The Nature of the Enzymically Catalysed Binding of Glucose to the Protein Coat of *Colpoda steinii* Cysts

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The cyst coat of the protozoan *Colpoda steinii* is a protein rich in glutamic acid (Tibbs, 1966). In results not explicable as arising from bacterial contamination or adsorption, the coat has been shown, *in vitro*, to bind [U-¹⁴C]glucose in a reaction that appears to be enzymically catalysed (Tibbs & Marshall, 1969).

After acid hydrolysis of the glucose-labelled coat, much of the radioactivity may be extracted by ether, behaves chromatographically as laevulinic acid and reacts with 2,4-dinitrophenylhydrazine. This is the behaviour to be expected from glucose itself after heating with mineral acid, and shows that most of the hexose backbone is retained intact on binding to the cyst coat. This has been confirmed by using [1-¹⁴C]-, [2-¹⁴C]-, [6-¹⁴C]- and [U-¹⁴C]-glucose. For every atom of C-2 bound one atom of C-6 is also bound. By comparison there is some loss of C-1, which may be up to 50%. In accordance with this, the [U-¹⁴C]glucose uptake compared with the [2-¹⁴C]glucose uptake showed that more than 5 carbon atoms of the hexose were bound but not the complete 6.

The loss of some C-1 suggests that the bonding is not via glycosidic or aldol links or via a Schiff-base type of compound. Confirming the latter, m-pyruvate at pH 7.4 and 45° for 11 hr. did not remove any radioactivity from coat labelled with [U-¹⁴C]-glucose. Fragmentation of the bound molecule by periodate oxidation should result in the loss of those carbon atoms that are more than one removed from any involved in binding. None of the label was removed by 0.01 M-sodium periodate (pH about 4.5) for up to 20 hr. at room temperature, suggesting that several of the sugar hydroxyl groups may be involved in the linking of the glucose to the coat protein.

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Studies on the Particle-Bound Carbohydrate Sulphoamide Sulphohydrolase and Carbohydrate Sulphate Sulphohydrolase of *Flavobacterium heparinum*

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Previous reports from these laboratories (Lloyd, Law, Fowler & Embury, 1968; Lloyd, Fowler, Embury & Law, 1968) summarized preliminary observations on the carbohydrate sulphoamide sulphohydrolase (syn. sulphamidase) and carbohydrate sulphohydrolase (syn. O-sulphatase) of *Flavobacterium heparinum* N.C.I.B. 9290. The finding (Dietrich, 1968) that both of these enzymes were sedimented when sonicated preparations of the organism were centrifuged at 6×10^6 g-min. raised the possibility that both enzyme proteins were particle-bound. This view was supported by experiments that showed that the sulphamidase and O-sulphatase were both excluded during gel filtration on columns of Sepharose 2B (mol.wt. exclusion limit 25×10^6).

The effects of various pretreatments on the sedimentation behaviour, gel-filtration properties and activities of the sulphamidase and O-sulphatase were investigated with a view to 'solubilizing' the enzymes before further purification studies. Neither was obtained in a 'soluble' active form after extended sonication, acetone-drying or treatment with 25 mM-sodium acetate solutions, pH 7.5, containing sodium cholate (0.5–1.0%, w/v), sodium dodecyl sulphate (0.1–1.0%, w/v), Nonidet P.40 (1.0%, w/v) or Tween 80 (1.0%, w/v). On the other hand, treatment of enzyme-containing pellets with Triton X-100 (1.5%, w/v) at 4° for 10 min. resulted in the 'solubilization' of both enzymes. These were retained in the supernatant fraction after centrifuging at 6×10^6 g-min., were retarded on columns of Sepharose 2B and 4B (mol.wt. exclusion limit 3×10^6), but were still excluded from Sephadex G-200 even in Triton X-100 (1.0%, w/v) solution.

Preparations of the enzymes in Triton X-100 (1.0%, w/v) solution liberated inorganic SO_4^{2-} ions from 2-deoxy-2-[³⁵S]-sulphoamino-D-glucose 6-O-sulphate (O-sulphatase) and inorganic $^{35}\text{SO}_4^{2-}$ ions from 2-deoxy-2-[³⁵S]-sulphoamino-D-glucose (sulphamidase). However, removal of the detergent from the solubilized enzyme preparation by chromatography on DEAE-Sephadex previously suspended in 10 mM-tris-HCl buffer, pH 7.5, was accompanied by the inactivation of both enzymes. When the enzymes were chromatographed on DEAE-Sephadex previously equilibrated with 10 mM-tris-HCl buffer pH 7.5, containing Triton

X-100 (1.0%, w/v), approx. 50% of the sulphamidase originally applied to the column was recovered early in the elution. An overall purification of the sulphamidase of approx. 60-fold was achieved, although the preparation was still contaminated with O-sulphatase activity.

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The Linkage of Corneal Keratosulphate to Protein

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After exhaustive digestion with proteolytic enzymes, corneal keratosulphate contains aspartic acid as the predominant remaining amino acid. In view of the known alkali-stability of the linkage to protein, this finding led to the suggestion that the polysaccharide chains are linked *N*-glycosidically through the amide group of asparagine (Seno, Meyer, Anderson & Hoffman, 1965). Our aim has been to isolate the linkage fragment and determine its structure.

Corneal keratosulphate was hydrolysed in 1M-HCl at 100° for 15 min. After removal of the acid, the hydrolysate was adjusted to pH 2 and passed over Dowex 50 (X2). The material eluted by 0.25M-HCl was subjected to high-voltage electrophoresis at pH 6.5 and 1.9, permitting the isolation of a compound with the same mobility and ninhydrin-staining properties as 2-acetamido-1-(*L*- β -aspartamido)-1,2-dideoxy- β -D-glucose. The isolated and authentic compounds migrated identically on t.l.c. in two solvent systems and electrophoresis in borate buffer, pH 10. Incubation with glycopeptide amidohydrolase (Makino, Kojima & Yamashina, 1966), which specifically cleaves 2-acetamido-1-(*L*- β -aspartamido)-1,2-dideoxy- β -D-glucose, yielded *N*-acetylglucosamine and aspartic acid in the molar ratio 1.0:1.0. Further, the 1-dimethylaminonaphthalene-5-sulphonyl derivatives of the isolated and authentic compounds were electrophoretically indistinguishable.

The yield of isolated linkage fragment was approx. 10% of keratosulphate-bound aspartic acid.

Such a yield is consistent with the view that all aspartic acid residues are similarly linked to polysaccharide chains.

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Observations on the Enzymic Sulphurylation of Tyrosyl Derivatives

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An enzyme catalysing the sulphurylation of derivatives of *L*-tyrosine has been partially purified from rat liver and is believed to be separate from the enzyme responsible for the sulphurylation of simple phenols (Dodgson, Basford, Jones & Mattock, 1967). Kinetic experiments have now been carried out on this enzyme with adenosine 3'-phosphate 5'-sulphatophosphate and various derivatives of *L*-tyrosine as sulphuryl donor and acceptors respectively.

At pH 7.5 the K_m values for *L*-tyrosine methyl ester and adenosine 3'-phosphate 5'-sulphatophosphate were 0.3 mM and 0.007 mM respectively. The two values of K_m were independent of one another, and analysis of the experimental results (Dalziel, 1957) shows that the data are consistent with a rapid-equilibrium random Bi Bi reaction. A similar mechanism has been postulated for phenol sulphotransferase from guinea-pig liver (Banerjee & Roy, 1968).

Variation with pH in the K_m for *L*-tyrosine methyl ester and tyramine, with a fixed concentration of adenosine 3'-phosphate 5'-sulphatophosphate, shows that the enzyme is specific for an unprotonated α -amino group on the acceptor molecule. The ionization of this group is also apparent in the enzyme-substrate complexes and affects both K_m and V_{max} . *N*-Acetyl-*L*-tyrosine ethyl ester is not a good substrate for this enzyme, and the low rate of its sulphurylation is independent of pH in the range 7.0-8.2. Hence the enzyme appears to be specific for compounds having a free and unprotonated amino group. This suggestion had already been made on the basis of some preliminary investigations by Segal & Mologne (1959). Variations in K_m for adenosine 3'-phosphate 5'-sulphatophosphate with pH at a fixed concentration

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