used, thus obviating any anomalous isoenzymic forms that might otherwise be produced during prolonged tissue storage. Secondly, in certain lysosomal storage disorders (though not applicable here) gross accumulation, in brain or liver, of glycolipids and mucopolysaccharides may interfere with certain resident enzymes (Ho et al., 1973; Kint, 1974). Such a problem is less likely to be met with cultured cells where storage, if apparent, is less severe. Thirdly, the use of cultured cells for diagnosis is becoming increasingly important and, since variants of the classical forms of these diseases have and probably will be met, their expression in cultured cells must be better understood. This is particularly true when dealing with cultured amniotic-fluid cells where clinical evidence is of little value.


An Attempt to Purify N-Acetyl-β-hexosaminidases from Crude Extracts of Human Liver by Affinity Chromatography

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A variety of ligands have been used for the purification of human N-acetyl-β-hexosaminidases by affinity chromatography. A proteoglycan glycopeptide from chondroitin 4-sulphate proteoglycan coupled to Sepharose 4B was used by Dawson et al. (1973) for the partial purification of hexosaminidase A from liver, and the substrate analogues p-aminophenyl-N-acetyl-β-D-thioglucosamine coupled to succinylated (3-carboxypropionylated) diaminodipropylamino-agarose (Grebner & Parikh, 1974) and 2-acetamido-2-deoxy-β-D-glucopyranosylamine coupled to Sepharose 4B (Geiger et al., 1974) have been used for the chromatography of hexosaminidases from urine and placenta respectively. The method described by Geiger et al. (1974) appears to give the most satisfactory results, although the affinity-chromatography step was introduced after considerable purification and the separation of hexosaminidases A and B by conventional techniques.

It would be advantageous if affinity chromatography could be used for the purification of hexosaminidases from crude extracts, and we have tested the feasibility of this by using N-acetyl-d-glucosamine as a ligand. In all the affinity methods previously reported for hexosaminidases, elution of the enzyme from the gel has been non-specific, achieved either by using an alkaline buffer or by increasing the salt concentration of the column buffer. We have attempted specific elution of the enzyme with solutions of N-acetylglucosamine, which is a competitive inhibitor of the enzyme.

The 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine was prepared by the method of Bolton & Jeanloz (1962). This glucosylamine tetra-acetate was either coupled directly to CNBr-activated Sepharose 4B and then deacetylated with sodium methoxide in dry methanol (Grebner & Parikh, 1974) or condensed with N-(benzyloxy-
Table 1. Elution of N-acetyl-β-hexosaminidase from a column of 2-acetamido-N-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine coupled to Sepharose 4B by N-acetylglucosamine

<table>
<thead>
<tr>
<th>Conc. of N-acetylglucosamine (M)</th>
<th>% of applied activity recovered</th>
<th>Increase (fold) in specific activity compared with crude extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>44</td>
<td>41</td>
</tr>
<tr>
<td>0.1</td>
<td>46</td>
<td>5.3</td>
</tr>
<tr>
<td>0.5</td>
<td>62</td>
<td>4.7</td>
</tr>
<tr>
<td>1.0</td>
<td>80</td>
<td>4.0</td>
</tr>
<tr>
<td>0.1 M-Glucose</td>
<td>2</td>
<td>—</td>
</tr>
</tbody>
</table>

carbonyl)-ε-aminohexanoic acid, deblocked, deacetylated (Lotan et al., 1973) and then coupled to the CNBr-activated Sepharose 4B.

In each experiment 5 ml of an aqueous 10% (w/v) extract of post-mortem human liver was applied to the affinity gel (2 ml) equilibrated in 0.01 M-sodium phosphate buffer, pH 6.0, and the column washed with 20 ml of the column buffer before the elution of the retained activity.

When the ligand was coupled directly to the Sepharose, 72% of the applied activity was retained. It was difficult to remove this bound activity, only 5% being eluted with 10 mM-N-acetylglucosamine in the column buffer and a further 8% with 0.2 M-NaCl in buffer.

Interposition of the hexanoamide arm between the ligand and the gel increased the retention to 88% and made easier the removal of bound activity, 44% being eluted with 10 mM-N-acetylglucosamine and the remainder with the salt wash. Electrophoresis on Cellogel (Poenaru & Dreyfus, 1973) showed that the unretained activity and activity appearing in the N-acetylglucosamine eluate was predominantly of the B type. Lengthening the spacer by condensing the 2-acetamido-N-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine with the carboxyl group of the 6-aminohexanoic acid arm of Sepharose 4B was counter-productive, giving 75% retention and recovery of 3% with 10 mM-N-acetylglucosamine and 31% with the salt wash.

The results of increasing the concentration of N-acetylglucosamine in the eluting buffer are given in Table 1, which shows that, although increasing the sugar concentration removes more activity from the affinity gel, this is accompanied by the removal of other proteins bound to the column. The specific activity of the enzyme decreases tenfold when the concentration of N-acetylglucosamine in the eluting buffer is increased from 10 mM to 1 M.

The nature of the interaction between the contaminating proteins and the affinity gel is uncertain. There might be a large number of proteins in liver which are able to recognize β-N-acetylgalcosaminyl groups, but it is more likely that the contaminating proteins are non-specifically bound, possibly by hydrophobic interaction with the spacer arm. Non-specific binding of this sort has been described by Hofste (1973).

The column was very effective in retaining β-galactosidase, and 90% of the activity in the crude liver extracts was bound. Elution with 0.5 M-N-acetylglucosamine removed 10% of the activity and the remainder was eluted with 0.2 M-salt wash. It seems likely that the β-galactosidase is bound non-specifically to the affinity column, since the enzyme is not inhibited by a 10 mM solution of the free ligand 2-acetamido-N-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine. Hexosaminidase activity is inhibited 73% by the same concentration of ligand.

Hexosaminidase might also be bound non-specifically to the affinity column, but the relatively high retention of the enzyme by the ligand that we have used here is in marked contrast with the very low retention on similar columns substituted with L-fucosylamine or D-mannosylamine (R. Thorpe & N. C. Phillips, personal communication).
addition, the elution of the affinity column with 100mM-D-glucose (Table 1) was almost ineffective in removing the bound hexosaminidase.

The use of 2-acetamido-N-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine bound to Sepharose for the affinity chromatography of hexosaminidases in crude liver extracts results in moderate (40-fold) increases in specific activity when 10mm-N-acetylglucosamine is used as an eluent. The technique seems to be more effective with partially purified preparations such as those described by Geiger et al. (1974), but might be improved by specific elution with N-acetylglucosamine.

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Two Forms of β-N-Acetylhexosaminidase from Physarum polycephalum
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Physarum polycephalum is a myxomycete or true slime mould which, during the plasmodial stage of its life-cycle, can be cultured axenically in a semi-defined medium (Carlile, 1971). Extracellular ribonucleases (Braun & Behrens, 1969) and proteinases (Farr et al., 1974) have been detected in the medium, and we thought that glycosidases might also be present. We are interested in the possible role of glycosidases and their substrates in development, but in addition we thought that the medium might be a good source of glycosidases for use in structural studies on complex carbohydrates.

With 4-methylumbelliferyl glycosides as substrates, seven glycosidase activities were detected, of which β-N-acetylglucosaminidase and β-N-acetylgalactosaminidase were the highest. These were subsequently found to be purified together. The activity of β-N-acetylhexosaminidase in the medium, expressed per mg dry wt. of P. polycephalum microplasmodia, remains fairly constant during the exponential phase of growth, but increases 8-fold in 3 days after the stationary phase is reached. Electrophoresis of spent growth medium in starch gel at pH 7.0 produced two widely separated bands of β-N-acetylhexosaminidase activity, both of which migrated towards the anode.

A purification scheme was devised which included the separation of the two forms of β-N-acetylhexosaminidase by ion-exchange chromatography. The medium was first treated with an equal volume of ethanol to precipitate some of the polysaccharide slime (Farr et al., 1972), the presence of which renders column chromatography very difficult. This was followed by precipitation with 90%-satd. (NH₄)₂SO₄, and after dialysis the resuspended precipitate was adsorbed on a column of hydroxyapatite. The β-N-acetylhexosaminidase was eluted in 200mm-sodium phosphate buffer, pH 7.0, dialysed and then applied to a column of DEAE-cellulose equilibrated in 5mm-sodium phosphate buffer, pH 7.0. On application of a linear NaCl gradient, two fully separated peaks of β-N-acetylhexosaminidase were obtained. We have termed these peaks X and Y. Both peaks were concentrated and applied separately to a column of Sephadex G-150. This purification procedure resulted in a 145-fold purification of fraction X and 300-fold purification of fraction Y. When subjected to starch-gel electrophoresis the purified