The Isolation of Phosphorylhexosamine from the Rest Protein Fraction of *Escherichia coli* B

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In earlier communications [1,2] it has been shown that acid hydrolysates of the Schneider rest protein fractions from mammalian organs contain O-phosphorylserine and O-phosphorylthreonine. Phosphorylserine has also been isolated from the corresponding fraction of yeast [3]. In other microorganisms phosphorylated hydroxy-amino acids of different composition seem to dominate in the acid hydrolysates. From *L. casei* O-phosphorylmuramic acid has recently been isolated [4], and from *E. coli* in the same way O-phosphorylethanolamine [5].

It has now been possible to isolate chromatographically pure phosphorylhexosamine from the acid hydrolysate of the rest protein of *E. coli*. Previously published methods for cultivation and isolation have been followed[6]. The bacteria were harvested at the end of the lag phase. They had been cultivated for about 2 h on a medium containing 1 mC ^{32}P per liter to facilitate the identification of phosphorus compounds. After lyophilization the cells were disrupted in a Raytheon 50-watt 9 kc magnetostriction oscillator during continuous cooling. The cell walls were separated by fractionated centrifugation [6]. The elution curve of the acid hydrolysate of cell walls passing through a Dowex 50-column was given in a previous paper [5].

By paper ionophoretic analysis it was found that the material from peak No. III on the curve was a mixture of at least three ninhydrin-positive compounds which at pH 5 migrated slower than phosphorylserine or phosporylthreonine. Sufficient amounts of material for analysis of peak No. III was obtained by mixing radioactive bacteria with large amounts of unlabeled cells. For large scale cultivation the bacteria were incubated in a 1 000 liter tank on the same medium for about 20 h. The usual yield of fresh bacteria from a single cultivation was about 2.5 kg. Material from two cultivations was used in this study. The hydrolysates of the rest protein fractions were separated on 10 Dowex-50 columns. Column dimensions were 6.5 × 55 cm.

Preparative paper ionophoresis of all material from peak No. III was carried out for 4 h in a 0.1 N pyridine-acetate buffer of pH 5 using Whatman paper No. 3. Two large ninhydrin-positive spots were found close to each other and well separated from a weaker spot with a more rapid migration. The phosphorus contents were 2.7 %, 0.71 %, and 0.16 %, respectively. Material from the slowest moving spot with the highest phosphorus content was eluted from the paper with water and purified by chromatography first on a small Dowex-50 column as a single peak with 0.01 N HCl and then on a small (0.9 cm × 25 cm) Dowex-1 formate column where the material could be eluted with water. Two rather well separated peaks were observed. Only a few mg were obtained from the first fraction with a phosphorus content of 6.0 %. The second peak had a phosphorus content of 11.6 %. In all about 40 mg of material was obtained from 5 kg of fresh bacteria.

The identity of this material with phosphorylhexosamine was verified by paper ionophoresis at pH 5 and paper chromatography in two solvent systems[1] where the compound moved as a single green-coloured ninhydrin-positive spot parallel with glucosamine-6-phosphate. This was prepared by incubating a yeast hexokinase preparation with equimolar amounts of glucosamine and radioactive ATP followed by purification on Dowex-1 formate column. Elementary analysis of the compound from *E. coli* gave the following values (Found: C 27.5; H 5.8; N 5.2; P 11.6. Calc. for $C_6H_{14}O_8NP$ (259): C 27.8; H 5.5; N 5.4; P 11.9). The position of the phosphate group must probably be in the primary alcoholic group. Only a phosphate group attached here would seem to resist the acid hydrolysis used in the isolation procedure [7].

Phosphorylhexosamine may not be present only in the cell walls. Water soluble fractions of proteins were obtained after centrifugation of the vibrated bacteria at 105100 *g* for 30 min at 0°C and precipitation with ethanol or acetone [6]. The acid hydrolysates of these purified fractions also gave the same types of elution curves on Dowex-50 columns with peaks in the same column volumes as No. III from the hydrolyzed cell walls. The function of phosphorylhexosamine in bacterial phosphoproteins is not clear. It could connect some peptide chains by diester bonds as in casein or pepsin [8], or possibly represent the active site in a transphosphorylating mechanism. A preliminary note has been published that galactosaminephosphate should be present in the hydrolysate of bone tissue [9].

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Mass Spectrometric Studies on Amino Acid and Peptide Derivatives

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In the mass spectrum of a methyl ester of the type RCH_2COOCH_3 the most prominent peak is normally found at *m/e* 74 and is due to a rearranged fragment $^1C_3H_6O_2^+$. A prominent peak due to the alkyl fragment R^+ is only observed when carbon atom 3 is quaternary. The mass spectrum of the methyl ester of an *α*-amino acid $RCHNH_2COOCH_3$ shows, on the other hand, a very large peak due to the ionized fragment $RCHNH_2^+$. The mass number of this fragment is different for all amino-acids except the isomeric valines and leucines.

The parent peaks are small but in the cases so far examined allow a direct determination of the molecular weight of the esters. A further prominent peak occurs at *m/e* 88 corresponding to the fragment $-CHNH_2COOCH_3^+$.

Although several free amino-acids and simple peptides can be brought into the gas phase without decomposition [2] compounds with the zwitterion structure have been found less suitable for mass spectrometric analysis than compounds in which either the amino group or the carboxyl is protected. It has been found possible to analyze peptides with protected amino- and carboxyl groups. Thus N-trifluoroacetyl-L-alanyl-L-phenylalanine methyl ester [3] gives an excellent mass spectrum with a parent peak at *m/e* 346 (calculated molecular weight 346.3).

The high-mass, high-resolution mass spectrometer thus seems to offer interesting possibilities in the analysis of amino-acids and peptides.

It is intended to extend this work to other amino-acid derivatives and to study the possibility of analyzing mixtures. As carried out at present one analysis requires about 100 micrograms of material.

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Infrared-spectroscopic Studies on Bile Acids

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The poor solubility of the bile acids in the nonpolar solvents required for infrared spectroscopy prevents their investigation in solution. We have therefore examined the spectra of the solid substances pressed in potassium bromide pellets. In the case of the bile acid esters which can be examined both in solution and in the solid state, the solid state spectra are found to be more distinct, owing to the fixed positions of the molecules in the crystal.

The spectra are sufficiently specific to allow identification of each bile acid by infrared spectroscopy. They are especially sensitive to the number and position of the hydroxyl groups in the skeleton. This is shown for the series of cholanic acid and its hydroxy derivatives, for coprostanic acid and its 3,7- and 3,7,12-hydroxy derivatives as well as for peptide conjugates of cholanic acid and its hydroxy derivatives with glycine and taurine. The length of the side chain affects not only the CH_2-vibrations but also those of the hydroxyl- and carboxyl group, indicating differences in the hydrogen bond pattern and/or different degree of hydrogen bonding in the crystallized compounds with different side chains.