Park & Strominger, 1957). Thus, though ester-linked alanine or glucosamine released by hot TCA may be specific indicators of teichoic acid, the low amounts of these constituents relative to wall phosphate suggest that the glucosamine content per ribitol phosphate unit might be variable. The lysine, glutamic acid, glycine and muramic acid of the wall, which are not associated with the teichoic acid, were released relatively slowly in hot TCA.

Table 2. *Distribution of amino sugars and alanine in fractions from Staphylococcus aureus*

Fraction	Muramic acid; % of total	Glucosamine (μ mole/100 mg. dry wt. organism)	Alanine (μ mole/100 mg. dry wt. organism)	Glutamic acid (μ mole/100 mg. dry wt. organism)
Hot TCA extract	< 3	6.5	12	Trace
Second hot TCA extract	0.5	1.3	3.8	Trace
Wall residue	96	5.2	15	7

Table 3. *Amino acid composition of the trypsin-resistant residue (mucopeptide)*

Preparation 1 was from normal organisms, and preparation 2 from organisms grown for 1 hr. in the presence of chloramphenicol (50 μ g./ml.); 6.0 mg. dry wt. of organism was used in each case.

Amino acid	μ mole in wall preparation	
	(1)	(2)
Alanine	0.53	0.58
Glycine	1.07	1.90
Glutamic acid	0.28	0.36
Lysine	0.44	0.57
Total amino acids recovered	2.32	3.68
Total before separation	2.44	4.02

(4) Treatment of the hot TCA-insoluble fraction with trypsin (under the conditions described in Table 1) converted about 95 % of the cell protein to soluble peptides. This fraction has not been further characterized. The progress of digestion by trypsin was followed by measurements of the optical density of the preparation (Fig. 2).

(5) The residue consists predominantly of the mucopeptide of the wall structure, as the following evidence indicates.

(a) After hydrolysis and separation of the amino acids by paper chromatography, 90 % of the ninhydrin-reacting material in the fraction is accounted for by alanine, glycine, glutamic acid and lysine, the characteristic amino acids of *Staphylococcus aureus* cell walls (Table 3). All the hexosamine of the hot TCA-insoluble fraction of the cell is present in this fraction (Table 4).

Table 4. Incorporation of ^{14}C -amino acids into the trypsin-resistant and trypsin-soluble fractions of *Staphylococcus aureus* Duncan during 45 min. growth in chemically defined medium

^{14}C amino acid	Final specific activity cpm/ μmole	Radioactivity of material insoluble in hot TCA, cpm		
		Total	Trypsin-soluble	Trypsin-resistant
L-Leucine	2,900	5,185	4,917	234
L-Proline	6,650	4,280	4,200	14
L-Phenylalanine	9,560	7,170	7,050	58
Glycine	3,750	24,400	8,700	15,700
L-Lysine	7,180	31,400	21,050	10,350
L-Alanine	1,410	8,700	4,320	4,300
L-Glutamic acid	8,100	29,950	20,100	5,850
Increase in hexosamine (μmole)		1.3	0	1.3

(b) Less than 5 % of the total radioactivity incorporated from ^{14}C -labelled non-wall amino acids into the hot TCA-insoluble fraction of growing organisms appeared in this fraction (Table 4).

(c) On treatment with lysopeptidase, an enzyme system which degrades the cell wall of *Staphylococcus aureus* (Salton & Ghuyssen, 1957), the turbidity of the material in this fraction decreased by 90 % (Fig. 2). From preparations

Fig. 2. Digestion of the hot TCA-insoluble residue of *Staphylococcus aureus* Duncan by trypsin followed by lysopeptidase, as measured by the change in optical density ($\log I_0/I$, 1 cm.) at 700 $\text{m}\mu$. The fraction from c.1 mg. dry wt. organism was incubated in 1 ml. with 500 μg . trypsin; a further 500 μg . trypsin were added at 1. After centrifugation (3000 g, 15 min.) and washing (at 2), the residue was resuspended with lysopeptidase (50 μg ./ml.).

containing ^{14}C in all the wall amino acids, 96 % of the radioactivity became non-sedimentable (3000 g for 80 min.) and 81 % became dialysable (Table 5).

(d) Electron micrographs showed that the material in this fraction consisted of structures which closely resemble cell walls prepared by orthodox methods. However, many of the structures seen in the electron microscope appear to have a further internal layer; it seems likely that, in many cases, this is the part of the wall which is in contact with the electron microscope grid, and which has become distorted as the wall collapsed on to the grid without, apparently, tearing (Plate 1).

Table 5. *Effect of digestion with lysopeptidase (50 $\mu\text{g./ml.}$) for 15 hr. on some properties of the trypsin-resistant fraction*

	Control	After lysopeptidase
1. Total cpm/ml. (^{14}C -alanine, ^{14}C -glycine, ^{14}C -glutamic acid and ^{14}C -glycine)	1520	1520
2. Non-sedimentable at 3000 g for 15 min.; cpm	43	1460
3. Cpm after dialysis against distilled water (24 hr.)	1500	1124
4. Suspension optical density at 700 m μ	0.43	0.03

Nature of the 'non-cell wall' material in the residue. The removal of that fraction of the residue into which the small degree of incorporation of non-wall amino acids occurred (Table 4) was attempted, without any success, by a number of methods. These included extraction, before or after hot TCA or trypsin treatment, with: methanol and/or ethanol: ether (1:1) for 60 min. at 45° or 10 min. at boiling point; 75 % (w/v) or 100 % phenol for 2 hr. at 5° or for 80 min. at 100°; 50 % (v/v) dimethyl formamide; acetone; 10 % (v/v) or 50 % (v/v) butanol for 2 hr. at 5°; 2 % (w/v) sodium dodecylsulphate for 2 hr. at 37°; replacement of TCA by perchloric acid (1 N, 4°, 15 hr.) for nucleic acid extraction; further digestion with pepsin (500 $\mu\text{g./ml.}$) for 14 hr. at pH 2.

The nature of this minor component of the trypsin-resistant fraction into which non-wall amino acids are incorporated is unknown; since a structure resembling an underlying layer can be seen in electron micrographs, it is conceivable that in some cases this might be part of the cytoplasmic (protoplast) membrane, although some of the procedures used in attempting to remove it were based on those which have been used to separate lipoprotein from the mucopeptide components of the cell wall of *Escherichia coli* (Weidel & Primosigh, 1958).

DISCUSSION

This extension of previous methods (Roberts *et al.* 1955) for the chemical fractionation of bacteria separates the mucopeptide of the cell wall of *Staphylococcus aureus*, which ordinarily accounts for at least 10 % of the dry weight of organisms, from other cellular fractions and enables estimation of the two separate

polymers of the wall. The resistance of the basic structure of bacterial cell walls, isolated by mechanical disintegration, to digestion by trypsin has been adequately demonstrated (Weidel, 1951; McCarty, 1952; Salton, 1953; 1956; Cummins & Harris, 1956). Hence, though trypsin may remove protein which is in some cases associated with the wall (such as the M-protein of haemolytic streptococci; Lancefield, 1948), the mucopeptide structure is not attacked. Earlier work (Salton, 1953) indicated that much of the protein of heated suspensions of *S. aureus* is not in a form susceptible to enzymic digestion. However, after treatment with hot TCA, which removes nucleic acids and teichoic acid, the protein becomes susceptible to tryptic digestion, and successful separation of the hot TCA-insoluble fraction into protein and mucopeptide is possible.

The fractionation procedure described here has been found suitable for use with *Staphylococcus aureus*. We have not investigated its applicability to other organisms, but there is no apparent reason why it should not be suitable for other Gram-positive organisms, at least. It has been applied to studies of the effects of antibiotics on the synthesis of cell-wall mucopeptide (Hancock & Park, 1958; Park, 1958*b*) and should be useful in experiments where a comparison is desired of the rates of synthesis of the major classes of high molecular-weight components of the cell (protein, nucleic acids, mucopeptide, teichoic acid).

We wish to thank Dr E. F. Gale, Dr K. McQuillen and the members of the M.R.C. Unit for Chemical Microbiology (Cambridge) for their interest and hospitality during the tenure by J.T.P. of a Senior Post-doctoral Fellowship of the National Science Foundation and by R.H. of an M.R.C. Scholarship.

We are indebted to Mr R. W. Horne (Cavendish Laboratory, Cambridge) for the electron micrographs, and to Dr M. R. J. Salton for a gift of lysopeptidase.

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EXPLANATION OF PLATE

Electron micrographs of cell-wall preparations obtained by the method described, after washing exhaustively with distilled water. The preparations were air-dried and photographed in the Siemens Elmiskop 1 microscope; the magnifications were: (a) $\times 13,000$; (b) $\times 49,000$.

(Received 28 July 1959)

J. T. PARK & R. HANCOCK—FRACTIONATION OF *S. AUREUS* CELLS

(Facing p. 258)