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Molecular Forms and Activities of Glycosidases in Cultures of Amniotic-Fluid Cells

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Ion-exchange chromatography or gel filtration demonstrated the presence of different molecular forms of nine lysosomal enzymes in cultured amniotic-fluid cells. The patterns of molecular forms were similar to those known from skin fibroblasts and liver tissue. During cultivation total enzyme activities fluctuated with the number of passages, without any consistent trend of increase or decrease, and without correlation to the dominating cell type in the culture.

A growing number of inborn errors of metabolism can be diagnosed antenatally by analysis of enzyme activities in amniotic-fluid cells (Burton *et al.*, 1974). Enzyme assay is considered to be better if cultured, rather than uncultured, amniotic-fluid cells are used (Nadler & Gerbie, 1969). There is, however, insufficient information available about the 'normal' enzymological properties of cultured amniotic-fluid cells. During foetal development the isoenzyme pattern of a particular enzyme may be significantly altered (Latner & Skillen, 1968). If lysosomal isoenzyme patterns in cultured amniotic-fluid cells from early pregnancy are different from those in postnatal tissues, this could give rise to diagnostic errors. This prompted us to investigate the isoenzyme spectrum of nine lysosomal enzymes in cultured amniotic-fluid cells from the second trimester of pregnancy. We also report on enzyme kinetics and activities of these enzymes.

Materials and Methods

Amniotic-fluid samples were obtained by transabdominal amniocentesis from women in the second trimester (16–17 weeks). The specimens were taken because of genetical indications (maternal age over 35 years or earlier history of chromosomal aberration) of abnormalities. In no case was there a history of inborn errors of metabolism involving lysosomal enzymes. Cell strains with normal karyotype from ten women were used in this investigation.

Generally 10–20ml of amniotic fluid was used for preparing the cell cultures. The amniotic-fluid cells were cultivated as follows.

The amniotic fluid was centrifuged at 1000*g* for 10min; the supernatant was removed and the cells were resuspended in 2ml of medium [70% Eagle's minimal essential medium with Earle's salts, 20% (v/v) foetal calf serum and 10% (v/v) human serum; Flow Laboratories, Irvine, Scotland, U.K.]. Cell suspension (1ml) was added to each of two Leighton tubes and bubbled with 5% CO_2 in air before stoppering the tubes and incubating them at 37°C. The medium was changed with 1.5ml of fresh medium every second or third day. Primary cultures were subsequently subcultured in 30 or 250ml Falcon plastic culture bottles (Falcon Plastics, Los Angeles, CA, U.S.A.) by using 0.25% trypsin solution (Flow Laboratories) in phosphate-buffered saline (10mM-sodium phosphate, pH7.4). Cells from each subculture were harvested by trypsin treatment at the stage of confluent monolayer. The cells were washed twice with 0.9% NaCl, centrifuged at 1500*g* for 5min at room temperature (20°C) and then frozen at −20°C until analysis, not more than 2 weeks later. No change in enzyme activity occurred during this time.

Before harvesting, the cell cultures were judged as predominantly fibroblastoid or epitheloid.

Enzyme assays

Enzyme assays were performed on crude homogenates. The cells from 30ml Falcon plastic culture bottles were homogenized in 4ml of ice-cold, distilled and deionized water by using an all-glass Potter–Elvejhem homogenizer.

Protein in the homogenates was assayed by the method of Lowry *et al.* (1951) with a suitable albumin solution (Kabi, Stockholm, Sweden) as protein standard. The protein contents of the cell homogenates used in the assays were between 20 and 100μg of protein/100μl of homogenate.

α-Fucosidase (*α-L-fucoside fucohydrolase, EC* 3.2.1.51). This was assayed with 150μl of 8mM-*p*-nitrophenyl α-L-fucopyranoside (Koch-Light

Laboratories Ltd., Colnbrook, Bucks., U.K.) in water, 150 μl of 1 M-sodium citrate buffer, pH 5.5, and 100 μl of cell homogenate. Incubation times were 0, 6 and 24h at 37°C. The reaction was stopped by the addition of 150 μl of 5% (w/v) trichloroacetic acid. The tubes were centrifuged (1000*g* for 10min), and 0.4ml of supernatant was mixed with 3ml of 0.2M-glycine buffer, pH10.7 (0.2M-glycine, pH adjusted with 1M-NaOH). Liberated *p*-nitrophenol was measured at 400nm with *p*-nitrophenol (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

N-Acetyl-α-glucosaminidase (*2-acetamido-2-deoxy-α-D-glucoside acetamidodeoxyglucohydrolase*, *EC* 3.2.1.50). This was assayed with 100 μl of 12.5mM-phenyl α-*N*-acetyl-D-glucosaminide (Sigma Chemical Co.) in water, 50 μl of 1M-sodium citrate buffer, pH4.5, and 200 μl of cell homogenate. The tubes were incubated for 0, 6 and 24h at 37°C. After incubation, 250 μl of Folin–Ciocalteu reagent (Kebo, Stockholm, Sweden) was added. After centrifugation (1000*g* for 10min), 400 μl of supernatant was mixed with 2ml of 0.4M-Na_2CO_3, and the E_{650} was read after 30min.

N-Acetyl-β-glucosaminidase (*2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase*, *EC* 3.2.1.31). A sample (50 μl) of 7.5mM-*p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Koch–Light Ltd.) in water, 50 μl of 1M-sodium citrate buffer, pH4.5, and 100 μl of cell homogenate were incubated together at 37°C for 0, 30, 60 and 120min. Subsequent procedures were as for α-fucosidase.

α-Galactosidase (*α-D-galactoside galactohydrolase*, *EC* 3.2.1.22). This was assayed with 100 μl of 7.5mM-4-methylumbelliferyl α-D-galactopyranoside (Koch–Light Ltd.) in water, 25 μl of 1M-sodium citrate buffer, pH4.5, and 25 μl of cell homogenate. The tubes were incubated for 0, 120 and 240min at 37°C. The enzyme activity was stopped by the addition of 3ml of 0.2M-glycine buffer, pH10.7, and fluorescence was read in an Aminco–Bowman spectrofluorimeter with an excitation wavelength of 365nm and an emission wavelength of 448nm. 4-Methylumbelliferone (Koch–Light Ltd.) in glycine buffer was used as standard.

β-Galactosidase (*β-D-galactoside galactohydrolase*, *EC* 3.2.1.23). A sample (100 μl) of 1mM-4-methylumbelliferyl β-D-galactopyranoside (Koch–Light Ltd.) in water, 25 μl of 1M-sodium acetate buffer, pH4.5, and 25 μl of cell homogenate were incubated at 37°C for 0, 30, 60 and 120min. The reaction was terminated and read as for α-galactosidase.

α-Glucosidase (*α-D-glucoside glucohydrolase*, *EC* 3.2.1.20). This was assayed with 100 μl of 1mM-4-methylumbelliferyl α-D-glucopyranoside (Koch–Light Ltd.) in water, 25 μl of 0.2M-sodium citrate buffer, and 25 μl of cell homogenate. Two different citrate buffers were used, one at pH4.5 for assay of acid α-glucosidase and one at pH6.0 for assay of the neutral enzyme. The tubes were incubated for 0, 60 and 120min at 37°C. Subsequent procedures were as described for α-galactosidase.

β-Glucosidase (*β-D-glucoside glucohydrolase*, *EC* 3.2.1.21). A sample (50 μl) of 1mM-4-methyl umbelliferyl β-D-glucopyranoside (Koch–Light Ltd.) in 10mM-histidine buffer (pH6.5, adjusted with 1M-NaOH), 25 μl of 0.2M-sodium citrate buffer, pH4.5, and 25 μl of cell homogenate were incubated at 37°C for 0, 120 and 240min. Subsequent procedures were as described for α-galactosidase.

α-Mannosidase (*α-D-mannoside mannohydrolase*, *EC* 3.2.1.24). A sample (50 μl) of 12.5mM-4-methylumbelliferyl α-D-mannopyranoside (Koch–Light Ltd.) in water, 10 μl of 1M-sodium citrate buffer, pH4.5 and 5.5, and 10 μl of cell homogenate were incubated together at 37°C for 0, 60 and 120min. Subsequent procedures were as described for α-galactosidase.

β-Glucuronidase (*β-D-glucuronide glucuronohydrolase*, *EC* 3.2.1.31). A sample (50 μl) of 10mM-phenolphthalein glucuronide (Sigma Chemical Co.) in water, 50 μl of 1M-sodium acetate buffer, pH5.0, and 100 μl of cell homogenate were incubated together 37°C for 0, 6 and 24h. The reaction was stopped with 3ml of 0.2M-glycine buffer, pH10.7, the samples were centrifuged (1000*g* for 10min) and liberated phenolphthalein was measured at 555nm with phenolphthalein (Sigma Chemical Co.) as a standard.

In all enzyme assays the enzyme activity measured was directly proportional to the amount of protein in the incubation tube within the concentration limits given in the Results section.

Separation methods

Gel filtration was performed by using Sephadex G-150 (Pharmacia, Uppsala, Sweden). The crude cell homogenate was centrifuged at 100000*g* for 60min and 4ml of the supernatant (40mg of protein) was applied to a column (70cm×3cm) of Sephadex G-150 and eluted with 0.01M-sodium phosphate buffer, pH7.0, containing 0.35M-NaCl. Pooled cells from five different strains were used for one separation experiment. The column flow rate was 20ml/h; 5ml fractions were collected.

For ion-exchange chromatography, 4ml of the ultracentrifuged and dialysed (against 0.025M-sodium phosphate buffer, pH7.4) cell-homogenate supernatant (40mg of protein) was applied to a column (24cm×0.9cm) of DEAE-cellulose (Whatman DE-52; W. and R. Balston, Maidstone, Kent, U.K.), equilibrated in 0.025M-sodium phosphate buffer, pH7.4. After elution with 60ml of 0.25M-sodium phosphate buffer, pH7.4, a linear gradient was established with 100ml of this buffer and 100ml of the same buffer containing 0.7M-NaCl; 1ml fractions were collected.

All procedures were performed at +4°C.

Results

Physicochemical characteristics of the various enzymes

The variations of activity with respect to pH in the homogenates of the cultured amniotic-fluid cells are shown in Fig. 1. The apparent Michaelis–Menten constants and the percentage of the theoretical maximum rate obtained at the actual substrate concentrations are shown in Table 1. The enzyme activity measured was proportional to time and the amount of protein in the test tube with the homogenate protein concentrations used (20–100 μg of protein/100 μl of homogenate).

Activities of the different enzymes

Enzyme activities were assayed in fifteen different

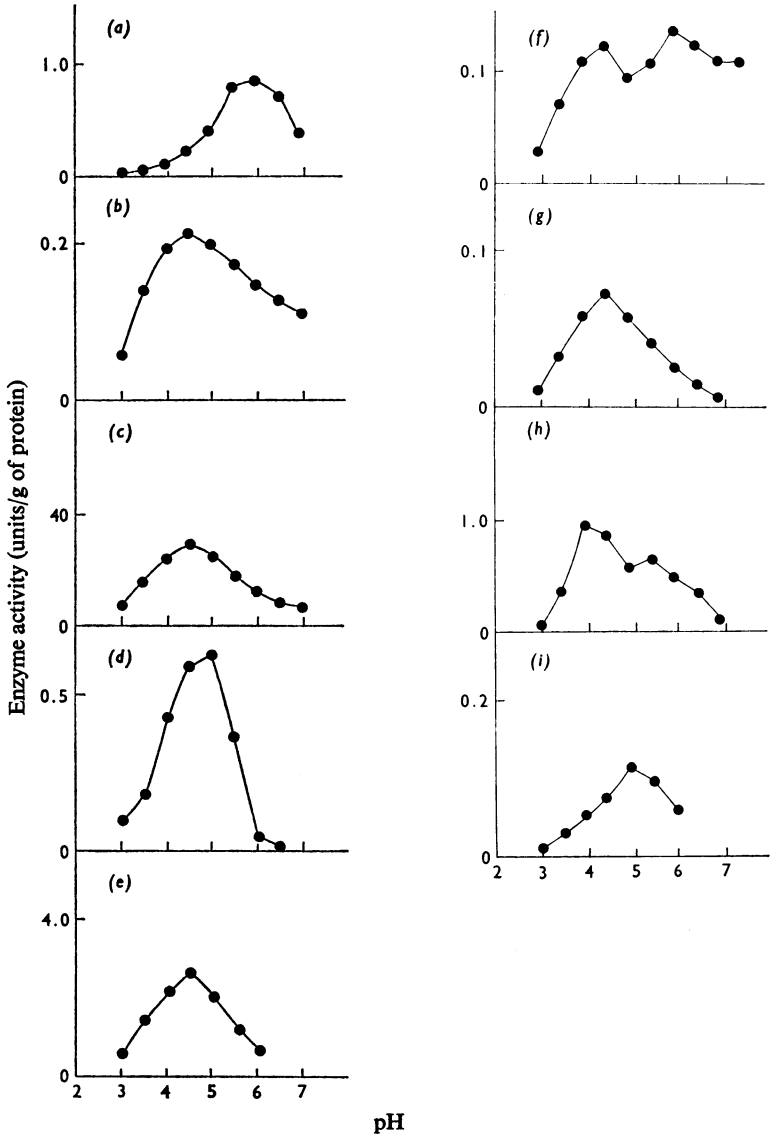

Fig. 1. *pH-dependence of the enzyme activities*

(*a*) α-Fucosidase, (*b*) *N*-acetyl-α-glucosaminidase, (*c*) *N*-acetyl-β-glucosaminidase, (*d*) α-galactosidase, (*e*) β-galactosidase, (*f*) α-glucosidase, (*g*) β-glucosidase, (*h*) α-mannosidase, (*i*) β-glucuronidase. Activity was determined as described in the Materials and Methods section, and is given as units/g of protein (1 unit = 1 μmol of substrate split/min).

Table 1. *Kinetic properties of some enzymes in cultured amniotic-fluid cell homogenates*

The methods used are described in the Materials and Methods section. Actual substrate concentration refers to the concentration in the incubation mixture. % $V_{max.}$ means the percentage of maximum velocity obtained at the actual substrate concentration.

Enzyme	Actual substrate concentration (mM)	pH and buffer used for K_m analysis	K_m (mM)	% $V_{max.}$
α-L-Fucosidase	3.01	5.5 (citrate, 1M)	0.30	91
N-Acetyl-α-D-glucosaminidase	3.57	4.5 (citrate, 1M)	0.12	93
N-Acetyl-β-D-glucosaminidase	3.75	4.5 (citrate, 1M)	0.63	84
α-D-Galactosidase	4.99	4.5 (citrate, 1M)	3.3	70
β-D-Galactosidase	0.66	4.5 (citrate, 1M)	0.44	56
α-D-Glucosidase	0.66	4.5 (citrate, 0.2M)	0.22	80
		6.0 (citrate, 0.2M)	0.25	75
β-D-Glucosidase	0.50	4.5 (citrate, 0.2M)	0.62	40
α-D-Mannosidase	8.85	4.5 (citrate, 1M)	2.95	80
		5.5 (citrate, 1M)	4.30	70
β-D-Glucuronidase	2.50	5.0 (citrate, 1M)	1.00	67

Table 2. *Enzyme activities in amniotic-cell homogenates*

Enzyme activity is expressed as units/g of protein (1 unit = 1 μmol of substrate split/min). Range values for 30 experiments, mean±S.D. in the first three subcultures (P_1, P_2 and P_3) are given.

Enzyme	pH	P_1–P_3 Range	P_1–P_3 Mean±S.D.
α-Fucosidase	5.5	0.42–1.17	0.78±0.20
N-Acetyl-α-glucosaminidase	4.5	0.10–0.24	0.12±0.04
N-Acetyl-β-glucosaminidase	4.5	17.2–43.6	28.0±6.7
α-Galactosidase	4.5	0.20–0.71	0.42±0.18
β-Galactosidase	4.5	0.70–2.89	1.30±0.62
α-Glucosidase	4.5	0.21±0.96	0.5 ±0.27
β-Glucosidase	4.5	0.07–0.27	0.16±0.07
α-Mannosidase	4.5	0.61–1.1	0.74±0.10
β-Glucuronidase	5.0	0.19–0.39	0.26±0.04

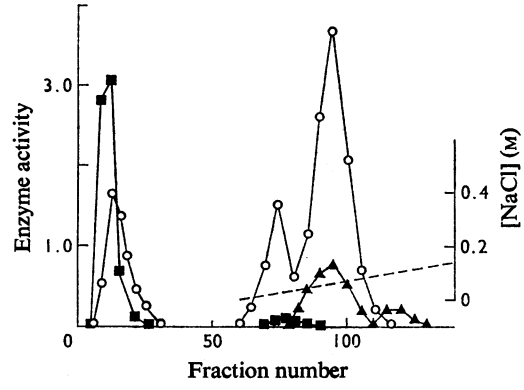

Fig. 2. *Ion-exchange chromatography of α-fucosidase* (○), *α-galactosidase* (▲) *and β-glucuronidase* (■)

Cell supernatant (4ml) containing 10mg of protein/ml was used as enzyme source. ----, NaCl gradient. Enzyme activity is expressed as μmol of substrate split/h. Enzyme assay and chromatography conditions were as described in the Materials and Methods section.

amniotic cell lines from ten subcultures (P_1–P_{10}). In Table 2, mean±S.D. and range are given only for the first three subcultures (P_1–P_3) as these are the ones mostly used for prenatal diagnosis. There was a fluctuation of activities with numbers of passages (P_1–P_{10}) within the limits given under 'Range' in Table 2. No consistent pattern of increase or decrease related to passage was noted for the enzymes investigated. No consistent correlation between the predominant cell type and amount of enzyme activity was found.

Molecular forms

Ion-exchange chromatography of cultured amniotic-fluid-cell preparations on DEAE-cellulose or gel chromatography on Sephadex G-150 demonstrated multiple molecular forms of all enzymes investigated (Figs. 2–6).

α-Fucosidase. The activity could be separated into three fractions by DEAE-cellulose chromatography (Fig. 2). Heterogeneity was also indicated by gel filtration (Fig. 5).

N-Acetyl-α-glucosaminidase. Two main fractions were present after gel filtration (Fig. 5). Two fractions, one broad adsorbed and one unadsorbed, were also present after ion-exchange chromatography, as seen in cultured skin fibroblasts (Hultberg *et al.*, 1975).

N-Acetyl-β-glucosaminidase. Two major fractions of about equal activity, one adsorbed and one unadsorbed, could be separated by ion-exchange chromatography (Fig. 4). The unadsorbed activity

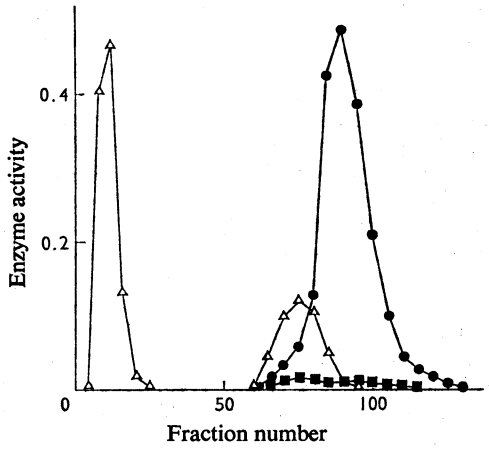

Fig. 3. *Ion-exchange chromatography of α-mannosidase* (△), *β-galactosidase* (●) *and β-glucosidase* (■)

Experimental conditions are as in Fig. 2. Enzyme activity is expressed as μmol of substrate split/h.

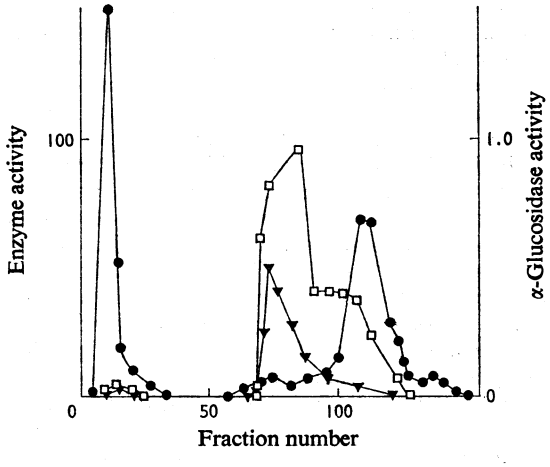

Fig. 4. *Ion-exchange chromatography of N-acetyl-β-glucosaminidase* (●), *α-glucosidase, pH*4.5 (□), *and α-glucosidase, pH*6.0 (▼)

Experimental conditions are as in Fig. 2. Enzyme activity is expressed as μmol of substrate split/h.

was heat-stable for 15 min at 60°C, whereas only 10% of the adsorbed activity was left after this treatment. Only one peak of activity was seen after gel filtration (Fig. 5).

α-Galactosidase. Ion-exchange chromatography showed a large fraction of α-galactosidase, followed by a smaller one (Fig. 2). The activity in the small

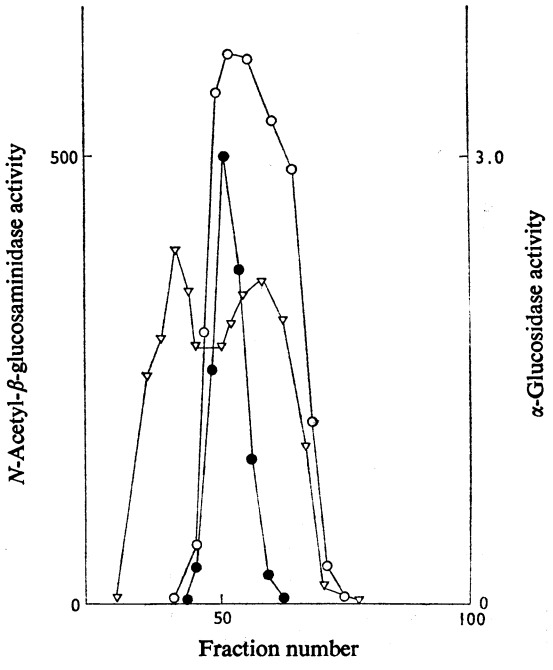

Fig. 5. *Gel filtration of α-fucosidase* (○), *N-acetyl-α-glucosaminidase* (▽) *and N-acetyl-β-glucosaminidase* (●)

Void volume is at fraction 33. Cell supernatant containing 10 mg of protein/ml was used as enzyme source. Enzyme activity is expressed as μmol of substrate split/h. Enzyme assay and gel-filtration conditions are described in the Materials and Methods section.

peak was heat-resistant (90% of the initial activity remained when the enzyme sample and buffer were incubated together for 10 min at 50°C), whereas the other form was heat-sensitive under the same conditions (10% of the initial activity remaining). After gel filtration only one fraction was present (Fig. 6).

β-Galactosidase. One peak of activity was observed after ion-exchange chromatography (Fig. 3), but two were present after gel filtration (Fig. 6).

α-Glucosidase. Ion-exchange chromatography gave a partial separation of the two forms of α-glucosidase; one with an acidic pH optimum and the other with a more neutral pH optimum (Fig. 4), as described by Hultberg *et al.* (1975). The neutral form of the enzyme was eluted as one peak from the Sephadex G-150 column (Fig. 6). The acidic form was retarded and distributed irregularly over a large elution volume.

β-Glucosidase. A broad peak of activity was found after ion-exchange chromatography (Fig. 3). Gel filtration gave distribution of the activity over a large elution volume.

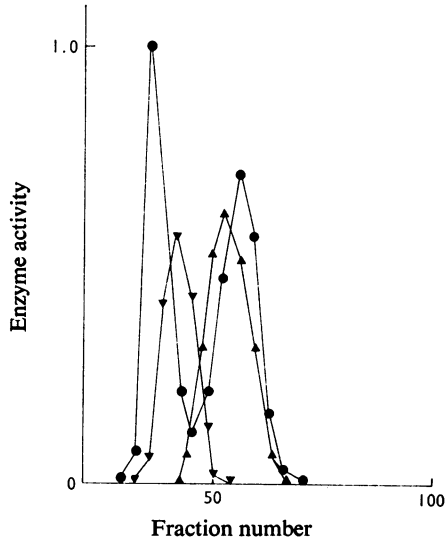

Fig. 6. *Gel-filtration of β-galactosidase* (●), *α-galactosidase* (▲) *and α-glucosidase, pH* 6.0 (▼)

Experimental conditions are as in Fig. 5. Enzyme activity is expressed as μmol of substrate split/h.

α-Mannosidase. Two fractions with acidic pH optima were present after ion-exchange chromatography (Fig. 3). Gel filtration gave one main peak of material with the same elution volume as for the liver enzyme (Hultberg & Öckerman, 1972).

β-Glucuronidase. The major part was unadsorbed on the DEAE-cellulose DE-52 column (Fig. 2), but there was a small adsorbed peak of material. Gel filtration gave one peak of enzyme with the same elution volume as for the liver enzyme (Hultberg & Öckerman, 1972).

Discussion

More and more diseases resulting from a known deficiency of a lysosomal enzyme have been detected in recent years. With the demonstration that these deficiencies can be observed in cultured amniotic-fluid cells, antenatal diagnosis for inborn errors of lysosomal-storage diseases has become practicable (Burton *et al.*, 1974). The need to have detailed knowledge of lysosomal enzyme assay has therefore arisen to avoid pitfalls in antenatal diagnosis.

Wiederschain *et al.* (1973) and Robinson & Thorpe (1973) have shown two forms of α-L-fucosidase in human tissues. In our experiments with cultured amniotic-fluid cells, ion-exchange chromatography revealed three fractions of the enzyme, as in liver tissue (B. Hultberg & S. Sjöblad, unpublished work).

α-D-Mannosidase had a biphasic pH–activity profile with pH optima of 4.0–4.5 and 5.5. Two major forms of this enzyme with acid pH optima were seen on ion-exchange chromatography. An activity comparable with α-mannosidase C (Carroll *et al.*, 1972) could not be demonstrated by using ion-exchange chromatography. Also α-D-glucosidase had a biphasic pH–activity profile with pH optima of 4.5 and 6.0. The two forms of the enzyme present in cultured skin fibroblasts (Hultberg *et al.*, 1975) are both present in cultured amniotic-fluid cells and were partly separated on ion-exchange chromatography.

For all enzymes investigated, the molecular forms found in cultured amniotic-fluid cells were similar to those found in postnatally obtained skin fibroblasts (Hultberg *et al.*, 1973, 1975), and liver tissue (Hultberg & Öckerman, 1970, 1972; Hultberg *et al.*, 1970).

The different enzyme activities in the cell strains investigated fluctuated with the number of passages. No consistent trend of increase or decrease with passage was observed. Neither did the dominating cell type in the cultures (epitheloid or fibroblastoid) influence the enzyme activities in any consistent way.

Some reports of enzyme activities in cultured amniotic-fluid cells have been published (Butterworth *et al.*, 1973*a*,*b*, 1974*a*,*b*; Sutherland *et al.*, 1974). Those authors found marked fluctuations of lysosomal enzymes with the number of passages. The fluctuations were unrelated to serum concentration in the medium, type of medium and the pH of the medium. Further, in their studies, enzyme activities showed no relationship to the time after subculture, when confluency was reached, and there was no correlation between gestational age and the activities of lysosomal enzymes in cells cultured from serial samples of amniotic fluid. Those authors conclude that cells cultured from any gestational age, and at any stage of culture up to the fifteenth passage, can be used as control material (with the possible exception of α-glucosidase, which may have higher activity in later passages). The fluctuations of enzyme activity with culture time and also in cells cultured from serial samples of amniotic fluid from the same woman result, however, in a wide range of normal values that would complicate identification of heterozygotes. Heterozygotes with partial enzyme deficiencies may also be mistaken for affected homozygotes.

Our results show that for diseases involving the enzymes investigated, cultured amniotic-fluid cells from early pregnancy can serve as a diagnostic material in the same way as postnatally obtained skin fibroblasts and liver tissue, since their molecular forms are similar. Knowledge of the different molecular forms will allow a more careful characterization of the deficiency state and will be helpful in detecting partial enzyme deficiencies and disease variants.

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